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Evaluation of baker's yeast strains exhibiting significant growth on Japanese beet molasses and compound analysis of the molasses types

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Cane molasses, most of which is imported, is used as a raw material for production of baker's yeast (*Saccharomyces cerevisiae*) in Japan. On the other hand, beet molasses is scarcely used for this purpose, but it can be of great advantage to cane molasses because it is domestically produced in relatively high amounts as a by-product of beet sugar processing. However, the yield of baker's yeast is sometimes low with Japanese beet molasses compared to imported cane molasses. For the production of baker's yeast with Japanese beet molasses, we evaluated *S. cerevisiae* strains, including industrial and laboratory strains, to group them according to the growth profile on beet and cane molasses. To discuss the factors affecting growth, we further analyzed the major compounds in both types of molasses. Beet molasses seems to contain compounds that promote the growth of beet molasses-favoring strains rather than inhibit the growth of cane molasses-favoring strains. It was assumed that α-amino acid was one of the growth promotion factors for beet molasses-favoring strains.

[**Key words:** Baker's yeast; Beet molasses; Cane molasses; Compound analysis; α-Amino acids]

Beet and cane molasses are used industrially as raw materials for biochemical processes, including ethanol fermentation from yeast, butanol fermentation from bacteria, and citric acid fermentation from mold (1). They also have traditionally been used as a main raw material for the production of baker's yeast (*Saccharomyces cerevisiae*).

To produce baker's yeast in Japan, most cane molasses is imported from Southeast Asia, including Indonesia and the Philippines, while beet molasses is obtained domestically from Hokkaido. The main compound in both types of molasses is sucrose. In addition to sucrose, both contain many similar compounds, such as amino acids, organic acids, and minerals (1). The concentration of these compounds in both types of molasses is variable in each sugar factory because they have different processing steps for the sugar purification. Moreover, this variation often occurs in an individual lot because the quality of sugar beet and cane crops are influenced by climate.

The yield of baker's yeast is variable depending upon the quality of molasses (2,3). In addition, in Japanese factories of baker's yeasts, the yield is sometimes low using Japanese beet molasses compared to imported cane molasses. The growth of baker's yeast is assumed to be affected by various compounds that are included in the molasses.

The growth-inhibitory factors affecting baker's yeast in beet and cane molasses have been reported. For example, lead(II) compounds, including lead(II) nitrate [$Pb(NO_3)_2$], may originate from sugar beet or sugar cane crops irrigated with contaminated water or grown in areas adjacent to high lead(II) emission areas, which have significant negative effects on growth (4). Acetic acid, butyric acid, and organochlorinated insecticides such as lindane and heptachlor also exert negative effects on baker's yeast growth (5).

In Japan, beet molasses is assumed to have the potential for more production than cane molasses because the amount of beet sugar manufactured in Hokkaido Prefecture is 466,000 tons. The amount of cane sugar manufactured at Kagoshima and Okinawa Prefectures is 164,000 tons annually (from October 1, 2010, to September 30, 2011) (6). However, as previously noted, imported cane molasses is mainly used since the yield of baker's yeasts is low when using Japanese beet molasses. Given the economic potential of Japanese beet molasses, we decided to search for *S. cerevisiae* strains that grow significantly on Japanese beet molasses compared to imported cane molasses. We defined three groups by the growth profile of yeast strains in the molasses medium: beet molasses-favoring strains (beet molasses type), cane molasses-favoring strains (cane molasses type), and non-typable. Moreover, to discuss why the strains exhibit significant growth on Japanese beet molasses, the major compounds of both types of molasses (sugar, amino acids, organic acids, and minerals) were compared.

MATERIALS AND METHODS

Microorganisms Twenty-six strains of *S. cerevisiae* were examined for their growth on beet and cane molasses. Five strains were preserved at Nippon Beet Sugar Mfg. Co., Ltd. (Tokyo, Japan) as baker's yeast (named as our stocks), 13 strains were derived from various baker's yeast except for those at Nippon Beet Sugar Mfg. Co., Ltd. (named as others), and five strains were isolated from wildflowers. For reference, one strain (ATCC 9080) was obtained from the American Type Culture

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Collection (ATCC, Manassas, VA, USA), and two strains (NBRC 0216 and NBRC 0224) were obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan).

Molasses Beet molasses was obtained from Nippon Beet Sugar Mfg. Co., Ltd. Cane molasses was imported from the Philippines. The total sugar contents (sucrose + glucose + fructose) of stock solutions of beet and cane molasses were 715 and 315 g/L, respectively. For the purpose of determining the amount of various compounds contained in the molasses, both types of molasses were diluted to 50 g/L of total sugar (the same as molasses medium).

