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Molecular Hydrogen Treatment of Sake Yeast and *kuratsuki* Bacteria Affects Sake Taste

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Abstract: To the best of our knowledge, there are no studies on the effects of molecular hydrogen (H_2) on microorganisms. In this study, we performed co-culture experiments using two microorganisms involved in sake brewing: sake yeast strain K1401 and the *kuratsuki* bacterium *Kocuria* strain TGY1127_2. The cells were suspended in water or water containing H_2 and statically incubated at 4 °C for 2 h before co-culture. Sake taste was estimated using a taste sensor. The taste of sake was affected by H_2 treatment of *kuratsuki Kocuria* as well as sake yeast. These results strongly suggest that H_2 treatment alters the physiology of *kuratsuki* bacteria and sake yeast. We showed that sake undergoes H_2 treatment of the microorganisms involved in sake brewing to boost its variety and meet the market demand.

Keywords: hydrogen treatment; *Kocuria*; *kuratsuki* bacteria; sake brewing; sake yeast

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1. Introduction

Molecular hydrogen (H_2) is used in medical treatments because it refreshes the respiratory chain on the mitochondrial membrane of human cells [1–5]. H_2 has been proposed to convert ubiquinone intermediates to ubiquinol, which increases the antioxidant capacity of the quinone pool and prevents the generation of reactive oxygen species [5]. However, to the best of our knowledge, there have been no studies on the effects of H_2 on microorganisms, which remains unclear. Because bacteria also have a quinone pool in their plasma membrane, we expected similar effects in the mitochondria of eukaryotes.

The present study investigated sake brewing and the microorganisms involved in this process. The sake yeast *Saccharomyces cerevisiae* is the most important microorganism used in sake brewing. Sake yeast converts sugar into ethanol. The final ethanol concentration is ~20%. Although beer and wine yeasts are also *S. cerevisiae*, sake yeasts differ phylogenetically [6]. Sake yeast generates chemical components during sake brewing that affect its flavor and taste [7–9]. Yeast strains produce different aromatic substances. The Brewery Society of Japan (*Jozo-Kyokai*) manages and sells sake yeast strains (*Kyokai* yeast strains), which were established in selected sake breweries in Japan because the flavor and taste of sake produced using naturally occurring yeasts are unstable and not always satisfactory [10].

The *kuratsuki* bacteria enter the sake production process and affect its flavor and taste [11–13]. Different sake breweries produce different *kuratsuki* bacteria [14]. The Japanese words "*kura*" and "*tsuki*" correspond to "sake brewery" and "inhabiting", respectively. Some ethanol-tolerant lactic acid bacteria (LAB; sake-spoiling bacteria) can grow in sake. During sake production, microorganisms such as *kuratsuki* bacteria die, but LAB do not. Some ethanol-intolerant LAB have been used in the traditional fermentation starter *kimoto* production process [15–17]. However, bacteria other than LAB have not been well studied for sake brewing. For example, co-culture studies of sake yeasts and bacteria other than LAB have not yet been performed. Different varieties of *koji*, rice, and sake yeast are used to produce sake with different flavors and tastes. *Koji* is made from steamed rice and is a *koji* mold that converts rice starch into sugar. We expect that the variety of sake will be further expanded by considering the types of *kuratsuki* bacteria.

We identified *Kocuria* isolates as *kuratsuki* bacteria at the Narimasa Sake Brewery [18,19]. The genus *Kocuria* belongs to the phylum Actinobacteria, which are not LAB. *Kocuria* isolates are classified into two different lineages [19]. Strains TGY1120_3 and TGY1127_2 belong to different lineages at the species level, and their genomic DNA sequences have been determined [19]. TGY1127_2 is more suitable for sake brewing than TGY1120_3 because of the comparison of their genes [19]. *Kocuria* strain TGY1127_2 lacks amylase, and no significant difference in Brix change was detected between the solutions of *koji* with and without TGY1127_2 [13]. Thus, although TGY1127_2 does not convert rice starch into sugar or sugar to ethanol, it does affect the flavor and taste of sake [12,13,18].

