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Fermentation characteristics and brewing potential of *kuratsuki* sake yeasts isolated from the Niigata Prefecture, Japan

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Abstract

The sake brewing industry continues to evolve in response to consumer preferences for novel flavors and fermentation characteristics. However, the genetic and phenotypic diversity of brewery landrace yeast (*kuratsuki* yeast) strains, particularly their regional brewing characteristics and potential for unique flavor development, remains poorly understood due to limited studies and challenges in distinguishing them from industrial strains. This study explored the phenotypic diversity of *kuratsuki* yeast in the Niigata Prefecture, which persists in sake breweries and is isolated outside the realm of industrial yeast selection. Small-scale brewing analysis demonstrated substantial variation in fermentation performance, including rapid and efficient sake production and unique flavor-compound profiles, highlighting their potential for introducing novel traits into brewing practices. Furthermore, several *kuratsuki* yeasts exhibited sufficient alcohol production capacity and desirable flavor characteristics during industrial-scale brewing, supporting their suitability for commercial sake production. The results of this study emphasize the crucial role of preserving brewing cultural heritage, including knowledge and resources related to yeast culture.

Keywords: alcoholic beverage, flavor-compound profile, phenotypic diversity, *Saccharomyces cerevisiae*, sake brewing

1. Introduction

Sake is a traditional Japanese alcoholic beverage produced through the prolonged fermentation of steamed rice using *Saccharomyces cerevisiae* and *Aspergillus oryzae* (*koji*), which is cultivated on steamed rice (Yoshizawa, 1999). *Saccharomyces cerevisiae* plays a central role in sake fermentation and the production of alcohols and a wide range of flavor compounds. Historically, each brewery relied on a uniquely adapted yeast strain (referred to in Japan as "*kuratsuki* sake yeast") that had been naturally domesticated within the brewery environment, resulting in a variety of sake flavors. However, in the last 80 years, industrial sake production has shifted toward the use of genetically similar yeast strains, particularly those belonging to the Kyokai no. 7 (K7) group, which includes the Kyokai no. 6, 7, 9, and 10 series, supplied by the Brewing Society of Japan. The K7 group of strains demonstrate a robust ability to ferment sake effectively, even under low-temperature conditions (Kitagaki & Kitamoto, 2013).

The K7 group is genetically distinct, sharing several common mutations that differentiate them from laboratory, wild, and wine/beer yeasts. Notably, the absence of *PHO3*, which encodes a constitutively expressed acid phosphatase, serves as a DNA marker to distinguish industrial sake yeast strains (Kuribayashi et al, 2014). Within the K7 group, *RIM15* is characterized by a unique single-nucleotide insertion (*RIM15*ins5067A) that is associated with enhanced alcohol fermentation capability (Watanabe et al., 2012). This mutation abolishes the function of the RIM15 protein kinase, which is crucial for regulating cell proliferation in response to nutrient availability. In addition, K7 group strains have various unique genomic features, such as the presence of *K7_02212* and deletions in the *PPT1* gene, which encodes a protein serine/threonine phosphatase, further distinguish this group (Hatakeyama et al., 2017). These phenomena may provide insights into the distinctive characteristics of K7 group strains.

Currently, however, the sake industry is undergoing rapid transformation, marked by a global rise in craft sake breweries (Kita, 2019). These breweries use various yeast strains to produce unique-flavored sakes, which distinguish them in a competitive market. Consequently, there is an urgent need to study and preserve *kuratsuki* yeast strains other than industrial brewing yeasts, especially in Japan, which is the birthplace of sake.

A notable example is the *S. cerevisiae* strain YS4, a *kuratsuki* yeast isolated from the Yoshinogawa Sake Brewery in Settaya, Nagaoka City, Niigata Prefecture, one of Japan's leading sake-producing regions (Fig. 1A). Established in 1548, this brewery is among the oldest in the Niigata Prefecture. The YS4 strain exhibits distinct brewing characteristics compared to the K901 strain, another member of the K7 group (Hatakeyama et al., 2020b). Similarly, the Kikumasamune sake brewery (Hyogo Prefecture, Japan) uses a yeast strain with distinctive brewing characteristics (Takao et al., 2018). Although considerable research has focused on industrial sake yeasts and their roles in sake production, studies on the brewing characteristics of *kuratsuki* strains remain limited. In contemporary sake production in Japan, K7 group strains, known for their excellent brewing qualities, are widely used to ensure consistent characteristics during sake production and to enhance the quality of the final product. Thus, with *kuratsuki* yeast strains gradually disappearing from sake breweries, isolating them has become increasingly difficult, and the biological/genetic diversity and potential of these yeast strains for sake fermentation remain largely unexplored. Furthermore,

no reports have described the regional brewing characteristics of the local sake yeasts used in each brewery, primarily because differentiating *kuratsuki* sake yeasts from K7 group strains is challenging owing to their largely similar physiological and genetic characteristics, as they belong to the same species, *S. cerevisiae* (Azumi & Goto-Yamamoto, 2001).

To address this, we previously developed a method for differentiating K7 group strains from other industrial sake yeasts (Kuribayashi et al., 2025; Kuribayashi et al., 2014). The aim of the present study was to isolate *kuratsuki* sake yeast strains from sake breweries in the Niigata Prefecture and assess their fermentation potential. Fermentation testing was conducted using various industrial brewing yeast strains to evaluate their suitability for safe sake brewing. Additionally, the fermentative properties of several *kuratsuki* yeasts were characterized on an industrial scale, resulting in the production of commercially viable sake with a unique flavor profile.

