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【Research Note】

Effect of Vibration Produced by Audible Sound on Alcohol Fermentation of Yeast

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Abstract

The effects of the vibration produced by audible sound on the alcohol fermentation characteristics of wine yeast (OC-2) were investigated. When a 1,000-Hz frequency vibration was applied to the culture broth, a slight increase in cell growth and alcohol production occurred. In contrast, with vibration at 4,000 Hz, a slight decrease in cell growth and alcohol production was observed. In addition, the composition of the organic acid produced by the yeast changed slightly with vibration. The contents of malic acid and acetic acid in the culture broth to which vibration was applied differed from those in the control culture (no sound application). However, sound produced at other frequencies tested did not affect yeast fermentation. Therefore, the effect of vibration produced by audible sound on alcohol fermentation varied according to the vibration frequency.

Keywords: audible sound, vibration, alcohol fermentation, *Saccharomyces cerevisiae*

Introduction

In the fermentation industry, various chemical, physical, and biochemical techniques have been used to control fermentation processes. Since the 1980s, research has been conducted on the effects of other factors, such as electromagnetism, pressure, and sound waves, on living cells (Grosse et al. 1988, Hara et al. 1990, Kojima et al. 1992, Okuno et. al. 1993, Rajnicek et al. 1994, Yaoita et al. 1990). Consequently, it has been observed that stimulation by these factors may lead to alterations in DNA synthesis, protein synthesis, membrane permeability, cell growth, and/or the three-dimensional structure of metabolites.

Numerous studies investigating the impact of sound waves on microorganisms, specifically ultrasound, have reported changes in microbial growth and metabolism (He et al. 2021, Joyce et al. 2003, Kisielewska 2012, Matsuura et al. 1994). In addition, a recent increase has been observed in the number of studies investigating audible sound stimulation (Adadi et al. 2021, Aggio et al. 2012, Banerjee et al. 2018, Gu et al. 2013, Harris et al. 2021, Shaobin et al. 2010). However, researchers not only differ in their methods of stimulating microorganisms with audible sound, but also few cases have measured or analyzed the propagation of sonic vibrations into the culture

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medium.

In winemaking, fermentation under the influence of music has been attempted to produce wines with a mellow and fine taste (Komatsu 1991). Currently, various Japanese sake and/or wines brewed under the influence of classical music are sold all over Japan. However, the mechanism by which sound-induced vibrations causes changes in liquor quality is not yet well understood. Furthermore, no studies have elucidated how music can be used to control yeast metabolism and alter metabolite production.

Yet, in fermentation systems, the vibrations of microorganisms induced by music or audible sound, appear to be considerably simpler and safer than other complex microbial control methods such as chemical addition, temperature control, and adjustment of dissolved gas concentration. (Schmid 2003). Therefore, the use of vibration produced by audible sound for microbial process control is considered highly practical.

In this study, a fundamental investigation into the effects of audible sound-induced vibration on microorganisms in liquid culture systems was conducted, with a focus on alcohol fermentation using wine-yeast. Specifically, this study examined the impact of vibration on cell growth, alcohol production, and the synthesis of certain organic acids.

Materials and Methods

Microorganisms and media

Saccharomyces cerevisiae OC-2 (wine yeast) was used. The preculture medium was composed of 1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract. For the main culture, modified Hayduck medium composed of 10% glucose, 0.25% L-asparagine, 0.1% KH_2PO_4 and 0.3% $MgSO_4{\cdot}7H_2O$ mixed in tap water (pH 5.3) was used.

Vibrational culture system

The fermenter consisted of a 500-mL Erlenmeyer flask made of polymethylpentene（TPX®）, polycarbonate (PC), or glass (G) were used, with a vibration exciter (Vibtone-28; ONKYO CORPORATION, Osaka, Japan) attached to the outside of the flask bottom (Fig. 1). Band noise signals of various frequencies (31.5 Hz, 63 Hz, 125 Hz, 250 Hz, 500 Hz, 1,000 Hz, 2,000 Hz, 4,000 Hz, 8,000 Hz, and 16,000 Hz) generated by the sound sources (PC and source files) were inputted into an amplifier (LP-V3Ⓢ; Lepy, China). The signal, amplified to 70% of maximum output, was continuously sent to a vibration exciter attached to the flask to stimulate the culture medium. Each flask was placed on a 5-mm-thick silicon plate to prevent propagating vibrations to and from other flasks.

