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A METHOD OF ENHANCING THE GLUCOAMYLASE ACTIVITY AND THE GERMINATION RATIO OF RICE-*KOJI* VIA EXPOSURE TO SOUND WAVES

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ABSTRACT

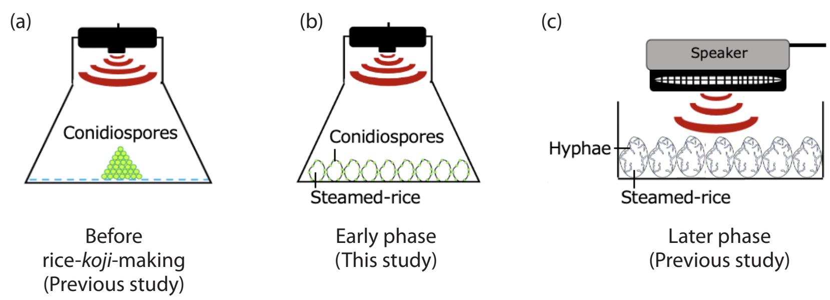
Research has shown that sound can influence microorganisms' growth and enzymatic activity. In this study, the glucoamylase activity of rice-*koji* was decreased when conidiospores of *Aspergillus oryzae* were exposed to sound irradiation during incubation. While this indicates an indirect effect of sound on glucoamylase activity, the possibility of a direct influence remains unclear. To explore this, rice-*koji* was prepared with direct sound irradiation at a sound pressure of 5.0 dB for 24 hours during the early stages of production. Results indicated a 1.3-fold increase in glucoamylase activity after incubation, with no change in hyphal weight. Sound waves at 16 kHz were also observed to enhance the germination ratio.

Keywords: *aspergillus oryzae* RIB40*,* rice-*koji*, glucoamylase activity, sound waves

INTRODUCTION

The microorganisms, such as *Aspergillus oryzae*, were called "*koji*-molds," and *koji*-molds secrete large amounts of hydrolases, such as a-amylase and glucoamylase [1]-[3]. There are many species of *koji*-molds. For example, *Aspergillus sojae* is utilised in the fermentation and maturation of Miso and soy sauce [4]. Thus, various fermented foods are produced according to the characteristics of *koji*-molds. The steamed rice that the *koji*-mold grows is called "rice-*koji*" and is used as a saccharification reagent in sake and awamori brewing [4]. In the brewing process, enhancing the enzymatic activity of rice-*koji* increases the production of glucose and amino acids, promotes the growth of yeast and lactic acid bacteria, and causes the flavour or alcohol contents of Japanese sake. The quality of the rice-*koji*-making process is affected by various environmental factors, such as temperature, humidity, and oxygen [5]. If the enzymatic activity is too high, it may lead to an increase in miscellaneous flavours and undesirable flavours. Controlling the enzymatic activity of rice-*koji* requires sensitive adjustments to environmental factors such as temperature and humidity [5].

Therefore, as a more straightforward method to control enzyme activity, a method to control the enzymatic activity of rice-*koji* via exposure to sounds was examined, which is one of the environmental factors. In the brewing industry, some cases incorporate sound irradiation techniques. For example, when sound



	(a) Before rice-*koji*-making (Previous study)	(b) Early phase (This study)	(c) Later phase (Previous study)
Phase of germination	✓	✓	—
Phase of hyphal elongation	—	✓	✓
Phase of production enzymes	—	✓	✓

Figure 1 Comparing the methods of irradiating sound waves to rice-*koji*

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waves at 200 Hz and 800 Hz were irradiated to *Saccharomyces cerevisiae* with intensities around 120 dB and 140 dB, cell growth was promoted, and the percentage of volatile compounds in the fermenter also changed [6]. Thus, incorporating sound irradiation technology is expected to shorten the fermentation period and improve flavour. By revealing the effect of sound waves on *koji*-molds in the same way as the case of yeast, sound irradiation technology is expected to shorten the rice-*koji*-making process and adjust the enzymatic activity of rice-*koji*.

In the case of bacteria and plants, it was reported that sound waves affect the ability to produce secondary metabolites such as ethanol and lactic acids and cell growth. For example, when a sound wave at 8.0 kHz was irradiated to *Escherichia coli* K-12 strain with an intensity of around 80 dB, the biomass increased, and cell growth was promoted [7]. Moreover, when a sound wave at 2.0 kHz was irradiated to a mung bean (*Vigna radiate*) with an intensity of around 90 dB, the stem and root were more elongated than that of a non-sound-irradiated mung bean [8]. While the effects of sound waves on organisms like bacteria and plants have been discussed, it remains unclear whether these organisms respond biologically to sound waves.