2. Materials and methods

2.1. Yeast strains and growth media

The Kyokai sake yeast strains, including classic strains (no longer distributed by the Brewing Society of Japan) such as K1, K2, K3, K4, K5, and K8; currently distributed strains of the K7 group (K6, K7, K9, and K10); and the shochu S2 and SH4 yeast strains, were obtained from the National Research Institute of Brewing. The laboratory strain S288C was purchased from Open Biosystems. The *kuratsuki* sake yeast strain YS4 was provided by the Yoshinogawa brewery located in the Niigata Prefecture (Fig. 1A; Hatakeyama et al., 2020b). To examine whether the brewing characteristics of *kuratsuki* sake yeasts differ from those of wild yeast strains found in nature, a wild yeast strain HG-3 was collected from Muramatsu Park (Gosen City, Niigata Prefecture) (Fig. 1A; Kuribayashi et al., 2024). Additionally, the wild yeast strain KY7 was isolated from a Japanese zelkova tree in the garden of Hiki Brewery (Niigata City), and SD1 was isolated from Sado City. These yeast strains were screened for the *PHO3* locus of *S. cerevisiae* using loop-mediated isothermal amplification (LAMP), as described by Kuribayashi et al. (2024).

Yeast cells were cultured in liquid yeast extract peptone dextrose (YPD) medium consisting of 1% yeast extract, 2% yeast peptone, and 2% glucose (w/v). Solid YPD plates were prepared by adding 2% agar to the YPD medium. Triphenyltetrazolium chloride (TTC) plates (consisting of TTC-basal medium, a high-phosphate solid medium containing 1% glucose, 0.2% peptone, 0.15% yeast extract, 0.1% KH_2PO_4, 0.04% $MgSO_4$, 0.027% citric acid, and 3% agar) were purchased from the Brewing Society of Japan. Spore plates (containing 0.5% sodium acetate and 2% agar) were used to induce sporulation. Growth medium containing canavanine, arginine, and ornithine (CAO) was used to positively select mutant yeast strains that do not produce urea (Difco; 0.17% yeast nitrogen base without amino acids or ammonium sulfate [Becton Dickinson Co., Sparks, MD, USA], with 10 mg/L L-canavanine [Sigma, St. Louis, MO, USA], 5 mM L-ornithine, 1 mM L-arginine, 2% glucose, and 2% agar) (Kitamoto et al., 1993).

2.2. Isolation of kuratsuki sake yeast strains

The locations of different sake breweries in the Niigata prefecture from which the

kuratsuki yeast strains YT1, YM3, KM1, HZ3, and OU5 were isolated are shown in Fig. 1A. Given the widespread reliance on the K7 group of yeasts for sake production, we developed a procedure to screen *kuratsuki* yeast strains that were distinct from K7 group strains (Fig. 1B).

First, bulk DNA from sake mash samples was extracted using the alkali-boiling method, and the *PHO3* gene of *kuratsuki* yeast strains was detected using our LAMP assay (Kuribayashi et al., 2014). *PHO3* is present in *kuratsuki* yeast but is absent in the K7 group of sake yeasts (Fig. 2). Next, yeast strains in the mash (which showed detectable *PHO3* locus) were inoculated on TTC plates and incubated at 28°C for 3 days. Candidate *kuratsuki* yeast colonies on the plates were selected using the diazocoupling stain method (Mizoguchi & Fujita, 1981). The K7 group strain colonies lacked acid phosphatase activity due to deletion mutations in the *PHO3* gene.

Isolated stained colonies were examined using polymerase chain reaction–restriction fragment length polymorphism analysis to detect the absence of the *RIM15*ins5067A mutation, which is characteristic of K7 group strains (Kuribayashi et al., 2025). PCR amplicons generated using the forward primer 5′-GGAAAGCGACCGACTACAGG-3′ and reverse primer 5′-CAATAGCACCAAGTTTTGGAAGCCAC-3′ were digested with the restriction enzyme CspCI (New England Biolabs, Ipswich, MA, USA). Using this series of selection methods, the isolated *kuratsuki* sake yeast strains were confirmed as *S. cerevisiae* through DNA sequencing of the 26S rDNA region, as described by Kawahata et al. (2007). The sequence data of the yeasts isolated in this study are presented in Supplementary Table S1.

2.3. Genetic properties

LAMP assays for *S. cerevisiae K7_02212* and *PPT1* were performed as described by Hatakeyama et al. (2017). The *FDC1*-K54* nonsense mutation, located in the *FDC1* gene encoding ferulic acid decarboxylase, was sequenced using the forward primer 5′-GTGCAATTATGAGGAAGGCCTATGAATCCCACTTACCAGCCCCGTTAAT-3′ and reverse primer 5′-TGCAGATGACACAGGAACAGTG-3′, as previously reported (Hatakeyama et al., 2020a). As one of the genes responsible for 4-vinyl guaiacol (4-VG) production, FDC1 protein converts ferulic and coumaric acids to their corresponding vinyl derivatives, resulting in the development of an off-flavor in sake.

2.4. Physiological profiles

Sporulation tests were performed by culturing yeast strains on YPD plates at 28°C for 3 days, followed by transfer to sporulation medium and incubation for an additional 1–2 days at 28°C. Killer toxin activity in *kuratsuki* sake yeast strains was evaluated via clear zone assays using Kyokai no. 901 cells (Brewing Society of Japan) as indicator cells (Hatakeyama et al., 2020b).

To validate the presence of mutations related to non-urea production in the collected yeast strains, we examined their ability to grow in CAO medium (Kitamoto et al., 1993). Briefly, 5 mL of YPD medium was inoculated with yeast strains and incubated at 30°C for 2 days. The resulting cells were harvested and washed with sterile water. Tenfold dilutions were then spotted onto CAO plates. Each culture was incubated at 30°C for 3 days, and yeast growth was assessed using a CAO plate.