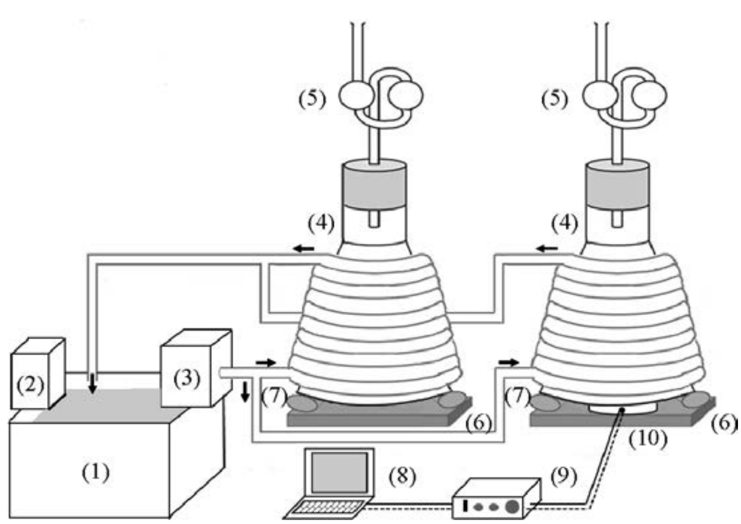

Fig. 1 Experimental Setup. (1) Water bath, (2) thermostat, (3) pump, (4) flask with water jacket, (5) fermentation airlock, (6) vibration damping sheet, (7) non-circular support ring with a notch, (8) audio playback PC, (9) amplifier, and (10) vibration exciter. Arrows indicate the directions of hot water flow.

Furthermore, to avoid the effects of vibrations from the motor of the thermostatic bath and the vibration exciter attached to the other flasks, the flasks were not kept in a thermostatic water bath for temperature control. Instead, the temperature of each culture medium was maintained by passing warm water at the appropriate temperature through a water jacket on the flask. The flasks were set up in an ordinary laboratory, and no attempts were made to insulate the flasks from being exposed to general routine noises. The reason for such a setup was based on the idea that installing a large commercial-scale fermentation tank in a quiet room would be impractical.

Cultivation

A 5-mL cell suspension obtained after precultivation for approximately 20 h at 30 °C was inoculated into a flask containing 400 mL of the main culture medium, and vibration caused by the band noise of various frequencies was applied to the culture broth. The main culture was performed at 30 ℃ under unstirred conditions with and without vibration stimulation in parallel. Data on the fermentation progress were obtained by conducting experiments comparing vibration and non-vibration conditions and repeated 3-4 times. From the obtained data, the mean value and standard deviation were calculated, and a cultivation progress chart was created based on those values.

Measurement of glucose uptake by yeast

The experiment was conducted following a previously reported method (Nakanishi et al. 1998). Specifically, yeast cells grown in preculture medium for 24 hours were washed twice with sterilized saline solution and then suspended in sterilized phosphate buffer (pH 7) containing 5% glucose. To determine the sugar uptake per unit cell by quantifying the amount of residual sugar in the cell suspension, it was necessary to inhibit the growth of yeast. Therefore, the cell concentration was set to a high value of 10^8 cells/mL. The cell suspension was incubated with or without vibration, and the residual sugar was assayed over a period of 4 days.

Measurement of yeast budding

This experiment was also performed under non-growing conditions for yeast, as described in the literature (Nakanishi et al. 1998). Specifically, precultured yeast cells were washed twice with sterile saline and then suspended in sterile tap water containing 0.1% KH_2PO_4 and 0.3% $MgSO_4 \cdot 7H_2O$ to a cell concentration of 10^8 cells/mL. The cell suspension was incubated with or without vibration, and the number of budding and non-budding yeast cells was measured over a period of 4 days using a Thoma hemocytometer and microscope.

Measurement of yeast dispersion in liquid medium

Yeast cells, cultivated in a preculture medium, were washed twice with sterilized saline solution and suspended in the medium to achieve a cell concentration of 10^8 cells/mL. The suspension was then incubated under the aforementioned conditions, and the turbidity of the cells was measured at the upper, middle, and bottom layers of the culture medium over an 8-h period.