Cultivation In grouping study, *S. cerevisiae* strains were pre-cultivated at 30°C for 12 h in 8 ml of yeast peptone dextrose (YPD) medium [glucose 20 g/L, polypeptone (Nihon Pharmaceutical, Tokyo, Japan) 20 g/L, and yeast extract (Oxoid, Basingstoke, Hampshire, UK) 10 g/L; pH 5.5] with shaking at 150 rpm. After pre-cultivation, 250 μl of culture medium was inoculated into 5 ml of laboratory molasses medium (carbon source 50 g/L, polypeptone 2 g/L, yeast extract 1.5 g/L, KH_2PO_4 1 g/L, and $MgSO_4 \cdot 7H_2O$ 0.4 g/L; pH 5.5) and grown for 12 h at 30°C with shaking at 150 rpm. Beet molasses and cane molasses were used as the sole carbon sources; the concentration of the carbon source was the sum of sucrose, glucose, and fructose. To prevent the Maillard reaction, sterilization was performed at 105°C for 5 min. Growth was monitored by measuring the turbidity with a Ubest-30 spectrophotometer (Jasco, Tokyo, Japan) as the optical density (OD) at 660 nm. The molasses growth type was defined using Eq. 1:

$$OD_{660} \text{ cultivated in beet molasses medium} - OD_{660} \text{ cultivated in cane molasses medium} \quad (1)$$

(>1.0: beet molasses type; <−1.0: cane molasses type; and from −1.0 to 1.0: non-typable)

In time-course studies, the strains were pre-cultivated in 8 ml of YPD medium with shaking at 150 rpm and 30°C for 12 h. After pre-cultivation, the yeast cells were harvested by centrifugation, washed, and suspended in distilled water to avoid the influence of metabolic products accumulated in the YPD medium. The suspension was adjusted at $OD_{660} = 20$; 250 μl was inoculated into the 5 ml of laboratory molasses medium, industrial molasses medium [carbon source 50 g/L, $(NH_4)_2SO_4$ 5 g/L, $NH_4H_2PO_4$ 1 g/L, and $MgSO_4 \cdot 7H_2O$ 0.5 g/L; pH 5.5], and synthetic medium [sucrose 50 g/L, casamino acids (Difco, Detroit, MI, USA) 3.05 g/L, and yeast nitrogen base without amino acids and ammonium sulfate (Difco) 1.7 g/L; pH 5.5] cultivated with shaking at 150 rpm and 30°C. Beet molasses, cane molasses, and sucrose were used as the sole carbon sources. Growth (OD) was monitored as described above.

To assess the relationship between OD value and growth yield, the strains were cultivated in 8 ml of YPD medium with shaking at 150 rpm and 30°C for 12 h. After cultivation, all of culture medium was inoculated into 100 ml of YPD medium with shaking at 150 rpm and 30°C for 12 h. After cultivation, all of culture medium was inoculated into 800 ml of YPD medium with shaking at 150 rpm and 30°C for 12 h. After cultivation, the yeast cells were harvested by centrifugation, washed, and suspended in distilled water. The suspension was dried at 105°C overnight, the yield (dry weight) was measured. OD was measured as described above.

High-performance liquid chromatography High-performance liquid chromatography (HPLC) was used to estimate the total sugar, amino acid, and organic acid contents in beet and cane molasses. Unless otherwise indicated, both types of molasses were suitably diluted and filtered through a 0.45 μm DISMIC-13CP disposable membrane filter unit (Advantec, Tokyo, Japan) before HPLC.

HPLC analysis of sugars and betaine (glycine betaine) was performed using a Shodex SUGAR KS-801 column (Showa Denko, Tokyo, Japan) and RID-10A detector (Shimadzu, Kyoto, Japan). The column was maintained at 80°C. The mobile phase consisted of 0.05 mM NaOH at a flow rate of 1 ml/min.

HPLC analysis of organic acids and pyroglutamic acid (2-oxo-pyrrolidone carboxylic acid; PCA) was performed following the method of Nagura et al. (7) using a Shodex Ionpac KC-811 ×2 column (Showa Denko) and L-7400 detector (Hitachi, Tokyo, Japan) with the absorbance at 445 nm. The column was maintained at 45°C. The mobile phase consisted of 2 mM HClO at a flow rate of 1 ml/min. The bromothymol blue (BTB) solution consisted of 0.2 mM BTB, 15 mM $Na_2HPO_4 \cdot 12H_2O$, and 2 mM NaOH at a flow rate of 0.5 ml/min.

