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Effects of sound waves on conidiospores of *Aspergillus oryzae* strain RIB40 and characterization of the enzyme activity of rice–*koji*

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It was reported that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores of yellow-*koji, Aspergillus oryzae,* at 16 kHz was decreased as compared with that of conidiospores that had not been sound irradiated. To reveal why the glucoamylase activity was changed, we observed the changes of the expression level of *glaB* encoding glucoamylase. We also determined to observe how the germination ratio of conidiospores and the hyphal weight of rice-*koji* were changed by sound waves. When conidiospores were irradiated with sound waves at 16 kHz, the expression level of *glaB* was decreased and the correlation between the expression level of *glaB* and glucoamylase activity was confirmed. When steamed rice was inoculated by sound-irradiated conidiospores, the conidial germination ratio 8 h after inoculation was 1.4 times higher than that of conidiospores that had not been sound irradiated, and the hyphal weight of rice-*koji* was 1.2 times higher than that of conidiospores that had not been sound irradiated. The correlation between the germination ratio and hyphal weight was also confirmed.

Key words: *Aspergillus oryzae* RIB40, glucoamylase activity, rice-*koji*; sound waves.

INTRODUCTION

In Asian countries, a microbial starter called *koji*, which is prepared with various microorganisms, is an important saccharifying agent in the production of fermented foods (Yamashita, 2021). *Aspergillus oryzae* and *Aspergillus sojae* are called *koji*- mold (Abe and Gomi, 2008), and *koji*-mold secretes large amounts of hydrolases, such as α-amylase and glucoamylase (Bennett, 2001). Rice-*koji* that *koji*-mold grown on steamed rice that secretes many enzymes from its hyphae, is widely used to make fermented foods unique to the region, such as Japanese sake and Chinese Shaoxingjiu (Nout and Aidoo, 2002).

The rice-*koji*-making process is affected by various environmental factors, such as light, temperature, and humidity (Okazaki et al., 1979). Among these environmental factors, the effects of sound waves on microorganisms have been highlighted in recent years. For example, it has been reported that when irradiated with low-frequency noise, bacteria representing *Staphylococcus* species exhibit resistance against antibiotic drugs, such as ampicillin (Kim, 2016). Furthermore, it has been reported that the growth of microorganisms involved in fermentation and brewing, such as *Saccharomyces* and *Lactobacillus* species, was enhanced by sound-wave radiation (Noguchi et al., 2011).

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In recent years, acoustic irradiation technology has been utilized in some breweries to improve quality. For example, it has been reported that the stimulation of the alcohol content of shochu is eased and aging is accelerated by irradiating with sound waves during periods of maturation (Noguchi et al., 2011). Despite the fact that the mechanism of the response to sound waves in *Aspergillus* species is unknown, it was reported that the glucoamylase activity of rice-*koji* was decreased as compared with that of rice-*koji* not irradiated when sound waves at 6.3 kHz were irradiated through the hyphal elongation stage of *koji*-mold 20 h after inoculation (Saigusa et al., 2015) (Figure 1B). Moreover, it was reported that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was not changed at 6.3 kHz and was suppressed 0.68 times at 16 kHz as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound (Matsumoto et al., 2021). These results suggested that the response to specific frequency sound waves was different between the stage of the hyphal elongation and the dormant stage of conidiospores. It was also suggested that the dormant stage of conidiospores of *A. oryzae* may respond to sound waves and may affect the enzyme activity of rice-*koji* prepared with sound-irradiated conidiospores. However, it is unclear how sound waves affect the growth of *koji*-mold, such as germination and hyphal elongation.

In this study, we determined to irradiate conidiospores with sound waves in the germination stage and observed how the germination ratio of conidiospores and the hyphal weight of rice-*koji* were changed by sound waves. Moreover, to reveal why the glucoamylase activity of rice-*koji* was changed by sound waves, we observed the changes of expression level of *glaB* encoding glucoamylase.

MATERIALS AND METHODS

Incubation of *koji*-mold on a solid medium

Yellow *koji*-mold, *A. oryzae* RIB40 NBRC100959, was purchased from the NITE Biological Resource Center (Tokyo, Japan). To suspend conidiospores, 1.0 mL of No. 707 liquid medium, described in the Data and Biological Resource Platform (https://www.nite.go.jp/nbrc/cultures/cultures/cultures.html), was prepared; the resultant aliquots were spread onto a potato dextrose agar solid medium (Nissui, Tokyo, Japan). The solid medium was sealed with surgical tape and incubated at 30°C for 5 days in incubator FS-620 (ADVANTEC, Tokyo, Japan).

Irradiation of conidiospores with sound waves and the preparation of rice-*koji*

Aliquots of 0.1 g of collected conidiospores were weighted exactly and added to a 50 ml Erlenmeyer flask with moistened filter paper at the bottom. Then the flask was covered with aluminium foil. The audio generator MINIRATOR MR2 (NTi, Tokyo, Japan) was used to generate sound waves. An earphone (YAZAWA Co. Ltd., Tokyo, Japan) was fixed to the head of the flask with vinyl tape. Conidiospores were irradiated by sound waves with 1.0, 6.3, and 16 kHz at 25°C for 24 h, and the power level was 5.0 dB (Figure 1A). For the control experiment, conidiospores incubated at 25°C in the absence of earphones, that is, not irradiated by sound waves, were prepared.

Aliquots of 100 g of commercial polished rice (*Oryza sativa* var. *Japonica* cv. *Hinohikari*) that was cultivated in Kumamoto Prefecture, Japan, were added to a 300-ml flask containing 30 ml of distilled water and left for 30 min. This aliquot was steamed at 105°C for 15 min using autoclave (BS-245, TOMY, Tokyo, Japan) to prepare steamed rice. After cooling to 40°C, 0.1 g sound-irradiated conidiospores were added to the flask. For the control experiment, 0.1 g conidiospores that had not been sound irradiated were added to the flask. The resulting mixtures were divided into two glass Petri dishes. Unless otherwise noted, rice-*koji* was made by incubating at 30°C for 36 h in a silence condition.

Extraction of enzymes from rice-*koji*

To extract the enzymes solution, 10 g of rice-*koji* was collected every 24, 36 after incubation, and was soaked in a mixture of 50 ml of 0.5% (w/v) sodium chloride solution containing a 0.2 M acetic acid buffer (pH 5.0) at 5°C for 18 h. The resulting extract was filtered and dialyzed with Visking tubing (5 nm, Nihon Medical Science, Osaka, Japan) against 1 L of a 0.01 M acetic acid buffer (pH 5.0) at 5°C for 24 h to remove the sugars or amino acid residue. This extract was used as an enzyme solution.

