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Fermentation and Sporulation Characteristics of *Saccharomyces cerevisiae* SHY111 Isolated from Korean Traditional Rice Wine

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Abstract Various alcohol yeast strains have been isolated from main mashes of Korean traditional liquors, and their genetic diversities were previously reported [23]. In this study, the strain SHY111, showing the highest alcohol production, was tested for its fermentation and sporulation characteristics. Additionally, its haploid cells were isolated and tested for their growth and fermentation patterns. The strain was identified as *Saccharomyces cerevisiae* based on its morphological and physiological characteristics. The sequences of the ITS (internal transcribed spacer) and 5.8S rDNA regions of *S. cerevisiae* SHY111 were found to be identical to those of *S. cerevisiae* that was obtained from through the yeast genome project. The maximum fermentation ratio obtained by the strain SHY111 (96.7%) was almost the same as that by *S. cerevisiae* Balyun No. 1 (96.5%) that was a little higher than that by *S. cerevisiae* KCCM11215 (95.8%). The strain was induced for sporulation in a sporulation liquid medium using log phase cells grown in different types of pre-sporulation media, and its haploid cells were obtained by spore dissection using a micromanipulator. The majority of the spores formed a small colony on a YPD agar plate, and the haploid yeast cells derived from the strain SHY111 showed a variety of growth and alcohol fermentation patterns. It was proposed that the fermentation patterns were related to their growth phenotypes in the most haploid strains, but possibly not in some strains.

Key words: Alcohol yeast, fermentation, haploid, Korean traditional liquor, *S. boulardii*, *S. cerevisiae* SHY111, tetrad analysis

Alcohol fermenting yeasts, which are also known as brewer's yeast, had been classified as a variety of species, which include *Saccharomyces cerevisiae*, *S. coreanus*, *S. ellipsoideus*, *S. sake*, *S. uvarum*, *S. vini*, etc., based on the type of liquors in the brewing of which they participate. However, the majority of alcohol yeasts are now classified as a single species, *S. cerevisiae* [1, 15, 19, 29].

Among Korean traditional liquors, the most typical ones are *Yakju* and *Takju*, which are similar to Japanese *Sake*. They are the most well known Korean liquors, which have been widely consumed for over thousands of years in Korea. Major alcohol yeast strains responsible in the fermentation of Korean traditional liquors have been classified as *S. coreanus*, but now they belong to *S. cerevisiae* [15]. It was first isolated by Saito in 1910 from *Nuruk*, a Korean unique koji for brewing Korean traditional liquors [20]. *S. cerevisiae* was also isolated from the main mash of *Takju* [13, 17]. Because of these reasons, it has been commonly believed that the two yeast strains are the dominant species responsible for the fermentation process of Korean traditional liquors.

There have been several reports on the morphological and physiological characteristics of the Korean traditional liquor yeast [13, 17, 25]. However, little is known about the genetic characteristics of Korean wild yeast strains and their haploid strains have never been isolated, although they can be used more easily for the strain improvement by genetic engineering. This might be due to the fact that microbiological studies conducted on Korean traditional liquors have been largely focused on the microflora in Korean *Nuruk* and main mashes [9, 17, 25, 30]. Our objective was to identify the dominant yeast strain participating in the fermentation of Korean traditional liquors and to isolate its haploid strains for the purpose of the strain improvement. In a previous paper [23], we isolated various alcohol yeast strains from Korean traditional liquors including *Yakju* and *Takju*. When classified by the Biolog Automated Identification System, they were found to belong mostly to *S. boulardii*.

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S. boulardii has been widely used in Europe to treat diarrhea in humans [20], and there have been many studies showing that this strain is effective in the prevention and treatment of many forms of diarrhea, especially antibiotic-associated diarrhea and colitis [3, 7, 21, 28]. However, this strain was never listed in the yeast identification books until 1997. In 1998, McCullough *et al.* [20] reported that the commercial strains designated as *S. boulardii* were not representatives of a separate species, and should be re-identified as isolates of *S. cerevisiae*. They could not differentiate the species *S. boulardii* from the strain *S. cerevisiae* by various methods, including amplification of the ITS region by polymerase chain reaction, which is known to be a useful tool for classification of yeast strains [6, 11, 16, 20, 24]. Moreover, Kurtzman and Fell [15] first listed *S. boulardii* as a strain of *S. cerevisiae* in their yeast identification book in 1998. Additionally, it was proposed that *S. boulardii* is an asporogenous strain of the species *S. cerevisiae*, because it does not produce any spores in repeated testing [20]. Despite these reports, the strain has been designated as *S. boulardii* by many investigators [3, 7, 8, 21, 28].