Generally, environmental conditions, such as culture conditions, affect bacterial properties. If the properties of *kuratsuki* bacteria are altered by H_2 treatment, the effects of *kuratsuki Kocuria* with and without such treatment on the taste of sake may differ. The current study aimed to confirm the effects of H_2 treatment of sake yeast strain K1401 and *kuratsuki Kocuria* strain TGY1127_2 on sake taste.

2. Materials and Methods

2.1. Cultivation of Microorganisms

The sake yeast *S. cerevisiae* (*Kyokai* yeast strain K1401) and *kuratsuki* bacterium *Kocuria* strain TGY1127_2 were used in this study. K1401 has been frequently used by sake breweries in Japan and was used in our experiments [13,18]. TGY1127_2 was isolated, classified, and used in our experiments [13,18]. K1401 and TGY1127_2 strains were grown using TGY medium (5 g/L tryptone, 1 g/L glucose, and 3 g/L yeast extract) and incubated at 25 °C for 12 h. Following pre-cultivation, the cells were separated by centrifugation and suspended in water or water containing H_2 (8 ppm; Ecomo International, Fukuoka, Japan). These four solutions, sake yeasts suspended in water (9.5×10^3 cells/mL), sake yeasts suspended in H_2-treated water (9.5×10^3 cells/mL), *kuratsuki Kocuria* suspended in water (1.5×10^5 cells/mL), and *kuratsuki Kocuria* suspended in H_2-treated water (1.5×10^5 cells/mL), were then statically incubated at 4 °C for 2 h. As a control, 290 mL of water was added as well as 60 g of *koji* (Isenou, Tokyo, Japan) and 10 mL of H_2-untreated sake yeast solution (9.5×10^4 cells). The following ingredients were added to 280 mL of water: 60 g of *koji*, 10 mL of H_2-untreated or H_2-treated sake yeast solution, and 10 mL of H_2-untreated or H_2-treated *kuratsuki Kocuria* solution (1.5×10^6 cells). Each mixed solution was statically incubated at 14 °C for 13 days. Brix and acidity were measured using a digital refractometer PAL-BX/ACID (ATAGO, Tokyo, Japan) at 0, 1, 3, 5, 7, 9, 11, and 13 days. For the Brix test, 0.3 mL of the sample solution was used; for acidity, 0.6 mL of the sample solution was diluted 20 times with water.

2.2. Estimation of Sake Taste

Sake taste was assessed using a taste sensor TS-5000Z (Intelligent Sensor Technology, Inc., Atsugi, Japan). The initial tastes, astringent stimulation, bitter miscellaneous taste, saltiness, sourness, and umami wre measured using the sensors AE1, CO0, CT0, CA0, and AAE, respectively. Each taste sensor has a different lipid membrane [20]. The strength of each taste is represented by the magnitude of its current value [20]. Aftertastes such as astringency, bitterness, and umami richness were measured by intensities in the second measurement after washing the sensors used in the initial taste measurement. Each measurement was repeated four times.

2.3. Statistical Analysis

Statistical analyses were performed using R software (The R Project for Statistical Computing, http://www.R-projet.org/ (accessed on 25 May 2023)). Bartlett's tests were performed before analysis of variance (ANOVA). Pairwise *t*-tests were performed using the Bonferroni technique of p-value correction when the ANOVA showed $p < 0.05$. The Kolmogorov–Smirnov test was performed to compare the Brix and acidity change patterns.

3. Results and Discussion

3.1. Effect of H_2 Treatment on Ethanol Fermentation of Sake Yeast

The H_2-untreated sake yeast strain K1401 was used as the control when measuring the Brix and acidity of the sake production process. The Brix and acidity of sake made with H_2-untreated sake yeast/H_2-untreated *kuratsuki Kocuria* were not significantly different from those of the control ($p > 0.05$ in the Kolmogorov–Smirnov test) (Figure 1). Additionally, there was no significant variance in the Brix and acidity of sake made with H_2-treated sake yeast/H_2-untreated *kuratsuki Kocuria*, sake made with H_2-untreated sake yeast/H_2-treated *kuratsuki Kocuria*, and sake made with H_2-treated sake yeast/H_2-treated *kuratsuki Kocuria* (Figure 1). These findings show that H_2 treatment of sake yeast and/or *kuratsuki Kocuria* did not significantly affect the fermentation of sake yeast, which was continuously maintained during sake brewing.

