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Characteristics of *Saccharomyces cerevisiae* isolated from fruits and humus: Their suitability for bread making

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ABSTRACT

The objectives of this study were to clarify whether the wild yeast isolated from fruits and humus is suitable for bread making. Using colony PCR, assimilation of carbohydrate and 18S rRNA sequencing, seven strains from among 70 samples were identified as *Saccharomyces cerevisiae*. The ethanol and CO_2 production by the 10-2 wild yeast strain were highest among the strains. The pH and utilized glucose of all strains were pH 3.00-3.60 and 99.99%, respectively. The total acid content of the 9-3 culture was the highest (82.7 mg/100 ml) among the seven strains. The acetic acid contents of 9-3 and 10-2 cultures were 56.8 mg/100 ml and 56.3 mg/100 ml, respectively. Our finding showed that the 9-3 and 10-2 strain isolated from fruits have abilities of fermentation suitable for bread making.

Keywords: *Saccharomyces cerevisiae*, colony PCR, wild yeast, organic acids, bread making

Introduction

Microorganism yeast is used in various fermented foods such as cheese (1, 2), bread (3, 4), vinegar, wine and sake (5, 6). The habitant distribution of the yeast is widely distributed, and it exists in every environment of the natural world such as water, soil, fruits and flowers (7-9).

Baker's yeast, or *Saccharomyces cerevisiae* is, an essential ingredient in bakery products produced by dough fermentation (4). In recent years, the idea of natural yeast bread produced using wild yeast instead of commercial dry yeast has attracted attention in Japan. For example, Shirakami-Kodama yeast, a strain of *S. cerevisiae* isolated in 1997 from leaf mold in the Shirakami Mountains and since used as a commercial baker's yeast has freeze resistance (10, 11). Yeast fermentation is not only to make dough rise, it can impart enhancing effects on quality, such as the generation of favorable flavor and aroma compounds (3, 12, 13).

The objectives of this study were to investigate whether any of the wild yeasts isolated from fruits and humus were suitable for bread making, and to clarify their fermentation characteristics. In this study, our group collected approximately 70 samples from fruits,

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leaf and soil in order to search for new sources of wild yeast. Seven *S. cerevisiae* were identified from these samples. We investigated these yeast strains for characteristics that are important to bread making: CO_2 and ethanol production, fermentation and assimilation of carbohydrates. In addition, we analyzed the amount of organic acid produced by culturing the yeast. These organic acids are associated with the flavor and taste of bread made with yeast (3, 9, 14). We compared the fermentation ability of the seven strains and analyzed the amount and types of organic acids by HPLC analysis.

Materials and Methods

Media

RE medium (0.67% nitrogen base [Difco Laboratories, Detroit, MI, USA], 1% raffinose, 8% (v/v) ethanol and 0.05% Chloramphenicol) was used to culture samples potentially containing yeast strains belonging to the genus *S. cerevisiae*. Color medium (0.172% yeast nitrogen base [Difco], 1% glucose, 3% sucrose, 0.3% methionine, 0.3% yeast extract and 2% agar) was used to isolate *S. cerevisiae*. After autoclaving, 1% aniline blue (2.5 mg/ml) and 1% ponceau R (5 mg/ml) were added to the color medium. YPD medium (1% yeast extract, 2% polypeptone, 2% dextrose, and 2% agar) was used to select a single colony of yeast.

YM medium (1% glucose, 0.5% pepton, 0.3% yeast extract and malt extract) was used to determine the fermentation characteristics of the isolated yeasts.

Sample collection

We collected 70 samples of plants from different locations in the City of Matsudo, Kyoto University and the fruit from a farm in Nagano from 2/08/2012 to 10/11/2012.

Isolation of yeasts

About 1 g of sample was transferred to 50 ml falcon tubes containing 3 ml of RE medium and incubated at 30°C for 48h. White turbidity in the medium indicate positivity; for these cultures, 0.1 ml of the suspension was spread on color medium plates and incubated at 30°C for 48h. After incubation, isolated colonies were directly examined and their purity was verified by visualizing yeast cells under a microscope. Colonies with distinct morphological differences such as color, shape and size were harvested from the color medium plates.