Analytical methods

For the analysis of vibrations propagated in the culture broth, an underwater sound-collecting microphone (H2a-XLR Hydrophone; AQUARIAN AUDIO PRODUCTS, Anacortes, WA USA), an audio interface (Rubix22; Roland, Shizuoka, Japan), and a PC with acoustic data analysis software (DSSF3 Light; Yoshimasa Electronic Inc., Tokyo, Japan) were used.

The glucose and ethanol concentrations in the culture broth were determined by high-performance liquid chromatography (HPLC; column, Shim-pack SCR-101N, Shimadzu Co., Ltd., Kyoto, Japan; column temperature, 55 ℃; detector, refractive index; carrier liquid, ion-exchanged water; flow rate, 0.8 mL/min). The content of organic acids in the culture broth was determined by HPLC (column, Shim-pack SCR-102, Shimadzu Co., Ltd.; column temp., 45 ℃; detector, conductivity detector; carrier liquid, 10 mM p-toluenesulfonic acid; buffer, 10mM p-toluenesulfonic acid + 0.2 mM EDTA).

Yeast cell concentration was quantified based on the turbidity of the culture broth at a wavelength of 660 nm measured using an absorbance spectrophotometer (U5100; Hitachi High-Tech Co., Ltd., Tokyo, Japan).

Significance tests for the data obtained from the fermentation test were performed using the Wilcoxon signed-rank test with EZR, which was designed for R (Kanda 2013). EZR is a modified version of R Commander specifically tailored to incorporate statistical functions commonly used in biostatistics.

Results and Discussion

Establishment of a vibrational culture system

Devices that vibrate with audible sound include speakers and vibration exciters. The former is a device that propagates sound through air vibrations (Dickason 2006) and can produce vibrations on the surface of a culture broth. However, speakers were not considered suitable for transmitting sound vibrations deep into the culture broth. Conversely, a vibration exciter is a device that applies a mechanical vibration to a test object to study the vibration characteristics of the object (Sakaba 1990, Tanaka et al. 2019). Therefore, a vibration exciter was considered effective in propagating vibrations throughout the culture medium in a flask. Therefore, a vibration exciter was used to produce vibration during alcohol fermentation by yeast cells in this study.

First, a flask suitable for vibrational culture was selected from among heat-sterilizable flasks made of different materials. A vibration exciter was set on the wall of the flask filled with distilled water, and vibration stimulation was performed while continuously changing the frequency of the applied sound. On the opposite side of the wall where the vibration exciter was attached, the propagated vibration was detected using an acoustic microphone, and the waveform data of the detected sound were analyzed. In the case of glass flasks, sonic vibration propagation at frequencies less than 1,000 Hz was negligible (Fig. 2). In contrast, in the case of polycarbonate or polymethylpentene flasks, the propagation of vibrations by sound in the lower frequency range was good. Moreover, the polymethylpentene flask showed the best propagation of sonic vibrations over a wide range of frequencies. Thus, it was decided to use polymethylpentene flasks for the vibrational culture of yeast. Incidentally, in fermentation facilities, it is considered important to experimentally determine whether vibrations are appropriately propagated through the liquid in the tanks during vibrations. Based on these measurements and both the tank material and the performance of the vibrator, the selection of the vibrators and the number of vibrators to be installed should be determined.