Determination of the hyphal weight of *A. oryzae* RIB40 cells in rice-*koji*

To reveal how the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores was changed, we measured the hyphal weight of rice-*koji*. Our procedures were carried out in accordance with the methods described in Masuda et al. (2009). To prepare extract containing enzymes, 2.0 g of ground dried rice-*koji* was weighed accurately, suspended with 10 ml of a 50 mM phosphate buffer (pH 7.0) and centrifuged at 2,270 × *g* for 10 min. The supernatant fluid was discarded. These processes were repeated four times to remove the ingredients. To degrade the cell wall of rice-*koji,* 10 mg of Yatalase (Takara Bio Inc., Kusatsu, Japan) was weighted accurately and added to the suspension; it was incubated at 37°C for 4 h with shaking. The resulting mixture was centrifuged at 2,270 × *g* for 10 min to collect the supernatant fluid, and 0.5 mL of the supernatant was added to 0.1 ml of a 0.8 M boric acid buffer and incubated in boiled water for 3 min. To cause a color reaction in N-acetyl-D-glucosamine, 3.0 mL of 10% (v/v) *p*-dimethyl-amino-benzaldehyde was added and incubated at 37°C for 20 min. Absorption was measured at 585 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Determination of the glucoamylase activity of rice-*koji*

To reveal how the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was changed, the glucoamylase activity of rice-*koji* was measured according to the official methods described in Taniguchi (1993). For an enzymatic reaction, 0.1 ml of properly distilled extract solution was mixed with 1.0 ml of 2.0% (w/v) soluble starch solution (Nacalai Tesque, Kyoto, Japan) and incubated at 40°C for 20 min. To cause a color reaction in glucose, 0.1 mL of a reaction mixture was added to 3.0 ml of a quantitative glucose reagent (Glucose CII Test Wako, FUJIFILM Wako, Osaka, Japan) and incubated at 40°C for 30 min. Absorption was measured

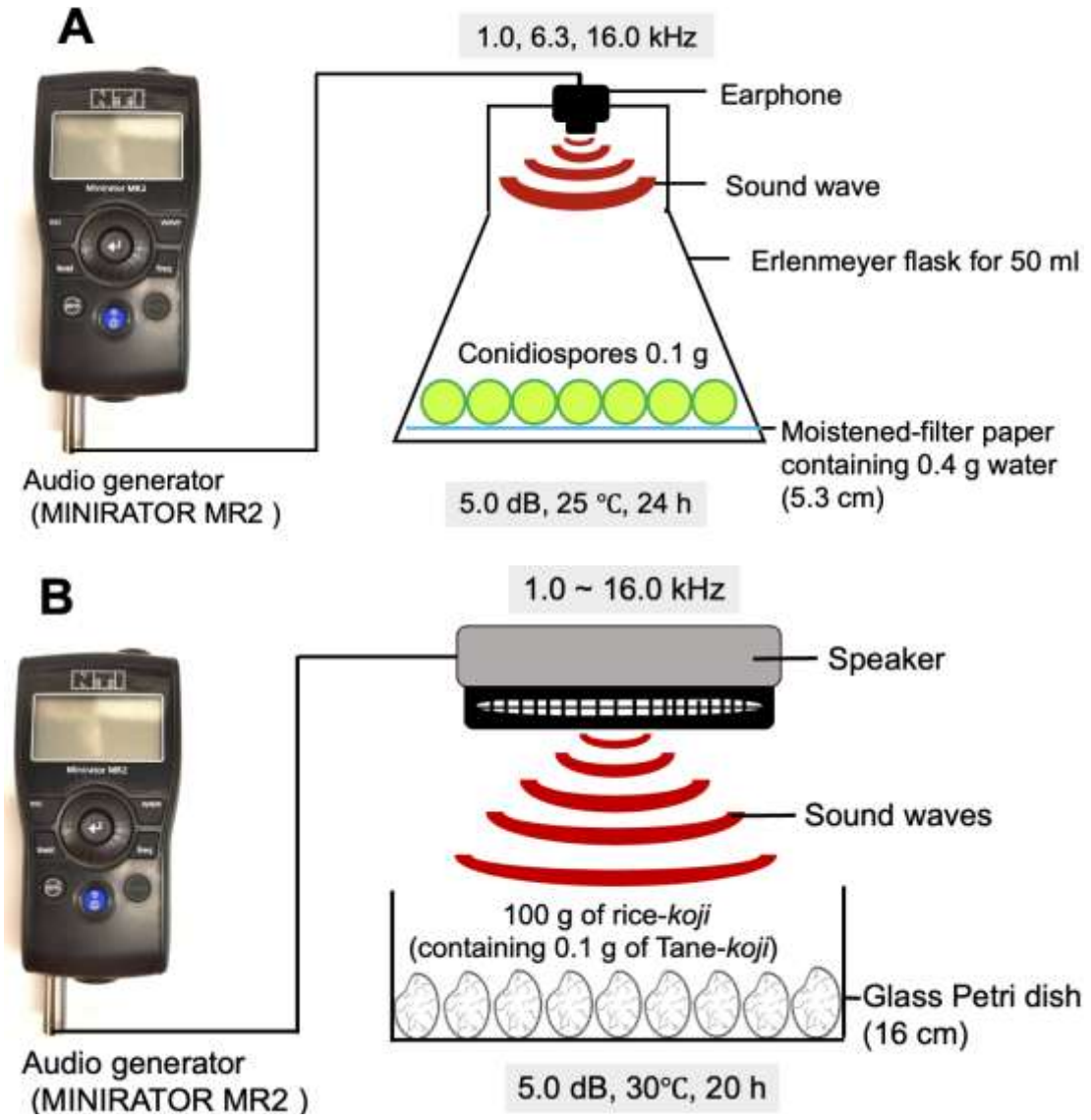

Figure 1. Methods of irradiating koji-*mold* with sound waves. “A” indicates the methods of irradiating *A. oryzae* RIB40 conidiospores with sound waves. To irradiate by sound waves, 0.1 g of conidiospores was aliquoted in a 50 ml Erlenmeyer flask with moistened filter paper on the bottom. The audio generator MINIRATOR MR2 was used to generate sound waves. The power level and frequency of sounds were adjusted. For the output of sounds, an earphone was connected to the head of the flask with vinyl tape. Conidiospores were irradiated with 1.0, 6.3, and 16 kHz sound waves at 25^{o}C for 24 h. “B” indicates the methods of irradiating conidiospores of Tane-*koji* that the stage of the hyphal elongation with sound waves. Rice-koji was prepared with 0.1 g of Tane-*koji* in Glass Petri dish of 16 cm in diameter at 30^{o}C for 20 h. After incubation for 20 h, sound waves was irradiated with the speaker that is connected to the audio generator MINIRATOR MR2 at 30^{o}C for 20 h. The power level and frequency of sounds were adjusted.
Source: Authors

at 505 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Determination of the acid protease activity of rice-*koji*