Therefore, this study revealed the effects of sound waves on rice-*koji*. The method of sound wave irradiation applied to *koji*-molds, as utilised in this research and previous studies, is illustrated in Figure 1. The phases that sound waves were irradiated directly were labelled as a check mark, and the phases that sound was not irradiated directly were labelled as "-." Previous studies involved irradiating sound waves at 16 kHz with an intensity level of 5.0 dB to conidiospores, preparing rice-*koji* using these sound-irradiated conidiospores (Figure 1a). It was found that the germination ratio of conidiospores was 1.4 times increased as compared with that of non-sound-irradiated conidiospores (that is, the control condition), and the glucoamylase activity of rice-*koji* was 0.87 times decreased as compared with that of the control condition [9]. The expression level of *glaB*, encoding glucoamylase, was reduced in sound-irradiated conidiospores at 16 kHz [10] was also reported. These results suggested that *koji*-molds responded to sounds and sound waves, indirectly affecting conidiospores' germination ratio and rice-*koji*'s glucoamylase activity. Then, the rice-*koji* was prepared under silent conditions after sound-irradiated-conidiospores were mixed with steamed rice and transferred to another container. Therefore, the change in enzymatic activity was considered an indirect effect of sounds. It is unclear whether sound waves affected the germination ratio of conidiospores and hyphal elongation and the ability to produce enzymes of rice-*koji* directly.

To improve a method of irradiating sounds to rice-*koji* and to assume a real brewing environment, sound waves were directly irradiated to rice-*koji* during the later phase of the rice-*koji*-making process, when *koji*-molds were expanding their hyphae and secreting enzymes in steamed rice (Figure 1c). It was found that when the sound wave at 16 kHz was irradiated, the glucoamylase activity of rice-*koji* was not changed as compared with that of the control. When the sound wave at 6.3 kHz was irradiated, the glucoamylase activity of the rice-*koji* was decreased significantly [11]. These results suggested that sound waves directly affected the glucoamylase activity of rice-*koji*, and different growing phases of *A. oryzae* showed different responses to specific frequencies of sounds.

The study confirmed the changes in the glucoamylase activity of rice-*koji*. In this method, sound waves were not irradiated since conidiospores were germinated and hyphae were expanded in steamed rice (the early phase of the rice-*koji*-making process). Therefore, it is unclear whether sound waves directly affected the germination ratio of conidiospores and hyphal elongation. In addition, while the condition of irradiating sound waves to decrease the glucoamylase activity of rice-*koji* has been clarified, the condition of irradiating sound waves to increase the glucoamylase activity of rice-*koji* has not yet been clarified.

This study aimed to assess the effect of sound waves during the early phase of rice-*koji* production and to identify conditions that enhance glucoamylase activity. Sound waves were directly irradiated to rice-*koji* while conidiospores germinated, hyphae expanded, and enzymes were secreted in steamed rice (Figure 1b).

MATERIALS AND METHODS

Incubation of *A. Oryzae* RIB40 Strain on a Solid Medium

Yellow *koji*-mold, *A. oryzae* RIB40 NBRC100959 strains, was purchased from the NITE Biological Resource Center (Tokyo, Japan). To suspend conidiospores, 1.0 mL of No. 707 liquid medium (5.0 g/L Polypepton, 3.0 g/L Yeast extract, 1.0 g/L $MgSO_47H_2O$), 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 medium (PDA medium, Nissui, Tokyo, Japan). PDA medium (4.0 g/L potato extract, 20 g/L glucose, 15 g/L Agar) was sealed with surgical tape and incubated at 30°C for five days in an incubator (FS-620, ADVANTEC, Tokyo, Japan).

The Making of Rice-*Koji* and Irradiating Sound Waves

Rice-*koji* with sound waves were prepared when the conidiospores germinated, and hyphae were expanded in steamed rice. To prepare steamed rice, 15 g of commercial rice (*Oryza sativa* var. *Japonica* cv. *Hinohikari*) was added into 50 ml of Erlenmeyer flask with 4.5 ml of deionised water, and this aliquot was steamed at 105°C for 15 min using an autoclave (BS-245, TOMY, Tokyo, Japan). After cooling at 45°C, 0.225 g of conidiospores collected from incubated *A. oryzae* RIB40 were mixed into steamed rice. The audio generator MINIRATOR MR2 (NTi, Tokyo, Japan) generated sound waves. An earphone (TS-100 BK, YAZAWA Co. Ltd., Tokyo, Japan) was connected to an audio generator and fixed to the head of 50 ml of the Erlenmeyer flask with vinyl tape at a distance of 80 mm from the conidiospores and this state was maintained during experiment.

In a previous study, the irradiating sound waves were reported at frequencies between 1.0 and 16 kHz to rice-*koji* in the later phase of the rice-*koji*-making process. Significant changes were shown, particularly at three frequencies of 1.0, 6.3, and 16 kHz [10]. In addition, this study also reported that the enzymatic activity of rice-*koji* that was prepared with sound-irradiated conidiospores was changed [9]. Therefore, to clarify the effects of irradiating sound waves on rice-*koji* during the early phase of the rice-*koji*-making process, sound waves at 1.0, 6.3, and 16 kHz were irradiated at 30°C for 24 hours. The intensity level of sounds was adjusted to 5.0 dB, and electric power was also adjusted to 4.5 W for the audio generator. Then, the height of the rice-*koji* was about 1.5 cm from the bottom to reach the sounds of the entire rice-*koji*. For the control experiment, the mixture was incubated at 30°C without earphones, which are not irradiated by sound waves.