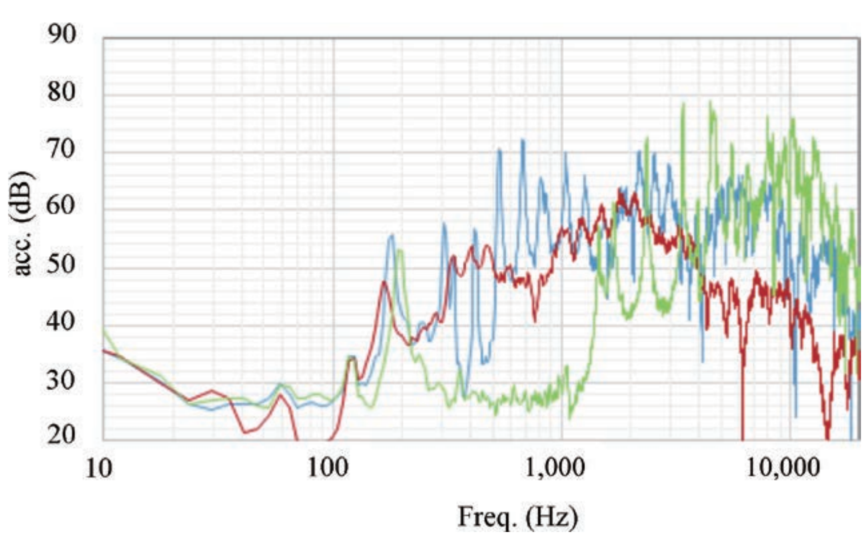

Fig. 2 The effect of flask material on the transmission of audible sound vibrations to the enclosed liquid. —, Polycarbonate; —, Polymethylpentene; —, Glass.

Second, the propagation properties of sound through water in a flask were investigated using band noises at 10 different frequencies. When vibrations were performed using band noises at 250 Hz, 500 Hz, 1,000 Hz, 2,000 Hz, and 4,000 Hz, frequency characteristic peaks of the band noise were detected in the waveform of the sound propagating in the water (Fig. 3). However, for vibrations induced by band noise produced at the other frequencies, the frequency charts of the sound detected in water were similar to those of ambient sounds, also known as background noise (i.e., non-vibration) (data not shown). Based on these results, alcohol fermentation by yeast under vibrational conditions was conducted using band noise with frequencies of 250 Hz, 500 Hz, 1,000 Hz, 2,000 Hz, and 4,000 Hz.

Alcohol fermentation of yeast with vibration produced by audible sound

To investigate the effect of vibration produced by audible sound on yeast alcoholic fermentation, band noises at different frequencies were applied to the main culture broth after inoculating a yeast cell suspension. The differences in glucose consumption, alcohol production, cell proliferation, and formation of several organic acids between fermentation with and without vibration were investigated. Stimulatory effects of vibration produced by band noise on fermentation were observed at 1,000 Hz and 4,000 Hz (Fig. 4). Vibration stimulation of the culture system using audible sound at 1,000 Hz had a pronounced effect on yeast glucose consumption, cell growth, and alcohol production, increasing them to approximately 10% higher than in the control culture (no vibration applied) (glucose consumption and alcohol production, p-value < 0.05). However, for cell density, although differences were observed in the measured values between the vibration and non-vibration conditions, the p-value was > 0.05. Therefore, no significant difference in cell growth was observed between the treatment and control conditions. Conversely, application of band noise at 4,000 Hz slightly delayed the glucose consumption of yeast, resulting in approximately 10% lower cell growth and alcohol production than in the control culture ($p < 0.05$).