To reveal how the acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores was changed, the acid protease activity of rice-*koji* was measured according to the official methods described in Taniguchi (1993). For the enzymatic reaction, 0.5 mL of properly diluted extract solution was mixed with 1.0 ml of 2.0% (w/v) casein solution (Nacalai Tesque, Kyoto, Japan) and incubated at 40°C for 60 min. To stop the enzymatic reaction, 3.0 mL of 0.4 M trichloroacetic acid solution (Nacalai Tesque, Kyoto, Japan) was added. The resulting mixture was filtered. To cause a color reaction in tyrosine, 1.0 mL of the filtrate was mixed with 5.0 ml of 2.2 M sodium carbonate solution and 1.0 ml of 0.9 N Folin-Ciocalteu Reagent (Nacalai Tesque, Kyoto, Japan). This mixture was

Table 1. Designed primers for each gene of *A. oryzae* RIB40

mRNA to be detected	Primer Name	Primer Sequence (5'-3')
AO090003000321	Aor_glaB_+488_FW	5'-TCGCATATGGCAACTCTCTG-3'
AO090003000321	Aor_glaB_+891_RV	5'-GCAGGGCTGGAATGTTGTAT-3'
AO090701000065	Aor_actA_+95_FW	5'-GTATCGTTCTGGATTCTGGTGAC-3'
AO090701000065	Aor_actA_+1333_RV	5'-AGAGATCCTTACGGACATCAACA-3'

Source: Authors

incubated at 40°C for 30 min in the dark. Absorption was measured at 660 nm with spectrophotometer U-1800 (Hitachi High-Tech Inc., Tokyo, Japan).

Extraction of total RNA from conidiospores

To reveal whether dormant stage of conidiospores respond to sound waves, we observed the accumulation of the expression product of *glaB* encoding glucoamylase. To extract total RNA, 0.1 g of sound-irradiated conidiospores was pulverized with a pestle and mortar cooled by liquid nitrogen. Total RNA was extracted according to instructions for the RNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan). The quantity of prepared total RNA was determined by using a BioPhotometer (Eppendorf, Japan).

Semi-quantitative RT-PCR and electrophoresis

Primers for gene-encoding glucoamylase (*glaB*) were designed using Primer3Plus (https://www.primer3plus.com/). Genome sequences were referenced in the *Aspergillus* Data Base (http://www.aspgd.org/), and are currently available in the Fungi Data Base (https://fungidb.org/fungidb/app). Normalization of the expression level of the gene was performed with gene-coding actine (*actA)*. The expression was detected by PCR using a pair of oligonucleotide primers listed in Table 1. KOD-Plus-Neo (TOYOBO Co., Ltd., Osaka, Japan) was used as the DNA polymerase for PCR. Degradation of the DNA of the total RNA solution was performed in accordance with the instructions for Deoxyribonuclease (RT Grade) for Heat Stop (Nippon Gene, Tokyo, Japan). Reverse transcription reaction was performed in accordance with the instructions for PrimeScript IV First Strand cDNA Synthesis Mix (Takara Bio Inc., Kusatsu, Japan). Semi-quantitative RT-PCR was performed in accordance with the instructions for the Takara PrimeScript RT-PCR Kit (Takara Bio Inc., Kusatsu, Japan). To perform electrophoresis, agarose gel containing 1.0% (w/v) agarose was prepared with 0.5 × TAE buffer. Electrophoresis was performed using a WSE-1710 Submerge-Mini (ATTO, Tokyo, Japan) at a voltage of 50 V for 30 min. Gene Ladder Wide 1 (Nippon Gene, Tokyo, Japan) was used as a ladder marker. GX GelRed Prestain Loading Buffer with Blue (Cosmo Bio, Tokyo, Japan) was used to stain samples, and the fluorescence of samples was detected by Gel Scene GS-GU (ASTEC, Fukuoka, Japan). To quantify the expression levels of *glaB*, qTOWER^{3}G Real-Time Thermal Cycler (Analytik Jena, Kanagawa, Japan) was used to perform real-time PCR. PCR reaction was performed in accordance with the protocol of TB Green Premix Ex Taq^{TM} II (Takara, Tokyo, Japan).

Microscopic observation of conidiospores

To reveal how the germination ratio of conidiospores was changed by sound waves, the conidiospores were observed microscopically in accordance with the protocol for the Thoma hemocytometer (Minato Medical, Tokyo, Japan). To incubate rice-*koji,* 0.2 g of sound-irradiated conidiospores was inoculated onto 10 g of steamed rice. To collect conidiospores, 0.5 g of the resulting rice-*koji* was recovered in test tubes at 0, 2, 4, 6, and 8 h after incubation and suspended with 0.2 ml of deionized water containing 0.1% (v/v) Tween 80 (Nacalai Tesque, Kyoto, Japan) and stirred. The supernatant obtained by stirring was collected and diluted threefold with prepared deionized water in another test tube. To observe the conidiospores under a glass cover, 20 µl of the resulting suspension was placed on the center of the Thoma hemocytometer. An Olympus BH-2 Microscope (Olympus, Tokyo, Japan) was used for microscopy.

Statistical significance test

The difference between the sound and non-sound conditions was examined by a statistical significance test (*t*-test) (Sakoda et al., 1954). Each value is presented as the mean ± standard deviation of three independent experiments (n = 3). The error range was calculated from the standard deviation of three samples. A significant difference was recognized when the one-sided test was less than 0.05 ($P < 0.05$).

RESULTS

Effects of sound waves on the expression level of *glaB*

During the germination stage, conidiospores were irradiated at 1.0, 6.3, and 16 kHz for 24 h on the moistened filter paper. Then, we determined to observe the changes in the expression level of *glaB* and compare it with that of conidiospores not irradiated by sound.

When conidiospores were irradiated with sound waves at 1.0 kHz, the expression level of *glaB* was increased as compared with that of conidiospores that had not been irradiated by sound. The quantitative result of real-time PCR indicated that the expression level of *glaB* was not changed (data not shown).

