Cambodian Rice Liquor Development Using *Rhizopus Oryzae*, *Saccharomyces Cerevisiae* and Alpha-amylase

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Abstract: *In Cambodia, rice liquors or Sra Sor is locally produced in large amounts every year. This traditional beverage is made from the combination of rice starch with different traditional starters consisting of molds and fermenting yeast. Recently, the quality and the ethanol yield of rice wine produced by traditional starter were notably low. Thus, the utmost concern is to improve the quality of the product in terms of physicochemical and sensory properties, yield, and to establish the quality control system throughout the process. The objective of this study was to improve the processing technique of rice liquor production by using Rhizopus oryzae with Saccharomyces cerevisiae and alpha-amylase. The physicochemical parameters including pH, acidity, total reducing sugar, glucose, maltose, lactic acid, acetic acid, ethanol, and methanol were investigated during fermentation and at the end of fermentation. The results showed that during fermentation, pH of all conditions decreased gradually over the time and reached the finally pH values around 3.09 ± 0.01 to 4.33 ± 0.04, while the concentration of acidity increased remarkably until the end of fermentation around 3.12 ± 0.064 g/l to 8.42 ± 0.45 g/l. This result was in accordance to ethanol production found highest of 9.66 ± 0.08 % (v/v) at the same condition. In conclusion, using alpha-amylase in the rice wine production process provided notable results with highest yield of ethanol and lower acidity by comparison to the control without enzyme. Thus, the mixture of R. oryzae, S. cerevisiae with the addition of alpha-amylase is a good process design for further study as well as industrial scale.*

1. INTRODUCTION

In Cambodia, rice is the main crop that plays an important role in the agriculture sector. Rice could be the raw material for many products such as rice powder, rice noodles, rice milk, dessert, and rice liquor (*Sra Sor*). *Sra Sor* is one of the most important alcoholic beverages for Cambodian culture (Kong & Chen, 2017). *Sra Sor* is made from a combination of rice starch with different traditional starters consisting of molds and fermenting yeast (Yamamoto & Matsumoto, 2011). This traditional beverage is generally obtained from the distillation process from rice wine products by different methods and equipment. Based on the study of Ly et al. (2018) most of the bacteria identified in Cambodian traditional dried starters (*Dombea*) were lactic acid bacteria including *Weissella cibaria*, *Pediococcus sp. MMZ60A*, *Lactobacillus fermentum*, and

Lactobacillus plantarum. Moreover, *Saccharomy cerevisiae* and *Saccharomycopsis fibuligera* were found to be dominated yeasts while the only amylolytic filamentous fungus was *Rhizopus oryzae* (Ly et al., 2018). Recently, the quality and the ethanol yield of rice wine produced by traditional starter has been notably low and the population of this traditional rice liquor has been enormously decreased. The low-quality rice liquors are characterized by acidic taste, spoiled aroma, muddy, smoky smell taste, cloudy, and undesirable color (Chim et al., 2018). More importantly, according to the report of Phnom Penh Post news in 2017, around 15 people were killed by the consumption of unknown source liquor products in Kampong Chnang province. High methanol content in liquor provokes death (Kong & Chen, 2017). Due to attribute to the fact that producers faced many problems and low income, lost customer trustability of buying their products. Thus, the utmost concern is to improve the quality of the product in terms of physicochemical and sensory properties, yield, and establish the quality control system throughout the process of operation. Quality improvement of these rice liquors

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would be one of the key strategies. Thus, the current study aimed to improve the processing technique of rice liquor production by using *R. oryzae* with *S. cerevisiae* and alpha-amylase. The addition of α-amylases is expected to improve liquefying as well as ethanol production during fermentation. To compare the effectiveness of the designed process, some physicochemical parameters such as pH, acidity, total reducing sugar, glucose, maltose, lactic acid, acetic acid, ethanol, and methanol were investigated during fermentation and at the end of the fermentation process. This study did not focus on the source of raw material and white rice (*Neang Minh*) was purchased from a local market. Moreover, *R. oryzae*, isolated from the traditional fermenting starter was used as liquefying and saccharifying agent, and commercial yeast *S. cerevisiae* as fermenting yeast. Only commercial alpha-amylase was used to enhance and improve liquefying and saccharifying during fermentation.

2. METHODOLOGY

2.1 Starter culture preparations

This study was conducted by using mold *R. oryzae* which is isolated from Cambodian traditional dried starters (*Dombea*) (Ly et al., 2018). *R. oryzae* strains were cultured in DRBC media (Dichloran Rose-Bengal Chloramphenicol). After 48 h of incubation at 37 °C, filaments and spores on DRBC were observed, then, they were collected and counted. This current study used only one concentration of 10^6 spores in 100 g of cooked rice before making koji. Spores were scratched and collected with sterilized peptone water (0.5 % NaCl and 1 % peptone) and counted with a microscope. Dried wine yeast (*S. cerevisiae*) was prepared to follow the instruction on the package by dissolving yeast with adding 50 ml of water at about temperature 38 °C to 41 °C. Alpha-amylases enzyme (Novozymes) were purchased from Denmark.