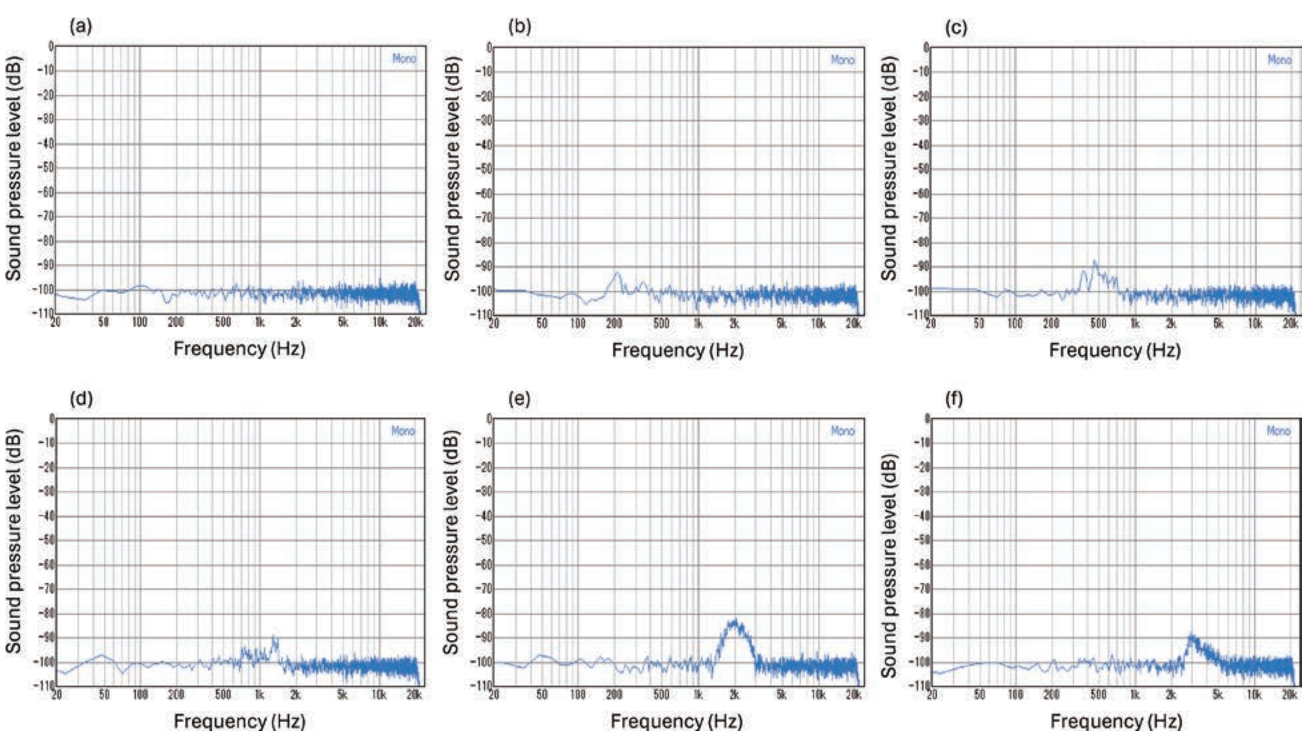

Fig. 3 The propagation of various vibrations with different frequencies through water in a flask. (a) control, (b) 250 Hz, (c) 500 Hz, (d) 1,000 Hz, (e) 2,000 Hz, (f) 4,000 Hz

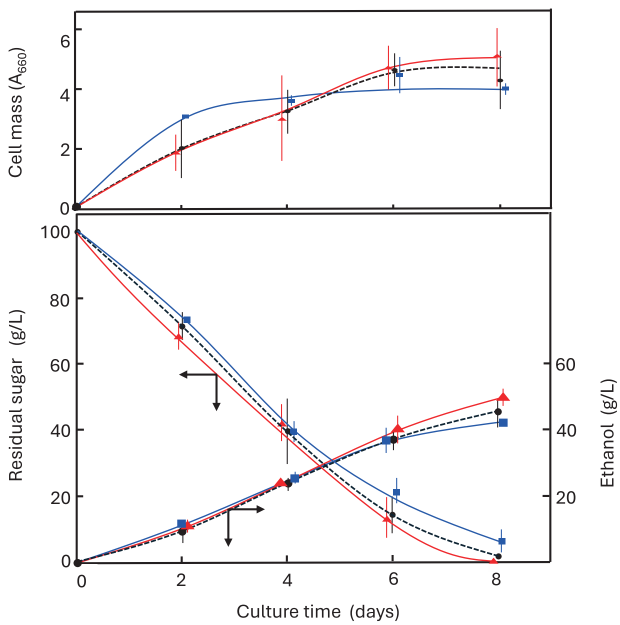

Fig. 4 Time course of ethanol fermentation.
Symbols: ●, control; ▲, 1,000 Hz; ■, 4,000 Hz

During alcohol fermentation, yeast also produces organic acids, which are affected by various culture conditions, such as dissolved oxygen (DO), dissolved carbon dioxide (DCO_2), temperature, and other factors (Nagai et al. 1992). The effects of vibration produced by audible sound (1,000 Hz or 4,000 Hz) were also observed in the composition of the organic acids produced (Fig. 5). When vibration from band noise at 1,000 Hz was applied, the changes in lactic acid and succinic acid production were minimal. Conversely, the acetic acid and malic acid concentrations at the end of fermentation increased approximately by 7% and decreased by approximately 10%, respectively, compared with those in the control ($p < 0.05$). In contrast, in the culture stimulated by vibration produced at 4,000 Hz band noise, an approximately 10% increase in malic acid production and a slight increase in lactic acid production were observed ($p < 0.05$), and little or no change was observed in the yields of other organic