When conidiospores were irradiated with sound waves at 6.3 kHz, the expression level of *glaB* was not changed as compared with that of conidiospores not irradiated by sound. The quantitative result of real-time PCR indicated that the expression level of *glaB* was decreased by 0.75 times as compared with that of conidiospores not irradiated by sound (data not shown).

When conidiospores were irradiated with sound waves at 16 kHz, the expression level of *glaB* was suppressed

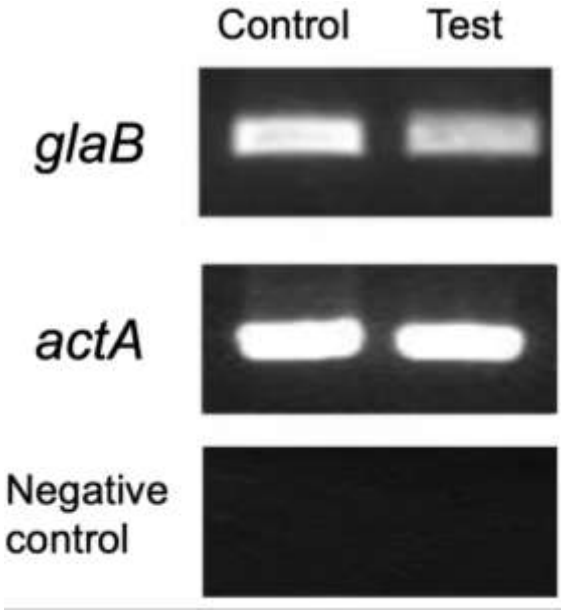

Figure 2. Effects of sound waves on the expression level of *glaB* encoding glucoamylase in *Aspergillus oryzae* RIB40. The gene expression levels were normalized by the expression level of *actA* and were evaluated via semi-quantitative RT-PCR analysis. The mRNA extracted from conidiospores not irradiated by sound is labeled “Control.” The mRNA extracted from conidiospores sound irradiated at 16 kHz is labeled “Test.” Total RNA of which reverse transcriptional reactions were performed without reverse transcriptase is labeled "Negative control" to ensure that contaminant DNA was not present in the samples.
Source: Authors

as compared with that of conidiospores not irradiated by sound (Figure 2). The quantitative real-time PCR result indicated that the expression level of *glaB* was changed by 1.28 times as compared with that of conidiospores not irradiated by sound (data not shown).

These results suggest that the expression level of *glaB* showed different changes in response to sound waves at different frequencies.

Effects of sound waves on glucoamylase activity

Rice-*koji* was prepared with conidiospores irradiated by sound waves during the germination stage for 24 h on moistened filter paper. Then we determined to observe the changes in the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores and compared with those of rice-*koji* prepared with conidiospores not irradiated by sound. Rice-*koji* was collected at 24 and 36 h after inoculation, and changes in the glucoamylase activity over time were observed during the rice-*koji*-making process (Figure 3).

In the case of sound waves at 1.0 kHz, the glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound (that is, in the control condition) were 164 ± 46.7 and 444 ± 83.4 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz, the glucoamylase activities were, respectively, 1.1, 1.1 times higher than those of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was not observed, and glucoamylase activity was enhanced at 24 and 36 h after inoculation. It was suggested that irradiating sound waves at 1.0 kHz may enhance the glucoamylase activity of rice-*koji.*

In the case of sound waves at 6.3 kHz, the glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound were 112 ± 33.3 and 314 ± 23.1 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, glucoamylase activities were, respectively, 0.9 and 1.2 times changed as compared with those of rice-*koji* made with conidiospores not irradiated by sound. An increase in the glucoamylase activity was observed during the first 36 h after incubation.

In the case of sound waves at 16 kHz, the

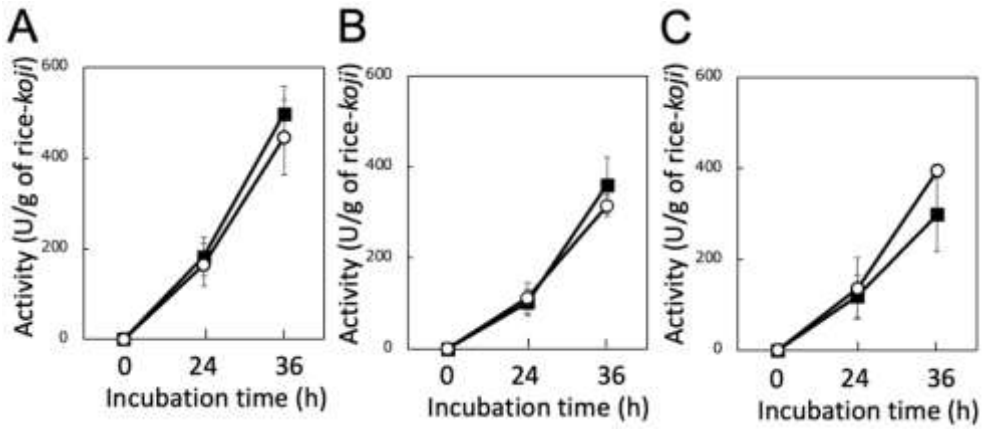

Figure 3. Changes in the glucoamylase activity of *A. oryzae* RIB40. Circles indicate the glucoamylase activity of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition). Black squares indicate the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate activity showing the color reaction of a 1 µg/ml glucose equivalent at 40℃ for 60 min from soluble starch, defined as (units/g of rice-*koji*). The horizonal axis indicates time after incubation to make rice-*koji* was started. Absorption was measured at 505 nm.
Source: Authors

glucoamylase activities of rice-*koji* prepared with conidiospores not irradiated by sound were 143 ± 62.3 and 391 ± 12.5 (units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 16 kHz, glucoamylase activities were, respectively, 0.9 and 0.8 times less than that of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was not observed, glucoamylase activity was decreased at 24 and 36 h after inoculation.

These results suggest that sound waves given to conidiospores during the germination stage affect the glucoamylase of rice-*koji.* Moreover, these results suggest that the effects of sound waves were reflected in the glucoamylase activity from 24 to 36 h after inoculation.

Effects of sound waves on acid protease activity

Before the rice-*koji*-making process, conidiospores during the germination stage were irradiated with sound waves for 24 h on moistened filter paper in advance. We determined to observe the changes of the acid protease activity of rice-koji prepared with sound-irradiated conidiospores as compared with those of rice-*koji* prepared with conidiospores that had not been irradiated by sound. Rice-koji was collected at 24 and 36 h after inoculation, and changes in the acid protease activity over time during the rice-*koji*-making process were observed (Figure 4).

In the case of sound waves at 1.0 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition) were 58.8 ± 9.90 and 90.7 ± 7.68 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz in advance, the acid protease of rice-*koji* was not changed at any time.