2.2 Experimental design

The experiment was divided into four conditions including one control (Table 1). The control condition is the process where α-amylase was not use in the process. Other three conditions, the enzyme was used and activated differently.

Table 1. Experimental design with code of conditions

Conditions code	Solid-state fermentation	Alcoholic fermentation
CRS	*R. oryzae*	*S. cerevisiae*
RAS	*R. oryzae* + α-amylases (N/AV)	*S. cerevisiae*
RSA/na	*R. oryzae*	*S. cerevisiae* + α-amylases (N/AV)
RSA/a	*R. oryzae*	*S. cerevisiae* + α-amylases (AV)

* *(CRS)* represented to control sample without used enzyme, *(RAS)* represented to the condition of using α-amylases during solid-state fermentation, *(RSA/na)* represented to condition of using α-amylases without heat activation prior to alcoholic fermentation process and *(RSA/a)* represented to the condition of using α-amylases with heat activitation prior to alcoholic fermentation process. N/AV represented non activiation of enzyme while AV referred to activiation of enzyme.
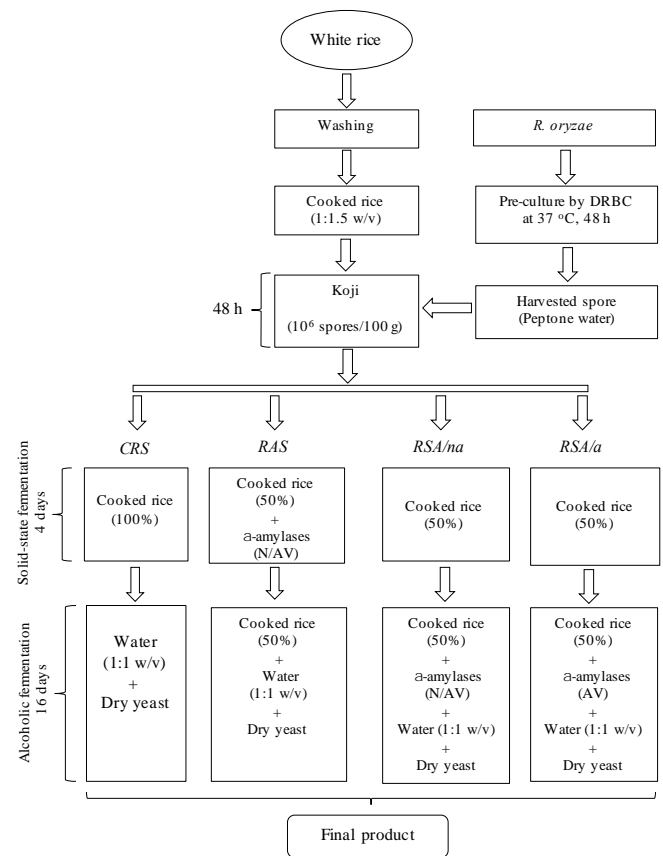

Fig.1. Flowchart of rice wine process

2.3 Koji preparation

Koji is a type of cereal that was inoculated with molds for two days or 48 h (Yoshizaki et al., 2010). Firstly, the rice grain was washed with tap water to remove an impurity substance and to ensure the rice grain was cleaned before cooking. Next, rice was introduced to cook with tap water with an optimal water ratio of 1:1.5 w/v (rice: water) based on initial rice weight to achieve the final moisture content at 65 % by electronic pot. After that, the cooked rice was

cooled at ambient temperature and it was placed in four different 1000 ml tanks and inoculated with R. Oryzae of 106 spores per 100 g of rice.

2.4 Solid-state fermentation

Solid-sate fermentation is a process technique for the production of microbial metabolites that performs on a solid substrate with low moisture content. After 48 h of koji incubation at ambient temperature, 4 kg of the cooked rice was added into 10 l of fermentable tanks for doing a controlled CRS sample which was mixed with the koji. RAS was the conditions in which 2 kg (50 %) of cooked rice was used to mix with koji into 10 l of fermentable tanks and RSA/na and RSA/a were the conditions of mixed 2 kg (50 %) of cooked rice with koji into 10 l of fermentable tanks. All of the processes of solid-state fermentation was taken 96 h respectively.

2.5 Alcoholic fermentation preparation

Alcoholic fermentation is a complex biochemical process which yeast converted sugar to ethanol, carbon dioxide, and other metabolic products. After 96 h of inoculated at room temperature, 4 l of pure water was added into fermentable tanks of control sample *CRS*. Then, 2 kg (50 %) of cooked rice plused with water were added into fermentable tanks of *RAS* and *RSA/na*. Alpha-amylase was added into fermentable tanks *RSA/na* without activated with a thermal process. For *RSA/a*, 2 kg of cooked rice were first activated with α-amylase at 75 °C for 20 min, then mixed well with solid substrate into 10 l of fermentable tanks. *S. cerevisiae* was added with the same ratio into fermentable tanks of all conditions respectively as showed in Fig. 1. The process of alcoholic fermentation took 16 days in control room temperature 30 °C.