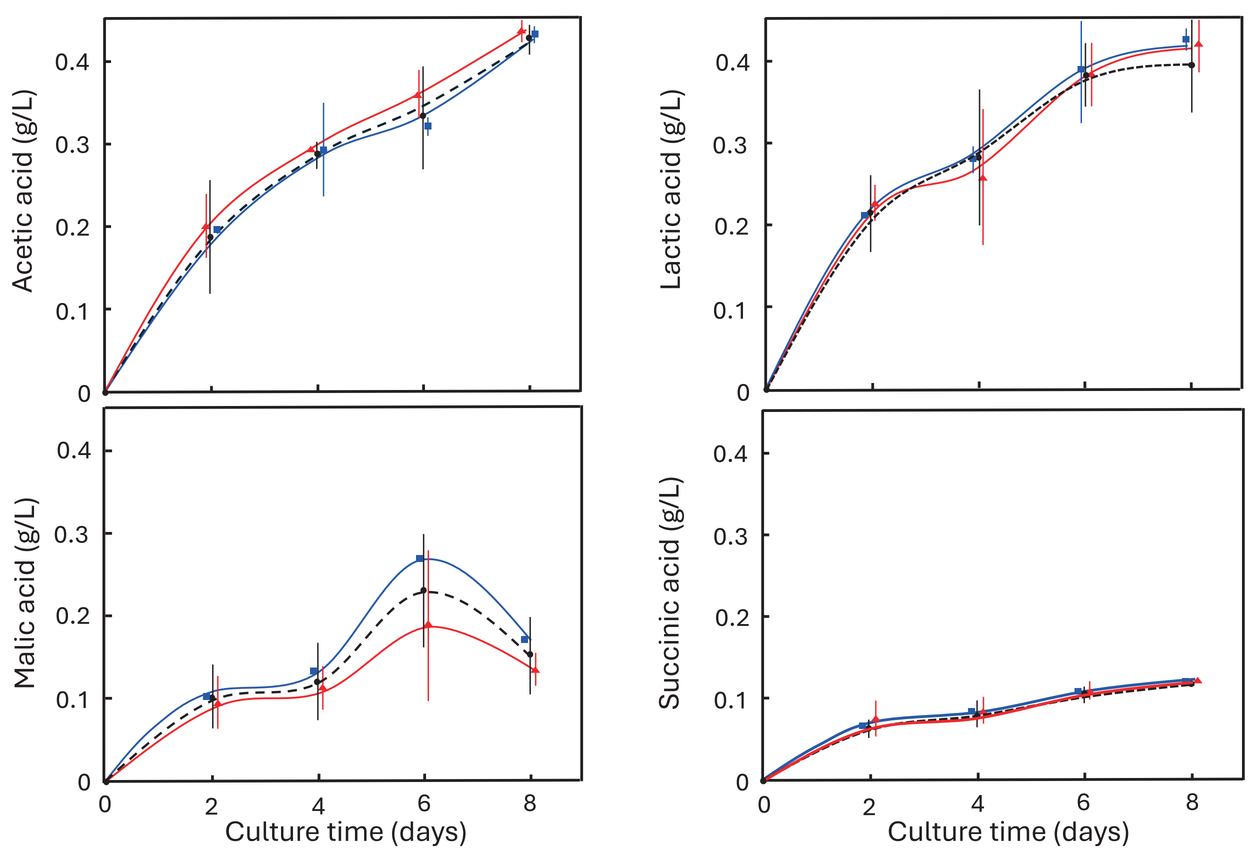

Fig. 5 Time course of organic acid production.
Symbols: ●, control; ▲, 1,000 Hz; ■, 4,000 Hz

acids produced.

These results indicate that vibrations produced by audible sounds affect the alcohol fermentation of yeast cells depending significantly on the frequency of the audible sound. However, the effects of sound frequency on yeast stimulation differed from those reported in other studies on vibrational stimulation of microbial cultivation. Some studies have reported that the growth of yeast, bacteria, algae, or plants can be stimulated by vibration, whereas others have found little to no effect (Adadi et al. 2021, Harris et al. 2021, Keramati et al. 2021, Shah et al. 2016, Shaobin et al. 2010). In addition, it has been reported that in the cultivation of shiitake mushroom fungi on agar media, vibrational stimulation at 1,000 Hz may either promote or inhibit growth, depending on the fungal species (Kobayashi et al. 2023). Other researchers have proposed that bacterial growth is influenced by sound, because sound may serve as a means of communicating the changes in environmental conditions to neighboring microorganisms (Matsuhashi et al. 2000). As mentioned earlier, these varying reports are mainly due to the differences in methods applied to expose the microorganism to sound waves.

