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Effect of temperature on saccharification and oligosaccharide production efficiency in *koji amazake*

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***Koji amazake*, prepared from rice *koji*, is a traditional Japanese sweet beverage. The main source of sweetness is glucose derived from rice starch following digestion by enzymes of *Aspergillus oryzae* during saccharification. The temperature of this process was empirically determined as 45°C–60°C, but no studies have systematically investigated the effect of temperature on saccharification efficiency. We addressed this in the present study by evaluating saccharification efficiency at various temperatures. We found that glucose content was the highest at 50°C (100%) and was reduced at temperatures of 40°C (66.4%), 60°C (91.9%), and 70°C (76.6%). We previously reported that 12 types of oligosaccharides are present in *koji amazake*; the levels of eight of these, namely nigerose, kojibiose, trehalose, isomaltose, gentiobiose, raffinose, panose, and isomaltotriose, were the highest at 50°C–60°C, whereas sophorose production was maximal at 70°C. Based on these findings, we initially performed saccharification at 50°C and then switched the temperature to 70°C. The maximum amount of each saccharide including sophorose that was produced was close to the values obtained at these two temperatures. Thus, oligosaccharide composition of *koji amazake* is dependent on saccharification temperature. These findings provide useful information for improving the consumer appeal of *koji amazake* by enhancing oligosaccharide content.**

[**Key words:** *Amazake*; Oligosaccharide; *Koji*; Saccharification; Sophorose]

Amazake is a traditional sweet Japanese beverage that is classified into two different types based on the raw materials and production methods used: (i) *koji amazake* made from rice *koji*, and (ii) *sakekasu amazake* made from *sakekasu* (sake lees) and sweeteners such as sucrose. The main source of sweetness of *koji amazake* is glucose, whereas that of *sakekasu amazake* is the added sugar. The production of *koji amazake* comprises the following steps: rice polishing, washing, soaking, and steaming; *koji* preparation; saccharification; and bottling.

The glucose that sweetens *koji amazake* is derived from rice starch digested by diastatic enzymes. Additionally, *koji amazake* contains oligosaccharides such as maltose (Glc(α1-4)Glc), isomaltose (Glc(α1-6)Glc), nigerose (Glc(α1-3)Glc), and kojibiose (Glc(α1-2)Glc) (disaccharides); maltotriose (Glc(α1-4)Glc(α1-4)Glc), isomaltotriose (Glc(α1-6)Glc(α1-6)Glc), and panose (Glc(α1-6)Glc(α1-4)Glc) (trisaccharides); and higher oligosaccharides (1–3). We previously reported that the disaccharides trehalose (Glc(α1-1)Glc), sophorose (Glc(β1-2)Glc), and gentiobiose (Glc(β1-6)Glc); raffinose (Gal(α1-6)Glc(β1-2)Fru); and two unknown trisaccharides are also present in *koji amazake*. Isomaltose, the most abundant oligosaccharide in *koji amazake* that has undergone complete saccharification, is generated through partial hydrolysis of starch by α-amylase and transglycosylation by transglucosidase (4). Other di- and trisaccharides in *koji amazake* are also generated by transglycosylation catalyzed by transglucosidase (5); these oligosaccharides have a sweetness (6) that differs from that of sucrose, whereas gentiobiose confers a bitterness (7) that is important for the taste of *koji amazake*. Moreover, rare di- and trisaccharides such as nigerose, kojibiose, gentiobiose, sophorose, and panose, which are unfermentable carbohydrates produced by microorganisms used in brewing that have purported benefits for human health (8), are generated during *koji* making, especially during saccharification (9). Thus, the saccharification process of *koji amazake* is not only important for digestion of starch into glucose, but also for the generation of other sugars.

The saccharification process is carried out in an open system; the temperature must be maintained above 50°C to prevent the growth of contaminating microorganisms such as lactic acid bacteria, but should not exceed 60°C to avoid inactivating diastatic enzymes such as α-amylase and glucoamylase in *Aspergillus oryzae*. The temperature range for saccharification (45°C–60°C) was determined empirically, and there have been no studies to date that have systematically investigated the effect of temperature on saccharification efficiency.