2.6 Determination of moisture content in cooked rice

The moisture content of cooked rice was determined by using oven drying following the standard method of AOAC (2012). Each aluminum plate was pre-dried in an oven (Memmert Beshickung/loading Modell 100-800, Germany) at the 105 °C for 1 h, cooled down in a desiccator for 15 minutes, and then weighted its mass (M_0). An amount of 2 g cooked rice (M_S) was put on an aluminum plate and dried in an oven at 105 °C for 24 h until the constant was obtained. Dried samples were kept in a desiccator for 15 minutes and then the mass was recorded each mass (M_1). Each experiment was conducted in triplicate and the moisture was determined by using the following equation.

$$\% \text{ Moisture} = [M_s - (M_1 - M_0)]/M_1]*100 \qquad (Eq. 1)$$

Where:

M_S = Mass of sample (g)
M_1 = Mass of sample after dried from oven (g)
M_0 = Mass of aluminum plate (g)

2.7 pH and acidity

pH parameter was measured by using a pH meter (OHAUS starter 300 Portable pH meter, USA). A volume of 50 ml of traditional rice wine sample was placed to the falcon and done by using a pH meter as mentioned above.

Total acidity (lactic acid equivalent) was measured by adding 10 ml of rice wine sample to 50 ml of deionized water and titrating with sodium hydroxide (NaOH) at concentration 0.1 N. During titration, the endpoint of acid-base was identified when the solutions turn to pink color and volume of titration NaOH was recorded as ml (AOAC, 1990).

$$X(\text{as acid lactic g/l}) = [(V_s - V_b)*N*F*E]/V \qquad (Eq. 2)$$

Where:
X: g/l of total acid as lactic acid
V_s: Titrated volume of NaOH for sample (ml)
V_b: Titrated volume of NaOH for blank (ml)
N : Normality of NaOH
E : Mass equivalent of lactic acid 90
V: Volume of sample (ml)

2.8 Total reducing sugar

DNS (dinitrosalicylic acid method) was used to determine reducing sugar in rice wine samples (Garriga, Almaraz, & Marchiaro, 2017). The standard curve was prepared by using the standard glucose solution (made from 0.1 g of anhydrous glucose dissolving in distilled water and raised the volume to 100 ml of distilled water). The standard glucose solution was then mixed with distilled water and Dinitro Salicylic Acid (to be a reagent) and the mixture was placed in boiling water for 5 min then cooling down at room temperature. Volume 7 ml of distilled water was added and mixed well to obtain a homogenization solution. These solutions were measured O.D at a wavelength of 540 nm by using a spectrophotometer. The value of O.D was then plotted in the function of glucose concentration. Reducing sugar in the rice wine sample was determined by 0.5 ml with distilled water 0.5 ml and reagent 2 ml. The procedure was the same as the method of standard glucose determination. The concentration of reducing sugar in the sample was determined by comparing it to the standard curve.

Table 2. Standard of total reducing sugar by using glucose

No.	STD glucose ml (g/l)	H₂O (ml)	DNS reagent (ml)	H₂O (ml)	Total (ml)	Wave length (nm)
1	0.2	0.8	2	7	10	540
2	0.4	0.6	2	7	10	540
3	0.6	0.4	2	7	10	540
4	0.8	0.2	2	7	10	540
5	1	0	2	7	10	540

2.9 HPLC analysis

A volume of 5 ml of each sample was carried out and centrifuged for 30 min at the speed of 2000 rpm to obtain a filterable sample. The supernatant was then separated from solid residue and separate by using a filter through a pore size 0.45 µm before direct-injected into HPLC. Parameters including maltose, glucose, lactic acid, acetic acid, and ethanol were auto-detected with the instrument, equipped with column Rezex RHM Monosaccharide OOH-0138-KO (300 x 7.8 mm), refractive index detector RID-20A. A volume of 5 µl was injected and carried with a mobile phase of H_2SO_4 with concentration 0.005 N at flow rate 0.6 ml/min, pressure 0.8 MPa, passed through the column at 60 ℃. Each sample took 25 min for analysis. The concentration of parameters; maltose, glucose, lactic acid, acetic acid, ethanol, and methanol was measured by calculating the peak area based on retention times and the peak was identify by comparing to an external standard. Standards were prepared into five dilutions to obtain the calibration curves. The concentrations of standards such as maltose, glucose, lactic acid, acetic acid, methanol and ethanol were carried out in the range from 0.01 to 2 g/100 ml, 0.035 to 7 g/100 ml, 0.01 to 2 g/100 ml, 0.01 to 2 g/100 ml, 0.75 to 15 % (v/v) and 0.01 to 2 % (v/v), respectively.

