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Development of flocculent *Saccharomyces cerevisiae* strain GYK-10 for the selective fermentation of glucose/fructose in sugar mills

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Advances in glucose/fructose-selective ethanol production have successfully enhanced raw sugar extraction from sugarcane juice by converting inhibitory substances (i.e., glucose/fructose) into ethanol, which is removed by subsequent operations in cane sugar mills. However, the commercial implementation of this breakthrough process in existing cane sugar mills requires a yeast strain that (i) can be used in food production processes, (ii) exhibits stable saccharometabolic selectivity, and (iii) can be easily separated from the saccharide solution. In this study, we developed a suitable saccharometabolism-selective and flocculent strain, *Saccharomyces cerevisiae* GYK-10. We obtained a suitable yeast strain for selective fermentation in cane sugar mills using a yeast mating system. First, we crossed a haploid strain defective in sucrose utilization with a flocculent haploid strain. Next, we performed tetrad dissection of the resultant hybrid diploid strain and selected GYK-10 from various segregants by investigating the sucrose assimilation and flocculation capacity phenotypes. Ten consecutive fermentation tests of the GYK-10 strain using a bench-scale fermentor confirmed its suitability for the implementation of practical selective fermentation in a commercial sugar mill. The strain exhibited complete saccharometabolic selectivity and sustained flocculation, where it maintained a high ethanol yield and conversion rate throughout the test.

[**Key words:** Ethanol; Inversion process; Reducing sugar; Selective fermentation; Yeast]

The glucose and fructose present in sugarcane juice (reducing sugars in sugarcane juice, RSS) interfere with cane sugar production. In particular, the RSS increase the syrup viscosity and inhibit sucrose crystallization, thereby increasing the amount of sucrose that cannot be recovered from molasses (1). Recently, we developed the inversion process for the selective conversion of RSS into ethanol prior to sucrose crystallization (2). This new milling process inverts the traditional production sequence, where raw sugar is extracted before ethanol production (Fig. 1). The inversion process facilitates the utilization of sugarcane that contains high amounts of reducing sugars, such as pre- and overmature crops or high-productivity cultivars, which may also facilitate the intensified use of land and a higher rate of cane sugar mill utilization (2). Furthermore, the traditional process can be transformed into the inversion process simply by adding a fermentation tank containing sucrose utilization-negative yeasts, thereby allowing simultaneous ethanol and sugar production.

The primary barrier to the commercial implementation of RSS-selective fermentation is that most of the saccharometabolism-selective yeasts are not *Saccharomyces cerevisiae* strains that have been approved as safe for use in the food industry. In general, the *S. cerevisiae* strains used in practical industrial applications secrete invertase outside the cell to degrade sucrose into reducing sugars (3), although some strains of *S. cerevisiae* that do not secrete invertase have been reported (4). There are two other practical obstacles to the commercial implementation of this method: The first is a lack of stability in terms of saccharometabolic selectivity during the consecutive utilization of yeast. Yeasts may start to utilize sucrose for several reasons, such as taking sucrose into the cell in conditions where reducing sugars are depleted (5). The second obstacle is the possible difficulty of separating the yeast from the juice throughout the process flow. Thus, if a flocculent-type strain is not used, an additional yeast separator must be employed after the fermentation reactor, which increases the cost. Previous studies of the implementation of selective fermentation in lactosucrose production (6) and the recovery of molasses (7) did not address these obstacles.

The results of laboratory-scale experiments performed using *Saccharomyces dairenensis* NBRC0211, *S. transvaalensis* NBRC1625, *S. rosinii* NBRC10008, and *Zygosaccharomyces bisporus* NBRC1131 indicated that these yeast strains are functional (8), but they are not suitable for practical use because of the three obstacles outlined above.

