Effect of *koji* making process on the quality of *koji* and liquor

製麹工程が麹や酒の品質に及ぼす影響

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Chapter 3 Additional moisture during *koji* preparation contributes to the pigment production of red *koji* by influencing gene expression

Chapter 1　Introduction

1-1 The role of molds for Asian brewing

In Asian brewing, molds are used as a source of starch hydrolases (Nagamine et al., 2003; Suganuma et al., 2007). Other liquors worldwide manufactured from cereals, such as whiskey and beer, are used the malt as a source of starch hydrolases. Thus, the use of molds as a source of starch hydrolases is a distinctive character of Asian liquor (Shiraishi et al., 2016).

In liquor manufacturing, the mold starters are inoculated on the steamed cereal, such as rice and barley as solid cultures. These solid cultures are referred to as "*koji*" in Japan. These molds produce many enzymes containing α-amylase and glucoamylase during growth on cereals. Next, the main material and yeast are added to *koji*, and the mixture begins the alcohol fermentation.

It is well known that the enzymes produced by molds contribute to the brewing of the liquor. In addition, it is also understood that the enzymes in *koji* contribute to form flavor compounds. Acid carboxypeptidase, protease, and lipase produced by molds

affect the level of amino and free fatty acids in the fermented mash. When yeast metabolites amino acids and fatty acid in the mash, it produces flavor compounds with higher alcohols such as isoamyl alcohol, isobutyl alcohol, and phenylethyl alcohol and esters such as ethyl hexanoate and isoamyl acetate. Thus, these activities in solid cultures like *koji* affect the liquor quality (Shiraishi et al., 2018). It is also reported that the flavor compounds produced by molds could be detected in liquor (Shiraishi et al., 2016; Rahayu et al., 2017). The second metabolites produced by molds also affect the characteristic flavor in each liquor. These results show that the quality of *koji* contributes to liquor quality.

1-2 White *koji*

In Japan, *koji*-molds that are primarily used for *koji* preparation are *Aspergillus oryzae* and *Aspergillus luchuensis*. *Koji* prepared by using *A. oryzae*, *A. luchuensis*, and *A. luchuensis* mut. *kawachii* (*A. kawachii*) are referred to as “yellow *koji*”, “black *koji*”, and “white *koji*”, respectively. Yellow *koji* is used for making *miso*, soy sauce, *sake*, and rice vinegar. Black *koji* and white *koji* have been exclusively used for making

shochu, Japanese distilled spirit. *A. luchuensis* and *A. kawachii* differ from *A. oryzae* in the high production ability of citric acid (Kadooka et al., 2020). Therefore, black *koji* and white *koji* contain a large amount of citric acid. Citric acid contributes to prevent microbial contamination during *shochu* brewing.

To be produced citric acid by *koji*-molds, black and white *koji* preparation has a specific process. Black and white *koji* preparations are described briefly. Polished white-grain rice is soaked in water, and then steamed. The steamed rice is inoculated with a *koji*-mold starter, Tane-*koji*. The inoculated rice is incubated for about 27 h at 35-38°C. This step is common process among yellow, black, and white *koji* preparations. Then, in the case of yellow *koji* preparation, the incubation temperature at the final step is maintained at around 38~39°C. On the other hand, in the case of black and white *koji* preparation, the incubation temperature is lowered and controlled at 35°C for additional 15 h. This final step refers to "*shimai-shigoto*" and is a specific process for black and white *koji*. This step could facilitate the production of citric acid by *koji* molds. Although it is well known that these preparation process of black and white *koji* are needed to produce enzymes and citric acid, other impact of the quality of *koji* is not investigated. In the *shochu* industry, white *koji* is preferred for the reason that more manageable than black *koji*, because the conidia number of white *koji* is less

than that of black *koji*. Thus, in this study, I focus on white *koji* and aimed to reveal the relationship between the quality of *koji* and the preparation process of white *koji*.

1-3 Red *koji*

Red *koji* is fermented rice using cultured *Monascus* spp. Red *koji* has been widely used in making alcohol, vinegar, and fermented soybean curd in Asian countries, including China and Japan. It can be presumed from the name of red *koji* that *Monascus* spp. is known to produce pigments such as red (monascorubramine and rubropunctamine), yellow (ankaflavin and monascin), and orange (monascorubin and rubropunctatin) (Patakova 2013; Chen and Johns, 1993; Feng et al., 2012). Red *koji* has also been used as a natural safe colorant for a long time. Therefore, pigment content is a critical index of red *koji* quality. Recently, it was revealed that *Monascus* pigments show hypolipidemic, antiobesity, antidiabetes, antimicrobial, anti-inflammatory, and antitumor biological activities (Lee et al., 2010; Yoshizaki et al., 2014; Kim et al., 2006; Hsu et al., 2012). These characteristics make *Monascus* pigments to be hotly considered in the medical community.

1-4 The research purposes and contents

Koji making is the first step in liquor production. The process of *koji* making differs from the spices and strains of mold. It is considered that these specific processes are adapted for the type of molds to gain the sufficient quality of *koji* for brewing for long time. Therefore, to understand the relationship between the process of *koji* making and the quality of *koji* is important to control the quality of liquor scientifically. In this study, I focus on the process of making white *koji* and red *koji*. I aimed to investigate the effect of the specific process of *koji* making on the quality of *koji* and liquor.

Specifically, this paper is composed of 4 chapters. In Chapter 1, I introduced the *koji* and the *koji* making process. In Chapter 2, I investigated the contribution of two α-amylases from *A. kawachii* to the microstructure of *koji* and the formation of flavor compounds. In Chapter 3, I investigated the effects of additional moisture during *koji* making on the quality of red koji as the growth, enzyme activity, and pigment production and the expression of the pigment biosynthetic and glycohydrolase genes. In Chapter 4, I summarized the content and results of this research.

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Chapter 2 Expression profiles of acid-stable α-amylase and acid-labile α-amylase of *Aspergillus kawachii* effect on the microstructure of *koji* and alcohol fermentation

2-1 Introduction

Shochu is a Japanese traditional distilled spirit made using *koji*. In *shochu* manufacturing, *A. kawachii* is mainly used as the starter *koji*-mold. During *koji* making, the fungus or it produces the required enzymes, like α-amylase and glucoamylase, but it also produces a large amount of citric acid in the latter part of *koji* making (Omori et al., 1994). Thus, the pH of the fermented mash of *shochu* is lowered by citric acid and results in preventing the contamination. *A. kawachii* produces two-types of α-amylase: one is an acid-labile α-amylase encoded by *amyA* gene (AKAW_11452) and the other is an acid-stable α-amylase encoded *asaA* gene (AKAW_02026) (Kaneko et al., 1996; Suganuma et al., 2007). During *koji* making, these two α-amylases have different manners of expression (Omori et al., 1994). The activity of acid-labile α-amylase is detected at an early stage of *koji* making. Then, its activity decreases and is absent in the final stage of *koji* making. However, the activity of acid-stable α-amylase is detected from the middle stage of *koji* making and quickly increases until the final stage. The

production of citric acid by *A. kawachii* during *koji* making occurs in a similar manner to that of acid-stable amylase (Omori et al., 1994). It has been strongly suggested that the decrement of acid-labile amylase activity goes hand in hand with low pH in *koji* by citric acid. All of the α-amylase activity in the fermented mash of *shochu* is thought to be as acid-stable amylase. Therefore, to date, the role of acid-labile amylase in *koji* has hardly been paid attention. However, I think that acid-labile amylase affects the microstructure of *koji* in the early stage of *koji* making. The microstructure of *koji* has not been observed over time, so far. I am interested in studying the contribution of the activities of the two α-amylases to the microstructure of *koji* and the formation of flavor compounds.

In this study, Investigated the influence of the complex expression profiles of two α-amylases from *A. kawachii* on the microstructure of *koji* and *shochu* brewing. Observations of the microstructure of *koji* and its relationship with flavor development play an important role in informing the management regarding brewing.

2-2 Materials and Methods

2-2-1 Materials, chemicals, and strains

Polished rice (Japonica rice) was purchased from Hombo Shoten Co., Ltd. (Kagoshima, Japan), and *A. kawachii* strains used in this study are listed in Table 2-1. *A. kawachii* control strain was defined to show the same auxotrophic background for comparison with the respective strains (Kadooka et al., 2019). *A. kawachii* strains were cultivated in minimal (M) medium (Barratt et al., 1965; Fungal Genetics Stock Center [Manhattan, KS]) with or without 0.21% (w/v) arginine and/or 0.15% (w/v) methionine. Yeast strain was Kagoshima-5, supplied by Kagoshima Prefectural Brewing Association.

2-2-2 Construction of **amyA**-*disruptant strain*

The *amyA* (AKAW_11452) gene was disrupted in *A. kawachii* SO_2 (Δ*argB*Δ*sC*) (Kadooka et al., 2016) by insertion of *argB* gene. A gene replacement cassette encompassing a homology arm at the 5′ end of *amyA* gene, *argB* marker gene, and a homology arm at the 3’ end of *amyA* gene was constructed by PCR (Takara Thermal

Cycler Dice Touch, Takara Bio Inc., Shiga, Japan) using the primer pairs amyA-FC/amyA-R1, amyA-F2/amyA-R2, and amyA-F3/amyA-RC, respectively (Table 2-2). For amplification of *argB* gene, the plasmid pDC1 was used as the template DNA (Aramayo et al., 1989). The resultant DNA fragment amplified with primers amyA-F1 and amyA-R3 was used to transform *A. kawachii* SO_2. The transformants were selected on the M agar mediums without arginine. Introduction of *argB* gene into *amyA* locus was confirmed by colony PCR method using the primer pair amyA-FC/amyA-RC (Fig. 2-1A). The constructed Δ*amyA* strain was transformed with *sC* gene cassette to use the same auxotrophic genetic background strain for the comparative study. The *sC* gene cassette was prepared by PCR using *A. kawachii* genomic DNA as the template DNA and the primer pair sC-comp-F/sC-comp-R (Table 2-2). The transformants were selected on the M agar medium without methionine.

2-2-3 Construction of **asaA** *disruptant strain and* **amyA** *and* **asaA** *double disruptant strain*

The *asaA* (AKAW_02026) gene was disrupted in *A. kawachii* Δ*sC* strain (Kadooka et al., 2020) and Δ*amyA* strain (Table 2-1) by insertion of *sC* gene from *A. kawachii* IFO

(NBRC) 4308 wild type strain. A gene replacement cassette encompassing a homology arm at the 5′ end of *asaA* gene, *sC* marker gene, and a homology arm at the 3’ end of *asa* gene was constructed by recombinant PCR using the primer pairs asaA-FC/asaA-R1, sC-F2/sC-R2, and asaA-F3/asaA-RC, respectively (Table 2-2). The resultant DNA fragment that was amplified with primers asaA-F1 and asaA-R3 was used to transform *A. kawachii* Δ*ligD* Δ*sC* strain and Δ*amyA* strain. The transformants were selected on M agar plates without methionine. Introduction of *sC* gene into *asaA* locus was confirmed by colony PCR using a primer pair asaA-FC/asaA-RC (Fig. 2-1B and 2-1C).

2-2-4 Construction of amyA, asaA, *and* glaA *triple disruptant strain*

The *glaA* (AKAW_08979) gene was disrupted in *A. kawachii* Δ*amyA*Δ*asaA* strain (Table 2-1) by insertion of *ptrA* gene. A gene replacement cassette encompassing a homology arm at the 5′ end of *glaA* gene, *ptrA* marker gene, and a homology arm at the 3’ end of *glaA* gene was constructed by recombinant PCR using the primer pairs glaA-FC/glaA-R1, glaA-F2/glaA-R2, and glaA-F3/glaA-RC, respectively (Table 2-2). For amplification of *ptrA* gene, the plasmid pPTR1 (Takara Bio, Shiga, Japan) was used as the template DNA. The resultant DNA fragment that was amplified with primers

glaA-F1 and glaA-R3 was used to transform *A. kawachii* Δ*amyA*Δ*asaA* strain. The transformants were selected on M agar plates with 0.1 μg/ml pyrithiamine. Introduction of *ptrA* gene into *glaA* locus was confirmed by colony PCR using a primer pair glaA-FC/glaA-RC (Fig. 2-1C). Because the size of PCR product from the *glaA* disruptant was similar to that from the parental strain, PCR products were digested with *EcoRI* for giving a unique digestion pattern for each strain.

2-2-5 Koji *making*

A. kawachii was grown on a potato dextrose agar plate containing 0.01% chloramphenicol until appearing conidia at 30°C for 48 h. Sterile water was added to the plate, and the plate was scraped to release the conidia. The polished rice 600 g was soaked in water for 1 h. The soaked rice was then steamed for 1 h and cooled to 45°C. The conidia suspension (1.0×10^7 spores / ml, 4 ml) was inoculated on the steamed rice. The inoculated rice was incubated over 42 h: first, a linear gradient of at 35°C -38°C over 19 h, with relative humidity (RH) of 95%, followed by 38°C for 8 h at RH of 95%, and finally 35°C for 15 h at RH of 90%. In addition, *koji* was mixed at 19, 23, 27, and 42 h, that are the mixing points of industrial *koji* making to cool *koji* and homogenize

mycelium growing conditions. *Koji* of 100 g were sampled just after inoculation and at 19, 23, 27, and 42 h.

2-2-6 Sugar analysis

Koji samples were freeze-dried and ground to powder. The powdered sample of 2 g was added to 10 ml of deionized water and maintained for 3 h at room temperature. Then, the suspension was centrifuged at 890 × *g* for 10 min at 4°C, and the supernatant was analyzed. The glucose content was measured by using high-performance liquid chromatography (HPLC) (Okutsu et al., 2012). HPLC conditions were as follows: pump, LC-20AD (Shimadzu, Kyoto, Japan); column, COSMOSIL Sugar-D (i.d. 4.6×250 mm, Nacalai Tesque, Kyoto, Japan); mobile phase, CH_3CN:H_2O (75:25); flow rate, 1.0 ml/min; and detector, RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Reducing sugar content was determined using a Somogyi-Nelson assay. Total sugar content was measured after acid hydrolysis using the Somogyi method (Yin et al., 2020; Kobayashi & Tabuchi, 1954).

2-2-7 Enzyme assays

Koji sample (2 g) was added to 10 ml of 100 mM acetate buffer (pH 5.0) containing 0.5% (w/v) NaCl, and was maintained at 4°C overnight. Thereafter, the obtained mixture was centrifuged at 890 × *g* for 10 min at 4°C, and the supernatant was analyzed as crude extract. Total α-amylase activity was determined using α-amylase assay kit (Kikkoman Biochemifa Co., Tokyo, Japan). Acid-stable α-amylase activity was measured following Omori *et al.* (1994). The crude extract was diluted with 50 mM glycine buffer (pH 3.0) and incubated at 30°C for 1 h. Then, this solution was diluted with 100 mM acetate buffer (pH 5.0), and α-amylase activity was measured in the same manner. The acid-labile α-amylase activity was calculated from the difference between the total activity and acid-stable amylase activity. One unit of α-amylase was defined as the amount of enzyme required to release 1 μmol of 2-chloro 4-nitrophenyl from 2-chloro 4-nitrophenyl 6-azide-6-deoxy-β-maltopentaside per minute at 37°C. Glucoamylase and α-glucosidase activities were determined using the saccharification power fractional quantification kit (Kikkoman Biochemifa Co.). One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmol of 4-nirtophenol from 4-nitrophenyl β-maltoside per minute at 37°C. Similarly, one unit of α-glucosidase was defined as the amount of enzyme required to release 1 μmol of 4-nirtophenol from 4-nitrophenyl β-glucoside per minute at 37°C. The absorbance of the

reaction mixture was measured using a UV/VIS spectrophotometer (Shimadzu UV-1700, Kyoto, Japan).

2-2-8 N-*acetyl glucosamine content*

The growth of *A. kawachii* in *koji* was estimated by measuring *N*-acetylglucosamine (GlcNAc) content (Miyamoto et al., 2020). A 0.5 g of *koji* powder sample was added to 5 ml of 50 mM phosphate buffer (pH 6.0). The sample was centrifuged at 890 × *g* for 10 min at 25°C. The precipitate was washed three times with phosphate buffer. The final precipitate was suspended in 2.5 ml Yatalase solution (Takara Bio Inc.) (1 mg/ml Yatalase in 50 mM phosphate buffer, pH 7.0) and incubated at 37°C for 6 h. After centrifugation at 10,000 × *g* for 10 min, 1 ml of the supernatant was filtered through a membrane. HPLC was conducted in the same manner as for the sugar (Glucose content) analysis.