Table 3. Parameter and concentration rang of standard

Parameters	Concentrations
Glucose	0.035-7 g/100 ml
Maltose	0.01-2 g/100 ml
Acetic acid	0.01-2 g/100 ml
Lactic acid	0.01-2 g/100 ml
Ethanol	0.75 % (v/v)-15 % (v/v)
Methanol	0.01-2 % (v/v)

2.10 Statistical analysis

Statistical analysis was done by using One-way ANOVA with Fisher's method to determine the mean, standard deviation and significant difference of samples. The statistical analysis of significantly different was set at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Optimization of moisture condition in cooked rice

Koji is also called solid culture produced by inoculation of rice with specific mold to get the right quality of rice liquors. During the koji fermentation, molds have a function to produce α-glucosidase that plays an essential role as starch digestion enzyme to convert starch to sugar which would be used by yeast for convertion to alcohol and carbon dioxide. To get the optimal of moisture content, two ratios of water 1:1.25 (w/v) and 1:1.5 (w/v) (rice:water), were studied.
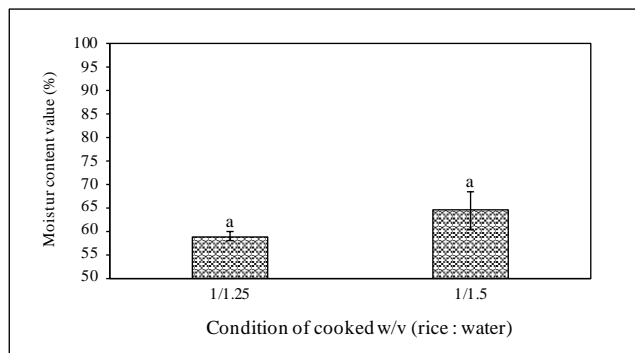

Fig.2. Total moisture content in cooked rice based on different ratios of water 1:1.25 and 1:1.5 w/v (rice:water). The same letter of alphabet means no significant difference between each ratio *(p >0.05).*

According to the result in Fig. 2, the moisture content was showed no significantly difference between the conditions of added water 1250 ml and 1500 ml into 1000 g of rice while the final moisture content of cooked rice at the condition of 1:1.25 (w/v) was 58.82 ± 1.03 % and the condition of 1:1.5 (w/v) was 64.32 ± 4.20 %. The condition of water added with ratio 1:1.5 (w/v) showed the greatest result of 64.32 ± 4.20 % of moisture content that was in the range of the targeted moisture content between 60 % to 65 %, which was the preferable conditions of growing koji according to previous studies (Ferreira, Montijo, & Martin, 2015; Ly et al., 2018). Thus, a water ratio of 1:1.5 (w/v) was chosen to conduct the further experiment.

3.2 Optimization of volume and duration of α-amylase activity

The optimization was divided into two parts between volume and duration of activating α-amylase with 100 g of cooked rice and 100 ml of water ratio 1:1 (w/v). The thermal process at 75 ℃ applied for activating α-amylase activity for all conditions (Whitehurst & Wiley, 2010).
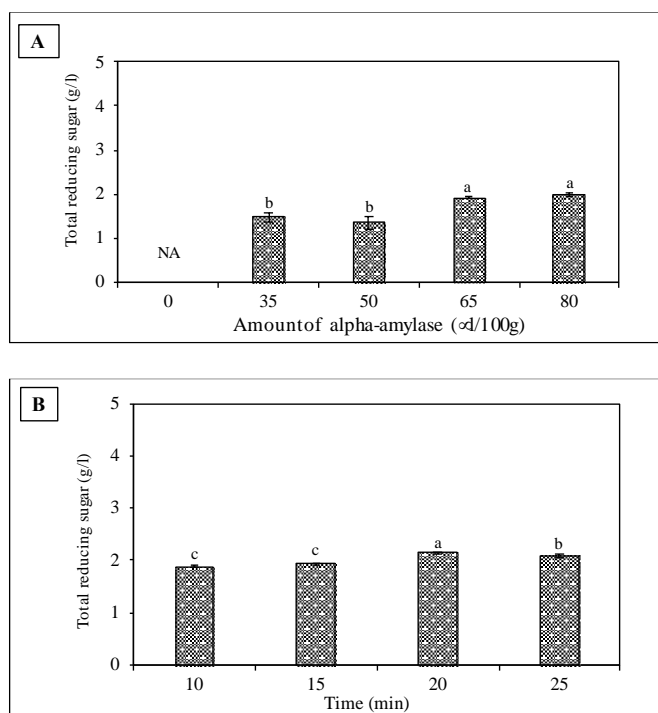
Fig.3. Total reducing sugar of the optimization between amount (A) and duration of α-amylases (B). Mean with different alphabetic letters were significantly different (*p<0.05*), NA "*Not Available*"

The enzyme α-amylase is an endoamylases that hydrolyzes the internal α-1,4-glycosidic linkage in starch. Starch must be processed in a way of gelatinized and liquefied. Gelatinization is a process of the swelling of the starch granules to reduce viscosity and to make starch susceptible to enzyme hydrolysis take place during saccharification, whereas liquefaction is the de-branching process that breakdown the intermolecular bonds of starch in both amylose and amylopectin during the presence of excess water and involve of heat in order to take up more water that known as a process of hydrolysis. Therefore, starch is rapidly broken down to produce glucose, maltose, oligosaccharides, or dextrins from the non-reducing ends of starch (Xiao, Storms, & Tsang, 2006). In Fig. 3(A), the result showed that total reducing sugar was observed between 0 ± 00 to 1.89 ± 0.04 g/l in presence of α-amylase from 0 μl up to 80 μl. The usage of α-amylase of 35 μl and 50 μl showed no significant difference of 1.43 ± 0.09 g/l and 1.32 ± 0.13 g/l, respectively, whereas the volume of 65 μl and 80 μl of α-amylase showed no significant difference and provided the highest amount of total reducing sugar (1.83 ± 0.01 g/l and 1.89 ± 0.04 g/l) by comparison to the two previous volumes. The 65 μl volume of α-amylase has been therefore chosen and applied for the whole experiment in terms of economical reason. The current findings also

showed the impact of starch hydrolysis by introducing α-amylase to improve sugar production.