Identification via colony PCR

Identification of *S. cerevisiae* was carried out according to the method described by de Melo Pereira et al. (15). The 25µl PCR mixture contained 12.5 µl PCR solution, 5 µl 2 mM dNTP mix, 0.075 µl (100 pmol/µl) of each primer and 0.5 µl KOD FX (Toyobo, Osaka, Japan). The laboratory yeast *S. cerevisiae* 288C, was used as the control for the genome analysis while the plate colonies were used as the templates. The sequences of the primers were ScHO-F (5'-GTTAGATCCCAGGCGTAGAACAG-3') and ScHO-R (5'-GCGAGTACTGGACCAAATCTTATG-3'). The PCR was initiated at 94°C for 4 min, followed by 35 cycles of the following program: 98°C for 10 s, 50°C for 1 min, and 68°C for 1 min. Amplification products were separated by electrophoresis on a 0.8% (w/v) agarose gel and stained with SYBR○,R Gold (Invitrogen, USA). A ladder marker (GeneRuler 100 bp DNA Ladder Plus) was used as a size reference.

Fermentation and assimilation of carbohydrate

The sugar fermentation test of the isolated yeasts was carried out based on the detection of gas formation in a Durham tube test (16). API ID 32°C (bioMerieux, France) identification kits were used for the identification of yeast strains, and assimilation of carbohydrates.

Identification of wild yeast by 18S rRNA

Nine microorganisms were sent to BEX CO., LTD (Tokyo, Japan) for identification via PCR using TaKaRa PCR Thermal Cycler Dice○,R Standard (TP650) and primers ITS1F (5'-GTAACAAGGT (T/C) TCCGT-3') and ITS1R (5'-CGTTCTTCATCGATG-3'). The 18S rRNA genes were sequenced using these same primers and the BigDye Terminators v1.1 Cycle Sequencing Kit in an

ABI Prism 3130Xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were used in a BLAST search of the DNA Data Bank of Japan (DDBJ).

Ethanol fermentation and analytical methods

Ethanol fermentation was carried out according to the method described by Dombek et al. (17). One hundred fifty mL of YM medium (20% glucose, 0.5% pepton, 0.3% yeast extract and malt extract) was placed in a 300-mL flask and adjusted to pH 5.5-6.0 with 1.0 N HCl prior to autoclaving. Yeast cells from YPD medium were transferred by means of sterile inoculation loops to the flasks. The flasks were incubated at 30°C for 7 days with a 150-rpm shaking rate. The flasks were weighed every 24 hours; the decrease in the volume of the flasks was assumed to be due to CO_2 production by alcoholic fermentation. Fermentation samples were centrifuged at 4,000 rpm for 10 min. The supernatant was removed and the total saccharide in the supernatant was determined as glucose by the Somogyi-Nelson method (18). The supernatant was transferred to a 100-mL volumetric flask, and the distillates to a 300-mL flask. The alcohol levels in the distillate were measured by the adjusting temperature to 15°C using a specified aerometer that floats in distillates.

Organic acid was analyzed by HPLC (HITACHI UV L-7405). A 1-ml sample was filtered through a 0.45-µm syringe filter, applied to a GL-C610H-S column (300 × 7.8 mm; flow rate, 0.5 ml/min, oven temperature, 60°C, injection volume, 20 µl, Hitachi Chemical CO, LTD, Japan), and UV absorbance was measured at 210 nm. All measurements were repeated two times for each treatment.

Results

Isolation and screening of yeast

Nine yeasts were isolated among the seventy samples from fruits, leaves and soil. The nine yeasts on the color medium were blue color and formed glossy colonies. Results of the colony PCR, 1-2, 9-2, 9-3, 9-6, 10-2, 14 and 16 was amplified 400 bp with *S. cerevisiae* by the ScHO primers (Data not shown). Furthermore, the patterns of assimilation ability of sugars in strains 1-2, 9-2, 9-3, 9-6, 10-2, 14 and 16 were agreed well with the metabolic characteristics of *S. cerevisiae* described by Hayford and Jespersen (19), and van der Aa Kuhle and Jespersen (20).