The vibrating culture method is considered versatile because it can be implemented by installing a vibrator onto an existing fermentation tank. However, the vibrator used varies among experimenters, and the testing environment also differs. Therefore, important aspects for establishing the vibrating method as a microbial controlling technology and for its future use in microbial culture systems are as follows:

(1) Increasing the number of studies of the effects of vibration on microorganisms.

(2) Actual measurement of the propagation of vibration into the culture medium.

These will make it easier to compare and evaluate the wide range of experimental results conducted by various researchers. We expect that research in this field will become increasingly active in the future.

Effect of vibration on glucose uptake and yeast growth

To investigate the factors contributing to the changes in fermentation behavior under vibrating conditions, the effects of vibration on two factors closely associated with the fermentation process, glucose uptake by yeast cells and yeast budding, were examined. First, the effect of vibration on glucose uptake was investigated under nongrowing conditions. It was found that the glucose in the cell suspension was gradually consumed, and almost all of it was consumed in about 4 days (data not shown). However, under vibrating conditions, no increase (1,000 Hz) or decrease (4,000 Hz) was observed in the glucose uptake of yeast cells compared with that in the non-vibrating system.

Second, the effects of vibration on the budding of yeast cells were also investigated under similar conditions. The budding ratio of yeast (number of budding cells / total cell number) with vibration at 1,000 or 4,000 Hz of band noise was approximately 30% (data not shown). This value was comparable to that obtained in the control system (no vibration stimulation applied), and a change in the number of budding cells was barely recognized with vibration. Furthermore, no significant microscopic differences were observed between the cells collected from the vibrated suspension and the cells collected from the non-vibrated suspension with respect to size and shape. Generally, it is well known that sound waves, especially ultrasonic waves, exert various physical effects on solid substances in liquids, such as removing dirt from objects and disrupting microbial cells. However, the physical effects of low-power audible sound vibration on microorganisms are considered to be extremely limited.

As mentioned in the previous section, in the current study, the change in glucose consumption of yeast during alcoholic fermentation under vibrating conditions was observed. However, in this section, vibrating stimulation by audible sound itself was found to have little effect on glucose uptake or budding of the yeast cell. In alcohol fermentation, it is not necessarily uncommon for yeast to change their growth, despite the fact that the budding ratio barely changes due to external stimulation. Similar phenomena have been observed in yeast alcohol fermentation with the application of electric current (Nakanishi et al. 1998).

Taking these factors into consideration, it is thought that audible sound vibrations during alcohol fermentation may have primarily affected cell division rather than budding of yeast cells (Goto 1989, Suzuki 1974), consequently leading to changes in cell growth and apparent glucose consumption.

Effect of vibration on the dispersion of yeast cells in the culture medium

The effects of vibration at frequencies of 1,000 Hz and 4,000 Hz on the dispersion of yeast in the culture medium were examined (Fig. 6).

In the upper layer, the turbidity of the yeast decreased rapidly within about 2 hours from the start of the measurement, regardless of the presence or frequency of vibration. Following the initial 2 hours of measurement, a decrease in yeast turbidity was observed in the middle layer. This delayed decrease in turbidity was attributed to the settling of yeast cells from the upper layers. In addition, in the vibration-stimulated system, the reduction in turbidity was somewhat delayed, regardless of the vibration frequency. Similarly, a delayed decrease in turbidity was observed in the lower layer, which was attributed to the settling of yeast cells from both the upper and middle layers. Furthermore, as seen in the middle layer, the decrease in turbidity was suppressed in the vibration-stimulated system. These results indicate that vibration, regardless of frequency, suppresses yeast sedimentation in the medium and maintains the dispersion of yeast cells in the culture for an extended period during the initial stages of alcohol fermentation.

Generally, weak agitation to delay the sedimentation of cells in the culture broth and/or provide trace amounts of oxygen aeration is effective in promoting alcohol fermentation by yeast (Katohda et al. 2006, Yoshizawa et al. 2002). Therefore, it is considered that the inhibition of yeast sedimentation by vibration (1,000 Hz and 4,000 Hz) also promotes fermentation. However, a slight delay, especially in the mid-to-late stages of alcohol fermentation (Fig. 4), was observed with 4,000-Hz of vibration, suggesting the potential effects of other factors on fermentation delay at this frequency.