Microscopic Observation of Conidiospores and Calculation of the Germination Ratio and the Area of Conidiospores

The sound waves revealed the change in the germination ratio of conidiospores by observing microscopically following the protocol of the Thoma hemocytometer (Minato Medical, Tokyo, Japan). 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 the start of the incubation on steamed rice and suspended with 0.2 ml of deionised water containing 0.1% (v/v) Tween80 (GR, Nacalai Tesque, Kyoto, Japan) and stirred. The supernatant obtained by stirring was collected and diluted threefold with prepared deionised water in another test tube. The conidiospores are observed under a glass cover with 20 ml resulting suspension placed on a Thoma hemocytometer. An Olympus BH-2 Microscope (Olympus, Tokyo, Japan) was used for microscopy. The germinated conidiospores were taken pictures using a USB microscope camera: Model MCU-10 (Kenis, Osaka, Japan). The magnification of the optical microscope was set to 40 × 100, and the total number of conidiospores was checked, whether between 30 and 50 cells in 4 × 4 squares. The total number of conidiospores and the germinated conidiospores was also counted. The germination ratio was calculated according to the following equation,

$$\text{The germination ratio (\%)} = \frac{\text{The number of germinated conidiospores}}{\text{The total number of conidiospores}} \times 100 \qquad (1)$$

The 4 × 4 squares of preparation were defined as one block, and the number of conidiospores in five blocks was counted following the protocol (https://minatomedical.com/01-ka101_m.html). The germination ratio in each block was calculated using Equation 1, and the average germination ratio at each of the five blocks was defined as the final germination ratio. As the germination ratio was changed, the area of conidiospores may also be altered. To observe the changes in the conidiospores area, the calculation uses the software "ImageJ" (https://imagej.net/ij/download.html).

Extraction of Enzymatic Solution from Rice-*Koji* and Dialysis

To extract the enzymatic solution, 10 g of rice-*koji* was collected 24 hours after the start of incubation and was soaked in a mixture of 50 ml of 0.5% (w/v) sodium chloride solution (GR, Nacalai Tesque, Kyoto, Japan) containing 0.2 M acetic acid buffer (GR, Nacalai Tesque, Kyoto, Japan) at 5°C for 18 h. The pH was adjusted to 5.0. The resulting extract was filtered with qualitative filter paper at φ 125 mm (No. 5C, ADVANTEC, Tokyo, Japan). The sugars or amino acid residue can be removed by recovering the filtration and was dialysed with visking tubes at 5.0 nm of pore size (Nihon Medical Science, Osaka, Japan) against 1,000 mL of 0.01 M acetic acid buffer (pH 5.0) at 5°C for 24 h. These extracts were used as a sample for measurement.

Determination of the Glucoamylase Activity of Rice-*Koji*

The glucoamylase activity of rice-*koji* that was prepared while irradiating sound waves changed was measured according to the method described by Taniguchi [12]. For the enzymatic reaction, 0.1 ml of properly extracted solution diluted five times with deionised water was mixed with 1.0 ml of 2.0% (w/v) soluble starch solution (GR, Nacalai Tesque, Kyoto, Japan) in the test tube, and incubated at 40°C for 20 min in the incubator. 2.0 N sodium hydroxide (GR, Nacalai Tesque, Kyoto, Japan) was added and left at room temperature for 30 minutes to stop the enzymatic reaction. 0.2 ml of 2.0 N hydrogen chloride solution (GR, Nacalai Tesque, Kyoto, Japan) was added for neutralisation. To cause a colour reaction in glucose, 0.1 mL of a reaction mixture was mixed with 3.0 ml of quantitative glucose reagent (Glucose CII Test Wako, FUJIFILM Wako, Osaka, Japan) in the test tube and incubated at 40°C for 20 min in the incubator. Absorption at 505 nm was measured with spectrophotometer U – 1800 to calculate the concentration of released glucose (Hitachi High-Tech Inc., Tokyo, Japan). The glucoamylase activity indicated the colour reaction of 1.0 mg/ml glucose equivalent at 40°C for 60 min from soluble starch and was defined as (U/g *koji*) of rice-*koji*.

Determination of the Acid Protease Activity of Rice-*Koji*

The acid protease activity of rice-*koji* that was prepared while irradiating sound waves changed was measured according to the method described by Taniguchi [12]. For the enzymatic reaction, 1.0 mL of properly extracted solution diluted five times with deionised water was mixed with 1.5 ml of 2.0% (w/v) casein solution (GR, Nacalai Tesque, Kyoto, Japan) in the test tube, and incubated at 40°C for 60 min in the incubator. 3.0 mL of 0.4 M trichloroacetic acid solution (GR, Nacalai Tesque, Kyoto, Japan) was added to stop the enzymatic reaction. The resulting mixture was filtered. To cause a colour reaction in tyrosine released from casein solution, 1.0 mL of the filtrate was mixed with 5.0 ml of 0.4 M sodium carbonate solution (GR, Nacalai Tesque, Kyoto, Japan) and 1.0 ml of 0.9 N Folin-Ciocalteu Reagent (GR, Nacalai Tesque, Kyoto, Japan) in the test tube. This mixture was incubated at 40°C for 30 min in the dark. Absorption at 660 nm was measured with spectrophotometer U-1800 to calculate the concentration of released tyrosine. The acid protease activity indicated the colour reaction of 1.0 mg/ml tyrosine equivalent at 40°C for 60 min from casein solution and was defined as (K U/g *koji*) of rice-*koji*.

