อิทธิพลของการลดขนาดเมล็ดข้าวเหนียวต่อการผลิตและสารให้กลิ่นของสาโท
Influence of size reduction of waxy rice kernel on Sato production and its volatile compounds

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บทคัดย่อ

เพื่อเป็นการลดค่าใช้จ่ายในการผลิตสาโท งานวิจัยนี้ศึกษาอิทธิพลของการลดขนาดเมล็ดข้าวเหนียวต่อการผลิตและคุณภาพของสาโท เมล็ดข้าวเหนียวถูกลดขนาดได้เป็น 4 ขนาดคือ 1.4-1.7 mm, 1.7-2.0 mm, 2.0-2.3 mm และ ขนาดเมล็ดเต็ม (ตัวอย่างควบคุม) *Aspergillus niger* TISTR 3257 ถูกใช้ในการผลิตโคจิที่อุณหภูมิ 30°C เป็นเวลา 7 วัน *Saccharomyces cerevisiae* SC90 ถูกใช้ในระหว่างกระบวนการหมักแอลกอฮอล์ที่อุณหภูมิ 30°C เป็นเวลา 8 วัน Crude amylase activity ที่สูงที่สุด (0.341 unit) พบในตัวอย่างโคจิที่ผลิตจากเมล็ดข้าวขนาด 2.0-2.3 mm ไม่พบความแตกต่างในรูปแบบการเจริญเติบโตของเชื้อยีสต์และปริมาณแอลกอฮอล์ (~10% v/v) ($p>0.05$) สาโทที่ผลิตจากเมล็ดข้าวเหนียวขนาด 2.0-2.3 mm มีความเข้มข้นของ ethyl butyrate, ethyl decanoate, diethyl succinate, phenyl acetate and 2-methyl-1-propanol, 3-methyl-1-butanol, phenethyl alcohol สูงกว่าในตัวอย่างควบคุมอย่างมีนัยสำคัญ ($p<0.05$) ผลการทดลองนี้แสดงให้เห็นว่าการลดขนาดของเมล็ดข้าวเหนียวลงไปที่ 2.0-2.3 mm ส่งผลกระทบต่อการผลิตสารให้กลิ่นของสาโท

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Abstract

To reduce the production cost of Sato, this study investigated the influence of using reduced size-waxy rice kernels on Sato production and quality. Reduced sized waxy rice was made to 1.4-1.7 mm, 1.7-2.0 mm, 2.0-2.3 mm, and full kernels (control). *Aspergillus niger* TISTR 3257 was used to make koji at 30 °C for 7 days. *Saccharomeces cerevisiae* SC90 was used during alcoholic fermentation at 30 °C for 8 days. The highest crude amylase activity (0.341unit) was observed in the koji made from 2.0-2.3 mm waxy rice. The growth pattern of yeast and alcoholic content (~10% v/v) did not show any difference among samples ($p>0.05$). The Sato made from waxy rice size 2.0-2.3 mm contained significantly higher concentration of ethyl butyrate, ethyl decanoate, diethyl succinate, phenyl acetate and 2-methyl-1-propanol, 3-methyl-1-butanol, phenethyl alcohol compared with those of the control sample ($p < 0.05$). This clearly shows that the reduction in size of waxy rice kernel to 2.0-2.3 mm influences the production of volatile compounds.