We addressed this in the present study by evaluating saccharification efficiency at various temperatures. Our findings provide basic information that can be used to improve the production process and quality of *koji amazake*.

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MATERIALS AND METHODS

Preparation of *koji amazake*, *sakekasu amazake*, and rice syrup *Koji amazake* was produced using rice *koji* and water. The rice *koji* in this study was a commercial product (*Koji*; Hakkaisan Co., Ltd., Niigata, Japan) made from rice with a polishing ratio 60%, and was stored at −25°C until use. Hot water (105 ml) adjusted to a temperature of 40°C, 50°C, 60°C, or 70°C was added to 50 g of *koji*; saccharification was then performed at a constant temperature for 8 h. In another experiment, saccharification was initially carried out at 50°C for 8 h and then at 70°C for 8 h for a total of 16 h. Enzymatic activity in rice *koji* is shown in Table S1. *Sakekasu amazake* was prepared by mixing 20 g of *Hakkaisan junmai-ginjyo sakekasu* (Hakkaisan Brewery Co., Ltd.), 65 ml of water, and 15 g of white sugar (National Federation of Agricultural Cooperative Associations, Tokyo, Japan). The alcohol present in *sakekasu* was volatilized by heating. The rice syrup was prepared by mixing 100 g of steamed rice, 130 ml of water, 1.5 g of Gluc SG, and 0.3 g of amano enzyme Cellulase A ("AMANO" 3; Amano Enzyme Inc., Aichi, Japan), followed by incubation at 60°C for 24 h.

Chemicals To determine sugar content, standards of D(+)-glucose, D(+)-maltose monohydrate, kojibiose, trehalose, gentiobiose, and D(+)-raffinose pentahydrate were purchased from Wako Pure Chemical Industries, Ltd. (Wako, Osaka, Japan). Isomaltose and panose were from Hayashibara Co. Ltd. (Okayama, Japan), and nigerose and isomaltotriose were from Funakoshi Co. Ltd. (Tokyo, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. To determine soluble vitamin content, thiamine hydrochloride (vitamin B1), riboflavin (vitamin B2), nicotinic acid and nicotinamide (vitamin B3; niacin), calcium(+)-pantothenate (vitamin B5), pyridoxal hydrochloride and pyridoxine hydrochloride (vitamin B6), (+)-biotin (vitamin B7), folic acid (vitamin B9), and cyanocobalamin (vitamin B12) were purchased from Wako. High-performance liquid chromatography (LC)-grade acetonitrile, methanol, and JIS Special Grade acetone were from Wako. Ammonia hydroxide (28%) was from Nacalai Tesque (Kyoto, Japan).

Analysis of sugars and soluble vitamins For sample preparation, *koji amazake* was pulverized immediately after saccharification using a mixer and centrifuged at 18,400 ×*g* for 5 min (himac CF16RN; Hitachi Koki Co., Ltd., Tokyo, Japan) at 20°C. The *sakekasu amazake* was centrifuged at 18,400 ×*g* for 5 min at 20°C. The supernatant was diluted with water. To separate sugars and soluble vitamins, ultra-performance (UP)LC analysis was performed with an Acquity H-Class UPLC system equipped with Acquity QDa detector and/or a 2998 photodiode array detector (Waters Corporation, Milford, MA, USA) as previously described (3). Sugar and soluble vitamin contents were determined with a standard curve of peak areas of reference standards.

Measurement of enzymatic activity The crude enzyme solution of rice *koji* was prepared by a modified version of the method established by the National Tax Administration Agency (10). Briefly, 10 g of rice *koji* was mixed with 50 ml of sodium acetate buffer (0.5% NaCl and 10 mM sodium acetate). *Koji amazake* was used without dilution. Each sample was pulverized with a blender for 1 min and incubated for 2 min at room temperature, then passed through filter paper (Advantec No. 2; Toyo Roshi, Tokyo, Japan). α-Amylase and glucoamylase activities in the supernatant were determined with α-amylase measurement and glucose forming activity assay kits, respectively (both from Kikkoman, Tokyo, Japan). β-Glucosidase activity was determined using a modified version of a previously described method (11). Briefly, 10 g of rice *koji* was mixed with 20 ml sodium acetate buffer. *Koji amazake* was used without dilution. Each sample were pulverized and filtered as described above. The reaction mixture consisted of 250 µl of 20 mM *p*-nitrophenol-α-D-glucopyranoside (pNPG) as substrate and 50 µl of 400 mM sodium acetate buffer (pH 5.0). A 500-µl volume of sample was added to the reaction mixture followed by incubation at 37°C for 15 min. The reaction was terminated by adding 1.2 ml of 300 mM sodium carbonate solution. The absorbance of pNP at 410 nm was measured with a spectrophotometer.