Determination of the Hyphal Weight of *A. Oryzae* RIB40 Cells in Rice-*Koji*

The hyphal growth of rice-*koji* that was prepared while irradiating sound waves changed was observed according to the method described by Masuda et al. [13]. Rice-*koji* at 24 hours after the start of incubation was collected and dried at 161°C for 30 min. Resulted dried rice-*koji* was ground with mortar. Two grams of ground dried rice-*koji* were weighed accurately and suspended with 10 ml of 50 mM phosphate buffer (GR, Nacalai Tesque, Kyoto, Japan) and centrifuged at 3,000 rpm for 10 min. Then, the pH was adjusted to 7.0. This centrifugation process was repeated four times to remove the ingredients. As described by Masuda, 10 mg of Yatalase (GR, Takara Bio Inc., Kusatsu) was used to degrade the cell wall of *koji*-molds and detect the product, N-acetyl-D-glucosamine (GluNAc). Then, 10 mg of Yatalase was weighed accurately, added to the suspension, and incubated at 37°C for four hours with shaking. The Yatalase activity was indicated as releasing 1.0 mmol of GluNAc per 1 minute from 0.5% (w/v) chitin solution at 37°C and was defined as 1 U. In this study, an enzymatic activity that is equivalent to 1.26 U was used per one measurement. The resulting mixture was centrifuged at 3,000 rpm for 10 min to recover the supernatant fluid. To cause a colour reaction in GluNAc that was released from the cell wall, 3.0 mL of 10% (v/v) *p*-dimethyl-amino-benzaldehyde (GR, Nacalai Tesque, Kyoto, Japan) was added to 0.5 ml of distilled supernatant and incubated at 37°C for 20 min. To calculate the concentration of released GluNAc, absorption at 585 nm was measured with spectrophotometer U - 1800. The hyphal weight was indicated as the amount of GluNAc contained in 1.0 g of rice-*koji* and was defined as (mg/g *koji*) of rice-*koji*.

Statistical Significance Test

The difference between the sound and non-sound rice-*koji* was examined by a statistical significance test (t-test) [14]. 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 independent experiments. A significant difference was recognised when the one-sided test was less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

The Effects of Sound Waves on the Germination Ratio of Conidiospores

When the conidiospores of black-*koji Aspergillus niger* N402 strains, which are a kind of *Aspergillus* species, were inoculated on a complete solid medium (CM medium), the germination of conidiospores was begun after six hours and hyphal elongation was begun at eight hours after the start of incubation [15]. When conidiospores of *A. oryzae* RIB40 strains that were non-sound-irradiated were incubated on steamed rice, the germinated conidiospores were observed two hours after the start of incubation. It was considered that steamed rice contains more abundant sugars, amino acids, vitamins, and inorganic salts than CM medium.