At the same procedure of α-amylase activation, the preferable durations were 10 min, 15 min, 20 min, and 25 min with the specific volume 50 μl. The result of total reducing sugar was observed between 1.80 ± 0.02 g/l to 2.06 ± 0.01 g/l in Fig. 3(B). The total amount of reducing sugar showed the lowest during the 10 and 15 min exposure with no significant difference (1.80 ± 0.02 g/l and 1.85 ± 0.02 g/l, respectively). Out of the four conditions, the total reducing sugar was the highest at the 20 min incubation. Thus, the condition of the enzymatic activation at 20 min was best in terms of energy savings and time consumption.

3.3 pH activity

pH is an important parameter in the process of each winery or ferment production. In particular, pH in winemaking could have a major impact on the final product quality if the pH levels of wine are not properly balanced, which could affected to the color, taste, aroma, and smell of the end products (Sensorx Inc., 2020). The result of the pH values of all wine conditions presented in Fig. 4. The result of pH values demonstrated a significant difference in all conditions and expressed as the concentration of free hydrogens ions containing in rice wine mush.
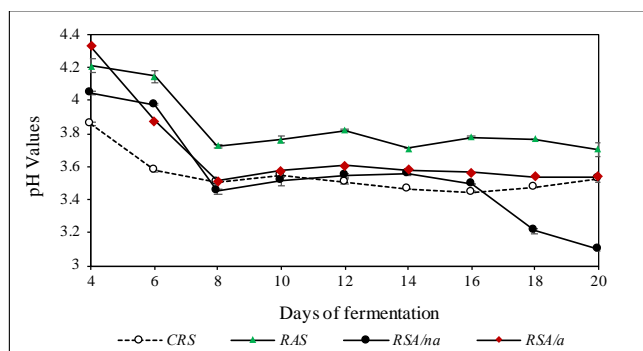
Fig.4. pH values of rice wine during fermentation with *CRS, RAS, RSA/na* and *RSA/a* conditions

The pH values of all conditions showed a decrease with the time during fermentation, respectively, which overall result of pH values was observed between 3.09 ± 0.01 to 4.33 ± 0.04 and the highest pH for *RSA/a* of 4.33 ± 0.04 was observed in Fig. 4 followed by *RAS* and *RSA/na*, whearese the pH-values for *CRS* of 3.85 ± 0.01 was significantly lower. After 6 days, pH-values were decreased remarkably followed by day 8, 10, 12, 14, 16, 18, and 20. At the end of fermentations, the pH-values of all conditions illustrate significantly difference while the highest pH-values were noticed for *RAS* of 3.70 ± 0.01 followed by *RSA/a* 3.54 ± 0.04, *CRS* of 3.53 ± 0.02, and *RSA/na* of 3.09 ± 0.01 was showed significantly lower. Based on the results, the

decreasing of pH values during fermentation was due to the hydrolysis of organic acid and the production of acidity (Chidi, Bauer, & Rossouw, 2018). Besides, the pH-values of rice wine between 4.33 ± 0.04 and 3.85 ± 0.01 are considered as the favorable conditions for *S. cerevisiae* while another study found the pH-values between 4.5 to 6.5 and *S. cerevisiae* has been reported to inhibit the growth at the pH-value exceed 8.0 (Peña al., 2015)

3.3 Influence of acidity during rice wine fermentation

Organic acid and total acidity are one among factors that plays a main role in the winery product that represents the sensory perception and directly influences the overall organoleptic characteristics of wine product (Chidi et al., 2018).
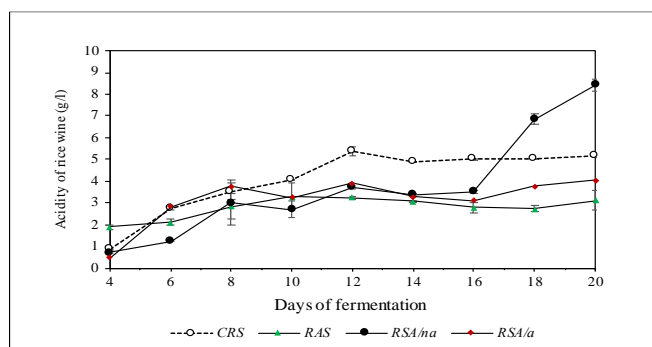
Fig.5. Acidity of rice wine during fermentation with *CRS, RAS, RSA/na* and *RSA/a* conditions