Color evaluation of *koji amazake* The color of *koji amazake* was analyzed with a CM-5 spectrophotometer (Konica Minolta Inc., Tokyo, Japan) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Effect of saccharification temperature on glucose production in *koji amazake* Saccharification of rice *koji* was carried out at 40°C, 50°C, 60°C, and 70°C for 8 h and saccharification efficiency was evaluated. The glucose and maltose contents at each temperature are shown in Fig. 1. Glucose content was highest at 50°C (100%) as compared to 66.4% at 40°C, 91.9% at 60°C, and 76.6% at 70°C. This is consistent with previous reports that the optimal temperature ranges for α-amylase and glucoamylase in *A. oryzae* are 50°C–55°C (12) and 50°C–60°C (13), respectively. Maltose level was about 14.5 times higher after 8 h at 70°C than at 50°C, corresponding to the decreases in α-amylase and glucoamylase activities to 4.1% and 2.1%, respectively, at the higher temperature (Fig. 2). Glucose was produced at 70°C (Fig. 1A), which was probably due to residual enzymatic activity. It is also possible that the method for measuring enzymatic activity used in this study did not detect all saccharification activity. Furthermore, the accumulation of maltose at 70°C (Fig. 1B) was likely due to residual endoglucanase activity, which

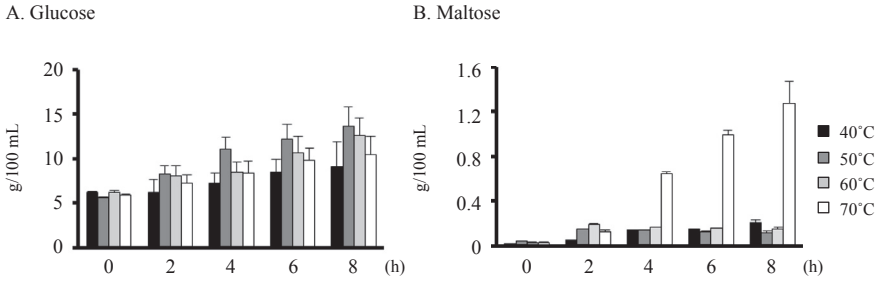

FIG. 1. Amount of glucose (A) and maltose (B) at various saccharification temperatures. Data represent mean ± SD (n = 4).

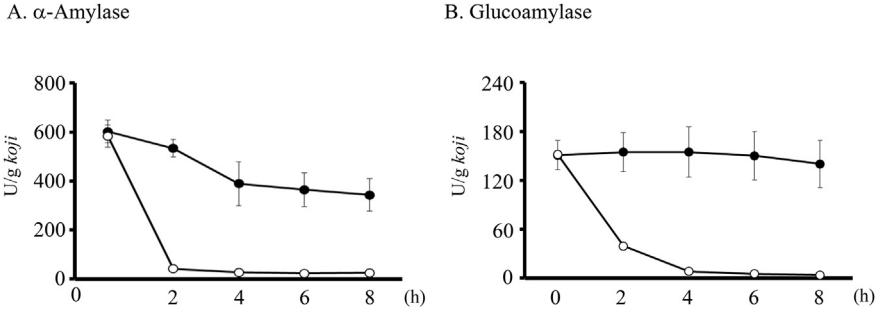

FIG. 2. (A) α-Amylase and (B) glucoamylase activities of *koji amazake* after 8 h at 50°C (filled circles) and 70°C (empty circles). Data represent mean ± SD (n = 3).

was weaker than the exoglucanase activity. Further investigation is necessary to determine the relationship between glucose amount and enzymatic activities in *koji amazake*. Nonetheless, our results confirm that the optimal temperature for highest saccharification efficiency is 50°C–60°C.