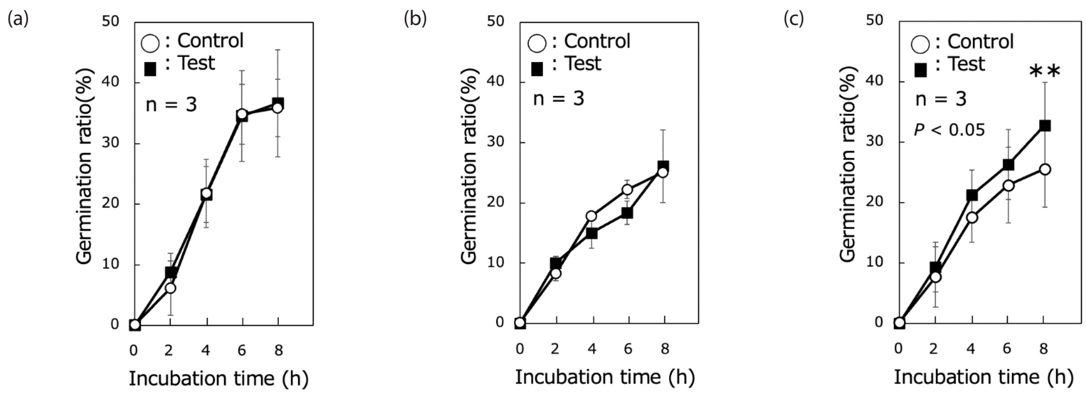

Figure 2 The effects of sound waves on the germination ratio of conidiospores

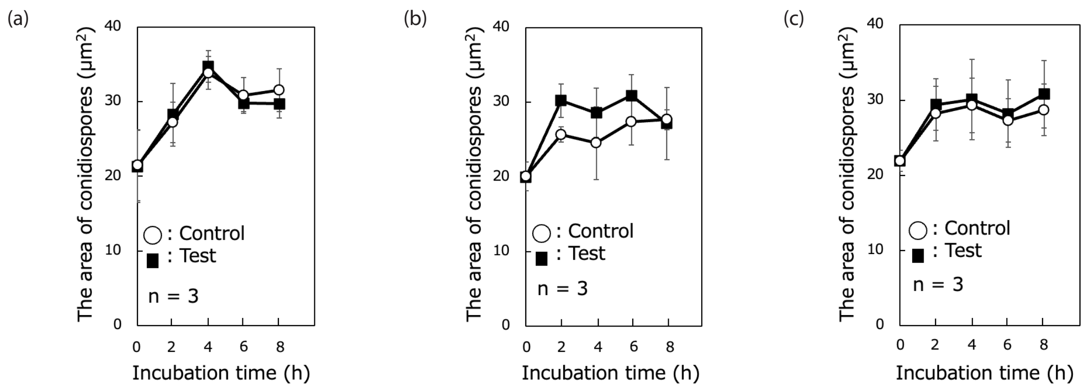

Figure 3 The effects of sound waves on the area of conidiospores

The changes in the germination ratio of conidiospores on steamed rice are shown in Figure 2. The white circle indicates the germination ratio of non-sound-irradiated conidiospores at each incubation time and is labelled as "Control." The black square indicates the germination ratio of sound-irradiated conidiospores at each incubation time and is labelled as a "Test." The changes in the germination ratio by irradiating sound waves at 1.0, 6.3, and 16 kHz were labelled as "a," "b," and "c", respectively. The horizontal axis represents the incubation time. The vertical axis represents the germination ratio of conidiospores (%). The error range was calculated from the standard deviation of three independent experiments. When a significant difference was recognised ($p < 0.05$), the asterisk (**) was labelled.

For sound wave at 1.0 kHz, the germination ratio of conidiospores that was not irradiated with sounds, that is, in the control condition, were 6.13 ± 4.50, 21.7 ± 5.6, 34.8 ± 4.9, and 35.9 ± 4.7%, at 2, 4, 6, 8 h after the start of incubation on steamed-rice, respectively (Figure 2a). When conidiospores were irradiated with the sound wave at 1.0 kHz, the germination ratio was 1.4, 1.0, 1.0, and 1.0 times changed compared to the control condition. The changes in conidiospores on steamed rice are shown in Figure 3. The white circle indicates the area of non-sound-irradiated conidiospores at each incubation time and is labelled as "Control." The black square indicates the location of sound-irradiated conidiospores at each incubation time and is labelled as "Test." The vertical axis represents the area of conidiospores (μm^2). When the sound wave at 1.0 kHz was irradiated, the conidiospores' area was not changed compared to that of the control condition at six h after the start of incubation (Figure 3a).

In the case of sound wave at 6.3 kHz, the germination ratio of conidiospores that of control condition were 8.35 ± 1.26, 17.9 ± 0.1, 22.3 ± 1.6, and 25.1 ± 0.2%, at 2, 4, 6, 8 h after the start of incubation on steamed-rice, respectively (Figure 2b). When conidiospores were irradiated with a sound wave at 6.3 kHz, the germination ratio was 1.2, 0.8, 0.8, and 1.0 times changed compared to those of the control condition, respectively. In addition, the area of conidiospores was higher than that of the control condition from 2 to 6 hours after the start of incubation (Figure 3b).

For sound wave at 16 kHz, the germination ratio of conidiospores that of control condition were 7.73 ± 5.01, 17.6 ± 4.1, 22.9 ± 6.3, and 25.6 ± 6.3%, at 2, 4, 6, 8 h after the start of incubation on steamed-rice, respectively (Figure 2c). When conidiospores were irradiated with the sound wave at 16 kHz, the germination ratio was 1.2, 1.2, 1.1, and 1.3 times changed compared to those of the control condition, respectively. In addition, when the changes in conidiospores were observed, the area of conidiospores was slightly higher than that of the control condition, and a positive correlation was confirmed (Figure 3c).

It was shown that when a sound wave at 1.0 kHz was irradiated, the germination ratio and conidiospores' area were less changed than that of other sound wave frequencies at 6.3 and 16 kHz. This was considered because the frequency (vibration number) is relatively low. Therefore, the physical vibrations exerted on the conidiospores were less, and the effect of sound waves on the germination ratio of conidiospores was relatively small. On the other hand, when the sound wave at 6.3 kHz was irradiated, the germination ratio increased over the first two hours compared with that of the control, and the area of conidiospores also increased. It was considered that the decreasing germination ratio was due to the fact that promoted germination of conidiospores caused the growth of its hyphae inside the steamed rice faster than that of control. Thus, conidiospores recovered less from centrifugation than from the control.

In addition, the area of conidiospores was more significant than that of other sound wave frequencies, and the effect of the physical vibration of sound on conidiospores was observed. An optimal sound wave at 6.3 kHz was considered for increasing the germination ratio and hyphal elongation. When the sound wave at 16 kHz was irradiated, a positive correlation was confirmed between the germination ratio and the area of conidiospores. Compared with the sound wave results at 6.3 kHz, although the germination ratio was significantly increased, the ratio of change in isotropic expansion of conidiospores was small. Therefore, it was possible that the conidiospores may show a frequency-specific response independently from the physical vibrations, and the germination ratio was increased.

The physical effects of sound on living organisms have also been reported for other organisms. When a sound wave at 800 Hz was irradiated to the *E. coli* K-12 strain with an intensity of around 100 dB, the cell size increased by 27.3% [7]. It was reported that the physical vibration of sound waves had altered the structure of cell membrane and cell membrane proteins, resulting in the increasing water flux into the cells, and the isotropic expansion of cells and cell growth were also promoted [16]. This study observed that the isotropic expansion of conidiospores was especially encouraged by sound waves at 6.3 kHz. The promoted isotropic expansion of conidiospores resulted in the faster germination of conidiospores and hyphal elongation.