2-2-9 Acidity of koji

Total acidity values were determined following the official methods of the National Tax Administration Agency, Japan (Brewing Society of Japan, 2006). Ten grams of *koji* was added to 50 ml deionized water. Ten ml of extract was titrated against 0.1 mol/L sodium hydroxide, with the indicator containing bromothymol blue and neutral

red, until it turned light green. Total acidity was represented in the titration volume of 0.1 mol/L sodium hydroxide.

2-2-10 Scanning electron microscope (SEM)

The grain was mounted on an aluminum stub using double-sided adhesive tape, with the broken surface oriented upwards. The samples were coated in a quick auto coater (JFC-1500, JOEL, Tokyo, Japan) with a layer of platinum in vacuum conditions. Coated samples were observed using a SEM (Miniscope TM3000, Hitachi High-Technologies Co., Tokyo, Japan).

2-2-11 Enzyme digestibility of steamed rice and **koji**

Steamed rice and *koji* were heated at 100°C for 1 h. Two g of sample was mixed with 10 ml of multiple enzyme solution (60 U/ml α-amylase, 24 U/ml amyloglucosidase, and 3 U/ml Peptidase R (Amano Enzyme Co., Japan)) in 0.1 M succinate buffer (pH 4.3) and incubated at 30°C for 24 h. The samples were centrifuged at 890 × *g* for 10 min at 25°C. Total sugar content in the supernatant was measured using the phenol-sulfuric acid method. Enzyme digestibility (%) was calculated as the ratio of the

hydrolyzed starch after the enzyme digestion to total starch contents in steamed rice or *koji* samples.

2-2-12 Fermentation test

Steamed rice and *koji* samples were heated at 80°C for 1 h. The 100 g sample was added to 180 ml of the same enzyme solution (60 U/ml α-amylase, 24 U/ml amyloglucosidase, and 3 U/ml Peptidase R (Amano Enzyme Co., Japan)) as that used for the enzyme digestibility test, except the buffer was changed to a 20 mM citrate buffer (pH 3.2). Then, 2 ml of the yeast seed culture was added to the fermented mash, and it was static incubated at 30°C for 7 days. The flowchart of fermentation test is shown in Fig. 2-2. The alcohol fermentation was monitored by measuring the amount of CO_2 gas that accompanied the alcohol fermentation. The total decrease in weight of fermented mash, which was measured as the difference between the initial weight and the weight after incubation, was used to determine the amount of CO_2 gas generated.

2-2-13 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was conducted according to the method reported in a study (Rahayu et al., 2017). The fermented mash of *shochu* was filtrated through paper filter (5.0 μm

PTFE Membrane). The filtrate sample was stirred on a magnetic stirrer coated with 0.5-mm polydimethylsiloxane (Twister, Gerstel K.K., Japan) at 1,200 rpm for 1 h at room temperature. Then the stir bar was washed with deionized water, dried with a tissue, and placed into a glass insert. The volatile compounds were desorbed from the stir bar using the temperature program of thermal desorption system (Gerstel TDS3 and Gerstel CIS4, Gerstel K. K.): 20°C for 1 min, 60°C/min to 260°C (hold for 1 min). In the meantime, cold trap was set to -150°C to cryofocus (Gerstel CIS4, Gerstel K.K.). After the desorption program was completed, the cryotrap was heated to inject the compounds into the GC analytical column: -150°C for 1min, 12°C/s to 270°C (hold for 2 min). The GC-MS system was equipped with an Inner Pure-WAX column (60 m × 0.25 mm I.D., 0.25-lm film thickness; GL Sciences Inc., Tokyo, Japan). The identification of volatile compounds was confirmed by comparing their mass spectra with the NIST05a mass spectral database and the RI values in the AromaOffice database (Nishikawa Keisoku Co., Ltd., Tokyo, Japan).

2-2-14 Statistical analyses

Statistical analyses were performed using SPSS software (IBM, NY, USA). Analysis of variance was performed to compare samples. Significant differences ($P < 0.05$) between means were determined using Tukey's multiple comparison tests.

2-3 Results and Discussion

2-3-1 Changes in the microstructure of rice grains and enzyme activities during koji *making*

The changes in *koji* were observed. There were no changes in the surface of steamed rice just after inoculation (i.e., at the incubation time of 0 h) (Fig. 2-3a). After incubation for 19 h, white spots were sparsely appeared on the surface, which was where *koji*-mold was growing (Fig. 2-3b). After incubation for 23 and 27 h, the area of white parts on rice surface were a drastic increase (Fig. 2-3c, 2-3d). Finally, after 42 h, the surface of *koji* was almost completely covered with the fungal mycelia of *A. kawachii* (Fig. 2-3e). The moisture content of *koji* decreased in inverse proportion to the growth (Fig. 2-4A), whereas glucose and reducing sugars increased until the incubation time of 27 h (Fig. 2-4B). There was no change in glucose and reducing sugars since that. Approximately 20% of starch was decomposed to reducing sugar as mainly glucose. Total sugar content gradually decreased. It was expected that sugar was used for the growth of *A. kawachii*. The acidity also increased in the latter part of *koji* making (Fig. 2-4C). Although high activity of acid-labile amylase was detected from the early to middle stages of incubation, the acid-labile amylase showed remarkable decrement since the latter half of incubation (Fig. 2-4D). On the other hand, the amount

of acid-stable amylase increased in the late stage (Fig. 2-4D). Glucoamylase and α-glucosidase also increased in the late stage (Fig. 2-4E, 2-4F).

At the beginning of *koji* making, the inside of *koji* was observed to maintain a gelatinized state through steaming (Fig. 2-3f, 2-3k). After 19 h of incubation, an interstice was observed at some places (Fig. 2-3g, 2-3l, arrowheads). This interstice increased and became larger by 23 h (Fig. 2-3h, 2-3m, arrowheads), and after 27 h, it appeared like a hole (Fig. 2-3i, 2-3n). Rice endosperm cells produce characteristic amyloplasts that are composed of compound-type starch granules. These rice-starch granules are 2–10 μm (Mitchell, 2009). Amyloplasts and endosperm cells in fresh plants are 10–20 μm and ~100 μm, respectively (Toyosawa et al., 2016). Although the accurate size of the starch granules and amyloplasts after cooking is unclear, it was expected that the spherical masses enclosed in an interstice were one granule or several granules of starch that had united as one. The mass enclosed in the largest gaps on *koji* at 27 h and 42 h was thought to be cells (Fig. 2-3i, 2-3j, enclosed box). From 27 h to 42 h, the mycelium increased and the spherical masses were broken more, and angularities in the particles were observed (Fig. 2-3o). Based on the results of enzyme activity, acid-labile amylase was found to be the primary contributor to the decomposition of starch in the early stage until 23 h of *koji* making. From 23 h, acid-stable amylase and acid-labile amylase decomposed the starch in a coordinated manner, and at the final stage, acid-stable amylase and glucoamylase had drastically decomposed the starch granules.

2-3-2 The properties of **koji** *prepared using control and disruptant strains*

AmyA, *asaA*, and *glaA* encode acid-labile amylase, acid-stable amylase, and glucoamylase genes, respectively. To reveal the relationship between the microstructure of *koji* and the expression profiles of these enzymes, we prepared *koji* using the control and single (Δ*amyA* and Δ*asaA*), double (Δ*amyA*Δ*asaA*), and triple (Δ*amyA*Δ*asaA*Δ*glaA*) disruption strains of *A. kawachii* (Table 2-1). These *koji* prepared using control, single, double, and triple disruption strains were named control-*koji*, Δ*amyA*-*koji*, Δ*asaA*-*koji*, Δ*amyA*Δ*asaA*-*koji*, and Δ*amyA*Δ*asaA*Δ*glaA*-*koji*, respectively. After incubation for 42 h, Δ*amyA*-*koji* or Δ*asaA*-*koji* showed growth of *A. kawachii* on the surface of the rice (Fig. 2-5a, 2-5b). However, both *koji* preparations showed poor growth of *A. kawachii* into the inside of rice (data not shown). The GlcNAc content derived from cell walls in fungi was measured as the mycelial mass. The GlcNAc content also showed that the disruptants of Δ*amyA* or Δ*asaA* could be grown at the same level as the control strain (Fig. 2-6A). The Δ*amyA*Δ*asaA* strain showed slight growth on the rice surface (Fig. 2-5c), and Δ*amyA*Δ*asaA*Δ*glaA*-*koji* was very hard, like raw rice grains, and no fungal growth was found on it (Fig. 2-5d). The GlcNAc content in both *koji* samples was correlated with *koji* appearance and was 2.8 and 8.0 times lower than that in the control strain (Fig. 2-6A). All strains showed nearly identical growth M agar plate containing glucose as the carbon source, while the growth

of Δ*amyA*Δ*asaA* and Δ*amyA*Δ*asaA*Δ*glaA* strains were slightly observed on M agar plate composed of an insoluble starch as the carbon source (Fig. 2-7). It was not observed the difference in the morphologies of every strain and the morphologies of the disruptants is similar with control-strain (data not shown).

In Δ*amyA*-*koji*, only the activity of acid-stable amylase was detected, and in Δ*asaA*-*koji*, only the activity of acid-labile amylase was detected (Fig. 2-6B, 2-6C). Thus, the expression profiles of α-amylase activities were compatible with the disruption of genes. The Δ*amyA*Δ*asaA*-*koji* and Δ*amyA*Δ*asaA*Δ*glaA*-*koji* showed 2% (<0.04 U/g dry *koji*) α-amylase activity in the control-*koji* (Fig. 2-6B, 2-6C). Glucoamylase and α-glucosidase activities compatible with the disruption of genes were also measured (Fig. 2-6D, 2-6E). In the final Δ*amyA*-*koji* and Δ*asaA*-*koji*, glucoamylase and α-glucosidase activities were moderately decreased (up to 75%) compared with those in the control-*koji* even though the growth was at the same levels (Fig. 2-6A, 2-6D, 2-6E). It was suspected that the growth of the single disruptants was delayed in the early and middle stages of *koji* making. The Δ*amyA*Δ*asaA* strain possesses *glaA* and α-glucosidase genes, but the activity of both decreased 10–20 times compared with the control strain. The poor growth of Δ*amyA*Δ*asaA* strain might be affected to the production of glucoamylase and α-glucosidase. The acidities of *koji* decreased markedly in Δ*amyA*Δ*asaA* and Δ*amyA*Δ*asaA*Δ*glaA* strains (Fig. 2-6F). Therefore, the production of citric acid might be decreased with the poor growth.

The Δ*amyA*-*koji* or Δ*asaA*-*koji* observed an interstice among the starch granules (Fig. 2-5e, 2-5f, 2-5i, 2-5j). However, the extent of degradation was inferior to that of the control-*koji* (Fig. 2-3j, 2-3o). The degree of degradation in the Δ*amyA*Δ*asaA*-*koji* was inferior to that of a single disruptant, but it showed a more degraded appearance than that of the Δ*amyA*Δ*asaA*Δ*glaA*-*koji* (Fig. 2-5g, 2-5h, 2-5k, 2-5l). These results show that acid-labile amylase and acid-stable amylase can work together to decompose the starch.

2-3-3 Effect of the microstructure of koji *on enzyme digestibility and fermentation*

In *sake* brewing, the enzyme digestibility of steamed rice grains strongly correlates with the efficiency of starch utilization during brewing (Okuda et al, 2009). To investigate the contribution of the microstructure of *koji* for brewing, we examined the enzyme digestibility of *koji*. The enzyme activities in *koji* were different between the strains as described above (Fig. 2-6). To only focus on the effect of the microstructure of *koji*, samples were previously heated to denature the enzymes in *koji*, and the commercial enzymes which was the equivalent activity with control-*koji* was used for digestion. Total enzyme digestibilities of *koji* samples were significantly higher by 1.1 and 1.5 times compared with steamed rice, except for the Δ*amyA*Δ*asaA*Δ*glaA*-*koji*, which had similar total enzyme digestibility to steamed rice (Fig. 2-8A). Among *koji*

samples, control-*koji* was most digested. The Δ*asaA*-*koji*, Δ*amyA*-*koji*, and Δ*amyA*Δ*asaA*-*koji* were most digested, in this order. The Δ*asaA*-*koji* was more digested than that of the Δ*amyA*-*koji*. However, the difference in enzyme digestibility was dependent on the content of reducing sugar in *koji*. The starch present within *koji*, where the fungal hyphae of mold did not reach, remained unchanged, and the decomposition of starch by the mold progressed in the limited part of *koji*.

The effect on alcohol fermentation in *shochu* brewing was investigated. Alcohol fermentation was monitored by measuring the integrated amount of CO_2 gas accompanied by alcohol fermentation (Fig. 2-8B, Table 2-3). After 24 h incubation, the generated CO_2 volume of control-*koji* was 1.5 times higher than in Δ*amyA*Δ*asaA*-*koji*, 2.2 times higher than in Δ*amyA*Δ*asaA*Δ*glaA*-*koji*, and 1.6 times higher than in steamed rice. Therefore, it was speculated that the fermentation speed of control-*koji* was faster than that of these *koji* samples, followed Δ*asaA*-*koji*, Δ*amyA*-*koji*, and Δ*amyA*Δ*asaA*-*koji* (Fig. 2-8B). The control-*koji*, Δ*amyA*-*koji*, and Δ*asaA*-*koji* were fermented significantly more than Δ*amyA*Δ*asaA*Δ*glaA*-*koji* and steamed rice throughout the fermentation period (Fig. 2-8B, Table 2-3). From 4 day, the fermentation in Δ*amyA*Δ*asaA*-*koji* was significantly higher than that of Δ*amyA*Δ*asaA*Δ*glaA*-*koji* and steamed rice (Fig. 2-8B, Table 2-3). There was no significant difference in alcohol fermentation between control-*koji*, Δ*amyA*-*koji*, Δ*asaA*-*koji*, and Δ*amyA*Δ*asaA*-*koji*.

These results showed that the microstructure of *koji* is proportionally affected the rate of alcohol fermentation.

2-3-4 Effect of the microstructure of koji *on the flavor compounds*

To investigate the volatile compounds in the fermented mash, It was measured 13 volatile compounds that were produced by yeast in the fermented mash: three higher alcohols, two acetate esters, six ethyl esters, and two medium-chain fatty acids (MCFA). The content of higher alcohols was significantly higher by 1.4 to 1.8 times in the fermented mash made using Δ*amyA*Δ*asaA*Δ*glaA-koji* than in mash made from other *koji* samples (Table 2-4). Moreover, the content of isoamyl alcohol was significantly higher in the mash made using Δ*amyA*Δ*asaA-koji* than the control preparation. Isobutyl alcohol and isoamyl alcohol trended to increase more in the mash made using Δ*amyA-koji*, Δ*asaA-koji*, and Δ*amyA*Δ*asaA-koji* than the control. Therefore, it seems that slow alcohol fermentation increased the production of higher alcohols by yeast. However, the results of the higher alcohol levels in the mash made using steamed rice were not confirmed with a significant difference compared with the control.

Takamine *et al.* (1990) showed that *shochu* prepared using a saccharifying enzyme and rice as alternative *koji* contains higher levels of higher alcohol than *shochu* prepared using *koji*. The higher alcohol is produced by yeast via the Ehrlich pathway or amino

acid synthesis (ÄYräpää, 1965). The synthesis of higher alcohols via amino acid synthesis pathways is stimulated when the initial amino acid level in the synthetic medium is low (ÄYräpää, 1965; Schulthess & Ettlinger, 1978). It was expected that the amino acid levels in the fermented mash were different between *koji* samples. In fact, the protease activity in *koji* samples was higher in the control-*koji*, Δ*asaA*-*koji*, Δ*amyA*-*koji*, Δ*amyA*Δ*asaA*-*koji*, and Δ*amyA*Δ*asaA*Δ*glaA*-*koji* in this order (Fig. 2-9A). Acid carboxypeptidase activity in *koji* samples was higher in the control-*koji*, Δ*amyA*-*koji*, Δ*asaA*-*koji*, Δ*amyA*Δ*asaA*-*koji*, and Δ*amyA*Δ*asaA*Δ*glaA*-*koji* in this order (Fig. 2-9B). The result of protease activity was consistent with the growth level of *A. kawachii* (Fig. 2-6A). This study confirmed that the protease activity was denatured by the heating treatment. Thus, the differences of protease activity in *koji* do not directly affect alcohol fermentation. Therefore, it was strongly indicated that the initial amino acid level in the fermented mash must be higher in control-*koji* than Δ*amyA*Δ*asaA*-*koji* and Δ*amyA*Δ*asaA*Δ*glaA*-*koji*, and it affected the level of higher alcohols.