HPLC analysis of amino acids without betaine and PCA was performed following the manufacturer's instructions using a #2619 PH column (Hitachi) and L-2420 detector (Hitachi) with the absorbance at 570 nm. The column was maintained at 57°C. The mobile phase used MCI Buffer L-8500-PH-1, PH-4, and PH-RG (Mitsubishi Chemical, Tokyo, Japan) at a flow rate of 0.4 ml/min. Ninhydrin solution was used with an L-8500 amino acid analyzer (Wako Pure Chemical Industries, Osaka, Japan) at a flow rate of 0.3 ml/min.

Inductively coupled plasma spectroscopy The mineral content (Ca, K, Mg, Na, and P) of beet and cane molasses was analyzed using inductively coupled plasma spectroscopy ICPE-9000 (Shimadzu) according to the Association of Analytical Communities official method 985.01 (8).

RESULTS AND DISCUSSION

Evaluating and grouping according to molasses growth type By use of the simple method for grouping, the 26 strains were classified into three groups according to their growth in laboratory molasses medium: beet molasses type, cane molasses type, and non-typable (Table 1). The beet molasses type contained six strains, the cane molasses type comprised nine strains, and non-typable consisted of eleven strains. In the baker's yeast strains of our stocks, the number of beet molasses type strains was the same as the number of cane molasses type strains. The baker's yeasts of others tended to belong to the cane molasses type and the non-typable. Wild type strains isolated from wildflower, which are sometimes used in breadmaking, tend to belong to the non-typable. The type strains from the ATCC and NBRC were of the beet or cane molasses type. In this way, some beet molasses type strains were obtained from various isolation spots.

For the purpose of confirming the molasses growth type, 1 strain of beet molasses type and 1 strain of cane molasses type (based on the values from Eq. 1) were selected from the 26 strains. The value of the selected beet molasses type strain (strain no. 5) was 2.7 while that of the cane molasses type strain (strain no. 2) was −6.6; both belonged to our stock. They were named the beet strain and cane strain, respectively.

Time-course study of representative beet and cane molasses type strains A time-course study under cultivation of laboratory molasses medium was performed (Fig. 1A–C). The beet strain exhibited significant growth compared to the cane strain on beet molasses medium for the entire time course. In contrast, the cane strain exhibited significant growth compared to the beet strain on cane molasses medium after 12 h. The cane strain also exhibited significant growth compared to the beet strain on sucrose medium after 12 h. In addition, to examine whether these two types of yeasts also show similar growth characteristics in the medium used for industrial yeast production, a time-course study under cultivation of industrial molasses medium, which consisted of low-cost nitrogen source, $(NH_4)_2SO_4$, and phosphate source, $NH_4H_2PO_4$, was also performed (Fig. 1D–F). The beet strain exhibited significant growth compared to the cane strain on beet molasses after 6 h. The cane strain also

TABLE 1. The grouping of molasses growth type.

Strain no.	*S. cerevisiae* strains	Value of evaluation	Grouping
1	Baker's yeast (our stocks)	0.0	Non-typable
2	Baker's yeast (our stocks)	−6.6	Cane molasses type
3	Baker's yeast (our stocks)	−3.6	Cane molasses type
4	Baker's yeast (our stocks)	1.2	Beet molasses type
5	Baker's yeast (our stocks)	2.7	Beet molasses type
6	Baker's yeast (others)	−4.4	Cane molasses type
7	Baker's yeast (others)	1.2	Beet molasses type
8	Baker's yeast (others)	0.2	Non-typable
9	Baker's yeast (others)	0.3	Non-typable
10	Baker's yeast (others)	−0.2	Non-typable
11	Baker's yeast (others)	0.4	Non-typable
12	Baker's yeast (others)	−2.0	Cane molasses type
13	Baker's yeast (others)	1.8	Beet molasses type
14	Baker's yeast (others)	−1.1	Cane molasses type
15	Baker's yeast (others)	0.2	Non-typable
16	Baker's yeast (others)	−1.7	Cane molasses type
17	Baker's yeast (others)	−4.0	Cane molasses type
18	Baker's yeast (others)	0.3	Non-typable
19	Wild type strains	1.7	Beet molasses type
20	Wild type strains	−0.2	Non-typable
21	Wild type strains	0.4	Non-typable
22	Wild type strains	1.0	Non-typable
23	Wild type strains	1.0	Non-typable
24	ATCC 9080	−1.8	Cane molasses type
25	NBRC 0216	−4.2	Cane molasses type
26	NBRC 0224	2.2	Beet molasses type

Beet and cane molasses were applied as the sole carbon source, and the growth type was determined based on the turbidity of the liquid culture. Value of evaluation: OD_{660} cultivated in beet molasses medium − OD_{660} cultivated in cane molasses medium (>1.0: beet molasses type; <−1.0: cane molasses type; and from −1.0 to 1.0: non-typable).