คำสำคัญ: สาโท, ข้าวเหนียว, สารให้กลิ่น

Keywords: Sato, waxy rice, volatile compounds

Introduction

Sato is a traditional Thai rice wine. It has a sweet taste with 10 to 15% (v/v) alcohol content. The production processes are similar to that of sake manufacture, although aging is not a common practice. Traditionally, Loog-pang or Chinese yeast cake (containing the mixtures of moulds, yeasts and herbs) used as a dry starter is mixed with steamed waxy rice (Sirisantimethakorm et al., 2008). Two processes including saccharification by moulds and alcoholic fermentation by yeasts occur simultaneously during solid state fermentation. Concurrently, moulds such as *Aspergillus oryzae, A. sojae* and *Rhizopus* spp. grow and produce both starch-digesting and protein-digesting enzymes (Aidoo et al., 2006; Chuenchomrat et al., 2008). After 5-7 days of the fermentation, sugary liquid is produced with small amount of alcohol. Therefore, water and sugar are added to adjust the total soluble solid (TSS) of the liquid to approximately 20-22 °Brix. During alcoholic fermentation, sugars and amino acids are fermented to ethanol, CO_2, organic acids and volatile compounds (Ribereau-Gayon et al., 2000). In addition, volatile compounds can be produced during solid state fermentation (Ito et al., 1990).

Due to the food crisis during 2007-2008, the cost of food and agricultural produces has increased significantly (von Braun, 2008; Singh, 2009). This included waxy rice, the raw material used in Sato production. An increase in the cost of waxy rice decreases profit margin of Sato producers. Therefore, it is very important to reduce production cost by using cheaper raw materials. Broken rice is a by-product from rice processing. It has been used as an adjunct for beer production. Suresh et al. (1999) reported that damaged rice (broken, attacked by fungi, or dirty rice) was used successfully to produce ethanol by simultaneous saccharification and fermentation using *Aspergillus niger* (NCIM 1248) and *Saccharomyces cerevisiae* VSJl. This type of fermentation is similar to processes occurring in Sato production. The price of broken waxy rice (1,150 baht/ 100 kg) is cheaper than that of full kernel (1,520 baht/ 100 kg) (Department of Internal

Trade, 2010). Potentially, it may be used as an alternative raw material for Sato production. However, there has no report about the use of broken waxy rice on Sato production. Therefore, this study investigates the influence of using reduced size waxy rice as raw material on Sato production and its quality.

Materials and Methods

Experimental plan

Sato was made from waxy rice that its size was reduced and classified to 1.4-1.7 mm., 1.7-2.0 mm., 2.0-2.3 mm., and full kernels (control) corresponding to sieve No. 12, 10, 8, 7, respectively. Pure culture of *A. niger* TISTR 3257 and *S. cerevisie* SC90 were used. Crude amylase activity and TSS in koji were measured. Alcohol content, eight volatile compounds and sensory evaluation in Sato were determined. In addition, the percentage of starch digestion at the end of both koji and alcoholic fermentation was quantified. All experiments were carried out in triplicates.

Size reduction of waxy rice

Waxy rice (purchased from a local market in Kancahnaburi, Thailand) was crushed by using a food processor (Panasonic, model MK-5086M). It was classified into 1.4-1.7 mm., 1.7-2.0 mm., 2.0-2.3 mm., and full kernels (control) using a series of sieves consisting of sieve No. 12, 10, 8, 7 (Retsch, Germany).

Sato production

Each size of waxy rice (100 g) was soaked in distilled water for 1 hour and then sterilized at 121 ^{o}C for 15 min in 500 ml Erlenmeyer flask. The sterilized rice was washed with sterile distilled water until the washed water was clear. Ten milliliter of spore suspension (10^{7} spores/ml) of A. *niger* TISTR 3257 (Microbiological Resource Centre, Thailand Institute of Scientific and Technological Research, Thailand) was inoculated into the washed rice. The incubation was carried out at 30 ^{o}C for 7 days. The resulted koji contained sugary liquid high in TSS. The sterile ammonium sulphate solution (1 g/l) was used to adjust TSS in each koji to 22 ^{o}Brix before *S. cerevisiae* SC90 was inoculated at 4% v/v (10^{7} CFU/ml). *S. cerevisiae* SC90 was previously reactivated for 24 hour in Yeast Malt Broth (YMB, Biomark, India) for 3 times. The alcoholic fermentation was carried out at 30 ^{o}C for 8 days.