Acetate esters and ethyl esters are responsible for the desired fruity and floral aroma in alcoholic beverages (Saerens et al., 2010). Both esters trended to be lower in the fermented mash made using the single, double, and triple disruptants than control in that order (Table 2-4). Especially, 2-phenylethyl acetate was significantly lower by 3.0 to 3.8 times in the fermented mash made using the triple disruptant than in the control. These results show that slow alcohol fermentation decreased the production of acetate

and ethyl esters by yeast. There was no significant difference the octanoic acid and decanoic acid contents among *koji* samples, but they also tended to decrease in the fermented mash made using the disruptants. However, the fatty acid levels in the mash made using steamed rice were 1.7 times higher than in the control.

Formation of acetate esters is dependent on the contents of acetyl-CoA and higher alcohol and the enzyme activity involved in the ester formation. In beer brewing, it is well known that the high-specific gravity of wort leads to the relative overproduction of acetate esters during fermentation (Anderson & Kirsop, 1974). In addition, there is a strong correlation between acetate ester levels and the gene expression levels of *ATF1* and *ATF2* (Alcohol Acetyltransferase Genes) of yeast (Saerens et al., 2008). The control-*koji* had the highest glucose content of 0.1 g/g dry *koji* (Table 2-5). It was twice that of Δ*amyA* and Δ*asaA* strains and seven-times that of Δ*amyA*Δ*asaA* strain (Table 2-5). The glucose content in the initial mash made using the control-*koji* was calculated to be about 5% (w/v). The 4% (w/v) glucose is able to induce the expression of *ATF1* (Verstrepen et al., 2003). The glucose content in *koji* is directly influenced on the initial glucose content in the mash. The glucose content of *koji* correlated with the quantity of acetate esters in the fermented mash. These results suggest that the partial digestion of starch in *koji* affected the glucose content in the fermented mash and resulted in accelerated production of acetate esters by yeast.

The formation of ethyl esters depends on the content of two substrates, acyl-CoA and ethanol, and the enzyme activity involved in the synthesis (Saerens et al., 2010). In addition, the content of MCFAs is the important factor in determining the MCFA ethyl esters (Saerens et al., 2006). In this experiment, the levels of two fatty acids, octanoic acid and decanoic acid, and the level of their ethyl esters weakly corresponded with each other (Table 2-4). The mechanism of the accumulation of MCFAs in the fermented mash is not yet clear. One researcher suggested that the long-chain fatty acids (LCFAs) accumulate and inhibit the acetyl-CoA carboxylase, which results in the inhibition of the elongation of fatty acids (Dufour et al., 2003). Then, acyl-CoAs are released during synthesis from the fatty acid synthesis complex, MCFAs are increased in the mash, and as a result, the MCFA ethyl esters increase (ÄYräpää & Lindström, 1977). Moreover, it is reported that the expression of *EEB1* encoding acyl-CoA: ethanol *O*-acyltransferase (EC 2.3.1.84) (Takahashi et al., 2017) and *FAS1* and *FAS2* encoding fatty acid synthase (EC 2.3.1.86) (Furukawa et al., 2003) contribute to the enhancement of MCFA ethyl esters. This study did not determine the reason for the increase in MCFA content in the mash made using the control strain. The results showed that the microstructure of *koji* affected the production of MCFAs by yeast. Conclusively, it was confirmed that acid-labile α-amylase and acid-stable α-amylase contribute not only the decomposition of starch during mash fermentation, but also the formation of the microstructure in *koji*.

It was showed that the different expression manners of two α-amylases cooperatively contributed to the microstructure of *koji*. In particular, it was revealed for the first time that acid-labile α-amylase plays an important role in the decomposition of starch in *koji* and also affects the flavor produced through its influence on alcohol fermentation. These results can provide the valuable information to understand how to control the alcohol fermentation and flavor.

2-4 Summary

Investigated the contribution of the complex expressions of two α-amylases, namely acid-stable α-amylase and acid-labile α-amylase, from *Aspergillus kawachii* to the microstructure of *koji* and the brewing. The acid-labile α-amylase was found to be the primary contributor to the decomposition of starch in the early stage of *koji* making. From the middle stage, both α-amylases decomposed the starch in a coordinated manner, and at the final stage, acid-stable α-amylase and glucoamylase decomposed the starch granules. Characterization of *koji* prepared by the single disruptions of acid-stable α-amylase or acid-labile α-amylase genes, double disruption of both α-amylase genes, and triple disruption of two α-amylase and glucoamylase genes in *A. kawachii* cells indicated that both α-amylases can work in a synergistic manner to decompose starch during *koji* making. The acid-labile α-amylase was found, for the first time, to play an important role in starch decomposition in *koji*. The rate of alcohol fermentation and ester contents in the fermented mash were higher the mash prepared the control strain, followed by single, double, and triple disruptants. These results indicate that the microstructure of *koji* plays a role in promoting alcohol fermentation and flavor development.

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Table 2-1. *Aspergillus kawachii* strains used in this study

Strain	Genotype	Source
Control	*ligD*$^{-}$ *argB*::*hph*::*argB sC*$^{-}$::*sC*	Kadooka *et al*. 2019
Δ amyA	*ligD*$^{-}$ *argB*::*hph sC*$^{-}$::*sC amyA*::*argB*	This study
ΔasaA	*ligD*$^{-}$ *argB*::*hph*::*argB sC*$^{-}$ *asaA*::*sC*	This study
ΔamyAΔasaA	*ligD*$^{-}$ *argB*::*hph sC*$^{-}$ *amyA*::*argB asaA*::*sC*	This study
ΔamyAΔasaAΔglaA	*ligD*$^{-}$ *argB*::*hph sC*$^{-}$ *amyA*::*argB asaA*::*sC glaA*::*ptrA*	This study

Table 2-2. Primers used in this study

Name	Sequence (5'-3')	Source
amyA-FC	GTGCTTGGCATTAGGGTTCG	This study
amyA-F1	GTAGTAGACCGAGCAGCCG	This study
amyA-R1	GCATGCAAGCTTTCGCGAGCCGGTTGCTATCCTCCA AGCA	This study
amyA-F2	TGCTTGGAGGATAGCAACCGGCTCGCGAAAGCTTG CATGC	This study
amyA-R2	GGGATGACAGCAGTAACGACAATTCGAGCTCGGTA CCCGG	This study
amyA-F3	CCGGGTACCGAGCTCGAATTGTCGTTACTGCTGTCA TCCC	This study
amyA-R3	ATAATAACTGCGCGGGGAGG	This study
amyA-RC	CTTGAGCTTGACTGCTCAGC	This study
sC-comp-F	CAATCACGCAAGCCGAGCTG	Kadooka *et al*. 2019
sC-comp-R	CTCACCGATGTAGGTCATG	Kadooka *et al*. 2019
asaA-FC	GCCGTGTCGACTTTCTTGGA	This study
asaA-F1	TATGGACCAGGCAGGACAGT	This study
asaA-R1	TGCTGCTGGGACACCATGACAACGGCCCAAACACA GCCTGACTAC	This study
sC-F2	CCGTTGTCATGGTGTCCCAGCAGCA	Kadooka *et al*. 2019
sC-R2	GTCCTCCTGGTATACGCCCAATTTG	This study
asaA-F3	CAAATTGGGCGTATACCAGGAGGACGGAGTACCCA GTCGGAGGTA	This study
asaA-R3	GGTACAGCCAGGTTGACCAT	This study
asaA-RC	GCCCCAAATACCACGAACAC	This study
glaA-FC	GAACATTGACATTCGGCGCC	This study
gla-F1	CTTTGGCGGAGGCTTTGAAC	This study
glaA-R1	ATGCAAGAGCGGCTCATCGTCAGTTGTCGCGTCTT GAGGG	This study
glaA-F2	CCCTCAAGACGCGACAACTGACGATGAGCCGCTCT TGCAT	This study
glaA-R2	CAATTCCGTCGGTCGCAATACGGGATCCCATTGGT AACGA	This study
glaA-F3	TCGTTACCAATGGGATCCCGTATTGCGACCGACGG AATTG	This study
glaA-R3	AGCTGCGTGATCAGCGAATC	This study
glaA-RC	GGTATTGTCGATCTGCCCGC	This study

Table 2-3. Time course of alcohol fermentation

	The integrated amount of CO_2 gas (g)																							
Strain	Day 1		Day 2				Day 3				Day 4				Day 5				Day 6				Day 7			
Control	0	13.1	±	2.9	a	23.6	±	3.9	a	30.3	±	3.9	a	34.5	±	3.4	a	37.0	±	2.8	a	38.4	±	2.2	a
Δ*amyA*	0	11.4	±	1.2	ab	21.3	±	3.0	ab	28.0	±	2.9	a	33.1	±	2.6	a	36.2	±	1.8	a	38.5	±	1.0	a
ΔasaA	0	11.0	±	0.6	acd	20.3	±	1.9	ac	26.9	±	2.8	a	31.5	±	3.2	a	34.8	±	2.7	a	37.2	±	2.3	a
Δ*amyA*Δ*asaA*	0	8.7	±	0.4	bed	17.6	±	0.1	bcd	25.1	±	0.4	a	30.0	±	0.2	a	33.7	±	0.4	a	36.3	±	0.2	a
Δ*amyA*Δ*asaA*Δ*glaA*	0	5.9	±	0.9	e	12.9	±	1.7	d	17.2	±	2.8	b	21.2	±	2.7	b	24.2	±	2.6	b	27.3	±	2.6	b
Steamed rice	0	7.9	±	0.1	de	15.5	±	0.4	cd	18.5	±	1.2	b	20.6	±	1.1	b	22.2	±	0.6	b	23.4	±	0.3	b

Values represent as mean ± standard deviations. *Koji* samples (n = 4), steamed rice (n = 3).

Different letters indicate statistically significant differences using Tukey's test at $P < 0.05$.

Table 2-4. The content of volatile compounds in the mash.

		Control			Δ*amyA*			Δ*asaA*			Δ*amyA*Δ*asaA*			Δ*amyA*Δ*asaA*Δ*glaA*			Steamed rice		
(Alcohols)																				
Isobutyl alcohol	mg/L	76.9	±	9.3 a	96.4	±	17.4 a	118.9	±	19.6 a	134.8	±	18.9 ab	183.9	±	49.5 b	118.8	±	29.2 a
Isoamyl alcohol	mg/L	218.1	±	44.9 a	235.5	±	21.2 a	284.7	±	8.9 ab	322.8	±	11.7 bcd	379.1	±	17.2 c	281.9	±	50.2 ad
2-Phenylethyl alcohol	mg/L	248.1	±	60.1 ab	205.4	±	45.6 a	243.7	±	8.5 ab	245.6	±	29.3 ab	396.1	±	74.8 c	345.8	±	27.3 bc
Total alcohol	mg/L	543	±	108 a	537	±	63 a	647	±	25 ab	703	±	22 ab	959	±	107 c	746	±	98 b
(Acetate)																			
Isoamyl acetate	μg/L	2862	±	454 a	2447	±	208 ab	2418	±	119 ab	1956	±	86 b	2037	±	530 b	851	±	157 c
2-Phenylethyl acetate	μg/L	2809	±	430 a	2252	±	239 b	2254	±	75 b	1056	±	65 c	744	±	265 cd	351	±	93 d
Total acetate	μg/L	5,671	±	361 a	4,699	±	256 b	4,672	±	134 b	3,012	±	139 c	2,781	±	762 c	1,201	±	21 d
(Ethyl esters)																			
Ethyl hexanoate	μg/L	115	±	7 ab	96	±	11 abc	102	±	14 abc	75	±	4 ac	60	±	8 c	139	±	45 b
Ethyl Octanoate	μg/L	61.7	±	3.4 a	63.5	±	2.7 a	59.7	±	9.2 a	52.7	±	4.8 ab	46.3	±	1.8 ab	39.7	±	16.5 b
Ethyl Decanoate	μg/L	3.4	±	1.5 ab	5.4	±	2.2 a	4.2	±	0.9 ab	3.9	±	0.6 ab	1.9	±	0.2 b	1.7	±	0.4 b
Ethyl palmitate	μg/L	88.6	±	9.8 abc	109.3	±	17.4 a	97.2	±	6.9 ab	80.8	±	14.6 bc	71.9	±	4.1 cd	51.6	±	7.2 d
Ethyl oleate	μg/L	25.7	±	4.2 a	12.0	±	1.5 bc	9.9	±	0.4 b	18.8	±	4.1 cd	22.1	±	2.7 ade	15.5	±	3.5 bce
Ethyl linoleate	μg/L	6.4	±	1.3 a	2.3	±	0.3 b	1.8	±	0.2 b	5.4	±	1.0 a	5.2	±	0.9 a	4.7	±	1.7 a
Total ethyl ester	μg/L	300	±	15 a	288	±	12 ab	274	±	13 ab	236	±	14 bc	207	±	8 c	253	±	57 ac
(Fatty acid)																			
Octanoic acid	μg/L	719	±	101 a	498	±	64 a	722	±	137 a	570	±	93 a	528	±	47 a	1293	±	585 b
Decanoic acid	μg/L	262	±	44 a	205	±	23 a	227	±	27 a	225	±	17 a	211	±	16 a	451	±	131 b
Total fatty acid	μg/L	981	±	142 a	703	±	84 a	950	±	164 a	796	±	91 a	739	±	41 a	1,743	±	682 b

Values represent as mean ± standard deviations (n = 3).
Different letters indicate statistically significant differences using Tukey's test at $P < 0.05$.

Table 2-5. Sugar contents in *koji* samples

	g/g dry *koji*					
	Control	Δ*amyA*	Δ*asaA*	Δ*amy*AΔ*asaA*	Δ*amyA* Δ*asaA*Δ*glaA*
Glucose	0.104 ± 0.021 a	0.059 ± 0.026 ab	0.049 ± 0.021 b	0.014 ± 0.004 b	0.004
Reducing sugar	0.167 ± 0.022 a	0.110 ± 0.032 b	0.088 ± 0.010 b	0.018 ± 0.002 c	0.010
Total sugar	0.893 ± 0.005 a	0.916 ± 0.032 ab	0.919 ± 0.010 ab	0.960 ± 0.005 b	0.951

Values represent as mean ± standard deviations of three independent experiments (n = 3).

The *ΔamyAΔasaAΔglaA* are represented as the mean of three measureents (n = 1).

Different letters indicate statistically significant differences using Tukey's test at $P < 0.05$.