The overall results in Fig. 5 showed an increase of acidity levels by the time of rice wine fermentations from days 4, 6, 10, 12, 14, 16, 18, and 20. The lowest total acidity was notice for RSA/a of 0.27 ± 0.00 g/l, followed by RSA/na of 0.70 ± 0.04 g/l, CRS of 0.90 ± 00 g/l, and RAS of 1.17 ± 0.03 g/l. On day 20th, the total acidity was significantly increased over time. The highest total acidity was observed for RSA/na of 8.42 ± 0.45 g/l, which showed a highly significant difference compared to CRS of 5.18 ± 0.06 g/l, RSA/a of 4.05 ± 0.26 g/l, and RAS of 3.11 ± 0.06 g/l. According to the results, RAS of 3.11 ± 0.06 g/l showed the greatest acidity, which agreed with the study of Boulton et al. (1999) that found that an average concentration acidity in rice wine was between 1.00 to 3.00 g/l. According to the study of Tay & Yang (2002), they reported that less than 50 % of acid produced during solid-state fermentation mostly was lactic acid. However, during alcoholic fermentation, there are several important organic acids produce by yeast hydrolysis that was associated with other bacteria such as succinic, pyruvic, and acetic acid (Bely et al., 2003). Another research conducted by Nunes et al. (2017) indicated that high productivity of acidity during alcoholic fermentation caused by glucose that has been broken down

and oxidized to pyruvate by yeast activity, then lactate was produced from pyruvate which caused lactic acid increase in high concentration (Chay et al., 2011).

3.4 Total reducing sugar

Reducing sugar analysis plays an important role in wine processing to know about the quantitative of fermentable sugar remaining in the wine to determine if the fermentation is complete (Zoecklein et al., 1990). By using the DNS method (dinitro salicylic acid), the overall results in Fig. 6 of total reducing sugar content were between 3.31 ± 0.09 g/l to 29.50 ± 0.03 g/l.
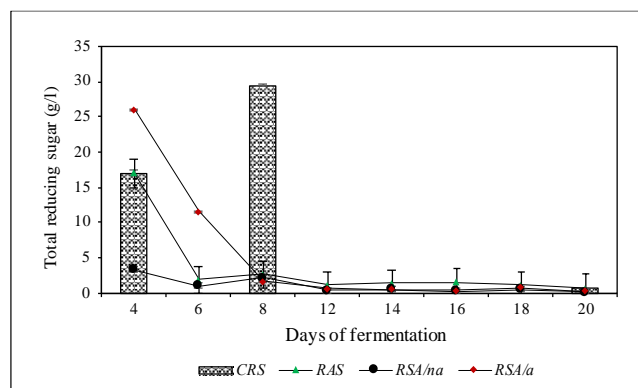
Fig.6. Total reducing sugar during rice wine fermentation with *CRS, RAS, RSA/na* and *RSA/a* conditions

At the started day, the highest production of total reducing sugar for *RSA/a* of 26.09 ± 0.33 g/l was observed in Fig. 6. Based on the statistical analysis, this result was noticed the highest among tested conditions followed by *CRS* of 17.06 ± 0.71 g/l, *RAS* of 16.97 ± 0.19 g/l, and *RSA/na* of 3.31 ± 0.085 g/l was significantly lower in Fig. 6. Whereas the conditions of *CRS* and *RAS* showed no significant difference while *RAS* was involved with the combination of non activated α-amylase with koji during solid-state fermentation. After day 6th, the result of total reducing sugar decreased remarkably except for *CRS* which noticed a significant increased of 29.50 ± 0.03 g/l. On day 8th, the overall results decreased respectively by the days until the end of fermentation. This result indicated that the fermentation completed due to the activity of yeast consumed substrate and produce ethanol with other by-products while reducing sugar remained in less amount at the end of fermentation. Meanwhile, *RAS* remained constant the high amount of reducing sugar of 0.73 ± 0.01 g/l, followed by *CRS* of 0.62 ± 0.11 g/l, *RSA* of 0.24 ± 0.00 g/l, and *RSA/na* of 0.17 ± 0.00 g/l. This result indicated that the use of activated enzyme with an optimal temperature 75 ºC and the mixed koji during solid-state fermentation had enhanced the enzyme digestion to hydrolyzed from koji that

leads to increased fermentable sugar and breaks down insoluble starch into maltose, dextrin, and smaller oligosaccharide (Sharma & Satyanarayana, 2013). These results agreed with the study of Suryawanshi et al. (2018) that found a higher amount of total reducing sugar was due to the pre-treatment process before alcoholic fermentation. The level of total reducing sugar that remained in the final day of fermentation was considered as in rank acceptable while compared to the study of Chinese rice wine which found the end levels of total residue reducing sugar level between 1.24 g/100 ml to 5.45 g/100 ml (Liu et al., 2014).