The Effects of Sound Waves on the Glucoamylase Activity of Rice-*Koji*

The changes in the glucoamylase activity of rice-*koji* at 24 hours after incubation on steamed rice are shown in Figure 4. The white bar indicates the glucoamylase activity of the non-sound-irradiated rice-*koji* and is labelled "Control." The black bar indicates the glucoamylase activity of rice-*koji* that was sound-irradiated and labelled as "Test." The changes of the glucoamylase by irradiating sound waves at 1.0, 6.3, and 16 kHz were labelled as "A," "B," and "C," respectively. The vertical axes indicate activity showing the colour reaction of 1.0 mg/ml of glucose equivalent at 40°C for 60 min from soluble starch, defined as (U/g *koji*) of rice-*koji*.

In each independent experiment, the glucoamylase activity of rice-*koji* that of control condition was 70.1 ± 5.0, 97.9 ± 7.0, 103 ± 5 (U/g *koji*) of rice-*koji* at 24 hours after the start of incubation on steamed-rice, respectively. When sound waves at 1.0 and 6.3 kHz were irradiated, the glucoamylase activity was 1.2 and 1.3 times higher than the control conditions (Figures 4a and 4b). When the

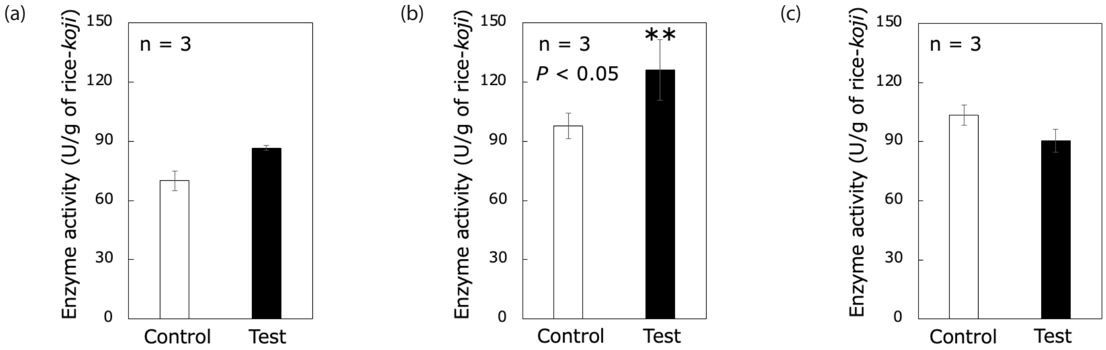

Figure 4 The effects of sound waves on the glucoamylase activity of rice-*koji*

sound wave at 16 kHz was irradiated, the glucoamylase activity was 0.9 times lower than that of the control condition (Figure 4c).

In particular, a significant difference was confirmed when a sound wave at 6.3 kHz was irradiated. It was considered that when sound waves were irradiated during the cell growth of *koji*-molds was active, the synthesis of biopolymers such as RNA and proteins was promoted in the cells. The cell growth in living organisms requires the accumulation of biopolymers such as nucleic acid molecules, proteins, and lipids within the cells. It was reported that when the chrysanthemum callus was irradiated sound wave at 1.0 kHz with an intensity around 100 dB for 60 min, the synthesis of RNA was promoted [17]. In the case of *Aspergillus* species, the expression level of genes such as *glaA, B* and *amyA, B, C,* which encode the glycolytic enzymes, were not changed during the conidial phase, and these genes were expressed since the start of the hyphal elongation phase of *koji*-molds [15]. We considered that the expression level of *glaB* in cells was enhanced by irradiating sound waves at 6.3 kHz when the cell growth was active, increasing glucoamylase activity. When a sound wave at 16 kHz was irradiated to rice-*koji*, the glucoamylase activity of rice-*koji* was 0.87 times decreased compared to that of control. This was considered because *koji*-molds showed frequency-specific responses. In the case of light, when the *A. oryzae* RIB40 strain was incubated while illuminating light at different wavelengths, the diameter of colonies was decreased by illuminating green light at 520 nm and blue light at 475 nm and not changed by illuminating red light at 630 nm [18]. It was due to some receptors that recognise only specific wavelengths of light at different wavelengths, and the expression levels of genes downstream related to cell growth and cell wall synthesis were regulated by responding to light. It is considered that because sound waves are recognised at different frequencies, the changes in glucoamylase activity differ depending on the frequency of sound.

Such frequency-specific responses have been observed in other organisms. This specificity of the frequency of sound waves has been observed in different organisms. It was reported that the cell growth of *E. coli* was promoted by irradiating sound waves at 1.0, 5.0, and 15 kHz, and then the ratio of changes of the cell growth was different for each frequency of sound [19]. In the case of plants, when a sound wave at 2.0 kHz was irradiated to the seeds of *Avena sativa L*., the a-amylase activity in the seeds was 1.7 times increased compared with that of non-sound-irradiated seed [20]. It may possess a common response mechanism to sound waves between plants and microorganisms. In most reports that reveal the effect of sounds on microorganisms and plants, sound waves were irradiated through the air at a high sound pressure of around 100 dB to microorganisms and plant cells in the cell growth phase. Unlike the method of irradiating sound waves in other research, the irradiated sound waves were determined to various growth phases of *koji*-molds with high-frequency sound waves of higher 1.0 kHz at a low sound pressure of 5.0 dB. The sound waves at only 5.0 dB of sound pressure changed the conidiospores' germination ratio and rice-*koji*'s enzymatic activity. In addition to the physical effects of sound waves, it was suggested that organisms, including *koji*-molds, may exhibit biological responses to sound waves.