2.5. Sake-brewing analyses

Small-scale sake-brewing tests were conducted to evaluate the fermentation capacities of Kyokai sake yeast, *kuratsuki* yeast, shochu yeast, and wild or laboratory strains, using 100 g of total rice for each test. The sake mash was prepared by adding rice, *koji*, and water, as described by Namba et al. (1978). Yeast cultures were initially grown in YPD medium. Fermentation was performed at a constant temperature of 15°C, and CO_2 levels were measured on the final day. After 16 days, each sake mash was centrifuged (4,440 *g*, 15 min, 4°C), and the supernatant was further refined to obtain sake.

An industrial-scale sake-brewing test was carried out using 200–1,500 kg of total rice from sake breweries in the Niigata Prefecture. Industrial sake production is based on fermentation with *kuratsuki* sake yeast residing in their own breweries. The sake was fermented using the standard *Ginjo* brewing method (using a low fermentation temperature and rice with a low polishing ratio) (Washizu and Yamazaki, 1974). Throughout the fermentation period, sake mash was sampled at regular intervals for component analysis. After fermentation, the sake mash was filtered, and the filtrate was obtained as sake.

The general properties of the sake and flavor compounds were analyzed following the standard method established by the National Tax Agency of Japan (https://www.nta.go.jp/law/tsutatsu/kobetsu/sonota/070622/01.htm) using headspace gas chromatography (GC-14A, Shimadzu, Kyoto, Japan). Urea contents were determined using a commercial assay kit (DetectX Urea Nitrogen Colorimetric Detection Kit, Ann Arbor, MI, USA) according to the method of Nagai et al. (2020). The glucose content was measured using a GA05 glucose meter (A&T Corporation, Kanagawa, Japan). We measured 4-VG content following a previously described method (Coghe et al., 2004) involving high-performance liquid chromatography (InertSustain C18 column; 250 mm × 4.6 mm, 5 μM, GL Sciences, Tokyo, Japan) and fluorescence detection (RF-20Axs, Shimadzu, Kyoto, Japan).

2.6. Statistical analysis

All statistical analyses were performed using Excel Toukei (Social Survey Research Information, Tokyo, Japan) which is a statistical software package, and using the statistical software 'EZR' (Easy R) (Kanda, 2013). Data obtained in the small-scale brewing test are expressed as mean ± standard deviation (Supplementary Table S2). As industrial-scale sake production was conducted only once at each brewery, the data were not statistically analyzed.

3. Results and discussion

3.1. Isolation of kuratsuki sake yeast strains

We screened for *kuratsuki* sake yeast strains, excluding those belonging to the K7 group, from sake mash obtained from various sake breweries in the Niigata Prefecture (Fig. 1A) and analyzed their genetic and physiological characteristics. Although this screening was performed for many breweries in the Niigata Prefecture, no *kuratsuki* sake yeast strains were initially isolated.

Next, we isolated *kuratsuki* sake yeast strains from high-foaming mash. In modern sake production, non-foaming sake yeast strains are frequently used, which has decreased the occurrence of high foam in sake mash. Prior to the development of the K7 sake yeast series of

non-foaming yeasts, foaming landrace yeasts were commonly used for sake production (Ouchi, 2010). Therefore, a high level of foam in sake mash is likely indicative of the presence of a unique yeast strain. Using mash that produced unexpectedly high levels of foam, we successfully isolated five *kuratsuki* sake yeast strains from five breweries.

Together with one strain previously isolated from the Yoshinogawa Sake Brewery in 2020, these six strains were subjected to genetic analysis (Fig. 2). The *kuratsuki* strains harbored *PHO3*, *K7_02212*, and *PPT1*, genes also present in classic Kyokai sake and shochu yeast strains. However, these isolates did not possess the genetic signatures of K7 group strains widely used in modern sake brewing, nor did they align with wild or laboratory yeast strains. Furthermore, the *RIM15*ins5067A mutation was only revealed in K7 sake yeast. Similar to *K7_02212*, the *FDC1*-K54* mutation variant was identified in all brewing yeasts, including *kuratsuki* yeast. In addition, sake breweries attempting to isolate their own sake yeast did not use the classic Kyokai sake yeast/shochu series. Therefore, these results suggest that our *kuratuski* sake yeasts maintain an original landrace different from classic/modern Kyokai sake yeast strains, shochu yeasts, and wild/laboratory strains. This distinction was further supported by physiological characteristics such as loss of spore-forming ability and acid phosphatase activity (Fig. 2). Loss of sporulation has been associated with the domestication of industrial beer yeasts (Ogata, 2019; Gallone et al., 2016), and K7 group yeasts are more evolved than *kuratsuki* sake strains in the sake brewing environment. Nonetheless, sporulation was observed in most *kuratsuki* strains, suggesting their utility in breeding programs via mating techniques and offering various applications within the brewing industry.

These *kuratsuki* yeasts do not interfere with sake production and can be used safely at industrial sites, because all yeasts in this assay were non killer toward *S. cerevisiae.*

Moreover, none of the yeast strains grew on CAO plates. In the Niigata Prefecture, non-urea-producing yeast strains, such as G9, G74, and G8, have been developed by the Niigata Prefectural Sake Research Institute (Kuribayashi et al., 2015) and are frequently employed in sake production. However, the *kuratsuki* strains in this study are clearly distinct from Niigata sake yeasts based on their inability to grow on CAO medium.

Collectively, these findings suggest that *kuratsuki* yeast strains from Niigata breweries are closely related to shochu/classic Kyokai yeasts, which cannot be used in these breweries. No reports have described the isolation of multiple brewery-specific yeast strains from a geographically restricted area, such as a prefecture; therefore, the results of this study provide valuable information for sake brewers. Furthermore, the isolated strains inhabit specific sake breweries, and each strain appears to have adapted uniquely to its respective brewery environment. For instance, strain YS4 exhibits non-sporulating and non-foaming phenotypes distinct from the other *kuratsuki* isolates (Hatakeyama et al., 2020b), although these traits remain to be fully characterized.