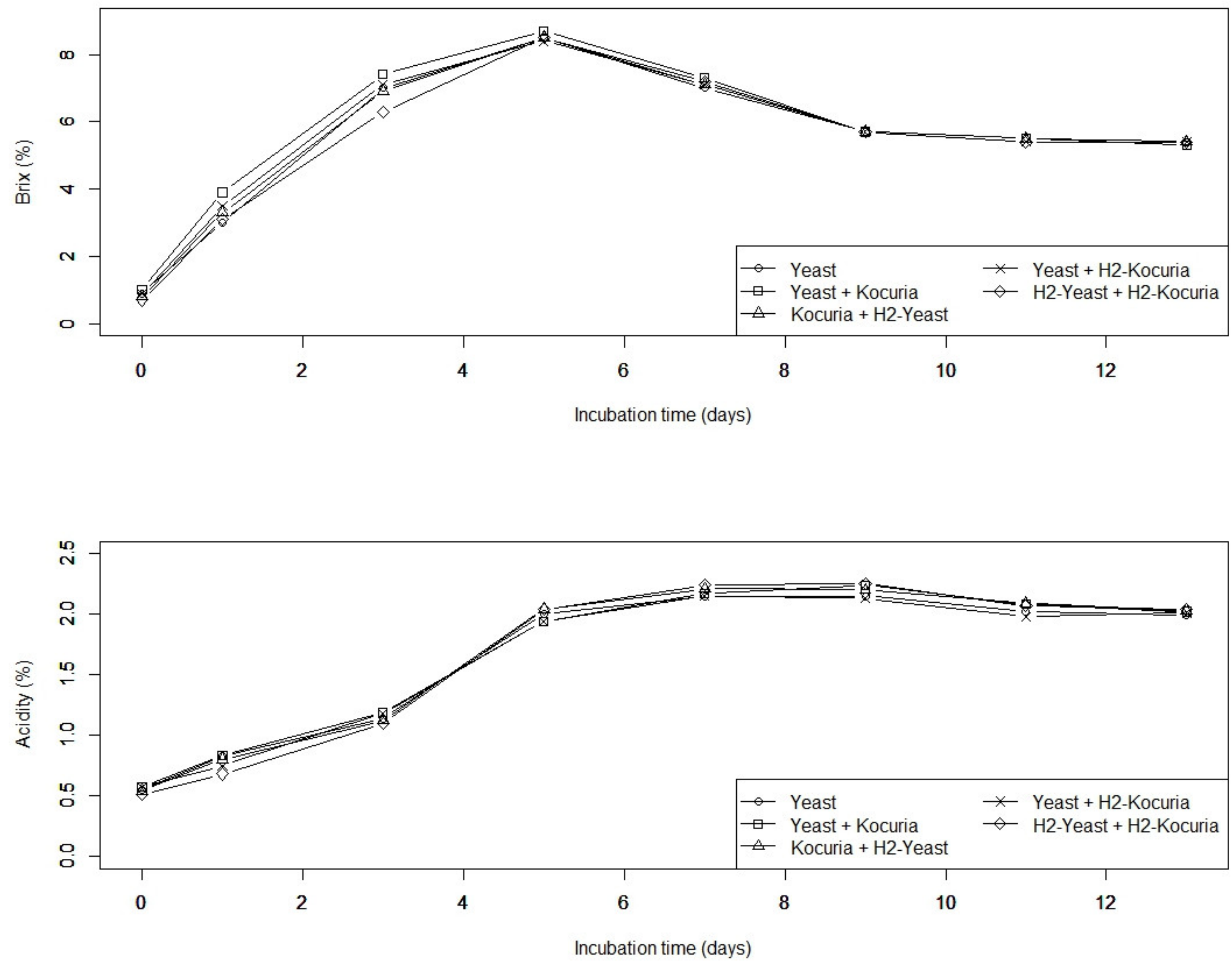

Figure 1. Brix and acidity of sake during brewing. *Koji* (60 g) and sake yeast solution (10 mL containing 9.5×10^4 cells) without H_2 treatment were added to 290 mL of water as a control. *Koji* (60 g), H_2-untreated or H_2-treated sake yeast solution (10 mL), and H_2-untreated or H_2-treated *kuratsuki Kocuria* solution (10 mL containing 1.5×10^6 cells) were added to 280 mL of water. Each mixed solution was statically incubated at 14 °C for 13 days. Brix and acidity were measured using PAL-BX/ACID (ATAGO, Tokyo) at 0, 1, 3, 5, 7, 9, 11, and 13 days.