In the case of sound waves at 6.3 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound were 49.2 ± 8.04 and 94.9 ± 3.89 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, the acid protease activity was 6.3 kHz in advance, and the acid protease of rice-*koji* was not changed at any time.

In the case of sound waves at 16 kHz, the acid protease activities of rice-*koji* prepared with conidiospores not irradiated by sound wave were 47.1 ± 4.83 and 83.7 ± 5.02 (K units/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 16 kHz in advance, the acid protease activity of rice-*koji* was not changed at any time.

Effects of sound waves on germination ratio

To reveal the effects of sound waves on conidiospores during the germination stage on the germination ratio of conidiospores, we determined to inoculate steamed rice with sound-irradiated conidiospores. We also compared that with the effects of conidiospores not irradiated by sound. The conidiospores were collected at 2, 4, 6, and 8 h after inoculation (Figure 5).

In the case of sound waves at 1.0 kHz, the germination ratios of conidiospores not irradiated by sound were 0.67 ± 0.12, 6.79 ± 7.64, 20.2 ± 5.22, 23.9 ± 10.7, and 26.6 ± 14.3%, respectively. When conidiospores were irradiated with sound waves at 1.0 kHz, the germination ratios were,

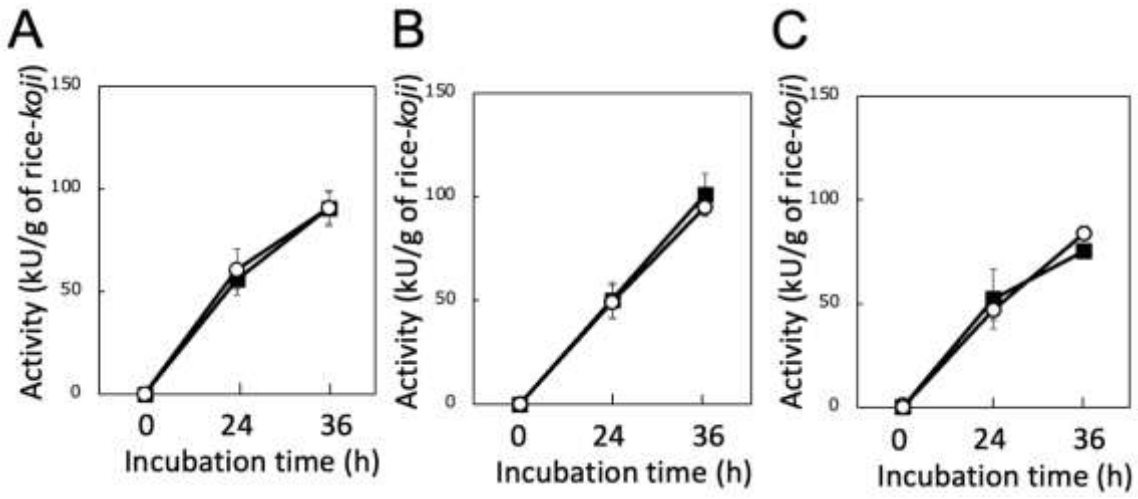

Figure 4. Changes in the acid protease activity of *A. oryzae* RIB40. Circles indicate the acid protease activity of rice-*koji* prepared with conidiospores not irradiated by sound (that is, the control condition). Black squares indicate the acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate the activity showing the color reaction of a 1 µg/ml tyrosine equivalent at 40°C for 60 min from casein, defined as (K units/g rice-*koji*). The horizonal axes indicate time after incubation to make rice-*koji* was started. Absorption was measured at 660 nm.
Source: Authors

respectively, 0.9, 1.6, 0.7, 0.7, and 1.0 times changed as compared with those conidiospores not irradiated. It was suggested that sound waves at 1.0 kHz may suppress the germination ratio from 4 to 6 h after inoculation and the effect of sound waves on the germination ratio was lost by 8 h after incubation.

In the case of sound waves at 6.3 kHz, the germination ratios of conidiospores not irradiated by sound were 0.81 ± 0.12, 2.42 ± 0.51, 8.77 ± 3.82, 10.4 ± 2.26, and 13.0 ± 0.87%, respectively. When conidiospores were irradiated with sound waves at 6.3 kHz, the germination ratios were, respectively, 0.7, 1.1, 0.9, 0.8, and 0.9 times changed as compared with those of non-irradiated conidiospores. It was suggested that sound waves at 6.3 kHz have a slight inhibitory effect on the germination ratio from 4h to 6 h after inoculation.

In the case of sound waves at 16 kHz, the germination ratios of conidiospores not irradiated by sound were 0.71 ± 0.17, 3.90 ± 2.62, 15.8 ± 5.94, 15.3 ± 5.07, and 19.2 ± 7.62%, respectively. When conidiospores were irradiated with sound waves at 16 kHz, the germination ratios were, respectively, 0.9, 0.8, 1.2, 1.5, and 1.4 times changed as compared with those of conidiospores not irradiated by sound wave. It was suggested that sound waves at 16 kHz may enhance the germination ratio from 4 to 8 h after inoculation. A significant difference was not observed.

These results suggest that sound waves given to conidiospores during the germination stage affect the germination ratio. Moreover, it was suggested that the effect of sound waves were reflected in the germination ratio from 4 to 6 h after inoculation, and the effect of sound waves at 16 kHz was maintained even after 6 h after inoculation.

Effects of sound waves on hyphal weight

To reveal the effects of sound waves on the hyphal elongation of conidiospores during the later stage of growing on steamed rice, we determined to observe the changes in hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores as compared with those of rice-*koji* that had not been irradiated by sound. Rice-*koji* was collected at 24 and 36 h after inoculation (Figure 6).

In the case of sound waves at 1.0 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave were 7.47 ± 1.61 and 15.2 ± 1.00 (µg/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 1.0 kHz in advance, the hyphal weight of rice-*koji* was not changed at any time.

In the case of sound waves at 6.3 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave were 5.59 ± 0.37 and 9.27 ± 0.53 (µg/g of rice-*koji*), respectively. When conidiospores were irradiated with sound waves at 6.3 kHz in advance, the hyphal weight of rice-*koji* was not changed at any time.