Analytical methods

Determination of crude amylase activity

Crude amylase activity in koji was measured using spectrophotometric method (DNS method). Ten gram of Koji was mixed with 10 mL phosphate buffer pH 6.8. Supernatant (centrifuge at 6000 rpm for 20 min) was separated from the mixture. The supernatant (5 ml) was mixed with 1% (w/v) starch solution (5 ml) and incubated at 37 ^{o}C for 30 min. The reaction was stopped by adding 10 ml of 1 N acetic acid. The mixture was centrifuged (6000 rpm for 20 min). Reducing sugar produced was quantified using DNS method. One unit of crude amylase activity was defined as the amount of reducing sugar produced per reaction time (Djekrif-Dakhmouche et al., 2006).

Monitoring yeast growth

During alcoholic fermentation, the viability of yeast was monitored using yeast malt agar (YMA, Biomark, India) at day 0, 2, 4, 6, 8 according to Atlas (2004) Samples were ten-fold serially diluted and spreaded on YMA plates. Plates were incubated at 30^{o}C for 2 days.

Determination of alcohol content

Alcohol content in Sato was measured by an alcoholometer. One hundred milliliter of Sato at the end of fermentation was distilled. The volume of distillate was adjusted back to its initial volume using distilled water prior to measurement. The measurement was carried out according to AOAC (2000) method number 957.03.

Determination of the percentage of starch digestion

The percentage of starch digestion after koji and alcoholic fermentation was monitored. Digested rice (both from koji and alcoholic fermentation) was washed and filtered through a Whatman filter paper No 1 (Kent, UK). The filtered residue was dried at 100 °C for 24 hours. Thereafter, the percentage of starch digestion was calculated from the initial rice weight.

Quantification of volatile compounds

Eight volatile compounds in Sato were quantified by a gas chromatography (PR21000, Perichrom) equipped with a 007-CW capillary column (30 m x 0.25 mm diameter, 0.25 mm film thickness (Quadrex, USA). These volatile compounds included 4 higher alcohols (3-methyl-1-butanol, 2-methyl-1-propanol, pentanol, and phenyl ethanol) and 4 esters (ethyl butyrate, ethyl decanoate, diethyl succinate, and phenyl acetate). These volatile compounds were extracted (liquid-liquid extraction) and quantified according to Ortega et al. (2001). Three microliter of the extracted sample were injected using 1:5 split ratio. The temperature of injector and detector was maintained at 200 and 250 °C, respectively. The oven temperature was programmed at 55 °C for 4 min, from 55 to 120 °C at 8 °C/min, held 10 min at 120 °C, from 120 to 200 °C at 10 °C/min, then held at 200 °C for 15 min.

Statistical analysis

Analysis of variance (ANOVA) and Tukey-HSD were used to analyze data (three replicates) using R statistical software (R Development Core Team, 2006).

Results and Discussion

Koji fermentation

Crude amylase activity and Total soluble solids

Crude amylase activity and total soluble solids (TSS) of koji made from steamed waxy rice reduced to size 1.4-1.7, 1.7-2.0, 2.0-2.3 mm and full kernel (control) using *A. niger* TISTR 3257 as a starter culture at 30°C were presented in Figure 1. Decreasing the size of rice to 2.0-2.3 mm significantly increased crude amylase activity ($p < 0.05$) to 0.341 unit. Koji made from the full kernel and rice reduced to 1.7-2.0 mm showed only 0.235 and 0.213 unit, respectively. Further decrease the size of rice to 1.4-1.7 mm decreased crude amylase activity drastically to 0.115 unit. This may suggest that the size reduction of waxy rice affects crude amylase production as well as its activity. In general, the production of amylase and other enzymes (celluloses, proteases, pectinases, etc) in solid state fermentation (SSF) system are related to growth of *Aspergillus* spp. Chutmanop et al. (2008) observed positive correlation between protease production and the growth of *A. oryzae*. Chou and Rwan (1995) also reported that the production of α-amylase, acid protease and neutral protease were associated with mycelial propagation of *A. oryzae* in extruded rice. Several factors have been reported to affect amylase production. Those factors include pH, nutrients, temperature, and aeration (Rahardjo et al., 2005a, 2005b; Kekos et al., 1987). In this research, the size reduction of rice should increase surface area providing easy accessibility to nutrients for *A. niger* TISTR 3257 to grow. This appears to be the case when the size of rice is reduced to 2.0-2.3 mm. However, decreasing rice size to 1.4-1.7 mm reduce the porosity of rice koji. This would decrease available oxygen which is necessary for the growth of *A. niger* TISTR 3257 and enzyme production. Similar relationship between particle size of substrate and enzyme production was reported by Pandey et al. (1995) and Krishna and Chandrasekaran (1996).