These ambiguities regarding the *S. boulardii* strain prompted us to characterize the strain SHY111 more carefully, which had been isolated from the Korean traditional liquor and identified as *S. boulardii* according to the Biolog Automated Identification System. In this study, the strain SHY111 was re-identified by the conventional methods [12], and its DNA sequences covering ITS and 5.8S rDNA regions were analyzed. Its alcohol fermentation and sporulation characteristics were investigated as well.

MATERIALS AND METHODS

Yeast Strains and Media

The alcohol yeast strain SHY111 isolated from the main mash for the Korean traditional rice wine has been previously described [23]. The other yeast strains, *S. cerevisiae* KCCM 11215 (formerly *S. coreanus*) and *S. cerevisiae* Balyun No. 1, were used for the control of alcohol fermentation. Yeast cells were grown at 30°C in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) and YNB (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose) media [26] with shaking (120 rpm) on a rotary shaker. For the alcohol fermentation, a YPD liquid medium supplemented with 15% glucose was used.

Yeast Identification

The yeast identification was performed according to the methods described by Kurtzman and Fell [15] based on its morphological and physiological characteristics.

DNA Sequencing

Previously, amplification and cloning of DNA fragments containing the strain SHY111 ITS and 5.8S rDNA regions were described in detail [23]. Plasmid isolation and purification were carried out by general methods [26]. The sequencing reaction was carried out using a forward (ALFred M13-40) and a reverse primer (ALFred M13) according to the AutoCycle Sequencing Kit (Amersham Pharmacia Biotech Co., Uppsala, Sweden). The PCR cycle program for DNA sequencing comprised of one cycle of 95°C (36 sec), 25 cycles of 95°C (36 sec)-50°C (36 sec)-72°C (84 sec), and then one cycle of 4°C for cooling. The reaction products were resolved on 7 M urea-5.5% polyacrylamide gel, which was read with an ALFexpress DNA sequencer that was supplied by the same company.

Alcohol Fermentation

Alcohol fermentation by the strain *S. cerevisiae* SHY111 and its dissected haploid cells was carried out at 30°C with shaking at 120 rpm on a rotary shaker in a 250-ml flask containing 100 ml of YPD medium supplemented with 15% glucose. Alcohol production was monitored every hour by reading the immersion refractometer scale of distillate of the culture broth and calculating the corresponding alcohol percentage by volume from the alcohol calculation table [2]. The alcohol fermentation ratio was expressed as the percentage of the actual alcohol content produced per theoretical amount of alcohol production under the conditions employed in this study. All experiments were carried out in triplicate at least three times.

Sporulation

Yeast sporulation was carried out in a liquid medium by the method of Codon *et al.* [5]. Pre-sporulation medium was composed of 0.8% yeast extract, 0.3% bacto-peptone, and 5% potassium acetate. Sporulation medium was composed of 1% potassium acetate, 0.1% yeast extract, and 0.05% glucose. Yeast cells grown in YPD medium for 24 h were inoculated into pre-sporulation medium and incubated at 22°C with shaking (120 rpm) on a rotary shaker. Cells were collected by centrifugation and washed with sterile 0.9% NaCl. The washed cells were resuspended in sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose), and then incubated at 30°C for 7 days for the induction of sporulation. Numbers of asci containing spores as well as vegetative cells were counted on a microscope. The sporulation ratio was calculated as the percentage of asci per total counts.

Tetrad Analysis

The yeast tetrad analysis was carried out as described previously by Kaiser *et al.* [12]. After the yeast cells were induced to sporulation in the liquid sporulation medium,

asci was harvested by centrifugation. The asci was washed twice with 0.9 M Sorbitol-0.1 M EDTA (pH 7.5), and resuspended in the same buffer. The resuspension was treated with 50 μl of 2.5 mg/ml Zymolyase and 50 μl of 0.28 M β-mercaptoethanol at 37°C for 30 min to remove the cell wall, and tetrads were directly dissected using a glass capillary on a micromanipulator (Singer Instruments Co. Ltd., Watchet, U.K.). The dissected spores were germinated and grown at 26°C for 5 days on an YPD agar plate, and they were then tested for their growth and alcohol fermentation.