3.5.1 Glucose and maltose production

Rice wine fermentations were divided into four conditions as mentioned by the involvement of incubation of *R. oryzae* in koji and α-amylase.
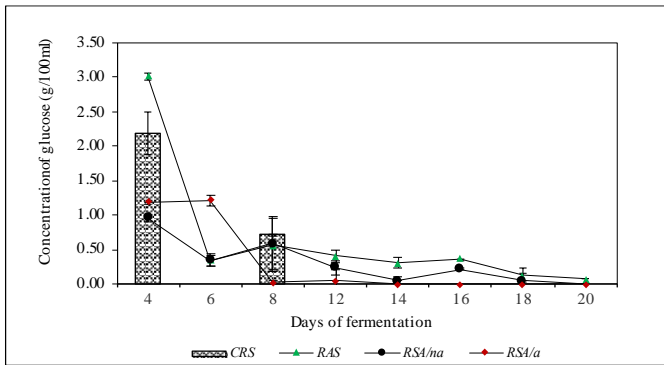

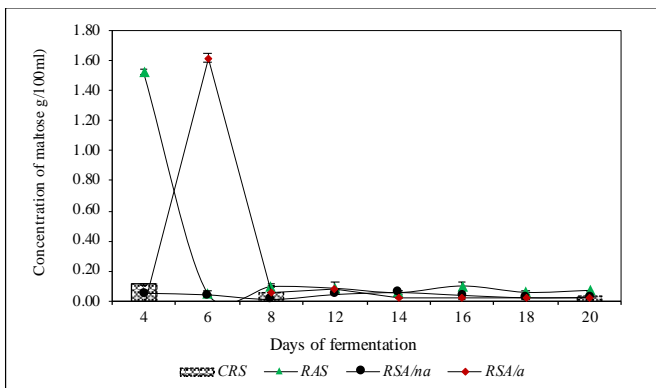Fig.7. Production of glucose during rice wine fermentation

Fig.8. Production of maltose during rice wine fermentation

Fig. 7 and Fig. 8 demonstrated the kinetic changing of glucose and maltose during the fermentation process for all conditions from days 4, 6, 8, 10, 12, 14, 16, 18, and 20. Based on the results, the highest production of glucose for *RAS* of 2.98 ± 0.04 g/100 ml was observed in Fig.7, and the highest production of maltose for *CRS* of 1.51 ± 0.02 g/100 ml was observed in Fig. 8, whereas the highest production of

glucose in Fig. 7 using α-amylase during alcoholic fermentation for *RSA/na* of 0.95 ± 0.06 g/100 ml and *RSA/a* of 1.186 ± 0.01 g/100 ml was significantly lower. After day 6th, the results of glucose found to decrease remarkability until the end of fermentation. Meanwhile in Fig. 8, the results of maltose production on day 6th found highly increased *RSA/a* 1.62 ± 0.03 g/100 ml then decreased by the time until the end of fermentations. Aidoo, Nout, & Sarkar (2006) reported that an increase of maltose and glucose concentration during the initial stage was probably caused by the activities of *R. oryzae* that has been hydrolyzed rice starch remained during fermentation into fermentable sugar for yeast consumption.

3.5.2 Production of lactic acid and acetic acid

Lactic acid is one of the main important factors that have a potential influence on wine quality which produced during fermentation and contribute to the total volatile acid with a higher amount about 90 % of total volatile acid found in Chinese rice wine by Liu et al. (2014).
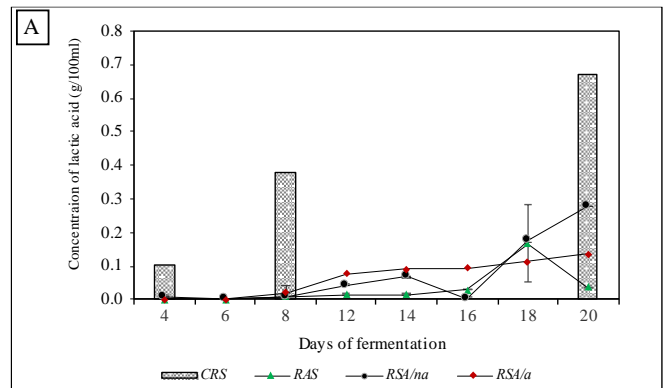

Fig.9. Production of lactic and acetic acid during rice wine fermentation, (A) showing about result of lactic acid and (B) showing about of acetic acid

Fig. 9(A) and Fig. 9(B) demonstrated the activities of lactic and acetic acid production during rice wine fermentations for all conditions from days 4, 6, 8, 12, 14, 16, 18, and 20. Based on the results above, the highest production of lactic acid for CRS 0.10 ± 0.00 g/100 ml was observed at started days in Fig. 9(A), while the other conditions were not showed the result of lactic acid production. The slight increase of lactic acid at the early stage is caused by the activity of *R. oryzae* produced during solid-state fermentation. However on day 8th, the production of lactic acid showed slowly increased until the end of fermentation, and *CRS* increased with an interesting yield of producing lactic acid more than the other conditions. This indicated the production of lactic acid was increased by yeast activity that consumed sugar and produced by-products

viz ethanol and other substance of organic acid. The statistical analysis demonstrated that all of the results showed a significant difference followed by *CRS* of 0.66 ± 0.02 g/100 ml, *RSA/na* of 0.28 ± 0.00 g/100 ml, *RSA/a* of 0.14 ± 0.00 g/100 ml, and the *RAS* of 0.04 ± 0.00 g/100 ml was significantly lower in Fig. 9(A). The result of lactic acid of all conditions was noticed highest results when compared to the study of Japanese sake rice wine which found between 0.01 g/100 ml to 0.05 g/100 ml (Kodama et al., 2002). However, acetic acid was observed absences for all conditions that involved α-amylase, while the condition of used the incubation of koji *CRS* showed the presence of acetic acid with a small amount from day 10th and sharply increased until day 20th.