After 48 h on the YPD agar plate, the isolated colonies of yeasts strain 1-2, 2, 9-2, 9-3, 9-6, 10-2, 14 and 16 were observed under-phase contrast light microscope. We chose four yeasts; 1-2, 2, 9-3 and 14 to compare the difference in the form of the yeasts (Fig. 1). As shown in a microphotograph of Fig. 1, the yeast strain 2 was oval and slightly longer than that of 1-2, 9-3 and 14. The yeast strain 9-2, 9-6, 10-2 and 16 were also the shape similar to 1-2, 9-3 and 14 (data not shown).

Characteristics of the wild yeasts

Table 2 shows the physiological characteristics of seven yeasts. All the strains fermented glucose, galactose, sucrose and raffinose. Although 1-2, 9-2, 9-3, 10-2, 14 and 16 assimilated glucose, galactose, sucrose, raffinose, methyl-α-$_D$-glucoside and palatinose, 9-6 did not assimilate palatinose. It was confirmed that these strains could use glucose and sucrose for bread making, but not maltose.

To characterize the fermentation ability of the seven strains isolated, we searched growth characteristics, CO_2 and ethanol production. Growth characteristics in the YM medium containing 20% glucose were assessed by measuring optical density (Figs. 2A and B). At 24 h after the start of the culture, 9-3 was found to have grown more quickly than the other strains. The optical density of 9-2 and 10-2 reached that of 9-3, approximately OD_{660} = 8, at 48 h after the culture was started (Fig. 2A). The growth rate of all strains was not changed after 96 h from the culture start. Ethanol production and glucose utilization were examined at 30°C for 5 d. Ethanol production by 10-2 was highest compared with other strains although the glucose consumption rate of all strains showed almost 100% (Table 3). Likewise, CO_2 production by 10-2 was highest among the strains (Fig 3A).