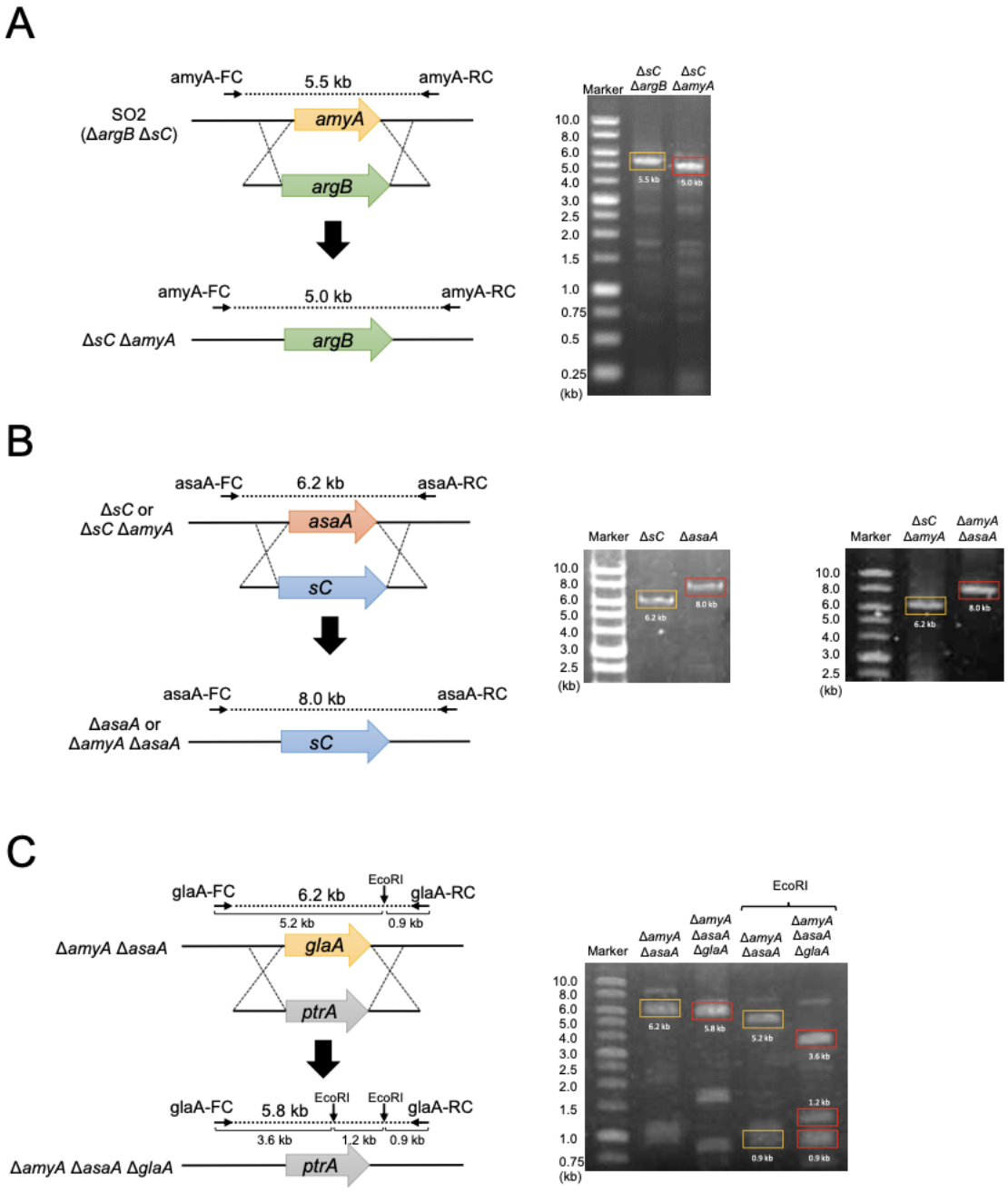

Fig. 2-1. Construction of *A. kawachii* mutants used in this study.

Disruption of *amyA* (A), *asaA* (B), and *glaA* (C). Results of electrophoretic analyses of the colony PCR products are shown in the panels to the right (A–C). PCR products of a primer set of glaA-FC and glaA-RC were digested with *EcoRI* before the electrophoresis (C). Boxes indicate putative sizes of amplicons.

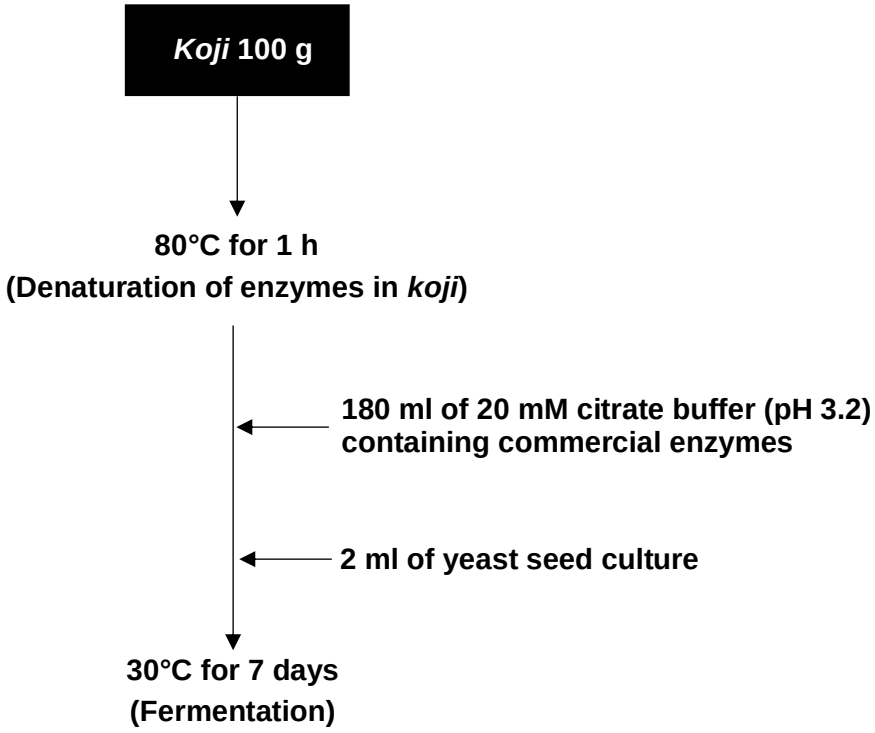

Fig. 2-2. The flow scheme of mash preparation for fermentation test.

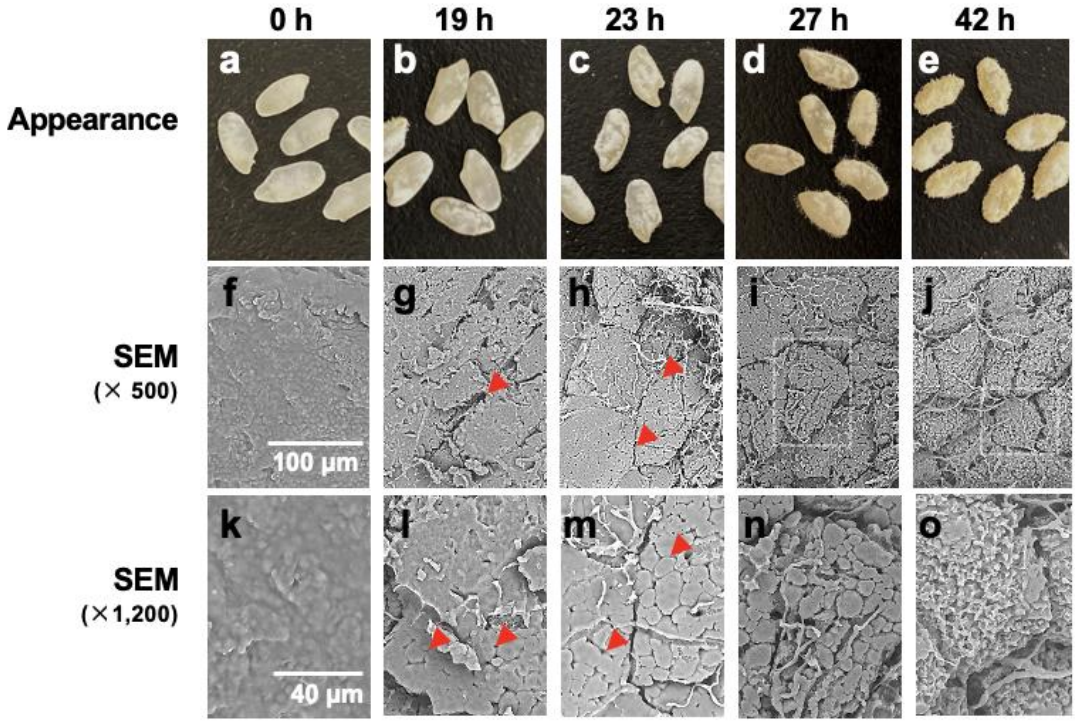

Fig. 2-3. Changes in the appearance of *koji* during *koji* making.

Koji was prepared using *Aspergillus kawachii* control strain. a–e shows the appearance of *koji*. f–j and k–o show scanning electron microscope micrographs of a cross-section of *koji* using magnifications of × 500 and × 1,200, respectively. The red arrowheads white enclosed boxes pointed an interstice and a mass enclosed in the largest gaps, respectively.

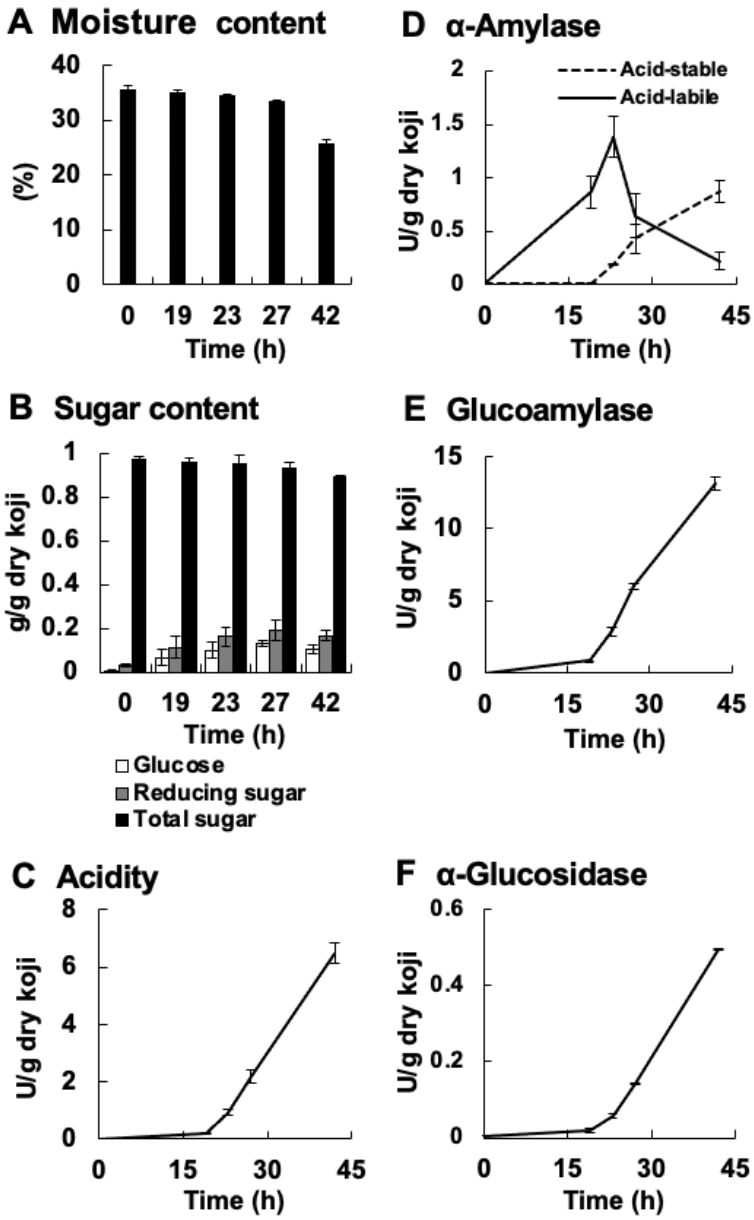

Fig. 2-4. Changes in the components and enzyme activity of *koji* during *koji* making.

Koji was prepared using *Aspergillus kawachii* control strain. (A) moisture content. (B) sugar content. Open bars, gray bars, and black bars show glucose content, reducing sugar content, and total sugar content, respectively. (C) acidity. (D) α-amylase. The solid and broken lines show acid-labile and acid-stable α-amylase, respectively. (E) glucoamylase. (F) α-glucosidase. Values are represented as the mean ± standard deviations (n = 3).

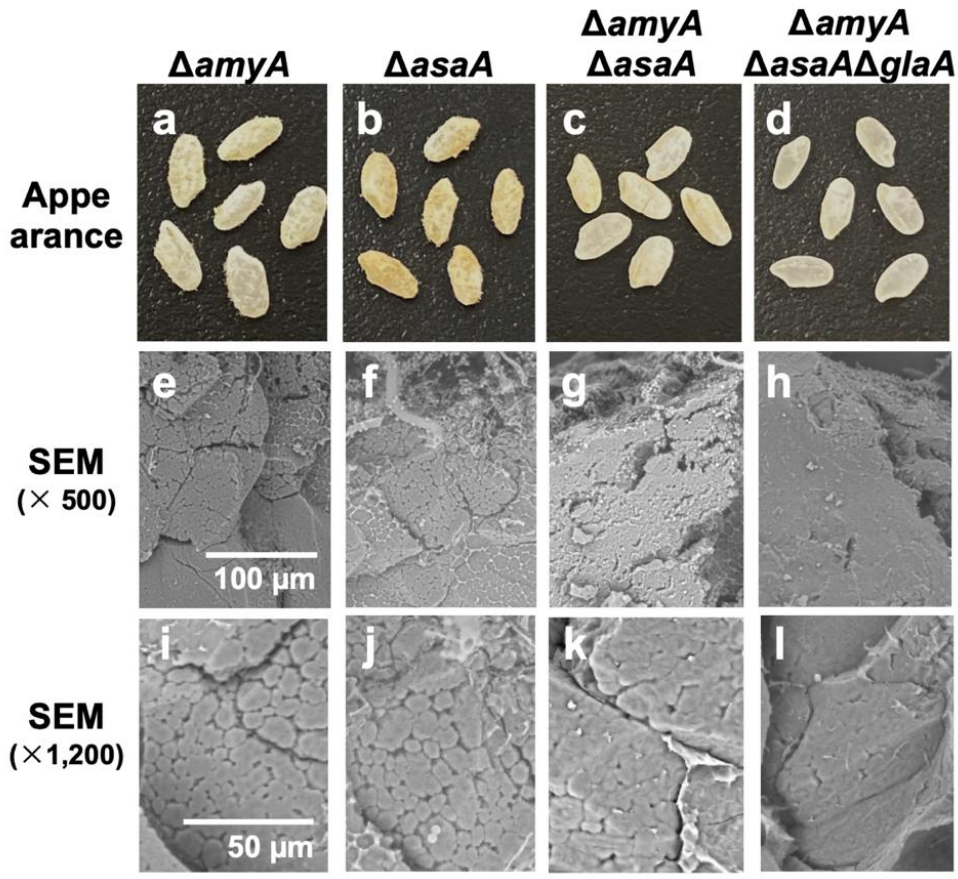

Fig. 2-5. Changes in the appearance of *koji* prepared by different strains.

Koji samples were sampled just after inoculation at 42 h. a–d shows the appearance of *koji.* e–h and i–l show SEM of a cross-section of *koji* at magnifications of × 500 and × 1,200, respectively.

