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Quantitative evaluation of *haze* formation of *koji* and progression of internal *haze* by drying of *koji* during *koji* making

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The construction of an experimental system that can mimic *koji* making in the manufacturing setting of a sake brewery is initially required for the quantitative evaluation of mycelia grown on/in *koji* pellets (*haze* formation). *Koji* making with rice was investigated with a solid-state fermentation (SSF) system using a non-airflow box (NAB), which produced uniform conditions in the culture substrate with high reproducibility and allowed for the control of favorable conditions in the substrate during culture. The SSF system using NAB accurately reproduced *koji* making in a manufacturing setting. To evaluate *haze* formation during *koji* making, surfaces and cross sections of *koji* pellets obtained from *koji* making tests were observed using a digital microscope. Image analysis was used to distinguish between *haze* and non-*haze* sections of *koji* pellets, enabling the evaluation of *haze* formation in a batch by measuring the *haze* rate of a specific number of *koji* pellets. This method allowed us to obtain continuous and quantitative data on the time course of *haze* formation. Moreover, drying *koji* during the late stage of *koji* making was revealed to cause further penetration of mycelia into *koji* pellets (internal *haze*). The *koji* making test with the SSF system using NAB and quantitative evaluation of *haze* formation in a batch by image analysis is a useful method for understanding the relations between *haze* formation and *koji* making conditions.

[**Key words:** *Koji*-making; *Haze* formation; Internal *haze*; *Koji* drying; *Aspergillus oryzae*]

Rice *koji* (molded rice) is used for the production of traditional fermented foods, such as sake (rice wine) and shochu (Japanese distilled spirit). It is made by solid-state fermentation (SSF) with steamed rice using the filamentous fungus *Aspergillus* spp. *Koji* has an important role in supplying of various hydrolytic enzymes. The process of *koji* production is called *koji* making. Mycelia grown on/in rice pellets containing rice structure collapsed by α-amylase look white because of the diffused reflection of light, called *haze*. Matsunaga et al. (1) reported that the degree of mycelial penetration into *koji* pellets (internal *haze*) is correlated to the amount of α-amylase production but not correlated to mycelia content and acid protease production. Sudo et al. (2) demonstrated that the correlation between internal *haze* and enzyme production was changed by water absorption for rice. For example, internal *haze* was barely formed at 70% water absorption for rice despite high mycelial content, while glucoamylase and α-amylase had low and moderate productivity, respectively. The amount of mycelial growth on *koji* pellets (surface *haze*) was correlated with mycelial content at 40% water absorption but not at 70% water absorption (2). For *koji* making with 70% water absorption, additional surface and internal *haze* were formed by decreasing water absorption by up to 40% at an earlier stage (approximately 8 h) of the culture (2). When the amount of internal *haze* was high, the *koji* possessed high activity of hydrolytic enzymes (3). In contrast, *koji* with a low amount of internal *haze* possessed high activity of acid protease and acid carboxypeptidase (4). These results suggest that the amounts of internal and surface *haze* have the potential to serve as indicators for favorable *koji* making, leading to improved quality of *koji*. Indeed, *haze* is actually recognized as an important indicator for estimating the quality of *koji* in the manufacturing setting of a sake brewery. Evaluation of the amounts of surface and internal *haze* has been performed by measurement of mycelial content of *koji* pellets polished on the outside (4). However, there is no simple method for this evaluation; thus, it is difficult to apply this method to *koji* making in a manufacturing setting.

To rapidly determine culture conditions for improved quality of *koji*, visual observations, which can simply evaluate *haze* formation, are suitable for *koji* making in a manufacturing setting. The amount of *haze* has been experimentally estimated by visual observation. Although Honda et al. (5) visualized and identified *haze* and non-*haze* sections separately on *koji* pellets based on brightness from illumination with transmitted light, this image analysis could not distinguish between surface and internal *haze*. In contrast, Matsunaga et al. (1) estimated the amount of internal *haze* from photomicrographs of cross sections of *koji* using reflected light.