RESULTS AND DISCUSSION

Identification of the Alcohol Yeast Strain SHY111

In the previous paper [23], several alcohol yeast strains were isolated from main mashes for brewing 4 different Korean traditional liquors (3 different kinds of *Yakju* and 1 *Takju*). Among them, the yeast strain SHY111 showed the highest alcohol production in YPD liquid medium that was supplemented with 15% glucose. Morphological and physiological characteristics of the strain SHY111 were investigated for its identification by the conventional methods. The strain showed the same morphological and physiological characteristics as those of *S. cerevisiae* (Table 1). Therefore, the isolated strain SHY111 was identified as a strain of *S. cerevisiae*. The strain was also tested for fermentation of 10 different carbohydrates and assimilation of 24 different carbon compounds including glucose, galactose, sucrose, maltose, raffinose, lactose, cellobiose, trehalose, starch, and inulin. It showed the same features as *S. cerevisiae*, with an exception of inulin assimilation (data not shown). The strain was found to assimilate inulin, which *S. cerevisiae* was unable to assimilate [1, 15, 19, 29].

These results prompted us to analyze its ITS regions in order to confirm whether or not it is genetically close to *S. cerevisiae*. The sequences of the ITS I (located between 18S and 5.8S rDNA) and ITS II (located between 5.8S and 26S rDNA) regions have been known to be useful in classifying the fungi [6, 11, 16, 20, 24]. PCR amplification of the strain SHY111 ITS as well as 5.8S rDNA regions and its cloning were previously described in detail [23]. Therefore, the sequences of the strain SHY111 ITS and 5.8S rDNA regions were analyzed using the recombinant plasmid, and compared with those of *S. cerevisiae* AB 972, which were acquired from the results of the yeast genome project [http://genome-www.stanford.edu]. The sequences covering ITS I, 5.8S rDNA, and ITS II regions were found to be exactly identical to each other, suggesting that the isolated strain SHY111 is genetically very close to *S. cerevisiae* AB 972, a yeast type strain (Fig. 1).

Table 1. Morphological and physiological characteristics of the isolate SHY111.

Classification	SHY111	*S. cerevisiae*
Morphological characteristics		
Cell shape	oval and round	oval and round
Cell size	3.5-5 × 6.5-7.5 μm	3.0-10.0 × 4.5-21
Vegetative reproduction	budding	budding
Ascospore	present (1-4)	present (1-4)
Pseudomycelium	absent	absent
True mycelium	absent	absent
Culture in YM medium		
Pellicle	absent	absent
Ring	absent	absent
Growth in YM agar		
Edge	entire	entire
Elevation	convex	convex, raised
Surface	smooth	smooth
Color	white creamy	white creamy
Physiological characteristics		
Growth	at 25°C, 30°C and 35°C	at 25°C, 30°C and 35°C
Gelatin liquefaction	-	-
Acid production	-	-
Urea hydrolysis	-	-
Ester production	+	+
Splitting of glucoside	-	-
Cycloheximide resistance	- at 100 or 1,000 ppm	- at 100 or 1,000 ppm

+, positive; -, negative.