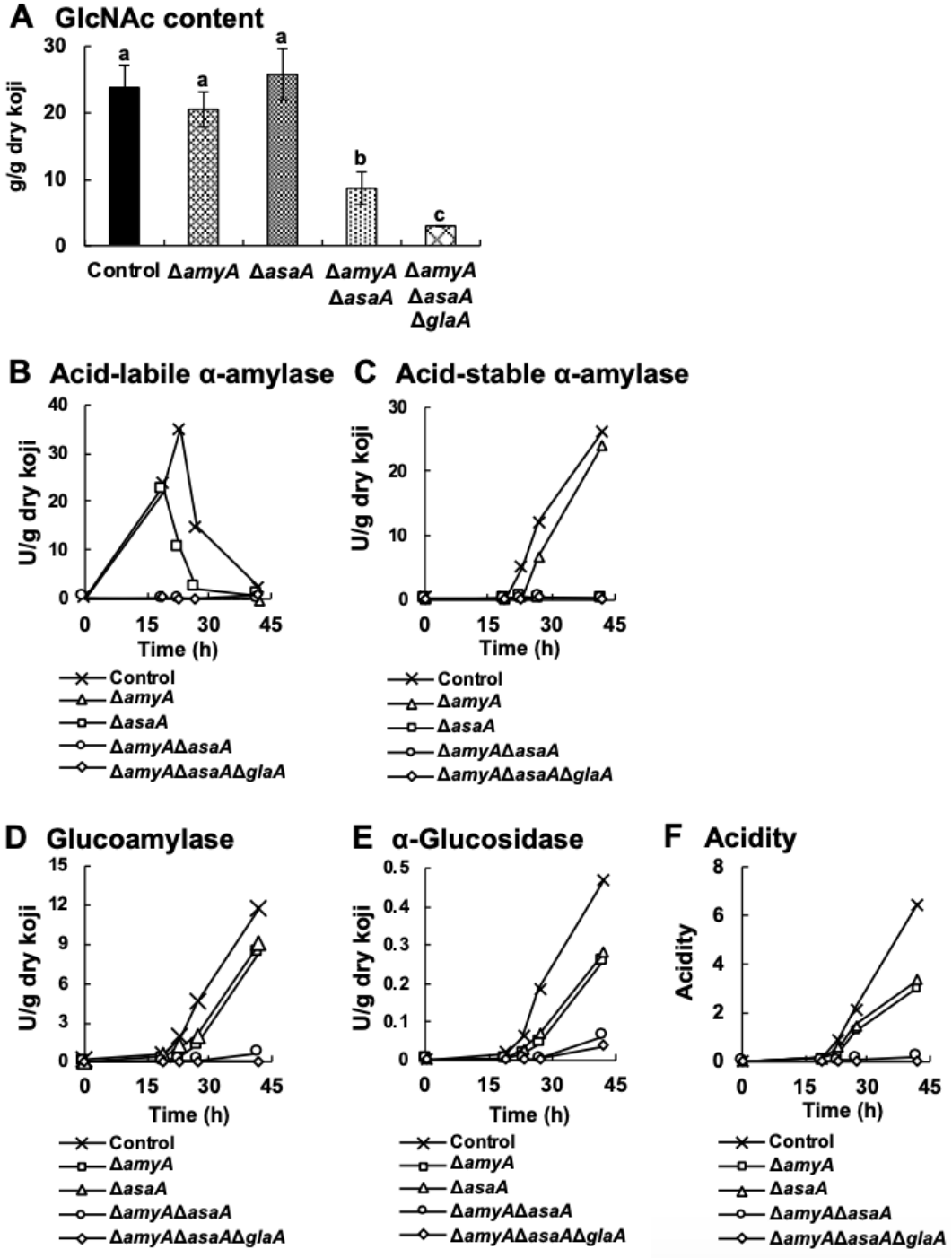

Fig. 2-6. Changes in *N*-acetylglucosamine content, enzyme activities, and acidity of *koji* prepared by different mutants.

Koji samples were sampled just after inoculation at 42 h. (A) *N*-acetylglucosamine (GlcNAc) content. The error bars indicate the standard deviations of three independent experiments. Different letters on the bars indicate statistically significant differences using Tukey's test at $P < 0.05$. (B) acid-labile α-amylase. (C) acid-stable α-amylase. (D) glucoamylase. (E) α-glucosidase. (F) acidity. Cross-marks, triangles, squares, circles, and diamonds show *koji* prepared using control, Δ*amyA*, Δ*asaA*, Δ*amyA*Δ*asaA*, and Δ*amyA*Δ*asaA*Δ*glaA* strains, respectively. Values are represented as the mean for steamed rice ($n = 3$) and *koji* ($n = 9$) samples.

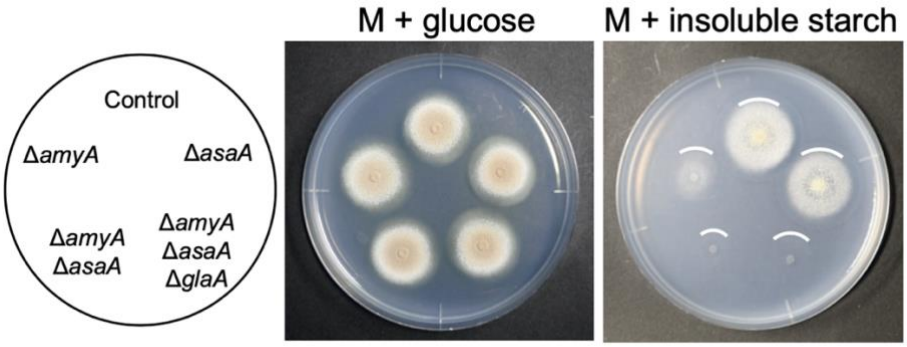

Fig. 2-7. Colony formation of *A. kawachii* control strain and four disruptants on M medium containing glucose or insoluble starch.

Conidia (10^4) were inoculated onto M agar medium containing glucose or insoluble starch and incubated for 5 days or 6 days at 30°C, respectively. The curved white lines indicated the end of colonies.

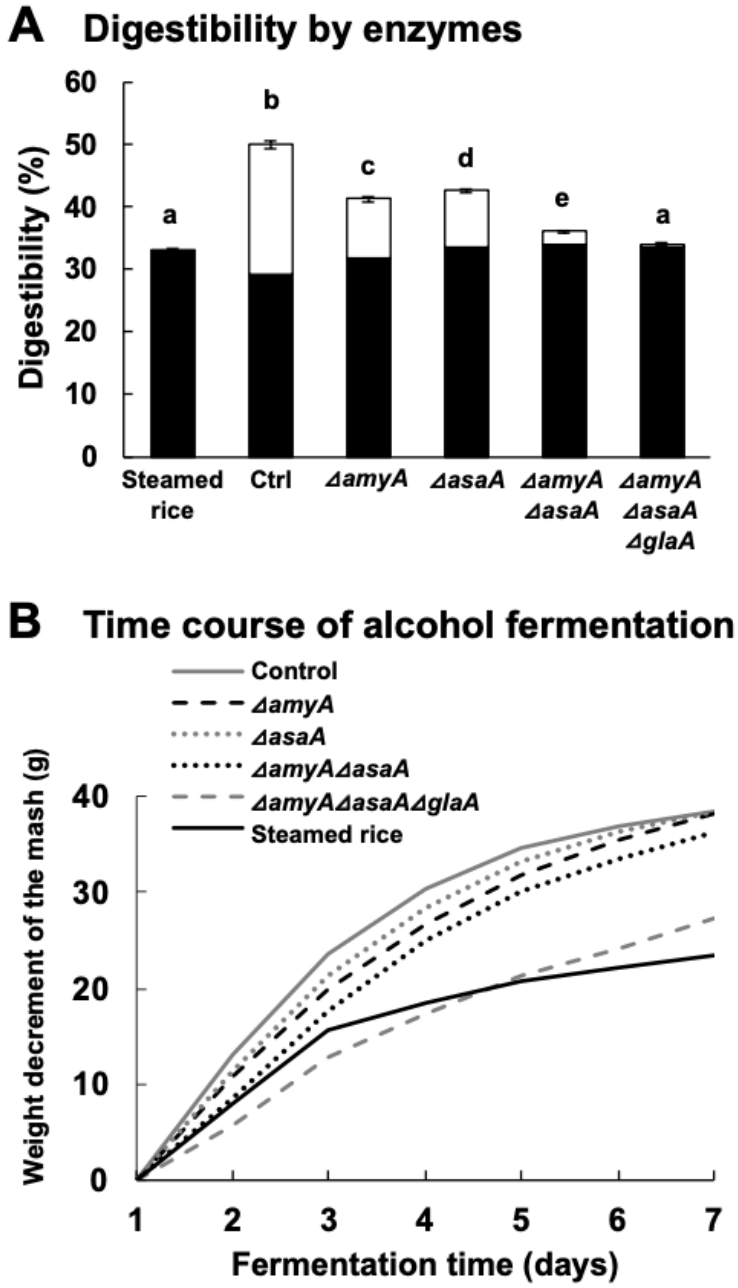

Fig. 2-8. Enzyme digestibility of *koji* and the progress of alcohol fermentation of the mash for *shochu*.

(A) The enzyme digestion test was conducted for 24 h at 30°C. Values represent as the mean for steamed rice (n = 3) and *koji* (n = 4) samples. White bars show the content of reducing sugar derived from *koji*. Black bars show the content of reducing sugar produced by enzyme digestion, which was estimated from the difference between the reducing sugar content after enzyme digestion and reducing sugar content in *koji*. The error bars indicate the standard deviations of total values of enzyme digestibility on independent experiments. Different letters on the bars indicate statistically significant differences using Tukey's test at $P < 0.05$. (B) Alcohol fermentation was monitored by measuring the integrated amount of CO_2 gas accompanied by alcohol fermentation.

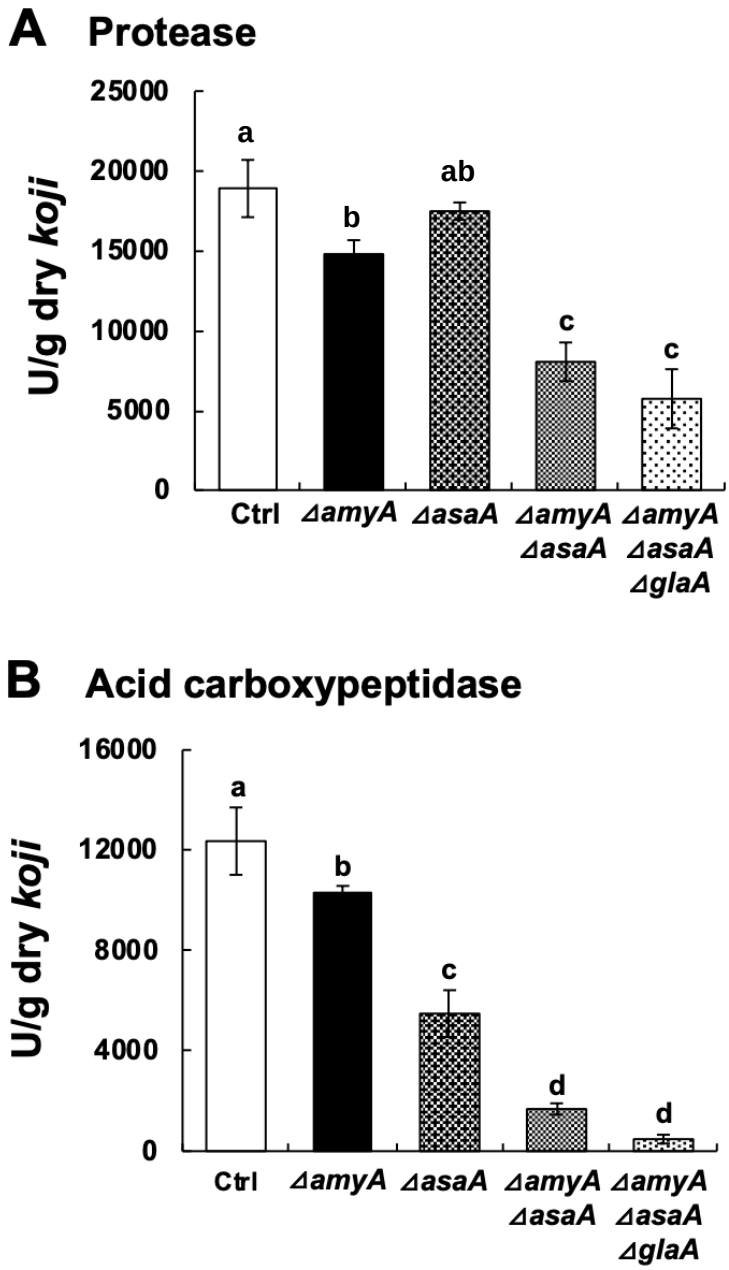

Fig. 2-9. Protease and acid carboxypeptidase activity in *koji* samples.

(A) protease activity. (B) acid carboxypeptidase activity. The error bars indicate the standard deviations of three independent experiments. Values represent as the mean (n = 3). Different letters on the bars indicate statistically significant differences using Tukey's test at $P < 0.05$. The acid carboxypeptidase activity was determined using an acid-carboxypeptidase assay kit (Kikkoman Biochemifa Co.). One unit of the acid carboxypeptidase was defined as the amount of enzyme required to release 1 μmol of L-alanine from carbobenzoxy-tyrosyl-L-alanine per minute at 37°C. Acid protease activity was determined using casein as a substrate by Folin-Ciocalteu method. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine in 60 min at 40°C.

Chapter 3 Additional moisture during *koji* preparation contributes to the pigment production of red *koji* by influencing gene expression

3-1 Introduction

Additional moisture in preparing red *koji* is a characteristic production process. There have been documents showing that moisture content is a key factor affecting the secondary metabolites of red *koji* (Pandey, 2003; de Carvalho et al., 2006； Johns et al. 1991).

Monascus pigments synthesis is conducted via polyketide and fatty acid synthesis pathways (Yang et al., 2015; Huang et al., 2018). In the polyketide synthesis pathway, a nonreducing fungal type I polyketide synthase encoded by *MpPKS5* and a reductase encoded by *mppA* are known to regulate the polyketide chromophore production (Balakrishnan et al., 2013; Bijinu et al., 2014). Meanwhile, in the fatty acid synthesis pathway, *MpFasA2* and *MpFasB2* encoded canonical fungal fatty acid synthases are involved in the synthesis of short-chain fatty acyl thioester for pigment synthesis (Balakrishnan et al., 2014). Pigment synthesis is coupled from both branches via enzymatic esterification by a trichothecene 3-*O*-aceyltransferase encoded by *mppB* (Huang et al., 2017). After that, the proteins encoded by *MpigE* (the homolog of *mppC*),

mppD, and *mppE* produce red, orange, and yellow pigment compounds, respectively (Liu et al., 2014; Balakrishnan et al., 2015; Balakrishnan et al., 2017; Huang et al., 2018). The *mppR1* and *mppR2* genes are regulatory genes for pigment biosynthesis (Balakrishnan et al., 2013). The expression levels of these genes are examined as per light, temperature, glycerol, sodium chloride, and inorganic nitrogen levels (Miyake et al., 2005; Huang et al., 2017, 2018; Chen et al., 2020; Hong et al., 2020). However, these experiments were conducted in the submerged culture. Gene expression regulation in SSF is hardly examined thus far. It has been revealed that SSF's initial moisture is affected by *Monascus* pigments production (Johns & Stuart, 1991). But, the underlying mechanisms by which moisture regulates *Monascus* pigments production remain unclear.

This study investigated the effects of additional moisture during *koji* making on the quality of red *koji* as per growth, enzyme activity, and pigment production, including the expression of the pigment biosynthetic and glycohydrolase genes. These knowledges will contribute to improve the quality of red *koji*.

3-2 Materials and Methods

3-2-1 Reagents, strains used, and materials

Red *koji* seed culture (*Monascus purpureus*) was purchased from Akita Konno Co., Ltd. (Akita, Japan). The reagents used in this study were commercially available special-grade reagents unless specified otherwise. Yatalase was obtained from Takara Bio Inc. (Shiga, Japan). Non-glutinous rice, *Oryza sativa* subsp. *japonica*, was cultivated in Japan and purchased from Hombo Shoten Co., Ltd. (Kagoshima, Japan).

3-2-2 Making koji *with or without additional moisture*

Red *koji* was prepared in our laboratory according to our previous study (Rahayu et al., 2017) with slight modifications (Fig. 3-1). Steamed rice was inoculated with a 10 g red *koji* seed culture. *Koji* making started from inoculation. The inoculated rice was then covered with a thick cloth and incubated at 35°C with a relative humidity (RH) of 95% at incubator (KCL-2000A, EYELA, Tokyo, Japan). After 48 h of inoculation, the sample was divided into two equal parts: one was continuously incubated and the other

was soaked in sterile deionized water at approximately 40°C (additional moisture). After thoroughly soaking it in water for 1 min accurately, the red *koji* was immediately taken out and then drained for 20 min. Then, it was returned to the incubator to resume the fermentation process under the same conditions (35°C, RH 95%). The entire process was completed in 96 h. The samples were mixed every 24 h for homogenization and temperature reducing. *Koji* samples were taken five times, specifically at 0 h (start of *koji* making process), 24 h, 48 h, 72 h, and 96 h for chemical analysis and enzyme assay. These *koji* making processes are considered fundamental in this study. Except for the soaking duration time, *koji* making was performed using the conventional method to reveal the effects of added moisture. The soaking duration time was either 1 min or 10 min. The preparation of red *koji* was repeated three times and analytical data were obtained from three separate experiments.

3-2-3 Moisture content

The moisture content of the samples was measured using an infrared moisture meter FD-720 (Kett Electric Laboratory, Tokyo, Japan).