In the case of sound waves at 16 kHz, the hyphal weights of rice-*koji* prepared with conidiospores not irradiated by sound wave 6.70 ± 1.54 and 11.4 ± 0.21 (µg/g of rice-*koji*), respectively. When conidiospores were

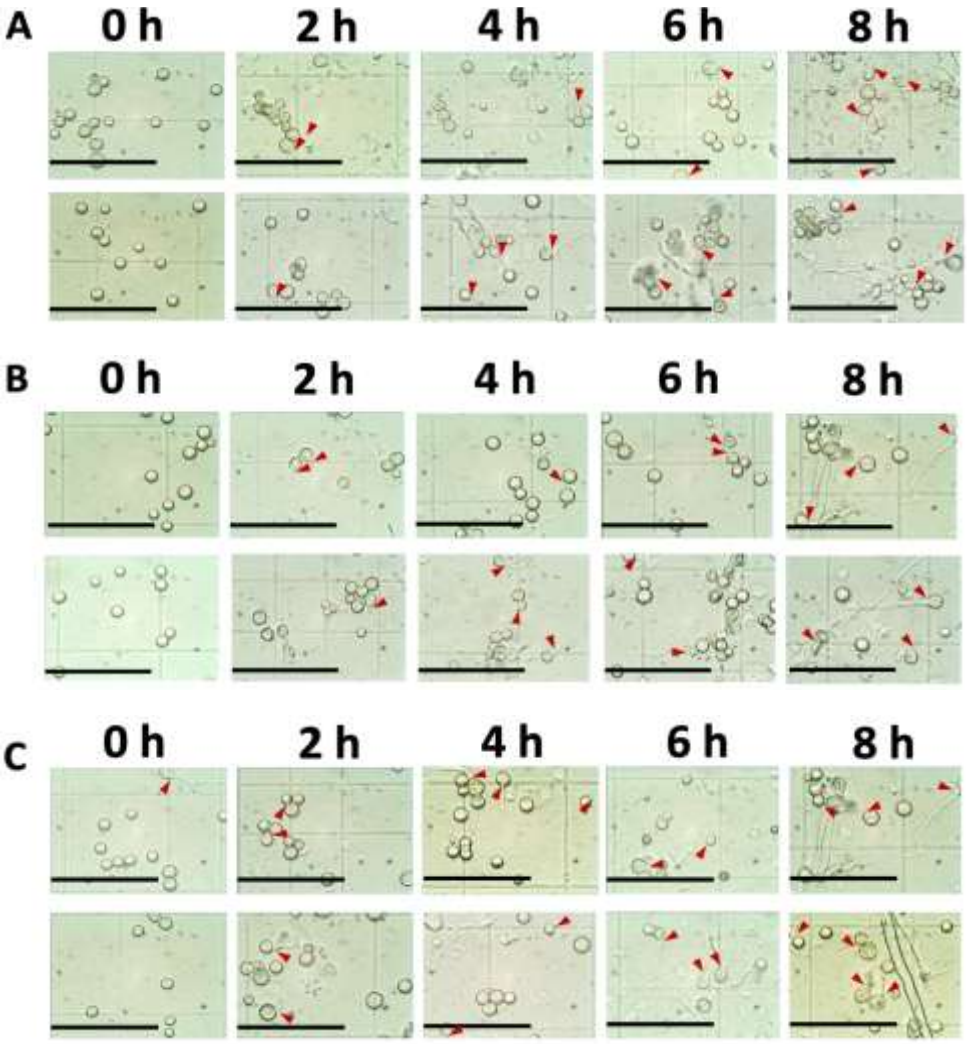

Figure 5. Changes in the germination ratio of conidiospores of *A. oryzae* RIB40. The upper panels show conidiospores not irradiated by sound (that is, the control condition) at 0, 2, 4, 6, and 8 h after the incubation to make rice-*koji* was started. The lower panels show sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. The black bars indicate 50 µm. The triangles indicate germinated cells.
Source: Authors

irradiated with sound waves at 16 kHz in advance, the hyphal weights were, respectively, 1.1 and 1.2 times higher than those of rice-*koji* prepared with conidiospores not irradiated by sound. A significant difference was observed ($P < 0.05$) in the hyphal weight at 36 h after incubation.

These results suggest that sound waves at 16 kHz given to conidiospores during the germination stage affect the hyphal elongation from 24 to 36 h after inoculation.

DISCUSSION

The effects of sound waves on hyphal weight, enzyme activities, and germination ratio at each frequencies are summarized in Tables 2 to 5. When the effects of sound waves on each parameter were compared, interesting correlations were found.

Correlation between the expression level of *glaB* and glucoamylase activity

It was reported that the expression level of *glaB* that codes glucoamylase was not usually changed from the dormant stage to the germination stage of conidiospores and was enhanced at the hyphal elongation stage, which is a later phase of conidiospores in *Aspergillus* spp. (Leeuwen et al., 2012). In the hyphal elongation

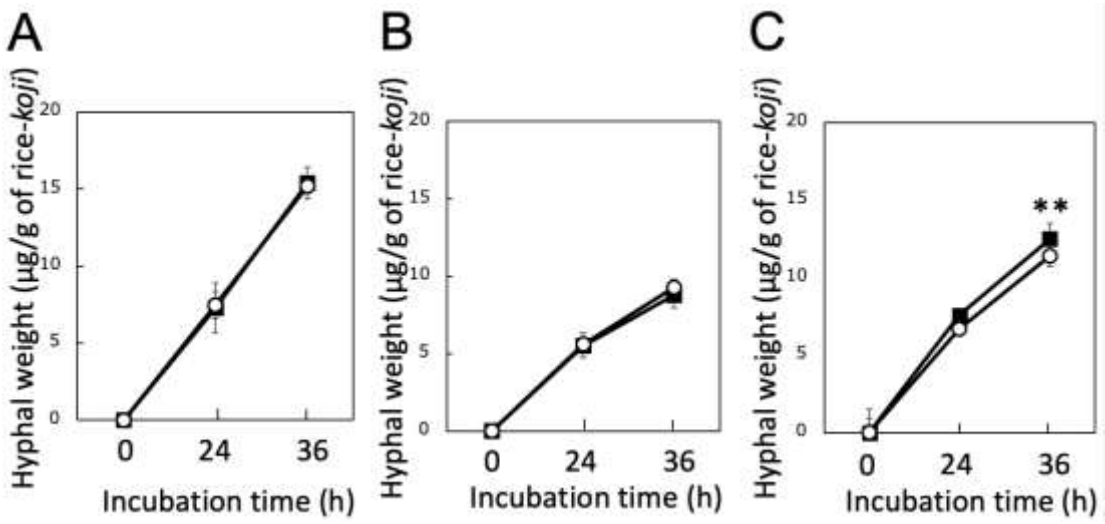

Figure 6. Changes in the hyphal weight of *A. oryzae* RIB40. Circles indicate the hyphal weight of rice-*koji* prepared with conidiospores that were not irradiated by sound (that is, the control condition). Black squares indicate the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores at frequencies of 1.0, 6.3, and 16 kHz (A, B, and C, respectively) for 24 h. Vertical axes indicate the hyphal weight contained in 1.0 g of rice-*koji*, defined as (µg/g of rice-*koji*). Asterisks (**) indicate a significant difference ($P < 0.05$). The horizonal axes indicate time after incubation to make rice-*koji* was started. Absorption was measured at 585 nm.
Source: Authors