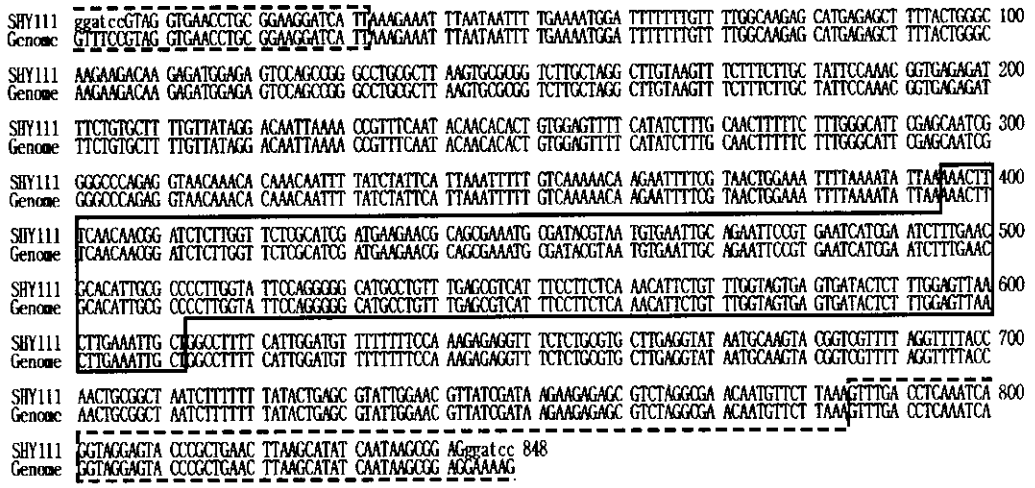

Fig. 1. Sequencing analysis of the Internal Transcribed Spacer (ITS) and 5.8S rDNA regions of *S. cerevisiae* SHY111.
The strain SHY111 sequences were aligned with those of *S. cerevisiae* AB 972 acquired from the results of the Yeast Genome Project [http://genome-www.stanford.edu]. The 5.8S rDNA region is boxed with the solid line, and the 3'-end of the 18S as well as 5'-end of the 26S rDNA is boxed with the dotted line in the figure.

Alcohol Fermentation by *S. cerevisiae* SHY111

The alcohol fermentation ratio by *S. cerevisiae* SHY111 was investigated in YPD medium supplemented with 15% glucose. When the alcohol fermentation was conducted at 20°C to 40°C, the maximum fermentation ratio was obtained at 30°C and 35°C after 36 h of fermentation, which was 96.7%. However, the increase of the fermentation ratio at 30°C was a little slower than that at 35°C. The strain showed almost the same alcohol fermentation patterns in a wide pH range of 4.0 to 9.0. The maximum fermentation ratio was obtained at pH 5.0 or 6.0, which was a little higher than those obtained at pH 4.0, 7.0, 8.0, and 9.0. Adjustment of the initial pH to 3.0 or 10 resulted in relatively slower fermentation than those in the pH range of 4.0 to 9.0 (data not shown).

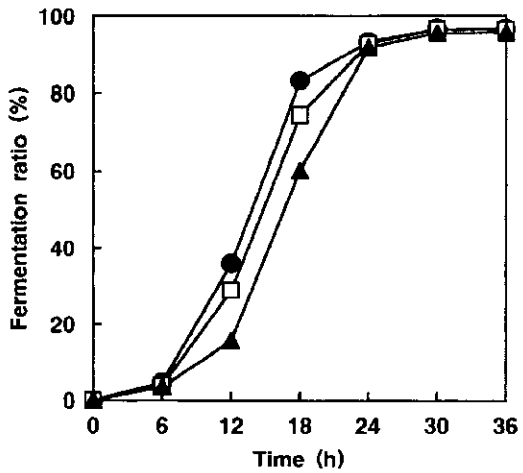

Fig. 2. Comparison of the alcohol fermentation ratio by *S. cerevisiae* SHY111 with those by *S. cerevisiae* strains Balyun No. 1 that has been used in the brewing industry for the production of alcohol, and *S. cerevisiae* KCCM11215 (formerly *S. coreanus*) that was isolated previously from *Nuruk*, a unique Korean koji.
●, *S. cerevisiae* SHY111; □, *S. cerevisiae* Balyun No. 1; ▲, *S. cerevisiae* KCCM11215.

The fermentation ratio by *S. cerevisiae* SHY111 at 35°C and pH 5.0 was compared with those by *S. cerevisiae* Balyun No. 1, which has been used in the brewing industry for the production of alcohol, and *S. cerevisiae* KCCM11215, which has been previously isolated from *Nuruk*, a unique Korean koji (Fig. 2). The maximum fermentation ratio was obtained after 30 h of fermentation by all three strains. However, the strain SHY111 showed faster increase in the fermentation ratio than the other two strains. The maximum fermentation ratio obtained by the strain SHY111 (96.7%) was almost the same as that by the strain Balyun No. 1 (96.5%) and a little higher than that by the strain KCCM11215 (95.8%).

