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Competitive advantage and tolerance of selected shochu yeast in barley shochu mash

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A shochu yeast strain, *Saccharomyces cerevisiae* BAW-6, was previously isolated from Kagoshima yeast strain Ko, and has since been utilized in shochu production. The BAW-6 strain carries *pho3*/*pho3* homozygous genes in contrast to the heterozygous *PHO3*/*pho3* genes in the parental Ko strain. However, absence of the *PHO3* gene per se cannot explain the fermentation superiority of BAW-6. Here, we demonstrate the growth advantage of the BAW-6 strain over the Ko strain by competitive cultivation in barley shochu preparation, where alcohol yield and *nihonshudo* of the former strain were higher than those of the latter strain. In addition, the maximum growth rate of BAW-6 was less affected than that of Ko by high Brix values of barley *koji* medium, suggesting that BAW-6 is less sensitive to growth inhibitory compounds derived from barley or barley *koji*. The tolerance of BAW-6 to growth inhibitory compounds, cerulenin and diethylstilbestrol (an H^+-ATPase inhibitor), was also higher than that of other yeast strains. Consistent with BAW-6′s tolerance to diethylstilbestrol in the presence of 8% ethanol (pH 4.5), H^+-ATPase activity, but not transcription of its gene, was higher in BAW-6 than in Ko. We conclude that the BAW-6 strain is associated with certain gene alterations other than *PHO3*, such that it can maintain cellular ion homeostasis under conditions of ethanol stress during the latter phase of fermentation.

[**Key words:** Shochu yeast; Barley shochu; Cerulenin; Diethylstilbestrol; H^+-ATPase]

The traditional Japanese liquor shochu is prepared from various ingredients, such as barley, sweet potato, rice, and buckwheat. The shochu *koji* used as a raw material for the first-stage fermentation contains a large amount of citric acid produced by a shochu *koji* mold, e.g., *Aspergillus kawachii*. The acidic conditions of the mash prevent undesirable bacterial contamination. The yeast typically used for shochu production is a homothallic diploid strain, which is taxonomically classified as *Saccharomyces cerevisiae*. The shochu production process involves two mash preparations: the first intended for yeast proliferation and the second for fermentation of the main ingredient. Typically, a portion of first mash is added to another first mash, which is termed *sashimoto* and is repeated throughout several months. After a two-month continuous culture with initial use of the Kagoshima yeast strain (Ko), we isolated a novel yeast strain from the barley shochu mash, which we named BAW-6. The alcohol tolerance of BAW-6 was higher than that of its parental strain Ko, and alcohol production in the latter phase of the second mash, in which yeast cells are highly stressed by alcohol, was considerably improved (1). Even though our initial selection marker for BAW-6 was the lack of constitutive acid phosphatase activity ($PHO3^-$ phenotype), the *PHO5/3* chimeric gene resulting from the deletion of a 1.9-kb region between the *PHO5* and *PHO3* tandem genes does not seem to be correlated with alcohol production (2). However, the change in phenotype of BAW-6 suggests the possibility of mutations in other genes that influence shochu production.

Recently, it was revealed that sake yeast exhibits high alcohol productivity due to the repression of gene expression mediated by the environmental stress-responsive transcription factors Msn2p and Msn4p (3), and a loss-of-function mutation in the *RIM15* gene, which encodes a putative upstream activator of Msn2p and Msn4p (4), resulting in a lack of stress response in the yeast. In sake production, the sake yeast is grown in a batch culture and is removed from the mash at the end of fermentation, whereas in the shochu preparation process, the shochu yeast is recycled many times, i.e., a small portion of shochu yeast culture is inoculated into the next batch as a starter, which is repeated many times. Due to the differences in environmental stress (fermentation temperature, fermentation duration, alcohol concentration, organic acid concentrations, and so forth) of sake and shochu mash, the high alcohol productivity of BAW-6 might be associated with a mechanism different from that in sake yeast. In this regard, a multi-drug resistant mutant of sake yeast was reported to be proficient in

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alcohol productivity (5,6). Moreover, Fukuda et al. isolated trichothecin-resistant mutants from sake and shochu yeasts that showed multi-drug resistance and superior alcohol productivity (7,8). Since BAW-6 exhibits high alcohol productivity, this strain may also have gained multi-drug resistance. In this study, we investigated the characteristics of the BAW-6 strain in order to determine whether it has a competitive advantage over Ko in barley shochu mash, and examined the potential for drug resistance as one of its stress tolerance traits.