Table 2. The ratio of changes in glucoamylase activity.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	2.71 ± 0.40
	Test	2.73 ± 0.38
6.3	Control	2.80 ± 1.01
	Test	3.53 ± 1.41
16	Control	2.71 ± 1.17
	Test	2.27 ± 0.89

"Control" indicates the glucoamylase activity of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores. Numbers indicate the ratios of glucoamylase activity increases produced from 24 to 36 h after inoculation to make rice-*koji*.
Source: Authors

stage of *koji*-mold, it was reported that the expression level of *glaB* was regulated by transcriptional factors, such as AmyR and FlbC (Gomi et al., 2000). According to Gomi et al. (2000), in eutrophic conditions, when transcriptional factor AmyR was activated by isomaltose, the expression levels of genes that codes starch hydrolytic enzymes, such as *amyA/B/C, glaA/B,* and *agdA,* were enhanced by activated AmyR. In this study, we suggested that during the germination stage, conidiospores were irradiated by sound waves at 16 kHz for 24 h on moistened filter paper; the expression level of *glaB* was suppressed as compared with that of conidiospores that had not been irradiated by sound. This result suggests that the expression level of *glaB* was unchanged from the dormant stage to the germination stage of conidiospores but was changed by irradiating sound waves. Quantitative results of real-time PCR indicated a 1.28-times changes in the expression level of *glaB* as compared with that of conidiospores that had not been irradiated by sound. Because the scale of changes was considered to be small, we would like to continue the experiment to confirm the decrease in the expression level of *glaB* by real-time PCR as well.

When the changes of glucoamylase activities of rice-

Table 3. The ratio of changes in acid protease activity.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	1.49 ± 0.28
	Test	1.62 ± 0.01
6.3	Control	1.93 ± 0.54
	Test	2.01 ± 0.67
16	Control	1.78 ± 0.25
	Test	1.45 ± 0.37

"Control" indicates the acid proteases of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the acid proteases of rice-*koji* prepared with sound-irradiated conidiospores. Numbers indicate the ratios of acid protease activity increases produced from 24 h to 36 h after inoculation to make rice-*koji*.
Source: Authors

Table 4. The ratio of changes in conidial germination.

Frequency (kHz)		Period (h)			
		0-2	2-4	4-6	6-8
1.0	Control	10.1 ± 1.08	2.97 ± 0.61	1.18 ± 0.24	1.11 ± 0.09
	Test	12.1 ± 6.50	2.03 ± 1.15	1.24 ± 0.25	1.44 ± 0.24
6.3	Control	2.97 ± 0.67	3.63 ± 2.27	1.19 ± 0.60	1.25 ± 0.20
	Test	4.78 ± 3.15	2.88 ± 0.93	1.05 ± 0.49	1.57 ± 0.23
16	Control	5.52 ± 5.75	4.06 ± 1.06	1.00 ± 0.08	1.25 ± 0.09
	Test	4.69 ± 0.67	5.99 ± 1.49	1.21 ± 0.09	1.20 ± 0.13

"Control" indicates the germination ratio of conidiospores not irradiated by sound. "Test" indicates the germination ratio of sound-irradiated conidiospores. Numbers indicate the ratio of increase in conidial germination from the start of the rice-*koji*-making process to 0, 2, 4, 6 and 8 h afterward.
Source: Authors

Table 5. The ratio of changes in hyphal weight.

Frequency (kHz)		Period (h)
		24-36
1.0	Control	2.03 ± 0.13
	Test	2.12 ± 0.25
6.3	Control	1.66 ± 0.07
	Test	1.59 ± 0.07
16	Control	1.65 ± 0.37
	Test	1.70 ± 0.20

"Control" indicates the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound. "Test" indicates the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores. Numbers the ratio of changes in hyphal weight of rice-*koji* from 24 h to 36 h after inoculation to make rice-*koji*.
Source: Authors

koji prepared with conidiospores not irradiated by sound were observed, it was found that the largest increase of glucoamylase activity was observed from 24 to 36 h after inoculation (Table 2). In the case of sound waves at 16 kHz, the ratio of increase in glucoamylase activity from 24 to 36 h after inoculation was suppressed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound.

In the case of rice-*koji* prepared with conidiospores not irradiated by sound, the expression level of *glaB* was relatively enhanced accompanied by the increase of glucoamylase activity of rice-*koji* from 24 to 36 h after inoculation. However, due to the irradiating sound waves at 16 kHz on conidiospores during the germination stage, the rice-*koji*-making process begin with a suppressed expression level of *glaB*. Therefore, it was considered that when sound-irradiated conidiospores were incubated in eutrophic conditions, such as those found in steamed rice, the glucoamylase activity of rice-*koji* was also relatively decreased as compared with that of rice-*koji* not irradiated by sound. These results suggest that sound waves given to conidiospores during the germination stage affect the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores.

Effects of sound waves on glucoamylase activity in relation to hyphal weight

From the result that the glucoamylase activity of rice-*koji* prepared with sound-irradiated conidiospores was changed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound, the hyphal weight was also relatively changed. Therefore, we determined to reveal the correlation between hyphal weight and the glucoamylase activity of rice-*koji.*

It was reported that hyphal weight usually began to increase 20 h after inoculation and continued to increase exponentially until 40 h after inoculation (Yoshii et al., 1973). After 40 h, the hyphal weight transitioned to the stationary phase, and the changes in hyphal weight slowed. In this study, we observed that the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound increased from 24 to 36 h after inoculation (Table 5). When conidiospores were irradiated by sound waves in advance, the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores was not changed as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. From this result, we found that sound waves given to conidiospores during the germination stage did not affect the hyphal cell growth of rice-*koji*. In the case of the effect of sound waves at 16 kHz, as compared with changes in the glucoamylase activity, as shown in Figure 3, glucoamylase activity was suppressed from 24 h after inoculation as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound, even though hyphal cell growth had not changed. The correlation between hyphal cell growth and the glucoamylase activity of *koji*-mold has been confirmed (Iwano et al., 2002). In this study, we observed that the glucoamylase activity in relation to the hyphal weight of rice-*koji* prepared with conidiospores not irradiated by sound increased over time. When rice-*koji* was prepared with conidiospores sound irradiated at 16 kHz, it was found that the glucoamylase activity in relation to the hyphal weight of rice-*koji* was suppressed over time during the rice-*koji*-making process. From these results, by irradiating conidiospores with sound waves, the ability to produce glucoamylase in relation to the hyphal cell growth of rice-*koji* was suppressed.