We developed an original SSF method using a wooden non-airflow box (NAB) with a moisture-permeable expanded polytetrafluoroethylene (ePTFE) membrane (6). This leads to a uniform drying state of the substrate, which promotes a high degree of reproducibility (6,7). In this study, we performed a *koji*

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making test by the SSF method and observed surfaces and cross sections of *koji* pellets using a digital microscope with bright and dark field illumination. The obtained high-resolution images reflected visible *haze* based on the same principle as previously utilized techniques (1), and surface and internal *haze* could be individually distinguished. We established a quantitative evaluation method of *haze* formation for use as an indicator for *koji* making, especially in batch production. Furthermore, the time course of *haze* formation and the influence of *koji* drying on *haze* formation during *koji* making were investigated to understand the details of *haze* formation.

MATERIALS AND METHODS

Fungal strain and culture substrate The *Aspergillus oryzae* RIB128 strain (sake *koji* mold) was used for this study. For preparation of conidiospore suspensions, the strain was grown on potato dextrose (PD) agar plates at 30°C for 10 days. Rice (*Omachi* produced in 2011 in Okayama Prefecture, Japan; polishing ratio, 60%; water content, 11.8%) was used as the culture substrate in *koji* making.

***Koji* making test by SSF system using NAB** Rice (200 g) absorbed with an appropriate volume of water (adjusted to approximately 33 wt% water absorption for rice) was steamed for 40 min. Rice for *koji* was prepared by inoculating a suspension (4 ml) of 1×10^5 conidiospores (in 0.2% Tween 80 and 0.9% NaCl) into the steamed rice for uniform adhesion of spores to that. The initial water absorption of the steamed rice was adjusted to 32–33 wt%. The steamed rice inoculated with conidiospores was incubated with 95% relative humidity (RH) in a non-airflow box (NAB) containing a circular stainless-steel sieve (Φ 190 × H 50 mm) in a temperature and humidity chamber (PL-3SP, Espec Corp., Japan) under static conditions. NAB is a wooden box (W 220 × D 220 × H 150 mm) that opens at the top and bottom sides, which are fitted with a moisture-permeable expanded polytetrafluoroethylene (ePTFE) membrane (Japan Gore-Tex Inc., Japan) (6). Culture conditions during *koji* making were controlled in the chamber with programmed temperature and humidity, as shown in Fig. 1A. The inoculated rice was cultured at 30°C for 24 h, followed by a gradual increase from 30°C to 40°C between 24 and 36 h, and held at 40°C for up to 48 h. RH was maintained at 95% throughout the culture. The deposition thickness of rice was approximately 2.5 cm.

Quantitative evaluation of *haze* formation *Haze* (surface and internal *haze*) of *koji* was observed using a digital microscope (DSX500, Olympus Corp., Japan), with exposure time of 1.00 ms, ISO speed of 200, non-epi-illumination, mix mode of bright and dark fields, and focus stacking mode to obtain an image with a large depth of field. For quantification of the surface *haze* rate on whole *koji* pellets, 100 pellets of *koji* were randomly selected and their surface was micrographed. For quantification of the internal *haze* rate to whole of cross sections of *koji* pellets, a cross section (approximately 0.5 mm in thickness) at the center of a *koji* pellet in a transverse direction was prepared from selected pellets using a sharp razor, and