Oligosaccharide production as a function of saccharification temperature *Koji amazake* contains a variety of oligosaccharides (1–3) present at low levels in *sakekasu amazake* and rice syrup, which are produced from steamed rice and diastatic enzymes (Table S2). We investigated the effect of saccharification temperature on oligosaccharide production and found that nigerose (Fig. 3A), kojibiose (Fig. 3B), trehalose (Fig. 3C), isomaltose (Fig. 3D), panose (Fig. 3G), and isomaltotriose (Fig. 3H) were most abundant at 50°C, whereas gentiobiose (Fig. 3E), and raffinose (Fig. 3F), were most abundant at 60°C. The difference in the amount of oligosaccharides at 50°C vs. 60°C may be due to the optimal temperature of the responsible glycosyltransferase. There are no reports on the production efficiency of these oligosaccharides in *koji amazake*; our data suggest that the optimal temperature for production of these oligosaccharides is 50°C–60°C, which is in accordance with the optimal saccharification temperature. However, sophorose level was 1.7 times higher at 70°C than at 50°C (Fig. 4A). Sophorose is formed by caramelization (14) and transglycosylation of β-glucosidase activity in another filamentous fungus (*Trichoderma reesei*) (15). To determine whether caramelization had occurred in this study, steamed rice was digested with α-amylase and glucoamylase at 50°C for 8 h to obtain the same amount of glucose (rice syrup) as that in *koji amazake*. The results showed that no sophorose was generated by caramelization (Table S2), which generally occurs at temperatures higher than 160°C but may be produced by transglycosylation of β-glucosidase. However, β-glucosidase activity at 50°C and 70°C was lost after 4 h (Fig. 4B). Some β-glucosidase enzymes in the genus *Aspergillus* are stable below 50°C (16,17). Here we observed only the degradation and not the transglycosylation activity of β-glucosidase. It is therefore necessary to develop a method for measuring β-glucosidase activity for sophorose production. Indeed, little is known about sophorose production in *A. oryzae*, which warrants further investigation.

Temperature shift during saccharification for glucose and oligosaccharide production Sophorose levels were increased at 70°C (Fig. 4A); however, the amounts of glucose and other oligosaccharides were low compared to those at 50°C (Figs. 1A and 3). Based on these findings, we carried out saccharification at 50°C for 8 h and then at 70°C for 8 h to determine whether sophorose and glucose levels would be maximized. The amounts of both sugars were unchanged after 16 h at 50°C and by the shift from 50°C to 70°C (Fig. 5). At the same time, sophorose (C) content was the same at 70°C as during the shift from 50°C to 70°C. Thus, the temperature shift from 50°C to 70°C during the saccharification process increased glucose and sophorose levels as compared to the reactions that proceeded at a constant temperature (50°C or 70°C). Our results demonstrate that it is possible to enhance sophorose production in *koji amazake* without affecting the production of other oligosaccharides by increasing the temperature of saccharification (data not shown). Further study is required to determine whether varying saccharification time would have comparable effects. The glucose level increased from 13.7 g/100 ml (Fig. 1A) to 15.1 g/100 ml (Fig. 5A) as saccharification time was increased from 8 to 16 h at 50°C. Similarly, sophorose level increased from 0.19 g/100 ml at 8 h (Fig. 4A) to 0.51 g/100 ml at 16 h (Fig. 5C) at 70°C. The functional importance of sophorose remains to be elucidated although it has been suggested to promote human health.

Effect of saccharification temperature on *koji amazake* quality Vitamin B is one of the main components of *koji amazake*. We measured the content of soluble vitamin B complexes in *koji amazake* (3); additionally, the thermal stability of soluble vitamin B complex was determined as previously described (18–22). In particular, thiamine (vitamin B1) is known to be heat-labile (18); we therefore investigated the stability of vitamin B