Interestingly, TSS of koji, clear sugary liquid, did not show the same trend as the results of crude amylase activity. There was no significant difference in TSS among koji made from rice reduced to size 1.7-2.0, 2.0-2.3 mm and the control sample. The TSS was in the range from 30-33^{o}Brix. It consists mostly of sugar. High concentration of sugars has been shown to inhibit amylase activity (Gangadharan et al., 2009). High TSS in koji made from rice size 1.7-2.0, 2.0-2.3 mm and full kernel should exert the same inhibition effect on crude amylase activity. When the size of rice was further reduced to 1.4-1.7 mm, the koji contained only 20.2 ^{o}Brix. Low crude amylase production as a result of unfavorable growth condition (limited aeration) for *A. niger* TISTR 3257 may be an explanation. Another possibility might be an increase in water adsorption during rice washing. This could possibly dilute TSS of the koji sample made from rice size 1.4-1.7 mm.

Alcoholic fermentation

Yeast growth

After finishing koji fermentation, the alcoholic fermentation was carried out subsequently. The growth of yeast was monitored during 8 days of alcoholic fermentation at 30^{o}C (Figure 2). The growth pattern of yeast in each sample was similar. This might be because it grew in similar fermenting medium (the initial total soluble solids was adjusted to 20 ^{o}Brix prior commencing alcoholic fermentation) and fermenting condition (30 ^{o}C). The yeast grew exponentially from day 0 (at ~5.2 log CFU/ml) to day 4 (to ~7.2 log CFU/ml). The cell number dropped slightly on day 6 (to ~7.0 log CFU/ml) and, then it decreased steeply from day 6 to day 8 (to ~6.0 CFU/ml). The results obtained indicated that decreasing in rice size did not affect growth pattern of yeast during Sato production.

Alcohol content

Table 1 shows alcohol content of Sato made from the four size of waxy rice. The alcohol content was in the range of 9.6 to 10.3 % (v/v). There was no difference in alcohol content among samples, although the values of crude amylase activity and TSS were different (Figure 1). However, the initial TSS in each sample must be adjusted to 20 ^{o}Brix prior alcoholic fermentation. This may explain why there was no difference in alcohol content among samples. In Sato production, the fermenting medium is high in sugars initially. Moreover, there is plenty of air on the surface of fermenting medium. These two conditions promotes Crabtree's effect, where yeast metabolizes sugars by the fermentative pathway even in the presence of air (Ribereau-Gayon et al., 2000). Theoretically, alcoholic fermentation of 20 ^{o}Brix of pure glucose would yield almost 10 % (v/v) ethanol. However, the sugary solution resulted from amylase activity during rice koji production does not contain only glucose. It is a mixture of mono-, di- and polysaccharides. Therefore, to produce 10% (v/v) of alcohol, starch must be further digested during alcoholic fermentation.