Sporulation of *S. cerevisiae* SHY111

When induced for sporulation by using pre-sporulation and sporulation agar plates described by Sherman [27], *S. cerevisiae* SHY111 was not able to produce any spores. Therefore, we tested various liquid pre-sporulation media for the induction of efficient sporulation of the strain (Table 2). The composition of pre-sporulation medium as well as the growth phase of yeast cells in the pre-sporulation medium were found to significantly affect its sporulation frequency. Stationary phase cells in 5 and log phase cells in only 3 out of 6 different pre-sporulation media could sporulate. Type A pre-sporulation medium could not induce sporulation regardless of the growth phase of the yeast cells. In the case of some pre-sporulation media, such as types C and E, log phase cells could not sporulate, although stationary phase cells could with a

Table 2. Sporulation frequency of *S. cerevisiae* SHY111 on liquid sporulation medium based on the composition of pre-sporulation media and the growth phase of the cells.

Pre-sporulation medium type*	Sporulation frequency (%)	
	Log phase cells	Stationary phase cells
A	0.0	0.0
B	5.6	5.3
C	0.0	6.5
D	18.9	13.5
E	0.0	2.9
F	18.5	5.8

*Pre-sporulation media types A and B composed of 1% yeast extract, 1% Bacto-peptone, and 1% glucose (A) or 1% potassium acetate (B). Types C and D media composed of 0.8% yeast extract, 0.3% Bacto-peptone, and 10% glucose (C) or 5% potassium acetate (D). Types E and F media contained 0.3% yeast extract, 0.35% Bacto-peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, and 1% glucose (E) or 1% potassium acetate (F). Yeast cells grown in the YPD medium were inoculated into various pre-sporulation liquid media and incubated at 22°C with shaking (120 rpm). Cells were harvested at log phase or stationary phase by centrifugation, and resuspended in an equal volume of sporulation medium after washing twice with 0.9% sterile NaCl. The cell suspension was then incubated at 30°C for 7 days, and numbers of asci were directly counted on a microscope. Sporulation frequency was expressed as the percentage of the number of asci per total counts.

poor ratio. However, in the case of the 2 different pre-sporulation media including types D and F, log phase cells showed a higher sporulation ratio than stationary phase cells. The highest sporulation frequency was obtained from the log phase cells in type D pre-sporulation medium, which was about 18.9%. The species *S. boulardii* is known as an asporogenous strain of the species *S cerevisiae*, because it does not sporulate [20]. However, *S. cerevisiae* SHY111 was found to have an ability to sporulate under the present conditions, and this suggests that the strain SHY111 is closer to the species *S. cerevisiae* than to the species *S. boulardii*.

Tetrad Analysis of *S. cerevisiae* SHY111

After the strain SHY111 was induced for sporulation by using pre-sporulation (Type D in Table 2) and sporulation media, tetrads were dissected with a micromanipulator (Fig. 3). The shape of the asci and acospores of *S. cerevisiae* SHY111 are shown in Fig. 3A. Most of the asci were found to contain only 2 or 3 spores and a few of them contained 4 spores, suggesting that sporulation is unstable in *S. cerevisiae* SHY111. When several sets of the dissected spores were germinated and grown on a YPD plate for 5 days, they showed various growth patterns (Fig. 3B). The growth phenotypes on a YPD agar plate were 1 fast-3 slow, 2 slow-2 very slow, all 4 slow, etc. In the case of the spores including 2a, 2b, 3a, 3b, etc., they could not form a colony. When observed on a microscope, they showed 2 or 4 cells, suggesting that they completed the cell division cycle only once or twice during 5 days of the growth period. The reason why their growth was extremely slow remains to be elucidated.