MATERIALS AND METHODS

Strains The laboratory diploid yeast BY4947 was provided by the National BioResource Project. The shochu yeast strain BAW-6 was preserved in our laboratory following a previous study (1). Two other shochu yeast strains, Kagoshima yeast (Ko) and SH-4 (RIB1019), were obtained from the Kagoshima Federation of Brewers Association and the National Research Institute of Brewing, respectively.

Media and growth conditions All the percentages of ingredients are expressed as weight per volume (w/v). The conventional complete medium for yeast culture is YPD (1% yeast extract, 2% polypeptone, 2% glucose). Synthetic dextrose (SD) medium contains 2% glucose, and 0.67% yeast nitrogen base without amino acids. A phosphate-rich medium contains 1% glucose, 0.2% polypeptone, 0.15% yeast extract, 0.1% KH_2PO_4, and 0.04% $MgSO_4 \cdot 7H_2O$. Agar was added to a final concentration of 2% for solid media. Barley *koji* medium was prepared by incubating 5 kg of barley *koji* at 55°C for 7 h after adding 1 g of Amano 2 enzyme for shochu (Amano Enzyme, Aichi, Japan) and 6 L of water. The solution of the incubated *koji* was then filtered through linen cloth, and the filtrate was used as barley *koji* medium after Brix adjustment. Brix values were measured using a portable refractometer (PAL-1; Atago). All the liquid stationary cultures and agar plates were incubated at 30°C, unless otherwise specified.

Competitive culture assay The BAW-6 and Ko strains were grown in 10 ml of barley *koji* medium at 30°C for 2 days, and 2×10^8 cells of each strain were added to a 300 ml conical flask containing 117 g of barley *koji* and 120 ml of water. After incubating at 25°C for 4–5 days, 1 ml of the culture was then used as a starter for a new batch of fermentation. This successive cultivation was repeated 4 times, and the culture was diluted and plated on phosphate-rich medium. After incubation at 30°C for 2 days, yeast colonies on the plates were subjected to diazo coupling (D.C.) staining for constitutive acid phosphatase (cAPase), as described by Toh-e and Oshima (9). The D.C. staining method turned colonies of BAW-6 white (cAPase negative) and those of Ko brown (cAPase positive) on the phosphate-rich medium. *Nihonshudo* and the alcohol content were measured using routine methods.

Proliferative activity test in barley *koji* medium Barley *koji* media with compounds of different concentrations were prepared with Brix values of 8, 12, or 18, glucose concentration of 7% or 10.5%, and citric acid concentration of 2% or 4%. The pH of all the media was adjusted to 3. Maximum specific growth rate (μ_{max}) was calculated after cultivating each yeast strain at 30°C with 30 shakings per minute (spm) in a temperature gradient incubator (TN-2612; Advantec).

Chemical tolerance assay Each of the yeast strains was cultivated in YPD medium at 30°C for 24 h with constant shaking, and the culture was diluted to an OD_{660nm} of 0.2, equivalent to 1×10^6 cells/ml. Media were supplemented with cerulenin, cycloheximide, clotrimazole and diethylstilbestrol to the final concentration of 0.63, 1.25, 2.5, 5, 10, 20, 40, and 80 μg/ml in solid SD medium with or without 8% ethanol (pH adjusted to 4.5). These supplemented SD plates were used for the application of 10 μl/spot of diluted yeast culture and were incubated at 30°C for 6 days. The minimum growth inhibitory concentration (MIC) was defined as the lowest concentration of the chemical that inhibited the growth of each yeast strain as judged by the naked eye.

Time course of yeast extracellular pH Cells in stationary phase were collected after cultivation of each strain in 10 ml of YPD medium at 30°C overnight with constant shaking. The collected cells were then washed 4 times at 4°C with 250 mM sorbitol solution and then diluted to an OD_{660nm} of 1.2 with 250 mM sorbitol. Ten milliliters of the diluted cell suspensions were incubated at 30°C for 5 min, and their pH was adjusted to 6.0. After the pH became stable, 250 mM glucose was added to the suspensions. The change in pH was monitored from the time when the glucose was added.

Diethylstilbestrol tolerance culture test A pre-culture of yeast strains grown in YPD medium was inoculated into YPD medium with 4% ethanol containing 0 to 20 μg/ml diethylstilbestrol, and the cultures were incubated at 30°C in a temperature gradient incubator (TN-2612; Advantec) with constant shaking.