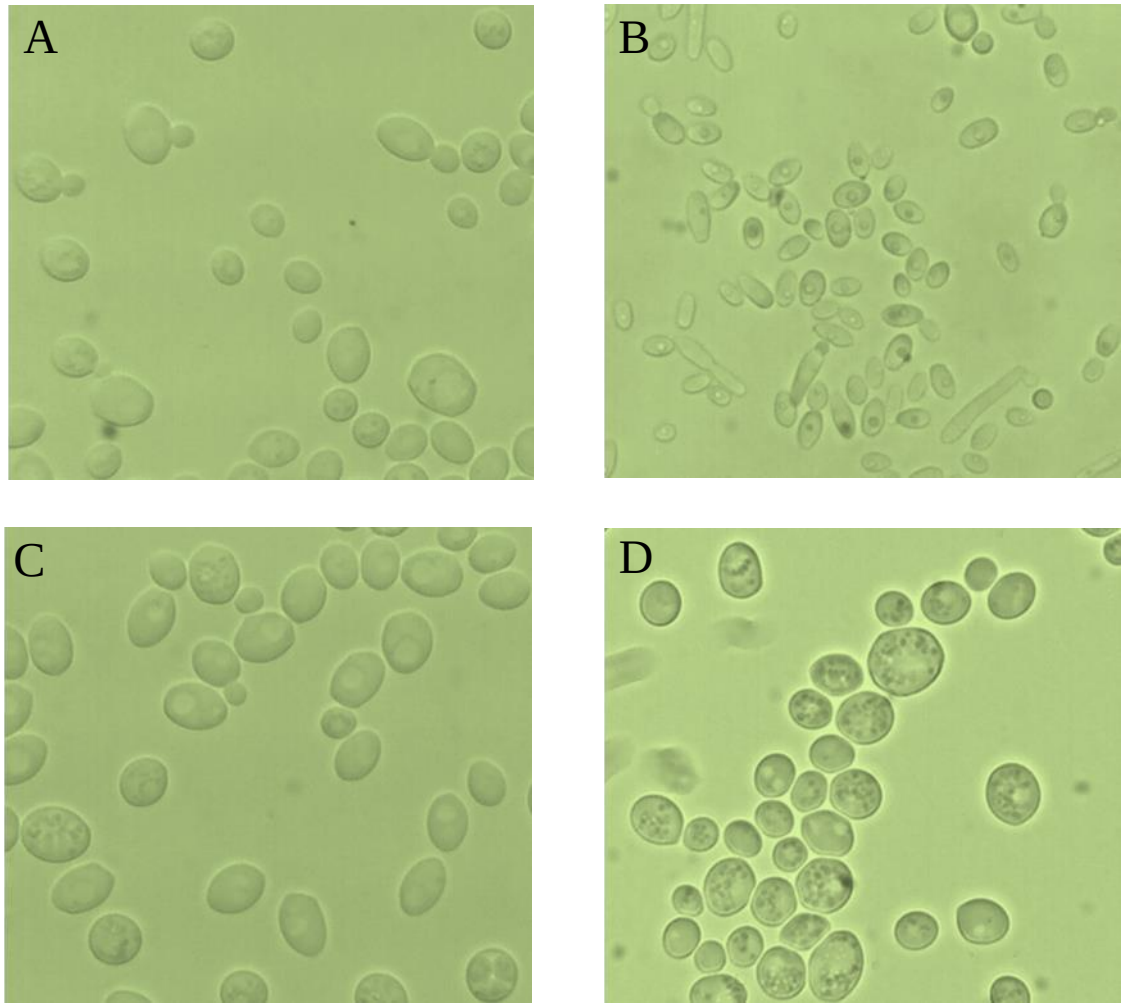

Figure 1. Microscopic images of yeasts (A) 1-2, (B) 2, (C) 9-3, (D) 14.

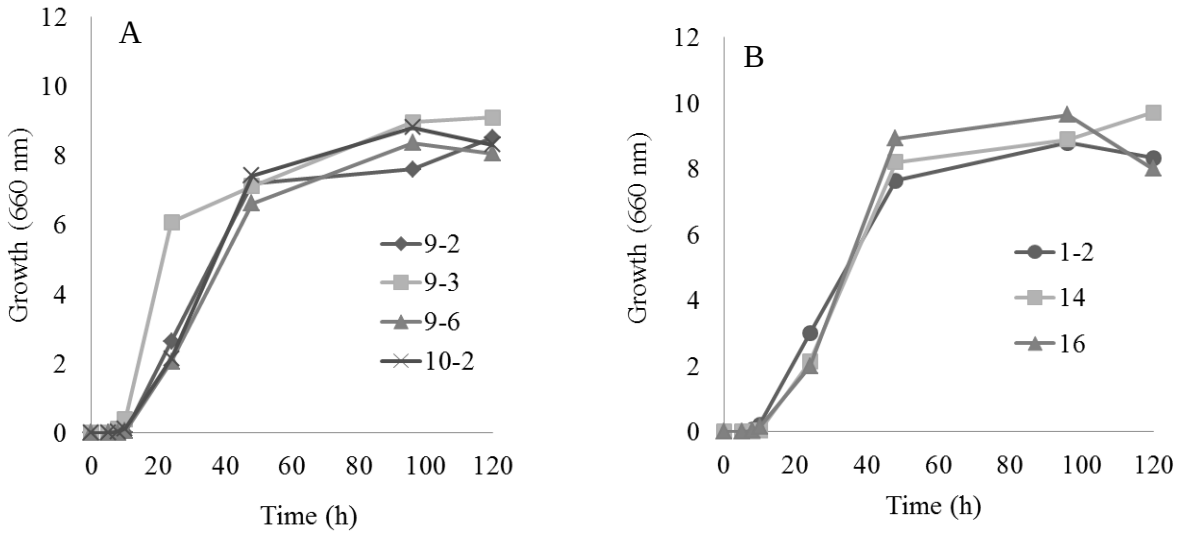

Figure 2. Growth characteristics of seven yeast strains. Seven yeast strains were cultivated by YM medium containing 20% glucose at 30°C for 5 days. In Fig. 2A and B, growth was monitored by measuring optical density at 660 nm at the indicated times.