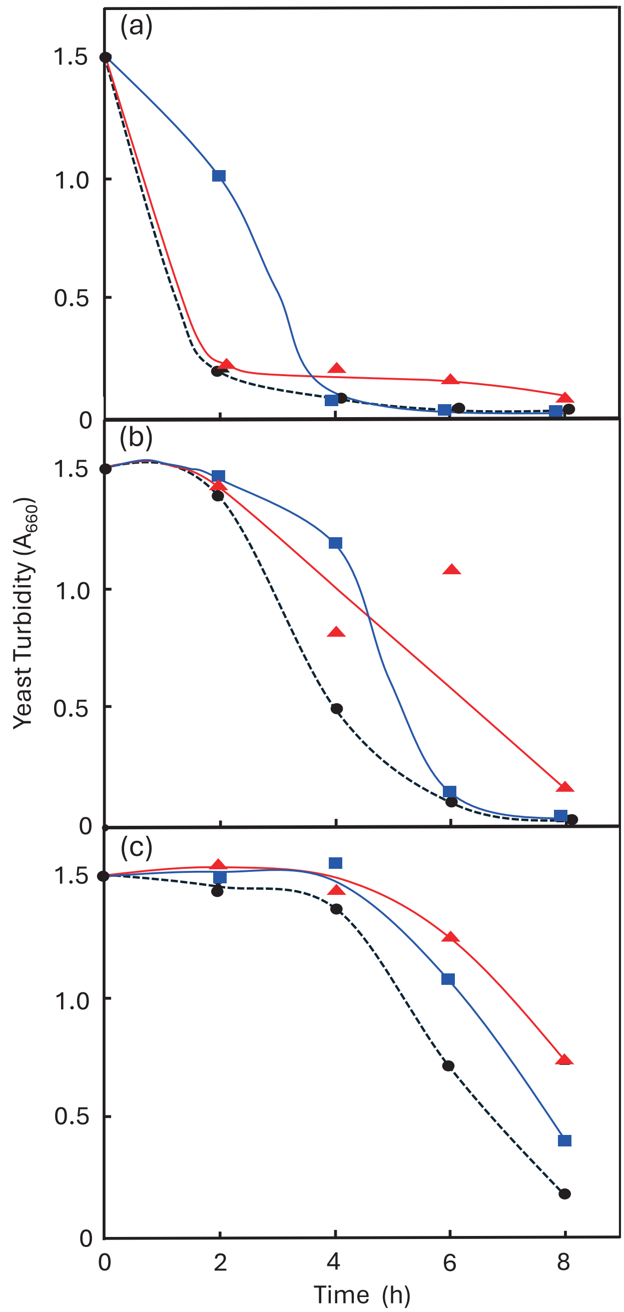

Fig. 6 Effect of vibration on dispersion of yeast cells in the Upper (a), middle (b) and lower (c) layers of the cell suspension. Symbols: ●, control; ▲, 1,000 Hz; ■, 4,000 Hz

The present study demonstrated that stimulation of yeast cultures with vibrations generated by audible sound results in subtle changes in aspects of yeast alcohol fermentation depending on the frequency of the vibration. However, this study could not elucidate the specific details of the effects of vibration on yeast. Therefore, further research is needed to clarify this aspect.

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可聴音による加振が酵母のアルコール発酵におよぼす影響

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要　約

可聴音による加振がワイン酵母（OC-2）のアルコール発酵におよぼす影響について検討した。1,000 Hzの可聴音による加振により、酵母菌体の増殖とアルコール生成がわずかに促進した。これに対して、4,000 Hzの可聴音による加振においては、菌体増殖とアルコール生成がわずかに遅延した。さらに、発酵に伴って酵母により生成される有機酸に関しても、主としてリンゴ酸および酢酸の生成が、加振培養系において対照系（非加振）と異なり、有機酸組成に変化が認められた。

しかしながら、他の周波数の可聴音による加振では、酵母の発酵にほとんど変化が認められなかったことから、可聴音による加振が酵母のアルコール発酵におよぼす影響は、周波数に大きく依存するものと推察された。