Table 1. Alcohol content of Sato made from waxy rice at different sizes

Size of waxy rice	% Alcohol (v/v)
Full kernel	9.8 ± 0.8^a
2.0-2.3 mm	10.3 ± 0.7^a
1.7-2.0 mm	9.6 ± 1.2^a
1.4-1.7 mm	10.1 ± 0.5^a

Mean ± standard deviation with the same superscript letter mean no significant difference ($p < 0.05$)

The percentage of starch digestion

The percentage of starch digestion during Sato production (koji and alcoholic fermentation) using the four sizes of waxy rice is shown in Table 2. The result showed that, during koji production, there was no difference in starch digestion among samples. The digestion was in the range of 71 to 79% (w/w, dry basis). Regarding the difference in crude amylase activity shown in Figure 1 and the percentage of starch digestion during koji fermentation (Table 2), this suggests that the low TSS in koji made from rice size 1.4-1.7 mm is likely to be due to the effect of dilution by the water adsorbed during rice washing. During alcoholic fermentation, rice starch was further digested. All three sizes of rice including 2.0-2.3, 1.7-2.0 and 1.4-1.7 mm were digested better than the control sample. However, there was no difference among reduced size samples, koji made from rice reduced to size 2.0-2.3, 1.7-2.0 and 1.4-1.7 mm. Teik et al., (2007) reported that increasing contact surface area between starch granules and yeast that was genetically modified to produce amylase increased starch hydrolysis. The reduction in rice size increases surface area for amylase to further digest during alcoholic fermentation. This may explain an increase in starch digestion in this research when rice size is reduced during alcoholic fermentation.

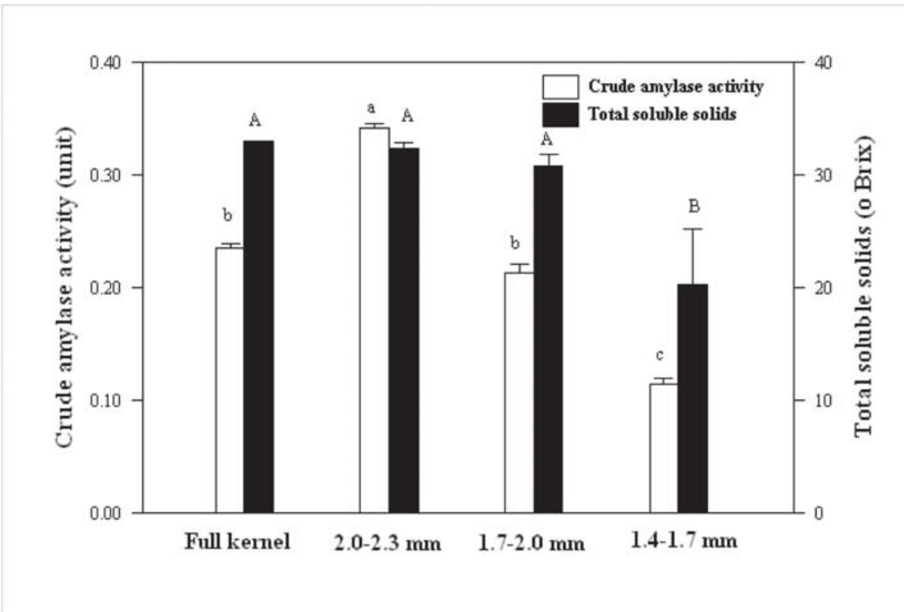

Figure 1. Crude amylase activity and total soluble solids of koji made from waxy rice at different sizes. Bars with the same letter (a, b, c or A, B) mean no significant difference ($p < 0.05$)