3-2-4 Enzyme activity

To extract enzymes in *koji*, red *koji* (10 g) was added to 50 ml of 10 mM acetate buffer (pH 5.0) containing 0.5% sodium chloride. The extract was then filtered using a filter paper (No.5C), where the obtained filtrate was used as an enzyme solution. The α-amylase activity was measured using an α-amylase activity measurement kit (Kikkoman Biochemifa Co., Tokyo, Japan). One unit of α-amylase activity was defined as the amount of enzyme required to release 1 μmol of 2-chloro-4-nitrophenol from 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-*β*-maltopentaoside per minute at 37°C. Saccharification power activity was measured using a saccharification power measuring kit (Kikkoman Biochemifa Co.). One unit of saccharification power activity is defined as the amount of enzyme required to release 1 μmol of 2-chloro-4-nitrophenol from 4-nitrophenyl *β*-maltoside per minute at 37°C. Enzyme activity was shown as a unit per gram of dry *koji*.

3-2-5 Measurement of N*-acetyl-β-d-glucosamine (GlcNAc) content*

Red *koji* (10 g) was dried using a freeze-dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Dry *koji* was then put in a plastic tube and heated at 100°C for 1 h to inactivate

the enzyme. After milling using a food grinder, the samples were sieved through a 5-μm sieve for analysis. Powder sample (0.5 g) was added to 5 ml of 50 mM phosphate buffer (pH 6.0) and suspended well. The sample was centrifuged at 890 ×*g* for 10 min at room temperature. The supernatant was discarded, and the precipitate was resuspended in 5 ml phosphate buffer and then centrifuged at the same condition. The supernatant was discarded again. This washing operation was performed three times. The precipitate was suspended in 2.5 ml Yatalase solution (50 mM phosphate buffer, pH 7.0, containing 1 mg/ml Yatalase, and then, it was incubated at 37°C for 6 h. The mixture was centrifuged at 13,700 ×*g* for 10 min at 4°C after incubation. The supernatant was filtered through a membrane filter (0.45 μm) and subjected to HPLC. GlcNAc was quantified by injecting 10 μL of each liquor sample into a Prominence HPLC system (Shimadzu Corp., Kyoto, Japan) under the following conditions: LC-20AD pump (Shimadzu Corp.); 4 × 250 mm i.d. Cosmosil Sugar-D column (Nacalai Tesque, Inc., Kyoto, Japan); mobile phase, acetonitrile:water (3:1); flow rate at 1.0 mL/min; oven temperature 40°C; detector, RID-10A refractive index detector (Shimadzu Corp.). A calibration curve was prepared using a GlcNAc preparation.

3-2-6 Pigment analysis

The pigment content is usually measured by the absorbance based on the visible spectra of *Monascus* pigments (Babitha, Soccol, & Pandey, 2007). Red *koji* (1 g) was added to 10 ml of 70% (v/v) ethanol and then extracted for 1 h at room temperature. The extract was centrifuged at 890 ×*g* for 10 min to collect the supernatant. The supernatant was diluted 20-fold with 70% ethanol. Absorbance at 410 and 510 nm was measured using a spectrophotometer (UV-1700, Shimadzu Corp., Kyoto, Japan). The absorbance unit multiplied by the dilution ratio was used as the pigment content index.

3-2-7 RNA extraction

Red *koji* was prepared using the fundamental method. The samples were divided into two equal parts after 48 h of incubation: one was soaked in sterile water and the other continuously incubated. The samples were collected at the time points of 10 min, 30 min, 1 h, 3 h, 6 h, and 9 h after soaking. Red *koji* samples without additional moisture were also obtained at the same time points. The obtained samples were immediately frozen in liquid nitrogen. The samples were ground using a sterilized mortar and pestle in liquid nitrogen and prepared for RNA extraction. Total RNA was extracted using the

RNA extraction kit (RNAs-ici!-S, RIZO Inc., Ibaraki, Japan). RNA extraction was performed in triplicate for each collected sample.

3-2-8 Quantitative real-time PCR (qPCR)

The primers for gene amplification of *MpFasA2*, *MpFasB2*, *MpPKS5*, *mppR1*, *mppB*, *mppC*, *mppD*, *mppE*, *mppR2*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 3-1. GAPDH was used as the housekeeping reference gene. Also, qPCR was performed in triplicate for each sample. Complementary DNA synthesis and qPCR were performed using One-Step TB Green Prime Script RT-PCR Kit II (Takara Bio Inc.) on a thermal cycler for real-time PCR (Thermal Cycler Dice Real-Time System, Takara Bio Inc.) for preincubation at 95°C for 30 s; this was followed by three steps： reverse-transcription (held at 95°C for 10 s), PCR reaction (40 cycles at 95°C for 10 s and 65°C for 60 s), and then dissociation (at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s).

3-2-9 Measurement of acetyl-CoA levels in red koji

Red *koji* was ground to a fine powder using a mortar and pestle in liquid nitrogen. The sample 100 mg was added 1 ml of cold 1 M $HClO_4$ solution. After suspending well, it was centrifuged at 10,000 × *g* at 4°C for 15 min. The supernatant was used for analysis. The acetyl-CoA level was measured using a PicoProbe acetyl-CoA assay kit (Fluorometric) (Abcam, Cambridge, UK). Fluorescence (Ex/Em = 535/587 nm) was measured using an InfiniteM200 FA (Tecan, Männedrof, Switzerland).

3-3 Results and Discussion

3-3-1 Effect of additional moisture during koji *making on red* koji *quality*

Prepared two red *koji* samples to confirm the effect of additional moisture on the quality of red *koji*. One was added with sterile water at 48 h of *koji* making (with additional moisture), while the other was prepared without water addition as a control (without additional moisture). In the final *koji* products (after the incubation time of 96 h), a clear difference was noted in terms of the appearance of red *koji* prepared with or without additional moisture (Fig. 3-1). After additional moisture, the moisture content of the sample (with additional moisture) increased by approximately 10 points (Fig. 3-2A). This difference in moisture content between the samples was maintained until the production was completed. Additionally, a time-dependent change in *koji* making was also examined for enzyme activities. α-Amylase activity tended to increase at 72 h of *koji* making and then decreased slightly at 96 h (Fig. 3-2B)**.** Finally, although the α-amylase activity showed a difference of approximately 1.5 times regardless of the additional moisture, no significant difference was confirmed. The saccharification power of red *koji* with additional moisture increased significantly from 48 h to 96 h in *koji* making **(**Fig. 3-2C**).** Red *koji* prepared without additional moisture has an increased saccharification power at 72 h, but the rate of increase slowed at 96 h of *koji* making.

The GlcNAc content derived from the fungal cell wall was measured as the mycelial mass. The GlcNAc level remarkably increased at 72 h, but additional moisture had no considerable effect on the red *koji* samples (Fig. 3-2D). It indicated that the mycelia growth rate of *M. purpureus* was similar during the red *koji* making with or without additional moisture.

The absorbances at 410 nm and 510 nm were used to measure the amount of yellow and red pigment, respectively (Zheng et al, 2009). The pigment production was observed at 72 h after incubation, and it continued accumulating throughout the fermentation period in both red *koji* samples with and without additional moisture (Fig. 3-2E). The red pigment content was 6 times more in samples with additional moisture than those without it at 96 h. Meanwhile, the yellow pigment showed 7 times difference between samples with and without additional moisture. From these results, it was confirmed that additional moisture had a promoting effect on pigment production.

Moisture content is considered to be one of the critical factors determining the yield of secondary metabolites in SSF (Pandey, 1992, 2003; de Carvalho et al., 2006). The importance of initial moisture content in SSF has been well investigated. Johns and Stuart (1991) examined the initial moisture content (38%–56%) for cultivating *M. purpureus* using rice as the substrate. Their findings show that as the pigment production increased, the moisture content also increased. In the case of using jack fruit

seed as the solid substrate, the initial moisture content ranged in 35%–60%, and 50% moisture content was identified as the most suitable for optimal pigment production (Babitha, Soccol, & Pandey, 2007). Our results showed that both initial and additional moisture contents are effective in promoting pigment production in SSF. The 56% moisture content in SSF was more effective in inducing pigment production by *M. purpureus* than 40% moisture content (Fig. 3-2E).

Our examination curried out the initial moisture content (approximately 40%) of rice inoculating *M. purpureus* was the general moisture content of the culture, and it can prevent the risk of bacterial contamination and rice grain combination (Hesseltine, 1965). Also, the 40% moisture content of steamed rice made it easier to thoroughly mix the seed culture. Therefore, the additional moisture used in SSF was considered an effective method to control the moisture content in red *koji*, which directly affected the quality of red *koji*.

3-3-2 The effect of additional moisture content on the quality of red koji

To reveal the relationship between the additional moisture content of red *koji* and the quality, red *koji* was prepared with different soaking duration times. The water

absorption levels associated with the soaking periods were confirmed. Red *koji* at 48 h in the *koji* making process was soaked in sterilized water for 1 min, 5 min, 10 min, 15 min, and 20 min, respectively, and it absorbed water rapidly in 1 min immediately after soaking (Fig. 3-3). A remarkable increase in water absorption was confirmed in 5 min. After that, the water absorption rate slowed and it reached a nearly constant level for approximately 10 min. Therefore, red *koji* was made in 1 min and 10 min of water addition duration time.

The moisture content of red *koji* at 48 h of preparation was 43.6% and those of red *koji* just immediately soaking for 1 min and 10 min increased by 56.5% and 60.64%, respectively. These moisture contents decreased with the incubation time. The final moisture contents were 48.0% and about 59.8% for red *koji* soaked for 1 min and 10 min, respectively (Fig. 3-4A). Red *koji* soaked in water for 10 min showed higher saccharifying power and α-amylase activity than that of 1 min (Fig. 3-4B, 3-4C); particularly, the saccharification power was 1.4 times higher (Fig. 3-4C). Conversely, the amount of GlcNAc was almost the same in both soaking duration times (Fig. 3-4D). The pigment content in red *koji* soaked for 10 min was approximately 1.4 times higher than that of the other sample soaked in water for 1 min (Fig. 3-4E). It can be concluded that there is a correlation between pigment and additional moisture content.

3-3-3 Expression of pigment and enzyme biosynthetic genes

The growth of fungi remained the same regardless of additional moisture, but the pigment production increased significantly (Fig. 3-2). It was suggested that the expression of genes related to pigment production by *M. purpureus* is promoted by high moisture content. Conducted qPCR of the pigment biosynthetic gene cluster to determine the effects of gene expression level (Fig. 3-5). The expression levels of ten genes of the FAS and PKS pathways (Fig. 3-6) related to pigment production, *MpFasA2*, *MpFasB2*, *MpPKS5*, *mppA*, *mppB*, *MpigE*, *mppD*, *mppE*, *mppR1*, and *mppR2*, including two genes of glycohydrolases (α-amylase and glucoamylase), were also assessed. In addition, red *koji* was confirmed to show the enzyme activity promotion after additional moisture. Hence, the genes related to enzyme production were also analyzed. And normalized the transcriptional levels to that of the *GAPDH* gene to standardize the results. The relative expression levels during *koji* making with and without additional moisture were monitored in the time course. It was noted the difference in terms of the color intensity of red *koji* after 24 h of additional moisture and after it was incubated for 72 h. Thus, it was considered that the gene expression changed within 24 h after additional moisture. Therefore, samples were collected before additional moisture (at 48 h incubation) and after water addition at 30 min, 1 h, 3 h, 6 h, 9 h, 12 h, 24 h, and 48 h.

The expression levels of the seven genes related to pigment production, *MpPKS5*, *MpFasA2*, *mppB*, *MpigE*, *mppD*, *mppE*, and *mppR2* were upregulated with additional moisture (Fig. 3-5). These seven genes were upregulated within 1 h after additional moisture, although the change was minimal. Specifically, the *MppR2* gene was first upregulated within 30 min after additional moisture. After the upregulation of *MppR2*, the gene expressions of *MpPKS5*, *MpFasA2*, *mppB*, *MpigE*, *mppD*, *mppE*, and *MppR1* were upregulated. *MppR1* and *MppR2* were the transcriptional regulator genes in *M. purpureus* (Balakrishnan et al., 2013). The mutant in the T-DNA insertion at the upstream of *MppR1* is an *albino*, which significantly repressed the transcription of *mppR1* and the nearby *MpPKS5*. However, *MppR2* has an unconfirmed effect on pigment production. Studies have shown that *MppR2* gene expression was downregulated rather than upregulated under cultivation conditions for high pigment production, whereas the gene expression of *MppR1* was upregulated similarly as the pigment production level (Balakrishnan et al., 2013; Chen et al., 2016). The contribution of *MppR2* to pigment production remains unclear. The gene expressions in the previous study were examined at 8 days of incubation period in a submerged culture. Therefore, our results implied that *MppR2* gene quickly responded to environmental changes.

The gene expression levels of *MpPKS5*, *MpFasA2*, *mppB*, *MpigE*, *mppD*, *mppE*, and *mppR2* increased after 1 h following water addition, reaching its maximum at 3 h compared to those without additional moisture. Then, gene expression level gradually decreased, and *MpFasA2*, *MpFasB2*, *MpPKS5*, *mppB*, *MpigE*, *mppD*, *mppE*, and *mppR2* in red *koji* prepared with additional moisture decreased more at 9 h, 12 h, and 24 h than that in red *koji* prepared without additional moisture. The expression levels returned to the same level as that of the red *koji* prepared without additional moisture after 48 h. Unexpectedly, gene expression levels of *MpFasB2*, *mppA*, and α-amylase showed no significant change (Fig. 3-5).

This study analyzed the relationship between the additional moisture and the expression of 10 genes related to pigment biosynthesis and 2 genes related to glycohydrolases.

3-3-4 Acetyl-CoA level in red koji

Acetyl-CoA is a crucial metabolite involved in central carbon and energy metabolism and it is the most important substrate in both pathways of a polyketide synthesis and fatty acid synthesis to synthesize the pigment (Yang et al., 2015). The proteins encoded

MpPKS5 and *MpFasA2* are used acetyl-CoA as the substrate. Therefore, it was expected that the acetyl-CoA level of red *koji* is increased by additional moisture. The acetyl-CoA level in red *koji* was significantly increased after 3 h, 9h, and 12 h of additional moisture (Fig. 3-7A). Acetyl-CoA is produced from glucose via pyruvate in the glycolytic pathway and citric acid cycle. In this experiment, the α-amylase activity and saccharification power in red *koji* tend to increase by additional moisture during the *koji*-making process was confirmed (Fig. 3-2B, 3-2C). Therefore, the glucose level was measured in red *koji* after additional moisture. And expected that the increment of acetyl-CoA level in red *koji* followed the increment of glucose level. However, the increment of acetyl-CoA and glucose levels in red *koji* was almost at the same time or the increment of glucose levels was later than acetyl-CoA (Fig. 3-7B). In addition, the upregulation of gene expression related to pigment production was observed before or at the same time with the increment of acetyl-CoA and glucose levels. Therefore, it was considered that the additional moisture accelerates both the gene expression related to pigment production and supply of the substrates, such as acetyl-CoA and glucose. The increment of glucose level in red *koji* followed the upregulation of glycohydrolase gene expression.

Yang *et al.* (2015) showed that *M. purpureus* YY-1 strain produced a larger amount of pigment when grown in rice medium (liquid culture) compared to sucrose–yeast extract medium despite that the YY-1 strain can grow approximately twice greater in

sucrose–yeast extract medium than rice medium. Furthermore, the gene expression levels in the pigment and acetyl-CoA synthesis pathways were higher in rice medium compared to sucrose–yeast extract medium. They concluded that the acetyl-CoA metabolic flux had shifted to pigment synthesis rather than cell growth when grown in rice medium. Our experiment also confirmed that *MpPKS5* and *MpFasA2* gene expressions, which used acetyl-CoA as one of the substrates, were upregulated with additional moisture (Fig. 3-6). Furthermore, it was observed that the acetyl-CoA and glucose level in red *koji* was increased by additional moisture (Fig. 3-7), however, the cell growth was not affected by it. These results are in agreement with Yang's work (2015). It suggests that additional moisture facilitates the switch shift from acetyl-CoA flux to pigment synthesis rather than cell growth of *M. purpureus*. Moreover, the upregulation of mppB, MpigE, mppD, and mppE affects pigment production.