The slow growth phenotype of some of the spores in Fig. 3B might be a result of poor germination or poor growth of the spores. Therefore, haploid yeast cells which were obtained from the tetrad dissection were tested for their growth patterns in YPD and YNB media (Fig. 4). There were dramatic differences found in their growth patterns and maximum cell masses, even among the haploid cells which were derived from the same tetrad set. Although it is difficult to explain the reason for their different growth patterns in the YPD medium, it is more likely due to the growth patterns of the haploid

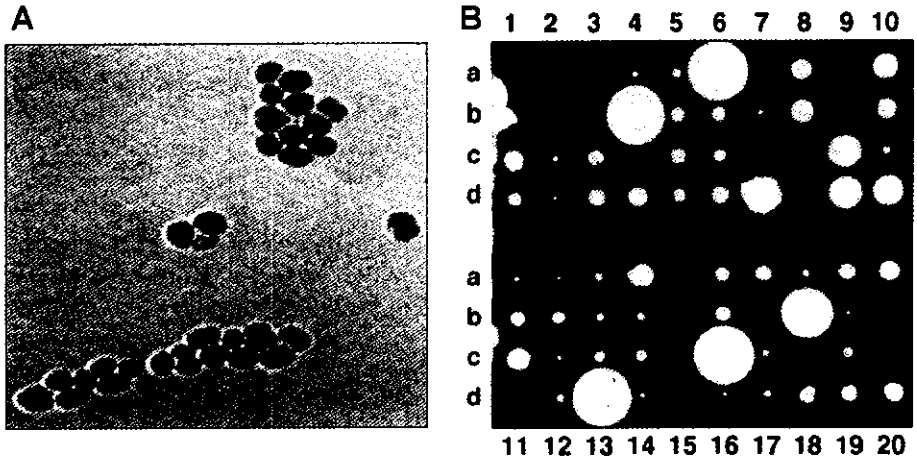

Fig. 3. Microphotograph of ascospores (×400) (A) and dissected tetrads (B) of *S. cerevisiae* SHY111.
Sporulation was induced at 30°C for 7 days in a liquid sporulation medium using log phase cells grown in the pre-sporulation medium (type D) as described in Table 2. After the tetrad dissection, the spores were germinated and grown at 26°C for 5 days on a YPD plate. Each asci was designated with a number, and four each colony derived from the spores in a single ascus was designated with alphabets a to d.

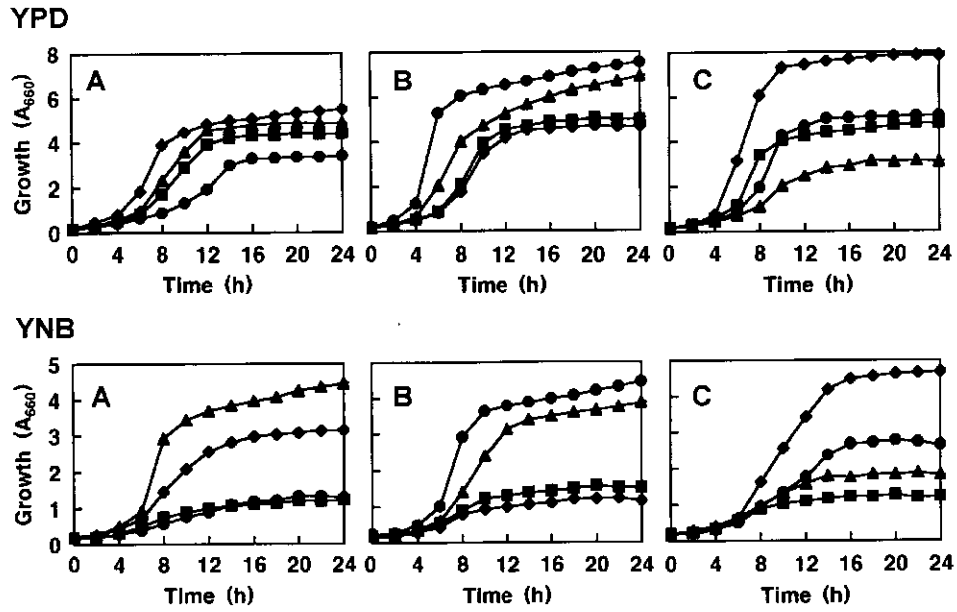

Fig. 4. Comparison of the growth patterns for several haploid strains derived from *S. cerevisiae* SHY111.
Three sets of the haploid cells derived from the *S. cerevisiae* SHY111 spores were grown at 30°C in both YPD (upper panels) and YNB media (lower panels), and their cell growth was monitored every 2 h at 660 nm. All the haploid cells were designated as a (●), b (■), c (▲), and d (◆) derived from tetrad sets 5 (A), 6 (B), and 10 (C) as seen in Fig. 3B.