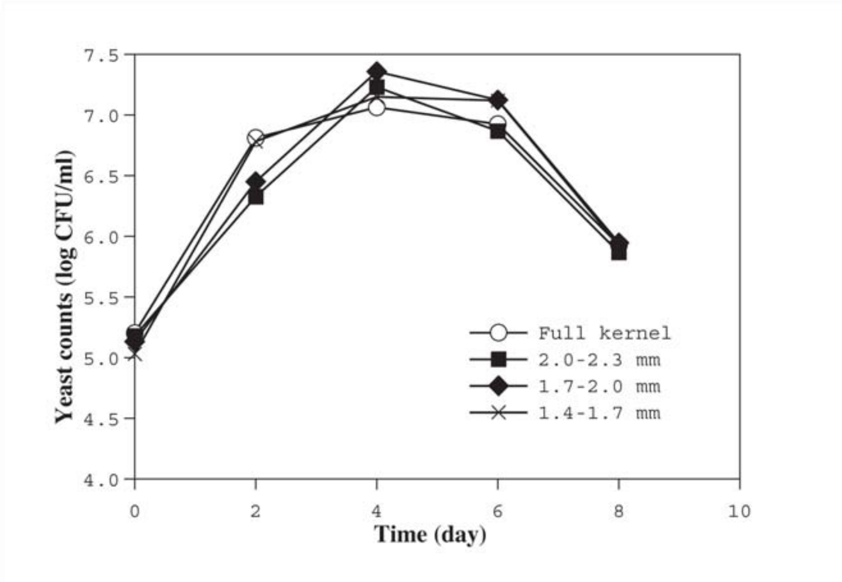

Figure 2. Yeast counts (log CFU/ml) during fermentation of Sato made from waxy rice at different sizes

Table 2. Starch digestion during Sato production using waxy rice at different sizes % Starch digestion means the percentage of starch digested comparing with the initial starch content expressed as dry basis

Size of waxy rice	% Starch digestion in koji	% Starch digestion in Sato
Full kernel	75.26±1.13[a]	78.13±1.12[b]
2.0-2.3 mm	71.16±1.49[a]	82.48±1.70[a]
1.7-2.0 mm	73.38±2.90[a]	82.85±1.44[a]
1.4-1.7 mm	79.55±2.37[a]	86.08±1.03[a]

Mean ± standard deviation in the same column with the same superscript letter mean no significant difference ($p < 0.05$)

Volatile compounds

Table 3 presented eight volatile compounds in Sato made from the four size of rice. These volatile compounds consisted of 4 esters (ethyl butyrate, ethyl decanoate, diethyl succinate, and phenethyl acetate) and 4 higher alcohols (3-methyl-1-butanol, 2-methyl-1-propanol, pentanol, phenyl ethanol). These compounds have been reported to have an odor impact on Sato (Sirisanthimethakom et al., 2008; Chuenchomrat et al., 2008). Generally, esters give fruity or floral aroma descriptor to alcoholic beverages (Ribereau-Gayon et al., 2000: Vanstrepen et al., 2003). Higher alcohols give unpleasant aroma-solvent-like odor descriptor. Reducing the size of waxy rice to 2.0-2.3 mm significantly increased concentration of ethyl butyrate, ethyl decanoate, diethyl succinate, phenethyl acetate, 3-methyl-1-butanol, 2-methyl-1-propanol and phenethyl alcohol ($p< 0.05$). However, further decrease rice size to 1.7-2.0 and 1.4-1.7 mm significantly increased concentration of phenethyl acetate, 3-methyl-1-butanol and phenethyl alcohol compared with the control sample ($p< 0.05$). On the contrary, the concentration of pentanol decreased with decreasing rice size. Nevertheless, the concentration of all volatile compounds determined was still higher than odor threshold. This suggests that all these compounds affect odor of Sato. In general, esters give floral and fruity aroma to alcoholic beverages such as wine, beer and sake (Vanstrepen et al., 2003). Higher alcohols at a suitable concentration (less than 300 mg/L) has been reported to give complexity to wine (Rapp and Mandery, 1986; Hazelwood et al., 2008). When the concentration of them are higher than 400mg/L, they give an unpleasant aroma (Ribereau-Gayon et al., 2000). These eight volatile compounds are produced during alcoholic fermentation by yeast (Garde-Cerdán and Ancin-Azpilicueta, 2008; Sirisanthimethakom et al., 2008). Catabolism of amino acids as well as sugars has been reported to be the route for higher alcohols production (Hazelwood et al., 2008; Garde-Cerdán and Ancin-Azpilicueta, 2008). Fatty acid ethyl esters (ethyl butyrate, ethyl decanoate, diethyl succinate) are formed by enzyme-catalyzed condensation of acyl-CoA and higher alcohols (Garde-Cerdán and Ancin-Azpilicueta, 2008). Acetate esters (phenethyl acetate, isoamyl acetate) are formed from the reaction of acetyl- CoA and higher alcohols. Therefore, the formation of esters during alcoholic fermentation depends on initial concentration of the two substrates (Vanstrepen et al., 2003). Higher alcohols, one of the substrate involved in esters formation, are formed from amino acids available in fermenting medium by Ehrlich pathway (Hazelwood et al., 2008). Amino acids are converted to α-keto acid followed by conversion to higher alcohols. Leucine, valine and phenylalanine are precursors of 3-methyl-1-butanol, 2-methyl-1-propanol and phenyl ethanol, respectively (Hazelwood et al., 2008). These amino acids are likely to be produced as a result of rice protein digestion by proteases produced during koji production. Negi and Banerjee (2006;2009) reported that protease was produced concomitantly with amylase by using *A. awamori* in a single bioreactor. In addition, they optimized both protease and amylase production by using evolutionary operation factorial design technique. Although, this study measured only crude amylase activity, the same condition that enhances crude amylases production (koji made from rice size 2.0-2.3 mm) should have the same effect on crude protease. This would result in an increase in amino acids concentration. Therefore, an increase in concentration of four esters and three higher alcohols in Sato made from waxy rice size 2.0-2.3 mm might be due to an increase in amino acids as a result of increasing crude proteases activity.