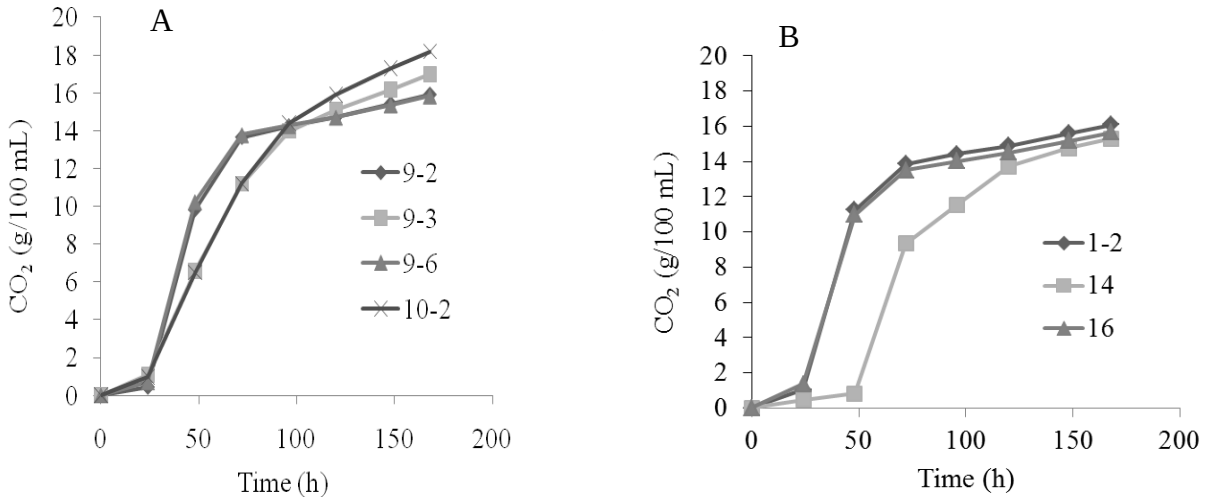

Figure 3. CO_2 production from seven yeast strains. Seven yeast strains were cultivated by YM medium containing 20% glucose at 30°C for 7 days.

Chemical characteristics of yeasts isolated from plants are shown in Table 3. The pH can be used as a marker for organic acids, carbon dioxide, and other substances produced during fermentation (15). The pH and utilized glucose of all strains were pH 3.00-3.60 and 99.99%, respectively. Therefore, it was shown that almost all the glucose in the YM medium was converted into alcohol by the wild yeasts. These results are similar to those of Kim et al. (6), who reported a culture pH of 3.8-3.9 in wild yeasts grown in YM medium. On the other hand, the total acid contents produced by wild yeast differed depending on the type of strains. The total acid content of our 9-3 strain was the highest (82.7 mg/100 ml) among these strains. After that, strains with the highest acid content were 10-2 (76.4 mg/100 ml) and 14 (71.2 mg/100 ml). Acetic acid was higher in these three strains (9-3, 10-2 and 14) than in the other strains. The acetic acid of 9-3 and 10-2 strains were 56.8 mg/100 ml and 56.3 mg/100 ml, respectively.

Discussion

We were to clarify whether the wild yeast isolated from fruits and humus is suitable for bread making. The yeast strains identified by 18S rRNA sequencing included 7 strains of *S. cerevisiae*, 1 strain of *Meyerozyma guilliermondii*, and 1 strain of *Cadida humilis* (Table 1).