In these experiments, the gene expression was temporarily downregulated at 9 h ~24 h after upregulation (Fig. 3-5). Studies on the expression profile on fungi in SSF are carried out over the past 20 years. Of them, *Aspergillus terreus* is well examined as the expression of lovastatin biosynthetic genes in SSF. *A. terreus* produces lovastatin with higher yield compared to the submerged fermentation (Barrios-González & Mejía, 2007; Tarragó-Castellanos & Barrios-González, 2017). The transcript accumulation levels of *lovE* (a transcription factor) and *lovF* (a polyketide synthase) in lovastatin

biosynthetic cluster were examined by northern blotting analysis in submerged fermentation and SSF. The lovastatin production proceeded at a high rate in SSF throughout the fermentation period. The transcript levels of *lovE* and *lovF* were highly accumulated on days 3 and 5 in SSF conditions. In this experiment, *lovE* and *lovF* transcript levels reduced at the latter part of fermentation. Although this study differed from our examination in point of the kind of fungi, examination period, and target-genes, the temporary downregulation of gene expression might often be observed under SSF.

In this study, it was revealed that additional moisture in red *koji* preparation is a simple and effective method to promote pigment and enzyme production. The regulatory mechanisms of additional moisture are expected to become a simple and powerful way to enhance the quality of functional foods produced with red *koji*.

3-4 Summary

Additional moisture in making red *koji*, *Monascus*-fermented rice, is a characteristic production process. To determine how additional moisture affects red *koji* preparation as per quality, this study compared the growth of *M. purpureus*, enzyme and pigment production, and related gene expressions using our findings. Thus, considered two kinds of red *koji*: one prepared with additional moisture at the middle part of the preparation and the other prepared without additional moisture. Our results showed that additional moisture did not promote the growth of *Monascus purpureus*, but it was significantly increased the pigment production (red and yellow) and tended to increase the α-amylase level and saccharification power. While adding a high amount of moisture (approximately 60% moisture content) promoted pigment production, it slightly repressed enzyme production. In contrast, adding approximately 50% moisture content promoted enzyme production. These findings showed that the additional moisture can affect the quality of red *koji* on the purpose. The expression of ten pigment biosynthetic gene clusters and two glycohydrolase genes in red *koji* after additional moisture was analyzed through real-time qPCR. Seven pigments genes were upregulated within 1 h after additional moisture, with *mppR2* being the first upregulated

gene within 30 min. The expression of genes as per pigment production quickly responded to additional moisture during SSF. Moreover, the acetyl-CoA which is a starting substrate for pigment content in red *koji* was increased within 3 h after additional moisture. This study first described the relationship between additional moisture and expression of pigment biosynthetic genes by *Monascus* spp. during red *koji* preparation.

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Table 3-1. Primers for real time-qPCR analyzing pigments biosynthetic and enzyme genes.

Primers	Sequences (5′→3′)	Reference
GAPDH-F	TGTACGTTCCGTTCCATTCC	This study
GAPDH-R	GCCACCTCTCCAGTCCTTGT	This study
MpPKS5 F	TGTCCGACGAGTTTCTGCAA	Huang et al., 2018
MpPKS5 R	TATCAACGCTGCTTGGGCAT	Huang et al., 2018
MpFasA2 F	ATGGATCGCCCGATCTTGTC	Huang et al., 2018
MpFasA2 R	CTTTGTCGAGTCCGCTGGAT	Huang et al., 2018
MpFasB2 F	CCTCCAGGGATTACAACCCG	Huang et al., 2018
MpFasB2 R	ATTCAATGCCAGGTGCTCCA	Huang et al., 2018
mppA F	TCCCGTTTCTTGGACGTGAG	Huang et al., 2018
mppA R	ACGTGCCATGGTTCTGTCTT	Huang et al., 2018
mppB F	CGTCTCGCCCGATAACTTCA	Huang et al., 2018
mppB R	TTGACAGACGGGTCGAAGTC	Huang et al., 2018
MpigE F	TCAAGAATCCACCGCACAGA	This study
MpigE R	GAGAAGAACGCCTGGGTCAA	This study
mppD F	TCAACACGGGAGATGCTGTC	Huang et al., 2018
mppD R	GCCAAAGGACAGGAGCAGAT	Huang et al., 2018
mppE F	CTTCCCGATGCCGTTGTGAT	Huang et al., 2018
mppE R	CGTCTCGTGGATCATCTCGT	Huang et al., 2018
mppR1 F	TCTGCAGTATGCCATGTGGG	Huang et al., 2018
mppR1 R	ATGGCACCGTCACTTAGCTC	Huang et al., 2018
mppR2 F	ACGAAACCCTCCATGACACC	Huang et al., 2018
mppR2 R	TGCAGACAGCCTTGTGGTAG	Huang et al., 2018
Mon-amylase-F	GAGATGGGCTACGACATTGGA	This study
Mon-amylase-R	TGGAGGAATTGAAGGGGTTG	This study
Mon-glucoamylase-F	CAAGTGCTGCGTCTGATTCC	This study
Mon-glucoamylase-R	CGTCTGGGCCGATGTTATTT	This study

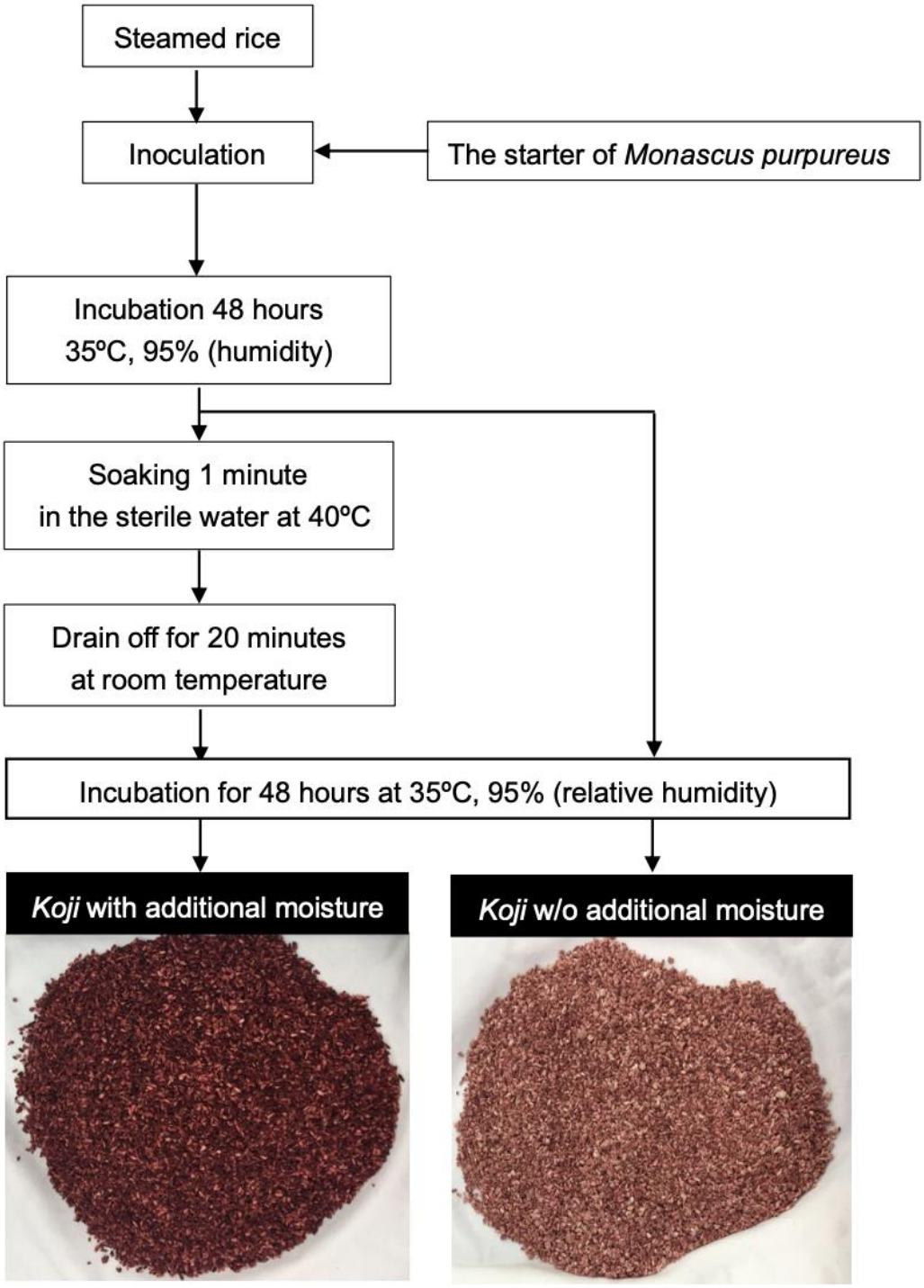

Fig. 3-1. The fundamental red *koji* making process in this study.
Red *koji* was prepared with or without (w/o) additional moisture.

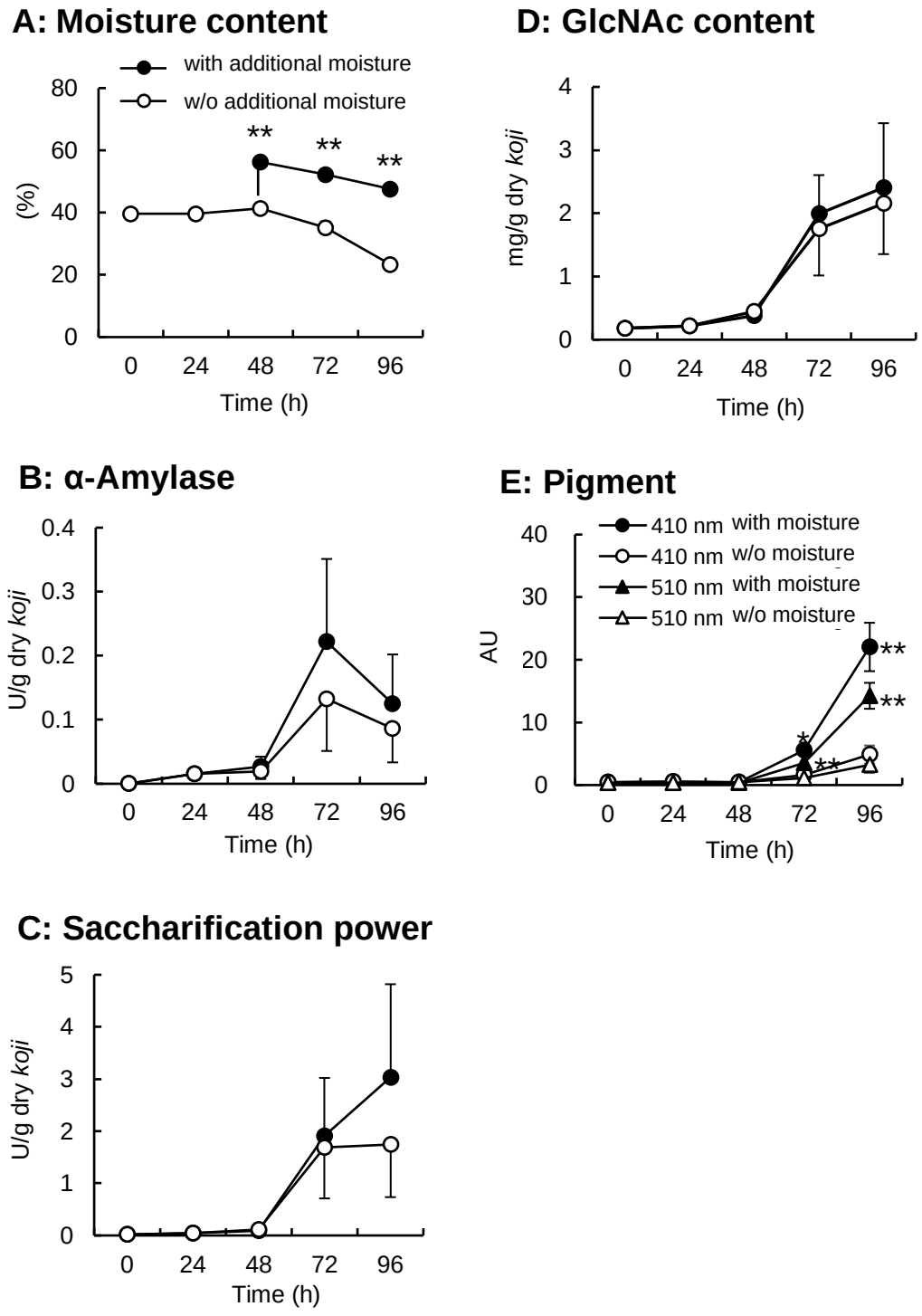

Fig. 3-2. Changes in moisture content, enzyme activity, and pigment production in red *koji* production.

Filled circles (●) and filled triangles (▲) represent red *koji* with additional moisture; while open circles (○) and open triangles (△) represent red *koji* without additional moisture. (A) moisture content; (B) α-amylase; (C) saccharification power; (D) *N*-acetyl glucosamine (GlcNAc) content; and (E) pigment. The error bars indicate the standard deviations of the three independent experiments. Significant differences (*$P < 0.05$, **$P < 0.01$) between means were determined using Student's *t*-test.

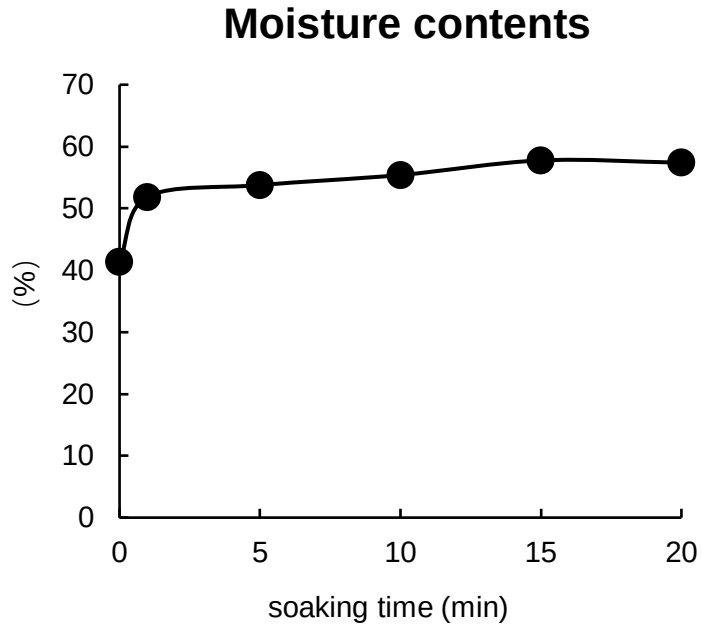

Fig. 3-3. The moisture contents associated with the soaking periods.

Red *koji* at 48 h in the koji making process was soaked in sterilized water for 1 min, 5 min, 10 min, 15 min, and 20 min, respectively.