The Effects of Sound Waves on the Acid Protease Activity of Rice-*Koji*

The changes in the acid protease activity of rice-*koji* at 24 h after incubation on steamed rice are shown in Figure 5. The white bar indicates the acid protease activity of rice-*koji* that was non-sound-irradiated and labelled as "Control." The black bar indicates the acid protease activity of rice-*koji* that was sound-irradiated and labelled as "Test." The changes in the acid protease activity by irradiating sound waves at 1.0, 6.3, and 16 kHz were labelled as "A," "B," and "C", respectively. The vertical axes indicate activity showing the colour reaction of 1.0 mg/ml of tyrosine equivalent at 40°C for 60 min from casein solution, defined as (K U/g *koji*) of rice-*koji*.

In each independent experiment, the acid protease activity of rice-*koji* that of control condition was 58.9 ± 8.6, 83.5 ± 7.1, 58.2 ± 9.5 (K U/g *koji*) of rice-*koji* at 24 hours after the start of incubation on steamed-rice, respectively. When the sound wave at 1.0 kHz was irradiated, the acid protease activity was 1.2 times higher than that of the control condition, and a significant difference was not confirmed (Figure 5a). When sound wave at 6.3 kHz and 16 kHz was irradiated, the acid protease activity was not changed as compared with that of the control condition (Figures 5b and 5c).

Throughout the experiment, no significant changes in acid protease activity were observed at any frequency. This is a desirable result,

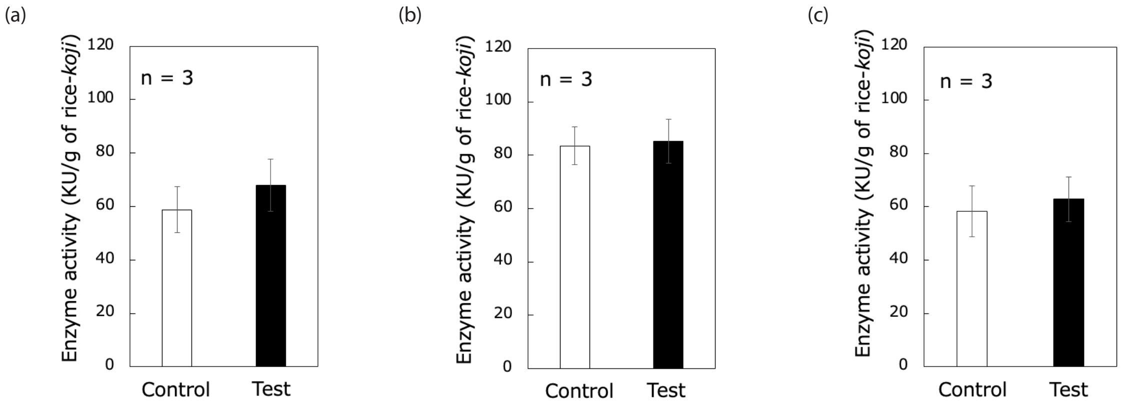

Figure 5 The effects of sound waves on the acid protease activity of rice-*koji*

especially in the brewing industry. In sake brewing, low acid protease activity is desirable because high acid protease activity may lead to unpleasant flavours and a deterioration in the taste of sake. Comparing with the results of glucoamylase activity mentioned above, it was found that the frequency of 6.3 kHz enhanced glucoamylase activity while the acid protease activity was not changed (Figures 4b and 5b). It was considered that even the same frequency may have different effects on the enzyme production mechanism. It was also considered that a sound wave at 6.3 kHz is optimal for sake brewing because this frequency increases the amount of supplied glucose while preventing unpleasant flavours and a deterioration in the taste of sake.

Increasing the acid protease activity of rice-*koji* could also be achieved through the brewing of soy sauce and mirin, as a high yield of amino acids is desirable in these processes. Future investigations into sound wave conditions at different frequencies may enhance the acid protease activity of rice-*koji* through sound wave irradiation.

The Effects of Sound Waves on the Hyphal Weight of Rice-*Koji*

The changes in the hyphal weight of rice-*koji* at 24 hours after incubation on steamed rice are shown in Figure 6. The white bar indicates the hyphal weight of rice-*koji* that was non-sound-irradiated and labelled as "Control." The black bar indicates the hyphal weight of rice-*koji* that was sound-irradiated and labelled as "Test." The changes in the hyphal weight by irradiating sound waves at 1.0, 6.3, and 16 kHz were labelled as "a," "b," and "c", respectively. The vertical axes show the hyphal weight contained in 1.0 g of rice-*koji*, defined as (mg/g *koji*) of rice-*koji*.

In each independent experiment, the hyphal weight of rice-*koji* that of control condition was 8.01 ± 1.13, 8.88 ± 0.49, 6.99 ± 0.67 (mg/g *koji*) of rice-*koji* at 24 hours after the start of incubation on steamed-rice, respectively. When a sound wave at any frequency was irradiated, the hyphal weight was not changed compared to the control conditions (Figures 6a, 6b, and 6c).