Table 1. Yeast strains isolated from plants

Strain No.	Plant	Species	Similarity (%)
1-2	Seed of peach	*Saccharomyces cerevisiae*	100
2	Apple fruit	*Meyerozyma guilliermondii*	100
9-2	Seed of Nectarine	*S. cerevisiae*	100
9-3	Seed of Nectarine	*S. cerevisiae*	100
9-6	Seed of Nectarine	*S. cerevisiae*	100
10-2	Leaves of apple	*S. cerevisiae*	100
14	Soil of Matsudo park	*S. cerevisiae*	100
16	Acorns	*S. cerevisiae*	100
30	Bitter melon (Goya)	*Candida humilis*	97

M. guilliermondii was used as starter for dough fermentation in combination with other strains (21). *C. humilis* was dominant species in sourdoughs for the production of durum wheat bran flour bread (22). It was reported that these yeasts have been used as baker's yeasts. The confirmation of the safety and practicability of the isolated yeast strains 2 and 30 in this study is still needed as the bread making. These results suggest that strains 1-2, 9-2, 9-3, 9-6, 10-2, 14 and 16 belong to *S. cerevisiae*, yeast commonly used in bread baking.

Table 2. Physiological characteristics of yeasts isolated from plants

Fermentation	1-2	9-2	9-3	9-6	10-2	14	16
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	+	+
Assimilation							
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	+	+
$_{L}$-Rhamnose	-	-	-	-	-	-	-
$_{D}$-Melibiose	-	-	-	-	-	-	-
$_{D}$-Xylose	-	-	-	-	-	-	-
$_{L}$-Arabinose	-	-	-	-	-	-	-
$_{D}$-Ribose	-	-	-	-	-	-	-
$_{D}$-Mannitol	-	-	-	-	-	-	-
$_{D}$-Glucosamine	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-
Methyl-α-$_{D}$-glucoside	+	+	+	+	+	+	+
Trehalose	-	-	-	-	-	-	-
Palatinose	+	+	+	-	+	+	+

Responses: +, = positive; - ,= negative

The main function of baker's yeast in bread baking is to increase dough volume by the evolution of gas during fermentation of available sugars in dough (4, 23). In this experiment, although it was observed that the 10-2 strain had liquid fermentation ability among these wild yeast strains, it was still unknown wheatear the 10-2 strain would be suitable for use in bread making.

As mentioned in the result, the highest acid content was 10-2 (76.4 mg/100 ml). Especially, the acetic acids of 9-3 and 10-2 were higher than in the other strains (Table 3). The acetic acid produced by these strains was higher than the acetic acid (0.45 mg/ml) in *yakju* (Korean liquor) made with Y88-4 (6). Bakery products have a very short shelf life. Sourdough has been used since ancient times and its ability to improve the quality and increase the shelf-life of bread has been widely described (24). It has been reported that some sourdough starters enhances the storage stability of the bread because lactic acid bacteria and yeast produce organic acids such as lactic acid and acetic acid that causes a lowering of pH (25, 26).

Sourdough bread is a fermented food made using lactic acid bacteria that has good flavor and antimicrobial activity. Moreover, Onishi et al. (26) have shown that sourdough consumption increase serum triglyceride and suppressed cholesterol levels in rats fed a high-fat diet containing the sourdough bread. It is possible that the 9-3 strain might be used as a starter for various fermented foods such as sourdough.

Table 3. Chemical characteristics of yeasts isolated from plant

Strain No.	pH	Utilized glucose (%)	Alcohol generation (%)	Acetic acid (mg/ml)	Propionic acid (mg/ml)	Isobutyric acid (mg/ml)	n-butyric acid (mg/ml)	Total (mg/ml)
1-2	3.20	99.99	9.55	28.6	15.8	0.51	3.07	47.98
9-2	3.17	99.98	9.56	29.2	6.94	0.77	2.81	39.72
9-3	3.20	99.99	8.48	56.8	22.9	ND	3.01	82.71
9-6	3.06	99.98	8.98	27.1	2.18	1.01	2.30	32.59
10-2	3.53	99.96	10.4	56.3	16.4	0.77	2.91	76.38
14	3.57	99.99	9.44	54.1	13.8	0.69	2.58	71.17
16	3.52	99.98	8.32	24.0	2.16	ND	1.89	28.05

In summary, it is quite probable that the 9-3 and 10-2 strains can be useful in the production of good-tasting bread because these strains showed ethanol- and CO_2-production ability. Further studies are needed to investigate the generation of aroma compounds by these seven wild yeasts and to evaluate the antifungal activities of the 9-3 and 10-2 strains.

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