3.2. H_2 Treatment Affects Sake Taste

According to the results of the TS-5000Z taste estimation, there was a significant difference ($p < 0.05$, in pairwise *t*-test) between sake made with H_2-treated sake yeast/H_2-untreated *kuratsuki Kocuria* and sake made with H_2-untreated sake yeast/H_2-treated *kuratsuki Kocuria* for astringent stimulation, bitter miscellaneous, saltiness, sourness, and umami tastes (Figure 2). A significant difference (in pairwise *t*-test) in astringent stimulation, bitter miscellaneous, saltiness, and sourness tastes was observed between sake made with H_2-untreated sake yeast/H_2-treated *kuratsuki Kocuria* and sake made with H_2-treated sake yeast/H_2-treated *kuratsuki Kocuria* (Figure 2). A significant difference (in pairwise *t*-test) in bitter miscellaneous and sourness tastes was observed between sake made with H_2-treated sake yeast/H_2-untreated *kuratsuki Kocuria* and sake made with H_2-treated sake yeast/H_2-treated *kuratsuki Kocuria* (Figure 2). These results indicated that H_2 treatment affected the properties of *kuratsuku Kocuria* and sake yeast.

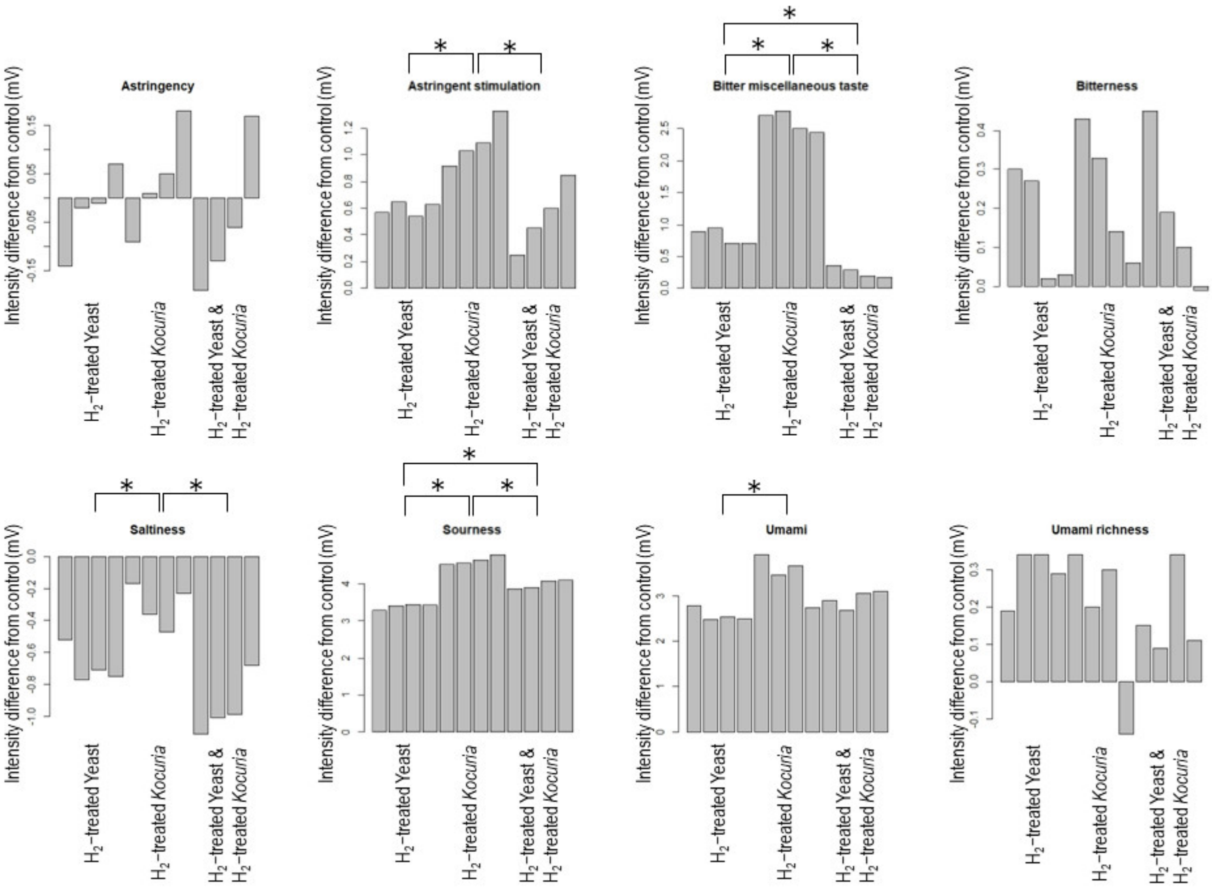

Figure 2. Difference between taste intensities of sake with and without H_2-treated bacteria. Taste intensities of H_2-treated sake yeast strain K1401 and/or H_2-treated *kuratsuki Kocuria* strain TGY1127_2 sake minus that of sake with H_2-untreated sake yeast and H_2-untreated *kuratsuki Kocuria* were used. Intensities of astringency, astringent stimulation, bitter miscellaneous taste, bitterness, saltiness, sourness, umami, and umami richness were measured using TS-5000Z. Each measurement was repeated four times. Each treatment has four bars from different experiments. Significant differences ($p < 0.05$, ANOVA) in analysis of variance were detected in astringent stimulation, bitter miscellaneous taste, saltiness, sourness, and umami. Asterisk (*) indicates significant difference ($p < 0.05$) in pairwise *t*-test.

H_2 treatment of sake yeast strain K1401 and *kuratsuki Kocuria* strain TGY1127_2 had an additive effect on saltiness; however, similar effects were not observed for other tastes (Figure 2). These results showed that the effect of H_2 treatment on sake yeast strain K1401 was not independent of that of H_2 treatment of *kuratsuki Kocuria* strain TGY1127_2. In other words, K1401 interacted with TGY1127_2 during sake production. The