3.5.3. Ethanol and methanol production

The ethanol production was increased remarkably by the time of fermentation for all conditions as shown in Fig. 10.
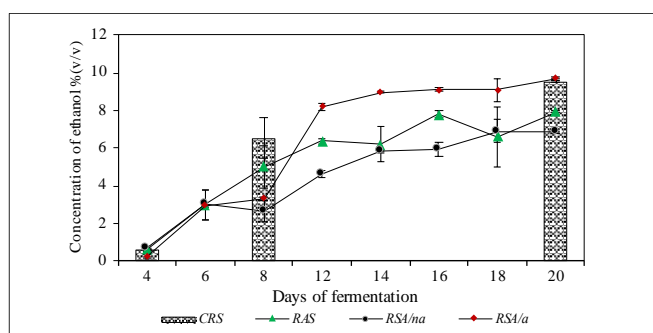

Fig.10. Production of ethanol during rice wine fermentation

On day 14, the results of all conditions showed a gradual increase and remained stable until day 20th. The highest production of ethanol for *RSA/a* of 9.66 ± 0.08 % (v/v) was observed in Fig.10, followed by *CRS* of 9.52 ± 0.02 % (v/v), *RAS* of 7.92 ± 0.03 % (v/v), and *RSA/na* of 6.91 ± 0.00 % (v/v) was significantly lower. According to the statistical analysis, the results of all conditions showed a significant difference, while the total reducing sugar was observed with the lower between 0.24 ± 0.00 g/100 ml to 0.73 ± 0.00 g/100 ml. Based on the result, the final residue of total reducing sugar showed a slight difference for (*CRS*, *RAS*) and (*RSA/na*, *RSA/a*). Regarding to this result, the metabolism of dried commercial yeast *S. cerevisiae* has a high potential in produced ethanol production. *RSA/a* provided high yields of ethanol production than the other conditions. This due to the use of koji with activated α-amylase that was an endo-action that can catalyze the hydrolysis of α-1, 4-glycosidic linkages and some branched α-1, 6-glycosidic linkages from the inner chains of starch and leads to the released of maltose, smaller

oligosaccharides, and dextrin as the main products of fermentable sugar for saccharify process (Sharma & Satyanarayana, 2013).
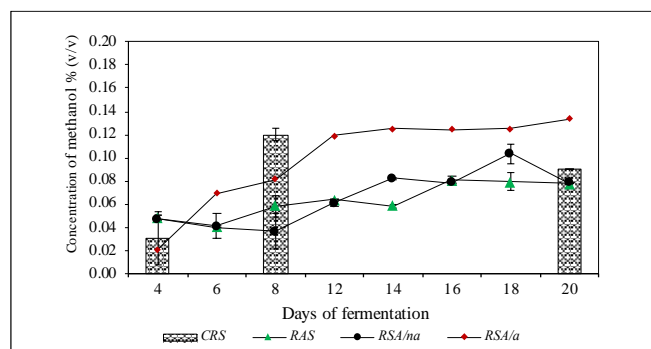

Fig.11. Production of methanol during rice wine during fermentation

According to Fig. 11, methanol production showed varying results for all conditions. On the started day of fermentation, the highest methanol production for *CRS* was observed, whereases the lowest found in the condition which involved with the enzyme. After, the yield of methanol increased until the end of fermentation, respectively. In contrast, for *CRS* conditions the result was decreased remarkably. At the end of fermentation, the highest yield of methanol was noticed with the highest result for *RSA/a* of 0.13 ± 0.00 % (v/v), followed by *CRS* of 0.1 ± 0.00 % (v/v), *RSA/na* of 0.08 ± 0.00 % (v/v), and *RAS* of 0.08 ± 0.00 % (v/v) was significantly lower. The contamination of methanol in rice wine is mostly caused by the hydrolysis of *S. cerevisiae* that led to the result of methanol produced during fermentation (Ohimain, 2016). However, the concentration of methanol during rice wine production at all tested conditions did not exceed 0.2 % (v/v), which is the standard limit in wine allowed by the European Commission (European Parliament and Council, 2008).

4. CONCLUSION

In conclusion, using α-amylase in the rice wine production process provided notable results in comparison to the control without using an enzyme. Obviously, this enzyme had enhanced starch hydrolysis into fermentable sugar for yeast consumption. Heating the mixture of rice with an optimal temperature of the enzyme provided the highest yield of ethanol with lower acidity. Thus, the mixture of *Rhizopus oryzae*, *Saccharomyces cerevisiae* with the addition of enzyme is a good process design for further study as well as industrial scale.

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