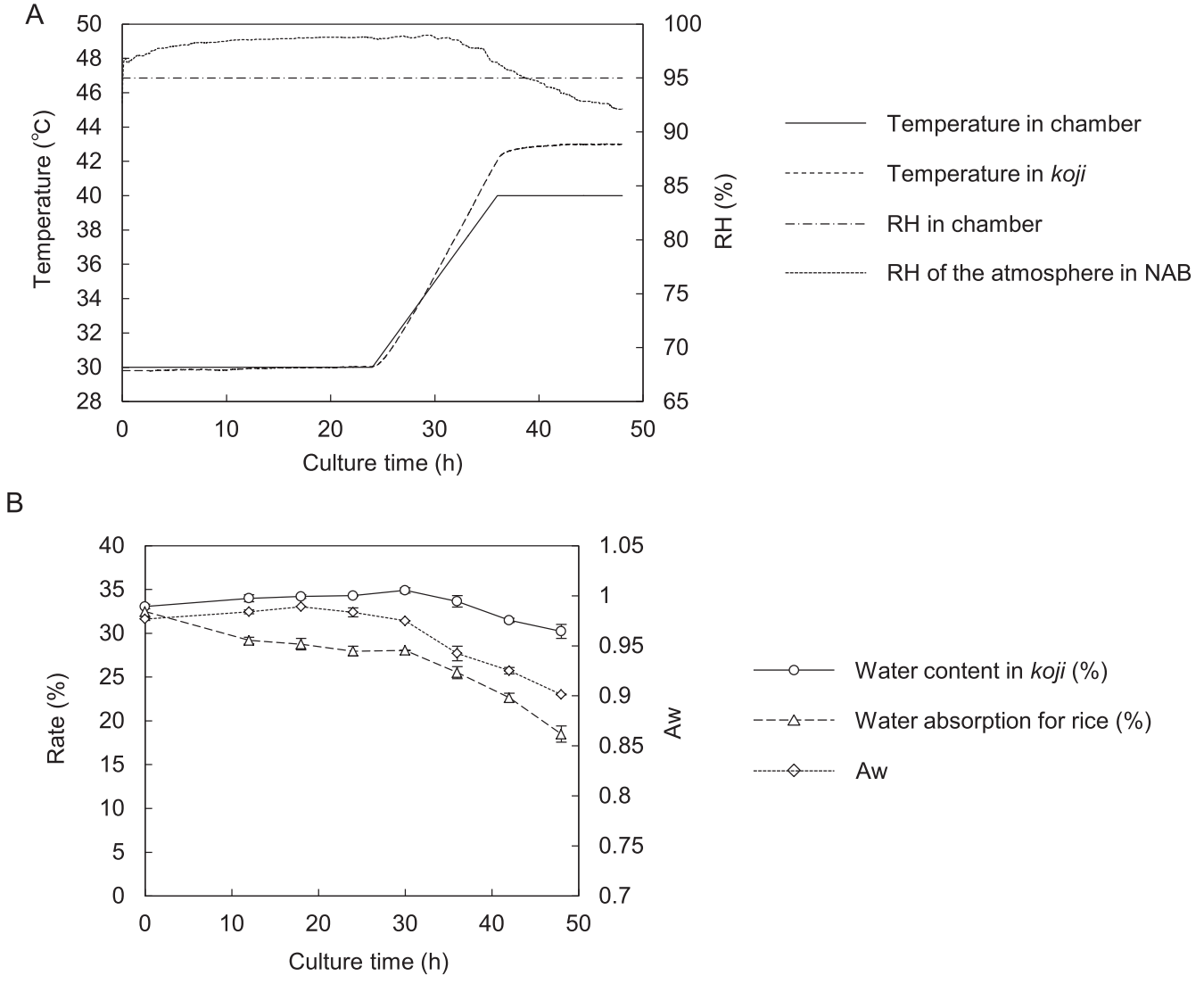

FIG. 1. Profile of measured parameters during *koji* making. (A) Culture scheme and profile of temperature and RH. The culture condition is named "base" in this report. The profile of temperature and RH were collected by data logger equipment (Thermo Pro GR-3000, Keyence Corporation, Japan; RTR-503, T&D Corporation, Japan). (B) Parameters associated with water in *koji*. Water absorption for rice indicates the ratio of water weight after the culture to rice weight before *koji* making. The presented values are averages of 10 independent cultures. Error bars indicate standard deviations (SDs).

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