3.2. Small-scale sake brewing

Small- and industrial-scale fermentation tests were performed to investigate the fermentative properties of the *kuratsuki* sake yeast strains isolated in this study. The fermentation profile for small-scale sake brewing is shown in Fig. 3A and Supplementary Table S2, and principal component analysis of these parameters is shown in Fig. 3B.

Hierarchical cluster analysis showed that the K7 group of yeasts, classic Kyokai sake strains, and wild strains clustered together to some extent, whereas the *kuratsuki* yeasts were dispersed. These results suggest that brewery-specific yeasts are enriched in the variation in sake brewing characteristics. Similarly, principal component analysis also showed that the classic Kyokai yeasts, K7 sake group, and wild yeasts are arranged together, whereas the *kuratsuki* sake yeasts are distributed uniformly. Furthermore, because the yeast strains KY7, SD1, and HG3 appeared to be independent of the other groups, we examined whether the wild yeast group had statistically significant differences in these brewing characteristics compared with the other strains. Statistically significant differences were detected using multivariate analysis of variance ($p < 0.01$), suggesting that there were clear differences in the overall brewing profiles between the wild and other yeast groups. This was also supported by the phylogenetic analysis of these yeast strains, which showed that wild and laboratory yeasts differed genetically from industrial brewing yeasts (Supplementary Fig. S1).

Unlike wild yeast strains, which produced 4-VG at concentrations exceeding the sensory threshold (52 μg/mL) (Utsunomiya, 2006), *kuratsuki* yeasts produced 4-VG at concentrations below this threshold, comparable to other industrial brewing yeasts (Fig. 4). Indeed, 4-VG levels in sake produced by wild strains were significantly higher than those in sake produced by industrial yeasts (Fig. 4). In alcoholic beverages such as sake and beer, 4-VG is considered an undesirable off-flavor compound. Failure to convert ferulic acid into 4-VG is often regarded as a marker of domestication in *S. cerevisiae* (Gallone et al., 2016). In this study, all *kuratsuki* yeast strains were genetically deficient in 4-VG production, harboring the *FDC1*-K54* mutation in the causative gene *FDC1* (Fig. 2). These findings indicate that sake yeasts are not naturally present in the environment; rather, they are found in breweries where they have undergone domestication. This possibility is further supported by the observation that 4-VG generation serves as a detoxification mechanism employed by *S. cerevisiae* in natural environments (Kerruish et al., 2024; Mukai et al., 2014).

Sake brewed with *kuratsuki* yeast strains exhibited considerable variation in alcohol concentration (12.9–18.4%) and flavor profiles (Supplementary Table S2). These findings indicate that *kuratsuki* yeasts show substantial phenotypic diversity, even within relatively small geographical regions, such as prefectures, and that they may therefore exhibit considerable variation depending on the specific brewery. Similarly, beer yeast in European farmhouses were found to possess phenotypic characteristics, even within the same genetic diversity group (Preiss et al., 2024). Although the limited sample size may have influenced our findings, a potential explanation for these results, specific to Japan, could be the impact of seasonal labor on traditional artisan groups. Niigata is home to a group of master brewers known as *Echigo-Toji*, one of Japan's three great master brewers (Nojiro, 1966). *Echigo-Toji* is a craftsmen group of master brewers originating in the Niigata Prefecture, who have been active in sake breweries throughout Japan. The *Echigo-Toji* group dates back to the Edo period (1603–1867) and developed as farmers engaged in sake brewing during the winter off season. Thus, the diversity of *kuratsuki* sake strains may have been influenced, in some way, by the relocation of craftsmen who carried yeast with them. HZ3 is markedly distinct from SD1, a wild yeast originating from Sado Island, both genetically and in its fermentation characteristics (Fig. 2, Fig. 3, and Supplementary Fig. S1). This observation makes it unlikely that HZ3 evolved naturally on the island. A reasonable assumption is that HZ3 was brought in

from another location. Gallone et al. (2016) raised this possibility based on the history of the spread of beer yeast with human migration. Additionally, the link between the *FDC1*-K54* mutation/*K7_02212* gene and the existing sake yeast strains implies that they likely stem from a single domesticated ancestor, reinforcing this theory (Fig. 2). Nonetheless, further investigations, such as the examination of the genomic diversity of a larger collection of *kuratsuki* yeast strains, are necessary to comprehensively test this hypothesis.

3.3. Industrial-scale sake brewing

Although the sake-brewing industry has traditionally focused on strains with high ethanol productivity and strong aromatic profiles, increasing consumer demand for low-alcohol and uniquely flavored sake has driven interest in novel strains (Kitagaki & Kitamoto, 2013; Sato et al., 2002). *Kuratsuki* strains exhibit potential for a wide range of applications in the sake-brewing industry. Therefore, to investigate whether these strains are suitable for commercial applications, the YT1, OU5, and KM1 strains were selected for sake-brewing tests in a sake brewery.