chemical compounds associated with the flavor of sake are mainly produced by sake yeast. H_2 treatment affected the physiology and metabolism of sake yeast, and H_2-treated *kuratsuki Kocuria* affected the interaction between the *kuratsuki* bacterium and sake yeast. Surprisingly, with astringent stimulation, bitter miscellaneous taste, sourness, and umami, the taste intensity of sake prepared with H_2-untreated sake yeast/H_2-treated *kuratsuki Kocuria* showed the highest change among the three sakes (Figure 2). This implies that sake yeast strain K1401 responds differently to H_2-treated and H_2-untreated *kuratsuki Kocuria*.

4. Conclusions

Although H_2 treatment in this experiment was performed at 4 °C for 2 h, there was a surprisingly significant difference of tastes between the presence and absence of H_2 treatment. In addition, although we expected an effect of H_2 on sake yeast, which is a eukaryotic microorganism, we were surprised to find that the effect of H_2 on the *kuratsuki* bacterium was greater.

H_2 treatment of sake yeast strain K1401 and/or *kuratsuki Kocuria* strain TGY1127_2 did not significantly affect ethanol fermentation during sake brewing. However, sake made with H_2-treated sake yeast and/or *kuratsuki Kocuria* had a different taste from sake made with H_2-untreated sake yeast and/or H_2-untreated *kuratsuki Kocuria*. This indicates that H_2 leads to changes in the physiology of sake yeast and *kuratsuki* bacteria. However, further research is required to fully elucidate this mechanism.

Our findings showed that H_2 treatment of sake yeast and *kuratsuki* bacteria affected the taste of sake. Various flavors and tastes of sake have been reported by varying the types of sake rice, *koji*, and yeast. Therefore, we propose using *kuratsuki* bacteria for sake brewing. Based on the results of the present study, we propose the use of H_2 to treat sake yeast and *kuratsuiki* bacteria during sake brewing. Sake undergoes H_2 treatment to boost variety and meet the market demand.

To the best of our knowledge, this is the first study to use H_2 in the production of fermented beverages. Future predictions indicate an increase in the global and Japanese demand for H_2. Thus, the need for clean energy and medical demands should be considered. Our proposal is to use H_2 in drinks and foods.

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