In this study, we developed a new *S. cerevisiae* strain that can be employed in the inversion process. We developed new strains with both sucrose utilization-negative and flocculent characteristics by interbreeding a strain that lacked the capacity for sucrose utilization with a flocculent strain. A promising strain was then selected in

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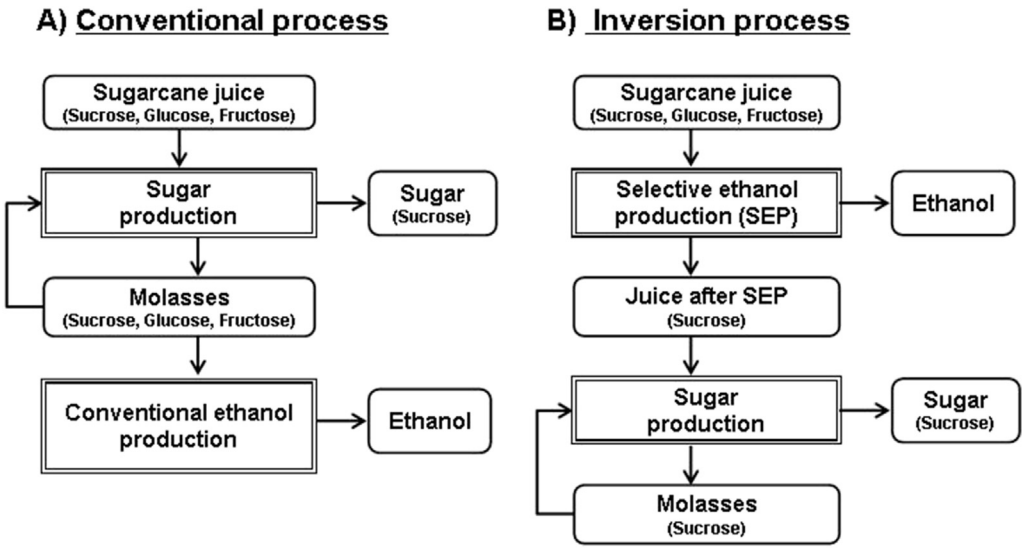

FIG. 1. Process of integrated sugar-ethanol production from sugarcane juice. (A) Conventional process, (B) inversion process.

laboratory-scale screening experiments and a bench-scale long-term stability test based on consecutive fermentation experiments.

MATERIALS AND METHODS

Yeast strains and medium The yeast strains used in this study were as follows: *S. cerevisiae* NBRC10055 (sucrose utilization-negative/flocculent-negative) and *S. cerevisiae* SDT (sucrose utilization-positive/flocculent-positive, NITE BP-1589). The yeast strains were cultured at 30°C in YPD medium [yeast extract 10 g L^{-1}, Bacto-peptone 20 g L^{-1} (Difco, Sparks, MD, USA), and glucose 20 g L^{-1}].

Yeast genetics and molecular biology methods Yeast genetics methods such as sporulation, tetrad dissection, and mating were performed as described by Amberg et al. (9). The genotype of the *MAT* locus was determined by PCR using the specific primers described by Huxley et al. (10). The mating test was performed according to a standard protocol described by George and Sprague (11). Mating between yeast cells was analyzed based on microscopic observations.

Primary screening Candidate yeast strains for use in the inversion process were first screened based on their lack of capacity to utilize sucrose in a fermentation test using a model medium. To evaluate the sucrose-negative and reducing sugars-positive phenotypes simultaneously, we measured the ethanol concentration using liquid medium instead of the plate medium.

Yeast strains were precultured in 1 mL of YPD at 30°C for 24 h. The precultured medium was centrifuged (5000 ×*g*, 5 min) to obtain the yeast cells. The yeast cells were then inoculated into 1 mL of the model medium [yeast extract 10 g L^{-1}, Bacto-peptone 20 g L^{-1}, glucose 15 g L^{-1}, fructose 15 g L^{-1}, and sucrose 120 g L^{-1}], which mimicked the typical composition of sugarcane juice, at 30°C for 24 h with reciprocating shaking (120 rpm). Next, the cultured medium was centrifuged (5000 ×*g*, 5 min) and the ethanol concentration of the supernatant was analyzed as described below.

Secondary screening The yeast strains selected by the primary screening procedure were then screened according to the following three criteria in 100 mL-scale fermentation tests (n = 3): (i) fermentation ability, (ii) flocculation characteristics, and (iii) retention of saccharometabolic selectivity after the depletion of reducing sugars.