RNA extraction and northern blot analysis Cells were grown in YPD medium, and were collected during both the logarithmic (OD_{660nm} = 0.2–0.3) and the stationary growth phase (OD_{660nm} = 1.0). The yeast RNA was extracted following the method by Schmitt et al. (10) The extracted total RNA (10 μg/ml) was denatured at 65°C for 15 min in a solution containing 50% formamide, 1× MOPS [20 mM 3-(*N*-morpholino)-propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA (pH 7.0)] and 0.17 volume formaldehyde (37% v/v). The denatured RNA was then separated by gel electrophoresis [1.5% agarose, 1× MOPS, 0.16 volume formaldehyde (37% v/v)], and transferred to a nylon membrane (Hybond-N; Amersham). The DNA primers ACT1F (5′-ACGGTCCCAATTGCTCGAGAGA-3′) and ACT1R (5′-GAAGACAGCACGAGGAGCGTC-3′) were used to detect the expression of the *ACT1* gene, whereas PMA1F (5′-TGTTTCAGCTCATCAGCCAAC-3′) and PMA1R (5′-ACCAGCTTGGAATTCTTGAACG-3′) were used to detect the expression of the *PMA1* gene. In order to synthesize the DNA probes for these genes, DNA fragments were labeled by using PCR DIG labeling mix (Roche) and a Veriti 96-well thermal cycler (Applied Biosystems). Hybridization was carried out at 42°C with hybridization buffer [5× SSC, 50% formamide, 50 mM sodium phosphate buffer (pH 7.0), 7% SDS, 2% blocking reagent, 0.1% lauroylsarcosine]. The detection was performed with a DIG Luminescent Detection Kit (Roche), and RNA bands were visualized using a luminescent image analyzer (LAS-1000; Fuji).

RESULTS AND DISCUSSION

Competitive culture of Ko and BAW-6 strains in barley *koji* medium The BAW-6 strain is a D.C. white isolate obtained

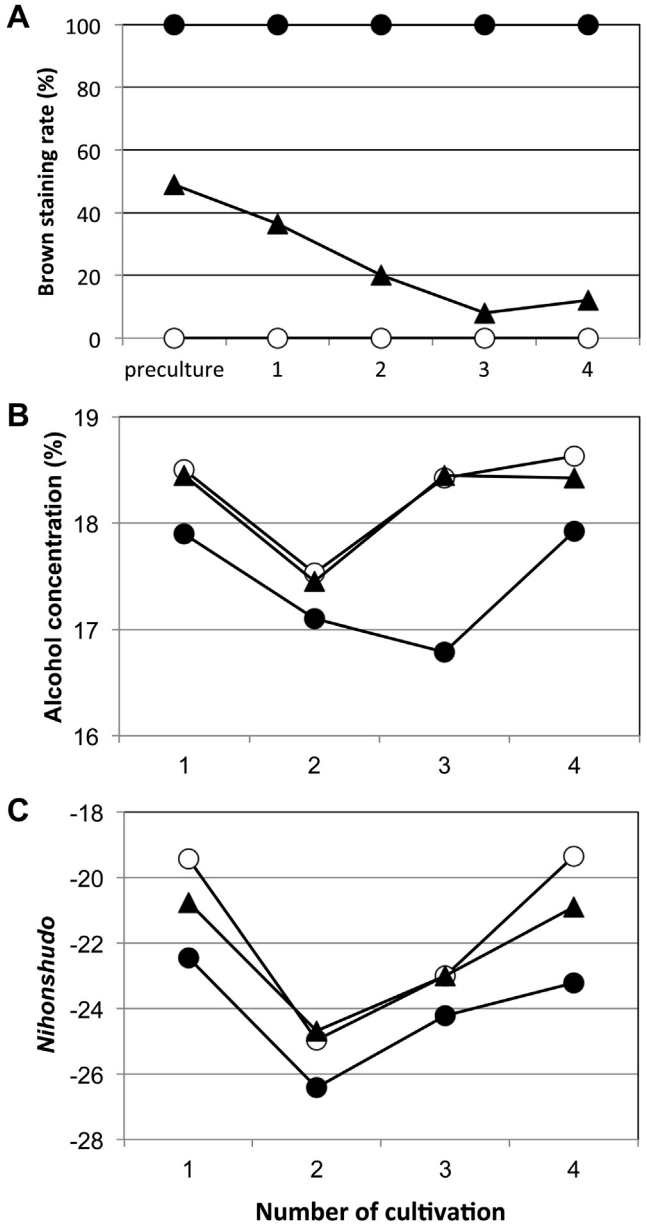

FIG. 1. Changes in D.C. staining rate (A), alcohol content (B) and *nihonshudo* (C) in barley fermentation mash of each sample of subculture. Symbols: closed circles, Ko; open circles, BAW-6; and closed triangles, mixed culture. Data are represented as the mean values of two experiments.