Moreover, according to the results shown in Figure 6, even though hyphal cell growth was not changed, the hyphal weight of rice-*koji* prepared with conidiospores sound irradiated at 16 kHz was increased from 24 h after inoculation as compared with that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. This result suggests that, by irradiating conidiospores with sound waves at 16 kHz, the increase in the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores may begin at a point in time before 20 h.

Effects of sound waves on acid protease activity in relation to hyphal weight

The effects of sound waves on acid protease activity in relation to the hyphal weight of rice-*koji* prepared with sound-irradiated conidiospores were investigated. It was reported that acid protease activity was usually increased from 16 h after inoculation and was actively enhanced from 20 to 32 h after inoculation, and then transitioned to the stationary phase, when the changes of acid protease activity became slower (Yoshii et al., 1973). In this study, we observed that the acid protease activity of rice-*koji* prepared with conidiospores not irradiated by sound was increased from 24 to 36 h (Table 3).

As compared with acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores, it was found that the ratio of increase of acid protease activity was changed from 24 to 36 h after inoculation. The effects of sound waves were different according to its frequency. In the case of 1.0 kHz, the ratio of the increase in acid protease activity was enhanced as compared with that of rice-*koji* prepared with sound-irradiated conidiospores from 24 to 36 h after inoculation. In the case of 16 kHz, the ratio of increase of acid protease activity was suppressed as compared with that of rice-*koji* prepared with sound-irradiated conidiospores from 24 to 36 h after inoculation. It was considered that the changes in the acid protease activity of rice-*koji* become slower 32 h after inoculation with or without irradiating sound waves.

As compared with the hyphal weight results, even though the hyphal elongation stage remained constant with and without sonication (Table 5), the ratio of the

increase in acid protease activity of rice-*koji* prepared with sound-irradiated conidiospores at 16 kHz was suppressed from 24 to 36 h after inoculation. This result indicated that the acid protease activity in relation to hyphal weight was changed by irradiating sound waves on the conidiospores during the germination stage.

Correlation between germination ratio and hyphal weight

From the results of Figure 6 and Table 5, when conidiospores were irradiated by sound waves at 16 kHz, the hyphal elongation of rice-*koji* prepared with sound-irradiated conidiospores took place earlier than that of rice-*koji* prepared with conidiospores that had not been irradiated by sound. We investigated the correlation between the germination ratio and hyphal weight.

When conidiospores of *Aspergillus* spp. were exposed to a eutrophic environment with adequate water and temperature, such as complete medium (CM), during the dormant stage for 2 h after inoculation, the conidiospores were reported to have moved into the isotropic growth stage after 4 h. The germination stage began after 6 h, and the polarized growth stage took place at the end of an 8 h period (Leeuwen et al., 2012). When conidiospores were irradiated by sound waves on moistened filter paper, the germination ratio was between 0.5 and 2.5%. These results showed that the sound-irradiated conidiospores on the moistened filter paper correspond to the isotropic growth stage while preparing for germination to the early germination stage. When steamed rice was inoculated with non-irradiated conidiospores placed on moistened filter paper for 24 h, the conidial germination stage was observed 2 h after inoculation, and the hyphal elongation stage was observed 6 h after inoculation (Figure 5). It was also found that the largest increase in the germination ratio was observed from 0 to 4 h after inoculation (Table 4). When conidiospores were irradiated by sound waves at 16 kHz during the germination stage on moistened filter paper for 24 h, it was found that the germination ratio increase was enhanced as compared with that of conidiospores not irradiated by sound from 4 h after inoculation on steamed rice. On the other hand, according to the result earlier stated (effects of sound waves on the germination ratio), the germination ratio of sound-irradiated conidiospores was enhanced as compared with that of non-irradiated conidiospores 4 h after inoculation on steamed rice. From these results, we suggested that by irradiating conidiospores with 16 kHz sound waves during the germination stage, their germination ratio was enhanced, and hyphal elongation occurred at an earlier stage than that of non-irradiated conidiospores. Therefore, we considered that the amount of hyphal weight during the same rice-*koji*-making may be enhanced.

It was also suggested that the expression level of genes related to germination, e.g., *flbC* and *medA* may be affected by sound waves. It was reported that *flbC* works as a positive regulator of *brlA,* and *med*A is a gene involved in the beginning of germination that was regulated by transcriptional activator BrlA (Leeuwen et al., 2012). The expression level of *medA* was increased during the germination process from 2 to 6 h after inoculation. When conidiospores were irradiated by 16 kHz sound waves, the germination ratio was increased from 2 h after incubation. Therefore, it was indicated that the expression level of *medA* also may be increased by sound-wave irradiation. An experiment designed to investigate the effects of sound waves on the expression levels of genes involved in the beginning of germination is being planned.

Conclusion

These results suggest that sound waves may be an important environmental factor in the rice-*koji*-making process. When conidiospores were irradiated by sound waves during the germination stage, the expression level of *glaB* was changed as compared with that of conidiospores not irradiated by sound. This result suggests that conidiospores may respond to sound waves during the germination stage.

Moreover, it was also suggested that the hyphal weight and enzyme activity of rice-*koji* prepared with sound-irradiated conidiospores were changed as compared with those of rice-*koji* prepared with conidiospores not irradiated by sound. This suggests that sound waves during the germination stage of conidiospores affected the hyphal elongation and enzyme activity of rice-*koji* when sound-irradiated conidiospores were added on steamed rice. In the case of sound waves at 16 kHz, the germination ratio of conidiospores was enhanced and hyphal elongation occurred at an earlier stage than that of conidiospores not irradiated by sound; and the ability to produce glucoamylase in relation to hyphal cells of rice-*koji* was suppressed.

These results may be applied to the preparation of rice-*koji* that exhibits the desired enzyme activity. We think these results can contribute to the development of a new technology of rice-*koji*-making that controls the hyphal elongation and enzyme activity of koji-*mold* via irradiating of sound waves. Further work is needed to elucidate the detailed mechanisms of the response to specific frequencies of sound waves. This research is expected to contribute to the practical application of adjunct technology that will improve the quality of brewed beverages.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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