The *kuratsuki* OU5 strain was used in an industrial-scale brewing test by fermenting 600 kg of total rice (*Junmai daiginjo-shu* grade, using *Koshitanrei* as the brewing rice, with a rice-polishing rate of 48%). The *kuratsuki* YT1 strain was subjected to an industrial-scale brewing test by fermenting 1,500 kg of total rice (*Junmai daiginjo-shu* grade, using *Yamadanishiki* as the brewing rice with a rice-polishing rate of 50%). The *kuratsuki* KM1 strain was subjected to an industrial-scale brewing test by fermenting 200 kg of total rice (*Junmai daiginjo-shu* grade, using *Koshiibuki* and *Gohyakumangoku* as brewing rice varieties with a rice polishing rate of 50%). In these scaled-up tests, all *kuratsuki* strains successfully produced the alcohol content and flavor profile necessary for commercialization (Fig. 5). Notably, *kuratsuki* strain YT1 underwent slow fermentation in the main mash, resulting in sake with a low alcohol content of 13.7%. KM1 exhibited intermediate alcohol production (15.8%) among the *kuratsuki* strains. The OU5 strain showed fermentation characteristics (alcohol content of 17.6% in the obtained sake) similar to those of K7 group strains (Kuribayashi et al., 2022), brewery yeast km67 (Takao et al., 2018), and *kuratsuki* yeast YS4 (Hatakeyama et al., 2020b). These brewing characteristics were similar to those observed in the small-scale tests (Fig. 3 and Supplementary Table S2); thus, *kuratsuki* sake yeasts can produce sake with various flavors and alcohol contents, even at an industrial scale. These results are crucial for the future of sake production, as the diverse characteristics observed strongly align with growing consumer demand for unique flavors.

The *RIM15* gene in the K7 group contains a characteristic single-nucleotide insertion (*RIM15*ins5067A) associated with its high alcohol fermentation ability (Watanabe et al., 2012). This *RIM15* mutation results in the loss of function of RIM15 protein kinase, which mediates cell proliferation in response to nutrients. However, the OU5 strains do not harbor the *RIM15*ins5067A mutation. Highly fermentable Hiroshima no. 6 sake yeast, such as *kuratsuki* yeast from the Niigata brewery, does not have the insertion mutation *RIM15*ins5067A, and it has been reported that *the MSN4* gene (involved in the alcohol stress response) is also different from that of K7 yeast (Yamasaki, 2021). This finding suggests that genetic mutations other than *RIM15*ins5067A may support high-alcohol fermentation in sake yeast. The three sake products additionally exhibited a marked contrast in their flavor

components (Fig. 5); however, this examination included a limited sample size with significant variations and a lack of studies to establish molecular events. Further studies on the genetic and phenotypic diversity of sake are required to understand these observations.

In conclusion, *kuratsuki* strains isolated from Niigata breweries displayed distinct fermentation properties and produced sake with unique flavor profiles. These results will be valuable for further practical improvement of sake yeasts for industrial-scale applications. In addition, the fermentation properties of *kuratsuki* yeast provide important information for future innovations in sake production.

Declarations of interest

None.

Disclosures

All experiments were performed in Japan and complied with current Japanese laws.

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Figure legends

Fig. 1 – Isolation of *kuratsuki* sake yeast strains from sake breweries in the Niigata Prefecture. A, map showing locations where yeast screening was successful. Years in parentheses indicate the founding dates of the respective breweries. White circles represent *kuratsuki* yeast isolated from the sake brewery; black circles denote wild yeast isolated from the natural environment. B, overview of the procedure used to isolate *kuratsuki* sake yeast strains from sake breweries.

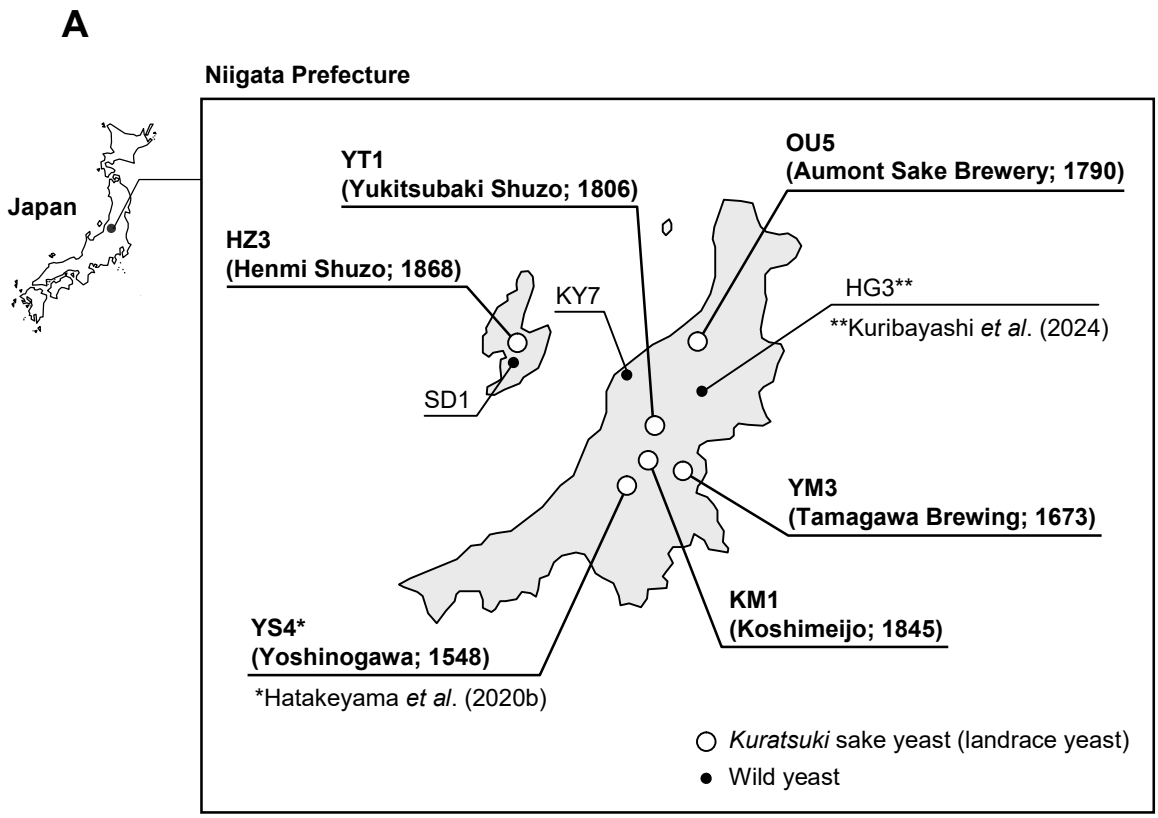
Fig. 2 – Genotypic and phenotypic characteristics of *Saccharomyces cerevisiae* strains used in this study. The *FDC1*-K54* mutation in the K2 strain displayed sequence heterogeneity, and a definitive consensus sequence could not be established.