Yeast strains were precultured in 3 mL of YPD at 30°C for 6 h. Next, all of the precultured yeast was added to 150 mL of YPD, followed by culture at 30°C for 18 h. The cultured medium was centrifuged (5000 ×*g*, 5 min) to obtain the yeast cells (wet weight = 1.0 g). The yeast cells were then inoculated into 100 mL of the model medium containing antifoaming agent [B-3022 (ADEKA, Tokyo, Japan) 10 g L^{-1}] in a flask with a fermentation lock, before incubating at 30°C or 40°C for 24 h with rotary shaking (120 rpm). The culture medium was centrifuged (5000 ×*g*, 5 min), and the ethanol concentration and saccharide composition of the supernatant were analyzed as described below. The ethanol concentration and saccharide composition (g L^{-1}) were measured at every hour during the first 6 h, and then at the 10, 20 and 24 h in each fermentation test. The maximum ethanol production rate was defined as the maximum value of the ethanol production per hour calculated from the ethanol concentration measured in the first 6 h. The ethanol yield was calculated as a percentage of the maximum amount of ethanol calculated from the measured ethanol concentration, to the theoretical amount, where we assumed that the reducing sugars in the model medium were completely converted into ethanol.

Tertiary screening Yeast strains selected by the secondary screening procedure were screened by the retention of sucrose concentration in 100 mL-scale of long-term fermentation.

Preculture and culture conditions were in the same method as secondary screening. However, the incubation time was extended to 168 h. To check whether sucrose consumption occurred after the depletion of reducing sugars, samplings of the culture medium were performed at every 24 h during the first 120 h and at 168 h.

Confirmation of the long-term stability The candidate strain selected by the screening process was tested to assess its long-term stability in consecutive fermentation experiments using bench-scale equipment.

The candidate strain was inoculated into 100 L of brown-sugar medium [brown sugar 134 g L^{-1} (Guanyinge brown sugar, China), glucose 14 g L^{-1}, and fructose 14 g L^{-1}] in a 200 L fermentor, where the inoculum composition of sucrose, glucose, fructose, and yeast cells were 110, 20, 20, and 50 g L^{-1}, respectively. Each batch fermentation experiment was performed at 30°C for 4 h. After fermentation, the fermented liquid was allowed to rest for 40 min and the flocculent yeast settled by gravity. Next, 82 L of the supernatant was removed from the fermentor and the 18 L of the settled yeast that remained in the fermentor was then used again for the next fermentation, where 82 L of fresh brown-sugar medium was added to the fermentor. This procedure was repeated 10 times. To confirm the long-term stability, we measured the concentration of ethanol, saccharides, and yeast in the fermentor at every hour and then calculated the residual yeast ratio, the maximum ethanol production rate, and the removal of reducing sugars. Residual yeast ratio in the fermentor was calculated by the equation:

$$Z = Y/(X + Y) \quad (1)$$

where X is the outflow yeast weight (g), Y is yeast weight (g) remaining in the fermentor after the supernatant was removed from the fermentor, and Z is residual yeast ratio in the fermentor. X was calculated from the recovered supernatant (L) and the yeast concentration (g L^{-1}). Y was calculated from the initial yeast concentration (g L^{-1}) of the next batch fermentation, because yeasts remaining in the fermentor were used in the next batch. The maximum ethanol production rate was calculated in the same way as the secondary screening. Removal of reducing sugars was calculated as a percentage of the consumption amount of reducing sugars during each batch to the amount of reducing sugars before each batch. The consumption of reducing sugars during each batch (g) was calculated by the concentration of reducing sugars (g L^{-1}) before and after the fermentation.

Flocculation capacity assay The capacity for flocculation was assayed as described by Smit et al. (12) and Kobayashi et al. (13), with some modifications. Yeast cells were subjected to preincubation in 5 mL of YPD (30°C, 48 h, 120 rpm), after which the harvested yeast cells were washed twice with 20 mM EDTA and twice with sterile Milli-Q water. The washed cells were resuspended in 1.25 mL of sterile Milli-Q water, or 1.25 mL of 20 mM calcium chloride solution, to obtain an absorbance at 600 nm (A_{600}) of 2.0. The absorbance was measured in a 1.0-cm cuvette using 1.0 mL of cell suspension. The cell suspension in sterile Milli-Q