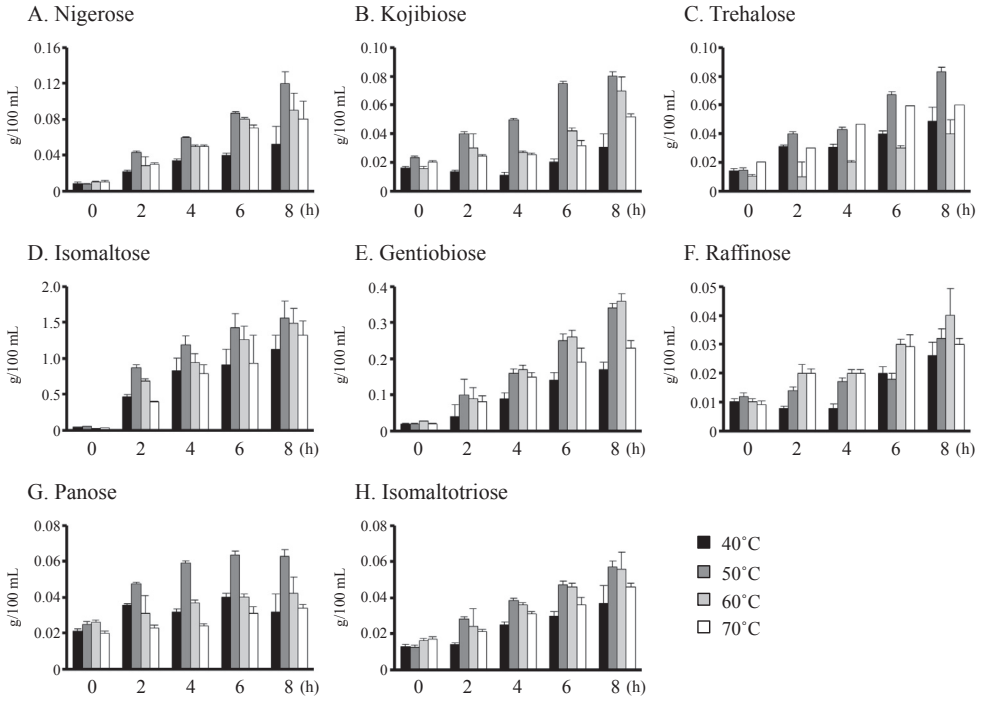

FIG. 3. Amount of oligosaccharides at various saccharification temperatures. (A) Nigerose, (B) kojibiose, (C) trehalose, (D) isomaltose, (E) gentiobiose, (F) raffinose, (G) panose, and (H) isomaltotriose. Data represent mean ± SD (n = 4).

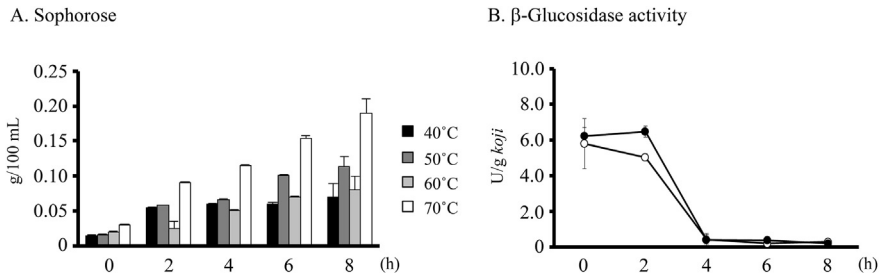

FIG. 4. (A) Amount of sophorose. Data represent mean ± SD (n = 4). (B) β-Amylase activity of *koji amazake* after 8 h at 50°C (filled circles) and 70°C (empty circles). Data represent mean ± SD (n = 3).