cells from the same tetrad set to be divided into two groups in the YNB medium; 2 high-2 low in their growth and the cell mass (compare c and d with a and b in the case of tetrad 5 in Fig. 4A YNB; a and c to b and d in the case of tetrad 6 in Fig. 4B YNB). It is interesting to note that haploid strain 10b with a reasonably good growth in the YPD medium showed the slowest growth in the YNB medium among 4 haploid strains derived from the same tetrad set (Fig. 4C in YPD and YNB). Unfortunately, the reason for these growth patterns can not be explained yet.

Alcohol Fermentation by Haploid Cells Derived from *S. cerevisiae* SHY111

Alcohol fermentation was conducted by some haploid cells derived from *S. cerevisiae* SHY111, and their fermentation ratios were investigated (Fig. 5). They showed various alcohol fermentation patterns. In the case of some haploid cells such as 5d, 6a, and 10b, alcohol fermentation occurred after 12 h and reached the maximum fermentation ratio after 24 h of fermentation, which was about 89.0%. Other haploid cells such as 6a and 10a showed a slow increase in the alcohol production and their fermentation ratio reached

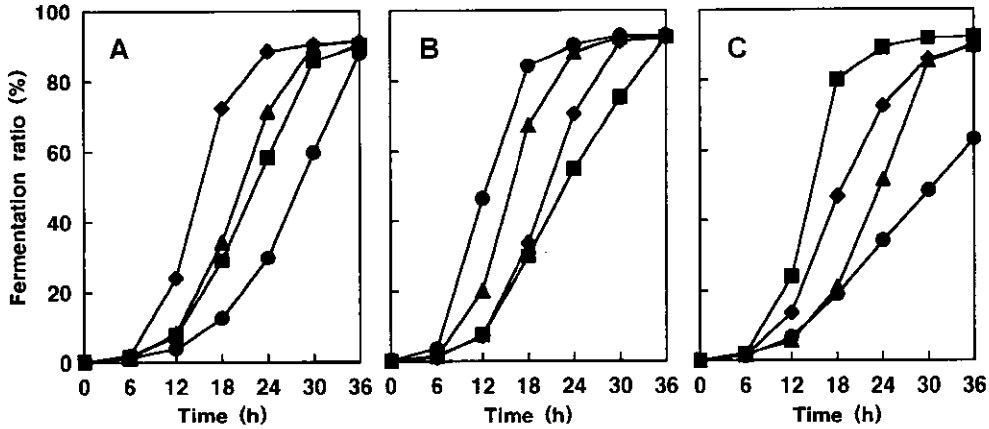

Fig. 5. Comparision of the alcohol fermentation patterns of some haploid strains derived from *S. cerevisiae* SHY111.
Three sets of the haploid cells derived from the *S. cerevisiae* SHY111 spores were used for the alcohol fermentation at 30°C in YPD supplemented with 15% glucose, and their fermentation ratios were monitored every 6 h. All the haploid cells were designated as a (●), b (■), c (▲), and d (◆) derived from tetrad sets 5 (A), 6 (B), and 10 (C) as seen in Fig. 3B.

only about 60% after 36 h of fermentation. However, further fermentation resulted in a similar or even a little higher fermentation ratio than those of the fast fermenter such as 5d, 6a, and 10b. Maximum alcohol fermentation ratios were obtained after 72 h of fermentation by haploid cells 5a and 10a, which were 90% by 5a and 88% by 10a. In the case of tetrad sets 5 and 6, slow growers such as 5a and 5b along with 6b and 6d showed relatively slower alcohol fermentation than with 5c and 5d as well as 6a and 6c (compare Figs. 5A and 5B with Figs. 4A and 4B). However, tetrad set 10 showed an alcohol fermentation pattern that was different from their growth phenotypes. Specifically, the haploid strain 10b, with a reasonably good growth in the YPD and the slowest growth in the YNB media among the spore set, showed the highest alcohol fermentation ratio. The strain 10a, with a reasonably good growth in YPD as well as YNB media, showed the lowest fermentation ratio during 36 h of fermentation (compare Fig. 5C with Fig. 4C).

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