Fig. 3 – Sake fermentation parameters in small-scale brewing tests. A, heatmap indicating the phenotypic diversity of CO_2 evolution in sake mash, the general components, and flavor-metabolite formation among six *kuratsuki* sake yeast strains, various industrial brewing yeasts, and wild/laboratory yeasts. The heatmap utilizes Z-scores for the color scheme, and the rows are organized through hierarchical clustering. B, principal component projections using the same set of sake-brewing phenotypes as in panel A.

Fig. 4 – Average 4-vinyl guaiacol (4-VG) production levels among the yeast strains studied grouped by population. Statistical analysis was performed using Dunnett test ($*p < 0.01$).

Fig. 5 – Industrial-scale sake production using *kuratsuki* sake yeast strains. A, OU5 strain in Aumont Sake Brewery. B, YT1 strain in Yukitsubaki Shuzo. C, KM1 strain in Koshimeijo. Each left panel shows the fermentation profile of *kuratsuki* sake yeast in the sake mash. Each radar chart on the right represents the flavor components of the resulting sake.

Supplementary Fig. S1 – Phylogenetic relationships of *kuratsuki* sake yeast and wild yeast strains isolated in the Niigata Prefecture. Genotyping via random amplicon sequencing and subsequent single-nucleotide polymorphism call analyses were conducted by Seibutsu-Giken (Kanagawa, Japan). Random amplicons were generated using 64 primers and sequenced on a DNBSEQ-G400 platform (MGI Tech, Shenzhen, China) with a paired-end read length of 150 bp. Sequencing adapters and low-quality bases were trimmed using Cutadapt software (version 4.0) and Sickle software (version 1.33), respectively. Sequences after 76 bases were deleted to ensure uniform read lengths for data analysis. Subsequently, within-population genetic indices, such as the proportion of polymorphic sites, nucleotide diversity, and inbreeding coefficient, were calculated using the Stacks software (version 2.62). Phylogenetic analysis was performed with RAxML software (version 8.2.9) using the GTR + G model and 100 rapid bootstrap replicates, as well as the *pgsumtree* package of Phylogears2 software (version 2.0.2015.11.30).



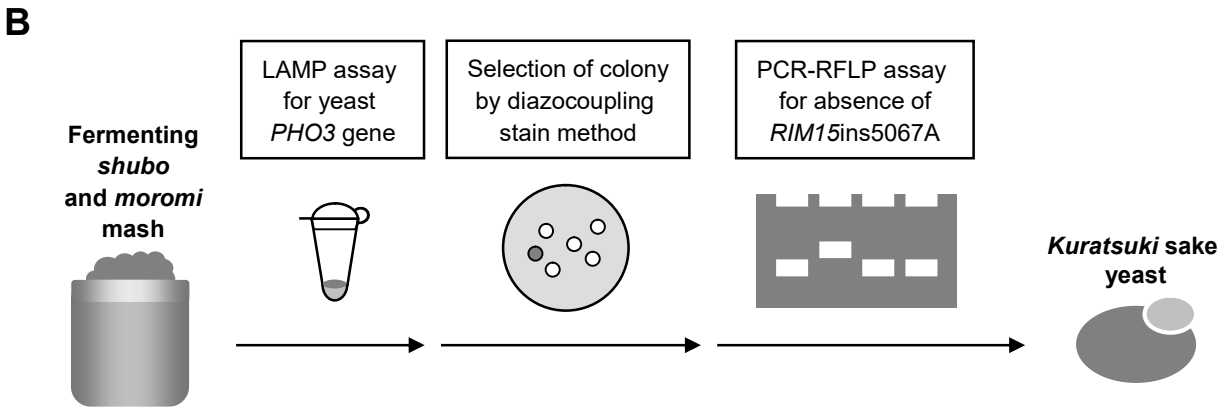


Fig. 1 Kuribayashi *et al*.