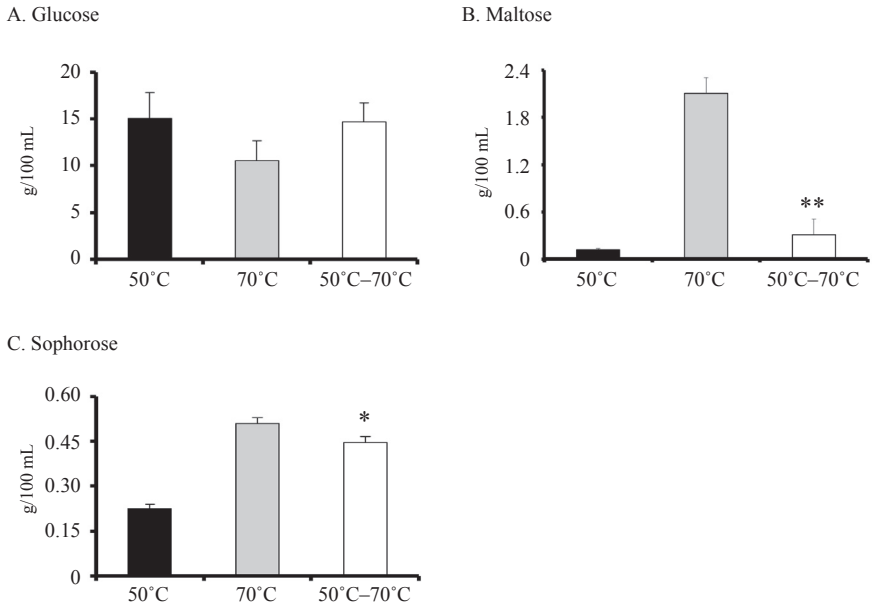

FIG. 5. Changes in saccharide production as a function of temperature during saccharification. (A) Glucose, (B) maltose, and (C) sophorose. Data represent mean ± SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (50°C).

complexes at various temperatures during saccharification. Although riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), and folic acid (B9) levels showed little variation across experimental conditions, thiamine (B1) and biotin (B7) contents decreased at 70°C and during the shift from 50°C to 70°C to 70% of the levels measured at 50°C (Table 1). Unexpectedly, biotin level was decreased by about 90% at 70°C, despite its known heat stability. While the reasons for this reduction require further investigation, we speculate that pH, oxidizing or reducing agents, and the moisture content of reaction components may be contributing factors. On the other hand, 25% of total biotin was lost during the shift from 50°C to 70°C as compared to that at 50°C.

TABLE 1. Concentration of soluble vitamins at different temperatures.

Vitamin B complex (μg/100 g)	50°C	70°C	50°C → 70°C
B1 (thiamine)	148.7 ± 14.3	102.9 ± 14.3**	107.6 ± 5.8
B2 (riboflavin)	428.1 ± 24.7	407.0 ± 31.0	415.2 ± 17.8
B3 (nicotinic acid and nicotinamide)	299.0 ± 24.1	279.4 ± 38.9	283.4 ± 39.7
B5 (pantothenic acid)	52.1 ± 2.5	55.3 ± 4.7	59.7 ± 2.2
B6 (pyridoxine)	39.9 ± 1.2	32.0 ± 2.6	31.3 ± 1.5
B7 (biotin)	1219.6 ± 26.6	112.2 ± 10.5*	918.4 ± 57.1*
B9 (folic acid)	218.2 ± 13.0	205.0 ± 10.5	221.3 ± 10.6
B12 (cyanocobalamins)	ND	ND	ND

Values represent means of quadruplicate experiments ± standard deviation. $^{*}P < 0.01$, $^{**}P < 0.05$ vs. control (50°C). ND, not detected.

TABLE 2. Color value of *koji amazake* after 16 h at different saccharification temperatures.

	50°C	70°C	50°C–70°C
L*	72.54 ± 0.52	68.81 ± 0.46*	70.64 ± 0.38
a*	−1.38 ± 0.03	−0.13 ± 0.05*	−0.71 ± 0.16**
b*	6.01 ± 0.37	8.84 ± 0.13**	7.35 ± 0.08**

L*, lightness; a*, redness; b*, yellowness. Values represent means of quadruplicate experiments ± standard deviation. $^{*}P < 0.01$, $^{**}P < 0.05$ vs. control (50°C).

When *koji amazake* is exposed to a high temperature for long periods of time, it loses its commercial value due to browning (Maillard reaction). We therefore analyzed the color of *koji amazake* prepared at each temperature. The rank order of browning was 70°C > 50°C → 70°C > 50°C (Table 2). The browning that was observed at 70°C was reduced when the temperature was shifted from 50°C to 70°C, indicating that the optimal temperature for saccharification not only increased sophorose content, but also improved the color and appearance of the product.

In this study, we confirmed that the saccharification and oligosaccharide production efficiency of *koji amazake* can be increased by varying the temperature. In addition, the amount of vitamin B complex was increased while the degree of coloration at each temperature was enhanced. These findings provide useful information for improving *koji amazake* production and its appeal to consumers by increasing the oligosaccharide content.

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