Table 3. Volatile compounds in Sato made using waxy rice at different sizes

Volatile compounds	Concentration (mg/l)				Odor threshold (mg/L)	Odor description
	Full kernel	2.0-2.3 mm	1.7-2.0 mm	1.4-1.7 mm		
Ethyl butyrate	18.00^{b}	54.75^{a}	12.62^{b}	14.54^{b}	0.4	Strawbery, apple, banana
Ethyl decanoate	7.10^{b}	60.19^{a}	9.41^{b}	6.83^{b}	0.2	Fruity, wine
Diethyl succinate	1.25^{b}	6.54^{a}	1.56^{b}	1.62^{b}	1.8	Fruity
Phenethyl acetate	1.05^{b}	2.98^{a}	2.78^{a}	1.82^{a,b}	0.25	Fruity, green apple, banana
3-methyl-1-butanol	146.25^{b}	235.01^{a}	180.95^{b}	156.20^{b}	60	Solvent
Pentanol	5.30^{a}	4.04^{b}	0.70^{c}	0.95^{c}	0.1	Phenolic, medicinal
2-methyl-1-propanol	49.35^{b}	86.90^{a}	55.03^{b}	61.05^{b}	75	Alcohol, nail polish
Phenethyl alcohol	39.05^{c}	61.46^{a}	67.09^{a}	53.40^{b}	14	Rose, honey

Mean ± standard deviation in the same row with the same superscript letter mean no significant difference ($p < 0.05$)

Conclusion

Reducing size of waxy rice to 2.0-2.3 mm was found to influence crude amylase activity and TSS of koji, starch digestion during alcoholic fermentation and volatile compounds. These effects are due to activities of both *A. niger* TISTR 3257 and *S. cerevisiae* SC90. The reduction of rice size to 2.0-2.3 mm promotes crude amylase activity. This treatment should also promote crude protease production resulting in increasing amino acids which are further used as precursors for higher alcohols as well as fatty acid ethyl ester production by yeast. An increase in eight volatile compounds in Sato made from rice reduced to size 2.0-2.3 mm might improve its sensory characteristics. These findings may suggest that broken waxy rice, which is a cheaper raw material, can be used instead of full kernel waxy rice to produce Sato with superior sensory characteristics.

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