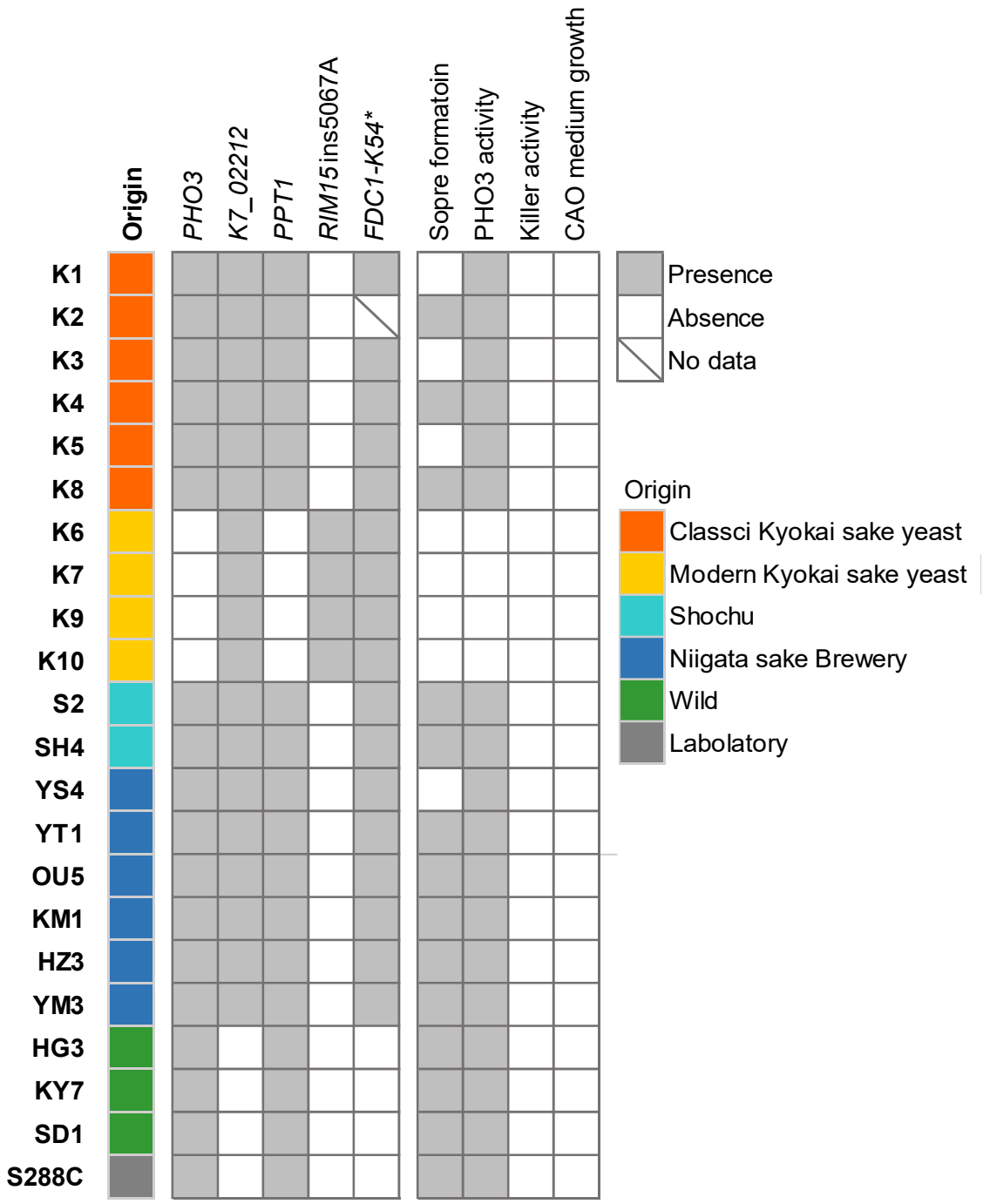
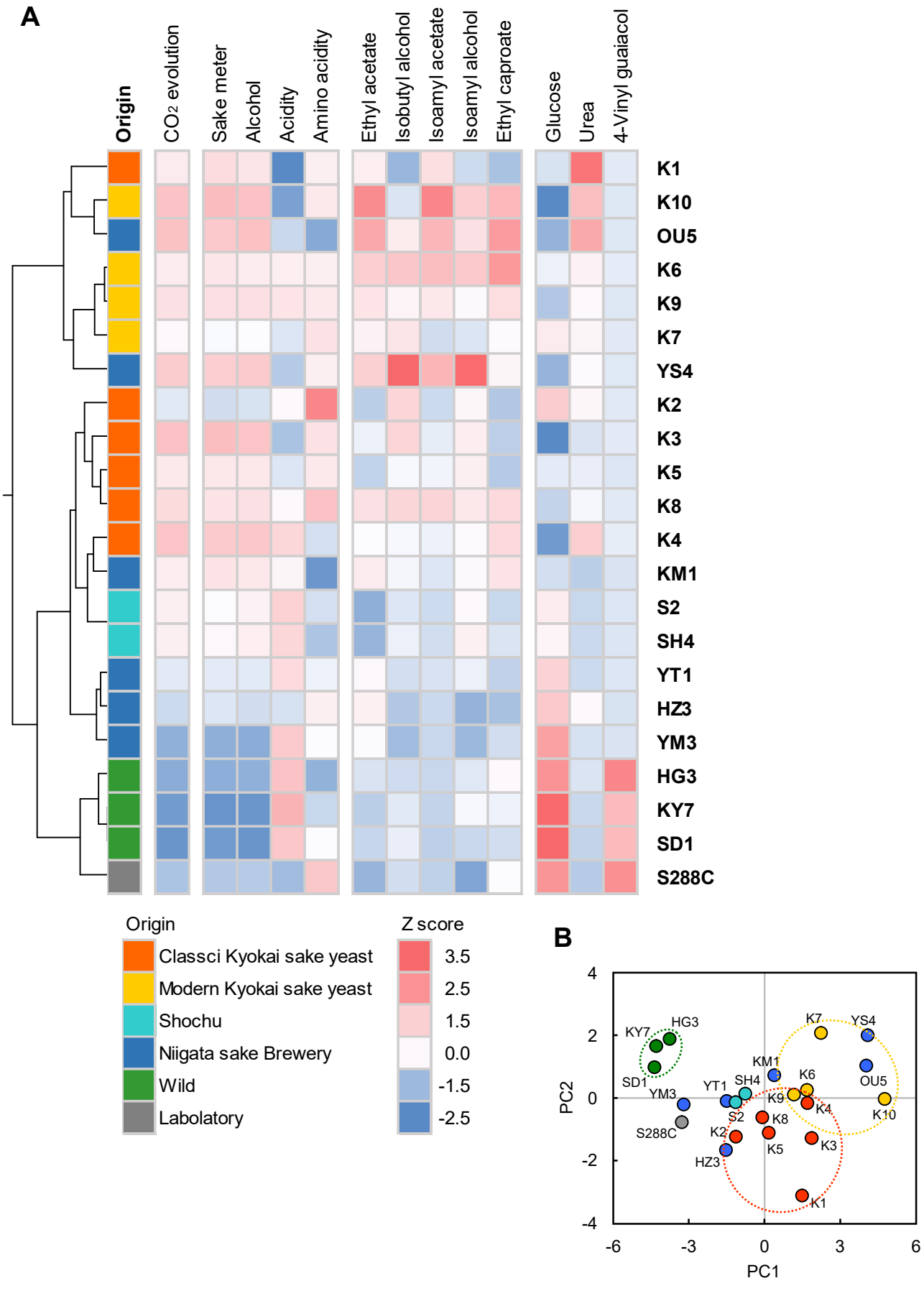


Fig. 2 Kuribayashi *et al*.