Throughout the experiment, no significant changes in the hyphal weight of rice-*koji* were observed at any frequency. These results suggested that while sound waves affected the germination ratio of conidiospores and the rice-*koji* glucoamylase activity, the overall rice-*koji* biomass was unaffected. Especially compared with the change of the glucoamylase activity in the sound wave at 6.3 kHz, the cell mass remained constant, and the glucoamylase activity increased (Figures 4b and 6b). From these results, it was considered that the amount of glucoamylase produced per hyphal cell may have been stimulated due to sound waves at 6.3 kHz.

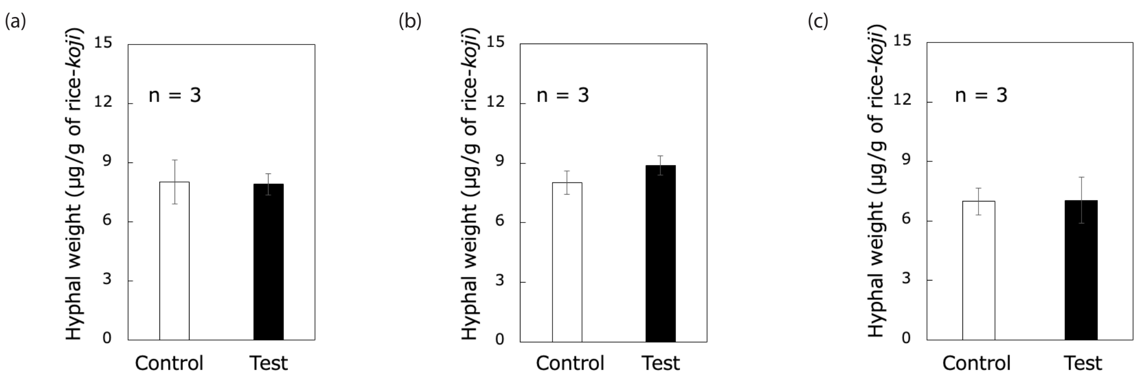

Figure 6 The effects of sound waves on the hyphal weight of rice-*koji*

The Potential Applications in the Brewing Industry

This study found that the effects of sound waves can be given to rice-*koji* directly or indirectly. The impact of sound waves on the germination ratio of conidiospores and enzymatic activity and the hyphal weight of rice-*koji* were summarised in Table 1. Each number indicates the relative values of each the germination rate (%), the glucoamylase activity (U/g *koji*) of rice-*koji*, and the acid protease activity (K U/g *koji*) of rice-*koji*, the hyphal weight (mg/g *koji*) of rice-*koji* that was non-sound-irradiated, to rice-*koji* that was sound-irradiated at 1.0, 6.3, and 16 kHz. When a significant difference was recognised ($p < 0.05$), an asterisk (**) was labelled.

Table 1 The effects of sound waves on *A. oryzae* RIB40 strain

	1.0 kHz	6.3 kHz	16 kHz
Germination ratio	1.01 ± 0.18	1.00 ± 0.10	**1.28 ± 0.26
Glucoamylase activity	1.24 ± 0.05	**1.29 ± 0.09	0.87 ± 0.05
Acid protease activity	1.16 ± 0.07	1.02 ± 0.05	1.01 ± 0.18
Hyphal weight	0.99 ± 0.10	1.06 ± 0.05	1.08 ± 0.14

The new method of irradiating sound waves can be applied to developing a new rice-*koji*-making process that will regulate the enzymatic activity of rice-*koji* in the future. For example, when conidiospores were irradiated with sound waves and added to steamed rice, the effects of sound waves can be given to rice-*koji* evenly and indirectly to decrease the glucoamylase activity when sound waves are irradiated. In contrast, in the early phase of the rice-*koji*-making process, the glucoamylase activity of rice-*koji* can be increased significantly. Enhancement of the glucoamylase activity of rice-*koji* is expected to shorten the saccharification periods degradation of dextrin that causes miscellaneous flavours of Japanese sake [21]. When the sound wave is irradiated during the later phase of the rice-*koji*-making process, the glucoamylase activity of rice-*koji* can be suppressed, thereby reducing the amount of supplied glucose and enabling the intentional extension of the fermentation period. Another planned experiment will involve irradiating sound waves during all stages of sake brewing. This includes alcohol fermentation, ageing periods, and the rice-*koji*-making process. The technology of irradiating sound waves may be applied to adjust the flavour of sake or *shochu*.

CONCLUSION

Rice-*koji* was prepared while irradiating sound waves through the early stage of the rice-*koji*-making process. Compared with the previous research, this study confirms the effects of sound waves on the germination ratio and enzymatic activities of rice-*koji*. Notably, the glucoamylase activity was significantly enhanced by irradiating sound waves at 6.3 kHz during the early phase of the rice-*koji*-making process for the first time. This study suggested that sound waves directly affected rice-*koji*'s germination ratio and glucoamylase activity. It was also indicated that even sound waves at the same frequency have different effects depending on the growth phase of *koji*-molds. When a sound wave at 16 kHz was irradiated to rice-*koji,* conidiospores were germinated, and hyphae were expanded. The germination ratio of conidiospores increased 1.3 times, and a significant difference was also confirmed. These results suggested that *koji*-molds responded to sound waves since the conidial phase and the germination ratio were changed. Controlling the enzymatic activity of *koji*-molds could be achieved by irradiating sound waves. This study provided insights that could inform research on how *koji*-mold responds to sound.

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