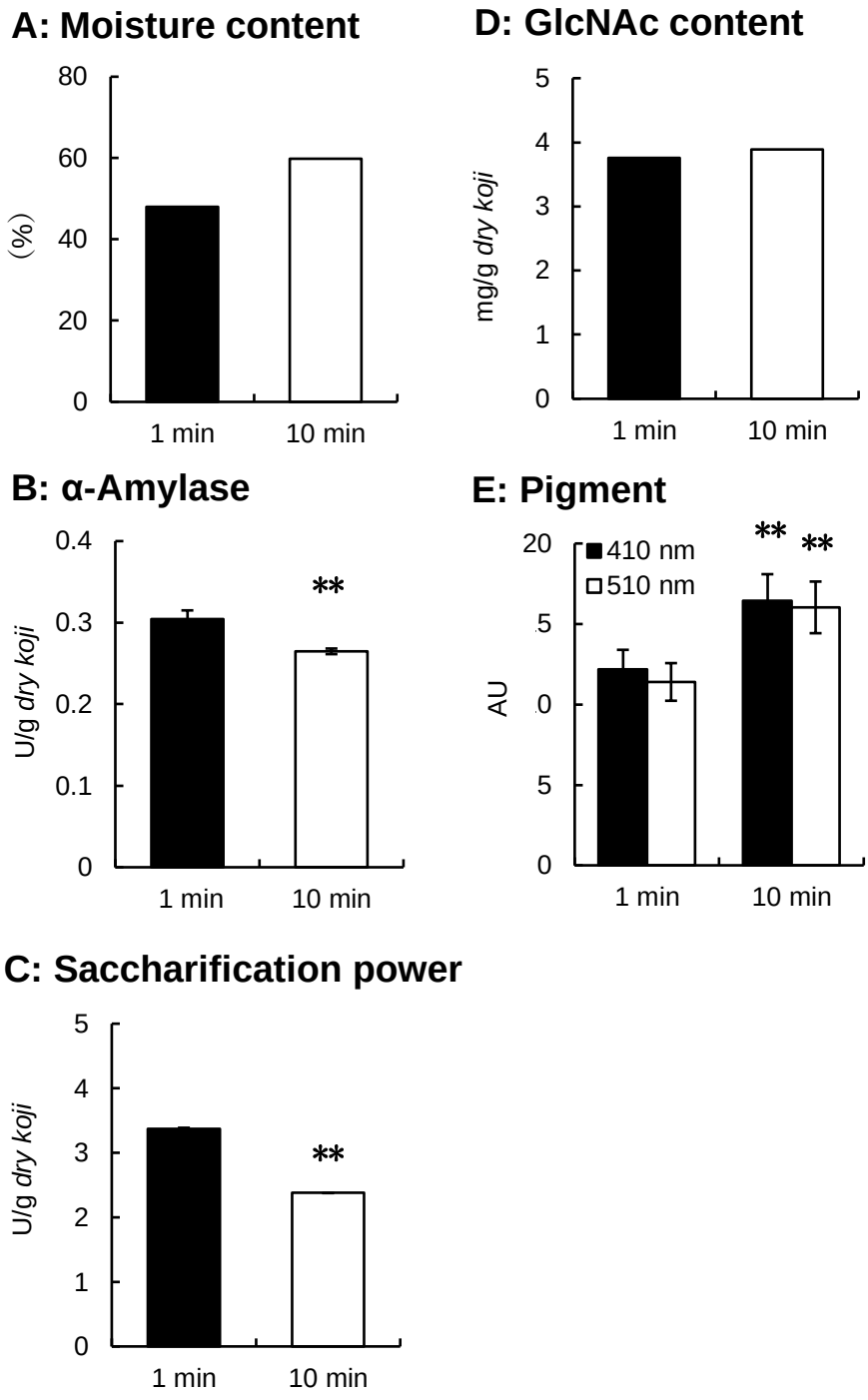

Fig. 3-4. Different properties of red *koji* with different additional moisture duration times.

(A) moisture content; (B) α-amylase; (C) saccharification power; (D) *N*-acetyl glucosamine (GlcNAc) content; and (E) pigment. The error bars indicate the standard deviations of the three independent experiments. Significant differences (**$P < 0.01$) between means were determined using Student's *t*-test. Moisture and GlcNAc contents were simultaneously measured.

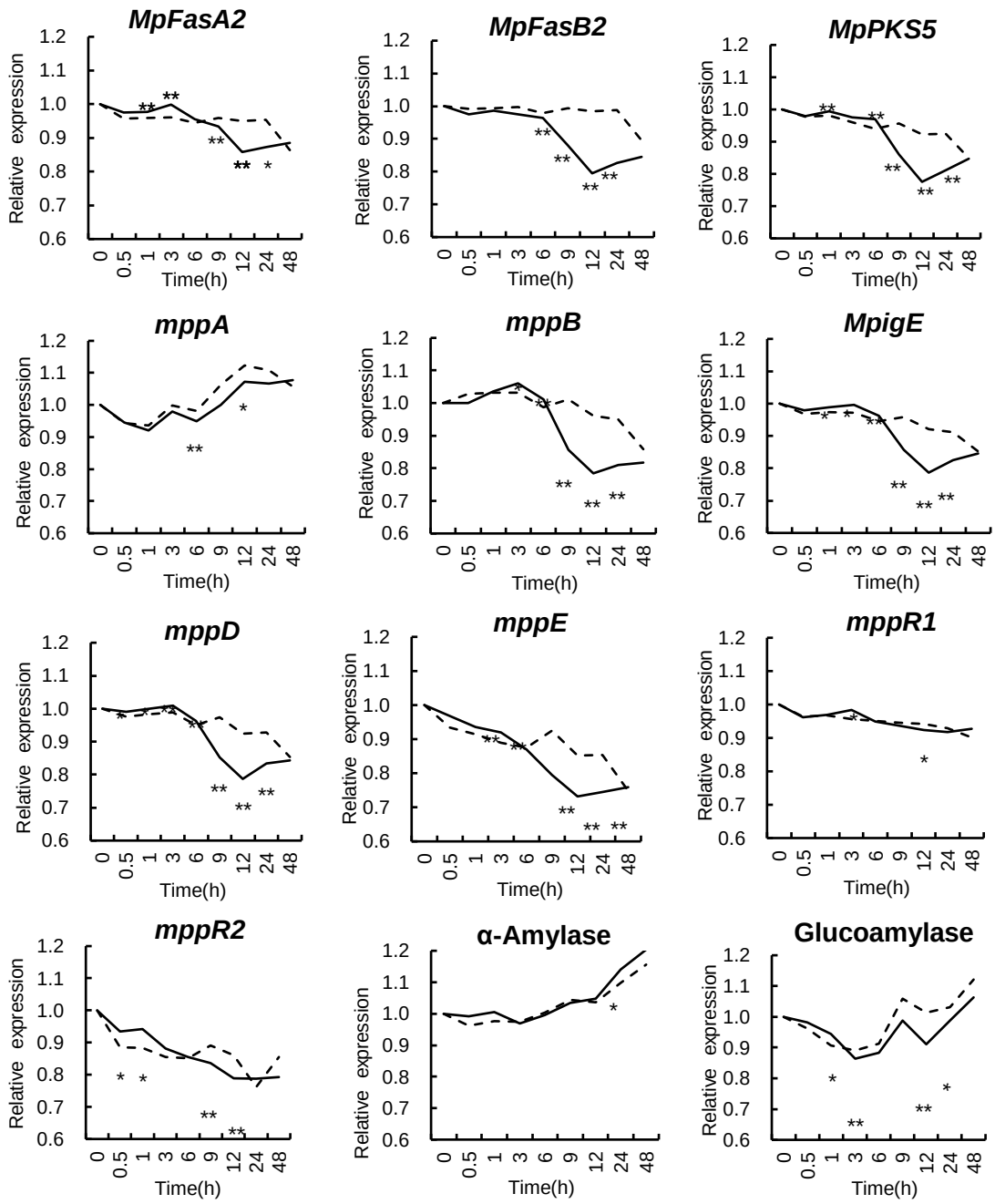

Fig. 3-5. The expression of genes related to pigment synthesis and enzyme production by *Monascus purpureus* after additional moisture.

Total RNA was prepared from the red *koji* samples after additional moisture. Transcriptional levels were normalized to that of the *GAPDH* gene. Solid and broken lines indicate the gene expression in red *koji* with and without additional moisture, respectively. Values represent the relative expression ratio of the sample without additional moisture at 0 h. Experiments were performed in triplicate. Significant differences (*$P < 0.05$, **$P < 0.01$) between means were determined using Student's *t*-test.

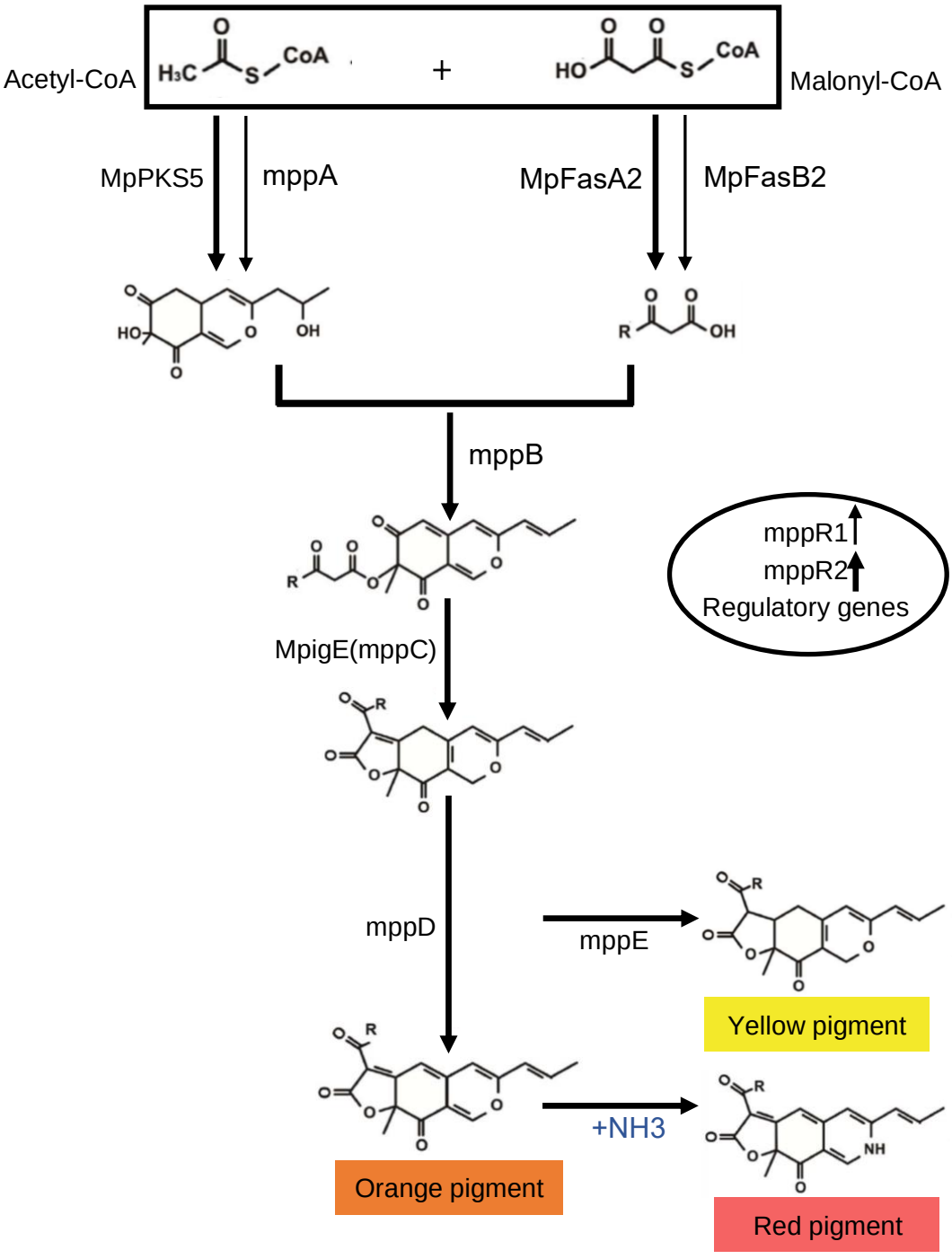

Fig. 3-6. A proposed explanation of the biosynthetic pathway of *Monascus* pigments.

The genes upregulated and no changes after additional moisture are shown by bold and thin arrows, respectively.

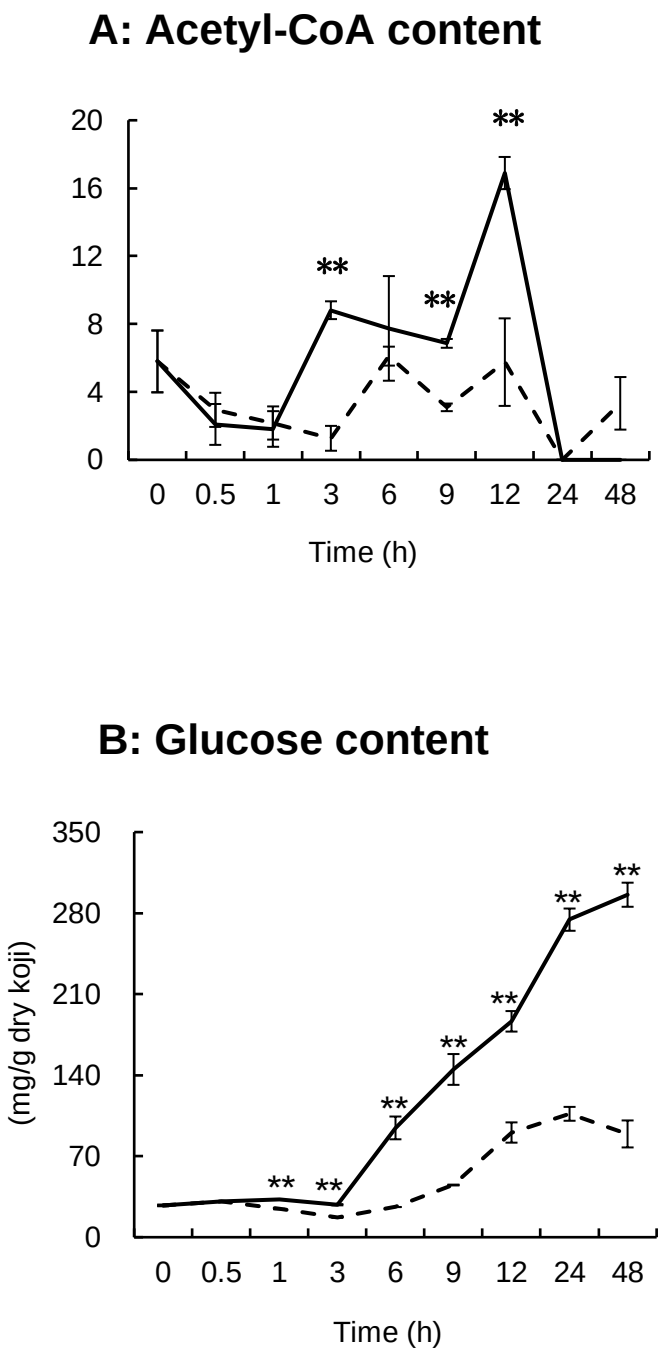

Fig. 3-7. Acetyl-CoA and glucose level in red *koji* after additional moisture process. (A) acetyl-CoA content and (B) glucose. Acetyl-CoA and glucose was prepared from the red *koji* samples after additional moisture. The soaking duration time was 1 min. Solid lines and broken lines indicated the gene expression in red *koji* with additional moisture and without additional moisture, respectively. These experiments were performed three times repeatedly. Significant difference (**P<0.01) between means were determined using Student's t-test.

Chapter 4 Conclusions

Fermentation of foods have been an empirical processing by artisans. Fermentation could increase the assimilability, nutrition, savor, palatability, and functional properties. In Asian fermented foods, the molds play an important role on the processing and contribute to form the attractive feature of foods. Thus, *koji* prepared by mold has an impact on the quality of fermented foods. In this study, we focused on the white and red *koji* and investigated the relationship between the *koji* preparation process and the quality of *koji* and fermented foods.

In Chapter 1, the manufacturing processes of white and red *koji* were introduced. In Chapter 2, the changes in rice microstructure and enzyme activity were observed over time during the manufacture process of *Aspergillus kawachii*, and investigated the contribution of the complex expressions of two α-amylases (acid-stable α-amylase and acid-labile α-amylase) from *A. kawachii* to the microstructure of *koji* and the brewing. The results indicated that both α-amylases can work in a synergistic manner to affect the microstructure of *A. kawachii* during *koji* making. Characterization of *koji* prepared by the disruptant strains in *A. kawachii* cells showed that the speed of alcohol fermentation and ester contents of the fermented mash were higher the mash prepared

the control strain, followed by single, double, and triple disruptants. These results indicate that the microstructure of *koji* plays a role in promoting alcohol fermentation and flavor development. In Chapter 3, we analyzed the influence of the addition of moisture during the manufacturing process on the quality of red koji. Comparing the red *koji* prepared with and without additional moisture during the manufacturing process showed that additional moisture promotes the pigment production. We found that the expression of gene clusters related to pigment synthesis was up-regulated after adding moisture. Acetyl-CoA, the substrate of *Monascus* pigments synthesis, has also increased significantly due to water stimulation. This is also regarded the additional moisture process as the main factor affecting pigment production. This research also laid the foundation for the stability of red *koji* quality.

In Chapter 4, Summarized the content and results of this research. These results can provide the useful information to understand how to control the alcohol fermentation and flavor.