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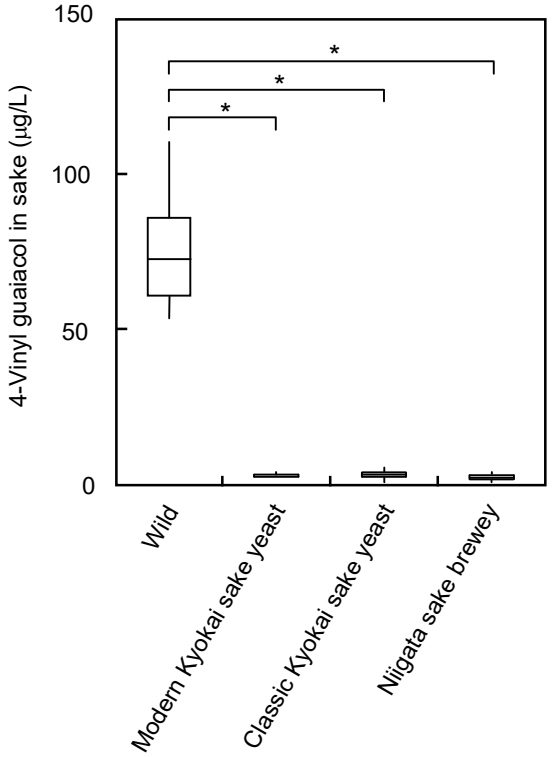

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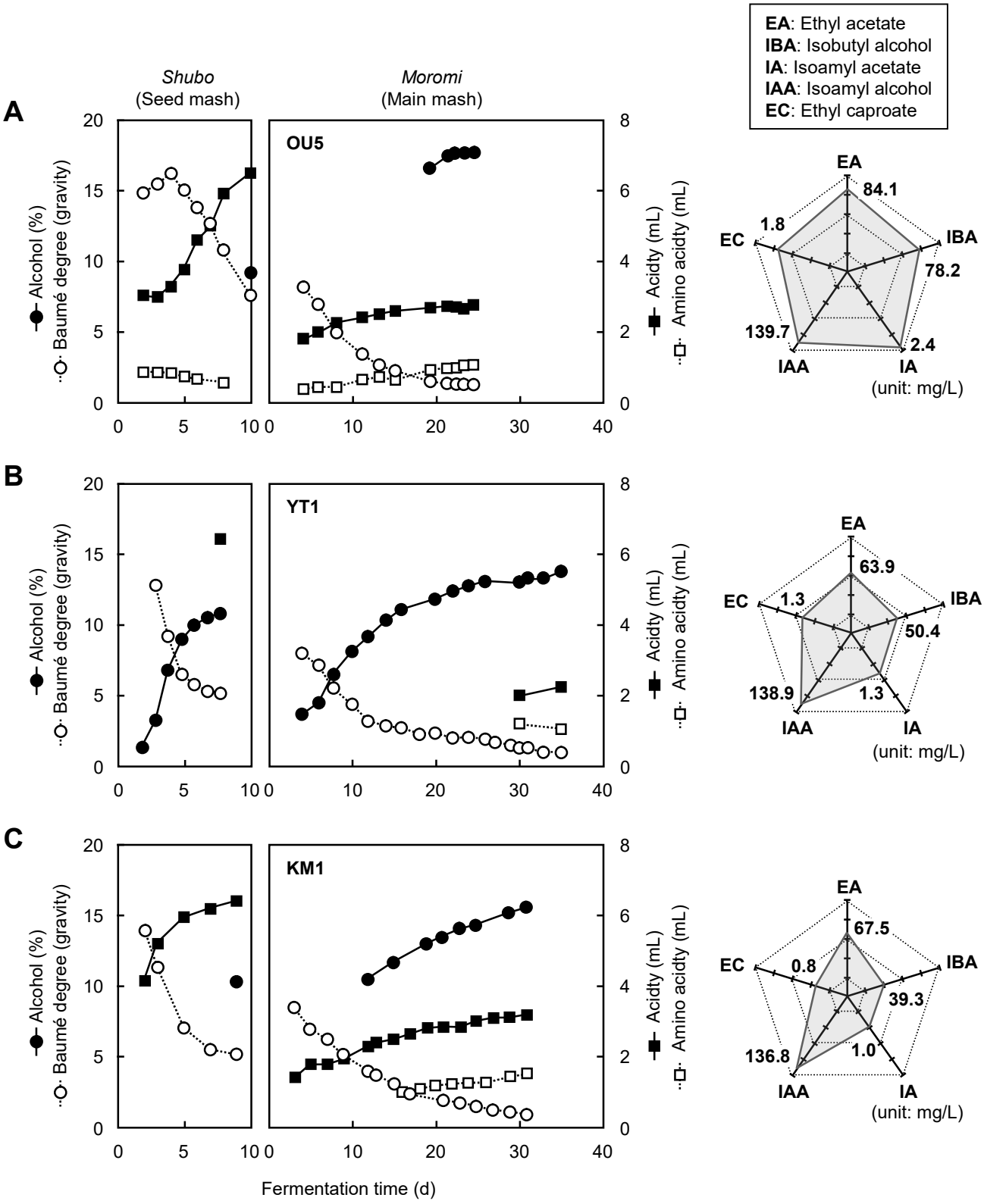

Fig. 5 Kuribayashi *et al*.