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Industrial Application of Artificially Induced Diploid Strains of *Torulaspora delbrueckii*

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Diploid strains of *Torulaspora delbrueckii* were tested for industrial application. Because the cell volume of the diploid strain was three times as large as that of the parental haploid strain, collection and subsequent dehydration to make compressed yeast cakes were greatly improved with the diploid YL3. The time required for dehydration of the diploid strain was shortened to 1/2.5 that of the parent strain under conventional conditions. Moreover, for the diploid cells frequent filter changes for dehydration were not required, which was the case with parental cells. Fermentation activity and tolerance to freeze-thawing in dough were succesfully inherited by the diploid strains. The diploid YL3 showed nearly the same activity as the diploid F31 in bread making. However, the endurance period of yeast cakes when stored at 30°C without softening to lead to liquefaction was much longer in YL3 (199 h) than in F31 (132 h). This superiority was ascribed to the fact that YL3 was induced through direct diploidization and had no genetic defect on chromosomes because the wild-type strain was employed as the parent, whereas F31 was obtained through protoplast fusion from two auxotrophic mutants and carried at least two mutagenized genes that were masked by heterolallelism.

In the accompanying report (2) we described new methods for the induction of formation of artificial diploids from the haploid yeast *Torulaspora delbrueckii* SANK 50268. SANK 50268 is an industrial strain used for bread making (3). Because the cell size of diploids is 2 to 3 times as large as that of the parent SANK 50268, one of the diploids was tested for dehydration on an industrial scale to compare it with SANK 50268. Some of the most important features of the yeast for bread making were also tested.

MATERIALS AND METHODS

Organisms. *T. delbrueckii* SANK 50268 and its artificially induced diploid strains YL3 and F31 were used throughout this study. YL3 was obtained through direct diploidization by treatment of regenerating protoplasts with dimethyl sulfoxide (DMSO), while F31 was induced through protoplast fusion, as described in the accompanying report (2). The organisms were grown in a 20-kl tank with aeration and feeding. The culture originally contained the following ingredients: seed yeast cells, 72 kg (wet weight); $MgSO_4 \cdot 7H_2O$, 4.3 kg; and water, 6,000 liters. To the culture was administered medium comprising 40% molasses (as glucose equivalent), 1.4% urea, and 0.16% $(NH_4)_2HPO_4$ to maintain a constant level of ethanol evolution; the pH was maintained at the 5.5 by addition of H_2SO_4 or Na_2CO_3. All the parameters, including oxygen transfer rate, were strictly controlled by a computer. After incubation for 16 h at 30°C, cells were harvested by centrifugation and washed 6 times with tap water. Then, the cells were pretreated in a 6% saline solution to facilitate filtration. The creamy cell suspension in pretreatment saline was dehydrated on a rotary drum with vacuum inside (Alfa-Laval dehydrator, Stockholm, Sweden). Starch was coated 15 to 25 mm thick on the rotary drum surface of the dehydrator, which served as the filter for yeast cells. The dehydrated yeast cell layer was moistened with a spray of distilled water to make compressed yeast cakes like those that are available on the market.

Endurance test for storage of compressed yeast cakes at 30°C. Twenty grams of compressed yeast cake was packed tightly in a petri dish (inner diameter, 45 mm; inner height, 18 mm) and kept at 30°C until the yeast cake began to soften. The endurance time was measured at intervals of 24 h, starting at 0 h by touching the yeast cake with a finger to test the rigidity.

Test for tolerance of yeast cell activity to freeze-thawing in dough and bread making. Dough and yeast cells were mixed and kneaded to obtain the following ratio of composition: wheat flour, 100 g; sucrose, 4 g; NaCl, 2 g; dough improver, 1.2 g; shortening, 4 g; yeast cells (compressed cake), 6 g; distilled water, 63 g. Mixing, molding, and subsequent baking were essentially based on the standard methods described by the American Association of Cereal Chemists, Inc. (1). The yeasted dough was first frozen rapidly and stored at −30°C and then at −20°C after removal of the CO_2 gas that had evolved during fermentation at 28°C for 30 min and subsequent molding. The molded, frozen masses of dough were taken out of the refrigerator at about 1-week intervals and thawed at 26°C for 90 min, followed by

TABLE 1. Hourly rates of dehydration to make compressed yeast cakes from the parent and diploid cells[a]

Strain	Saline pretreatment (h)	Dehydration (liter/m² per h)	Dehydration ratio
SNAK 50268	4.5	106.5 ± 14.7	1
YL3	1	261.2 ± 14.9	2.5

[a] The time required for saline pretreatment was measured only once, while the time for dehydration was the mean ± standard deviation of experiments repeated 23 times. The dehydration ratio does not include the time for saline pretreatment.

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TABLE 2. Time for compressed yeast cakes to soften on storage at 30°C

Strain	Time to softening (h)[a]
SANK 50268	176.4 ± 27.9
YL3	199.3 ± 30.4
F31	131.8 ± 41.7

[a] Results are means ± standard deviations of experiments repeated 30 times. Results are given as the time when the compressed yeast cakes began to soften after storage at 30°C.

kneading and a second fermentation at 38°C and in 90% moisture for 90 min in a standard metal box without a lid. A control portion of the dough that did not receive freeze-thawing treatment contained a one-third amount of yeast cells (2 g) and was baked directly after the second fermentation. For sweet bread the following alteration in dough composition was made: sucrose, 25 g; NaCl, 1 g; yeast cells, 3 g. Both types of dough were baked at 220°C for 25 min in an electric oven, and the resulting loaves were measured for volume by using a loaf volume meter with rapeseed replacement. The taste and flavor of the bread loaves were tested by 25 panelists by conventional methods.

RESULTS AND DISCUSSION

Hourly rate of dehydration to make yeast cakes. To compare the diploid YL3 strain with the parent SANK 50268 strain for the efficiency of the dehydration process, which was a difficult problem for the latter strain, both strains were cultivated under the same conditions in a 20-kl tank and dehydrated as described above. Yeast cream containing 520 kg of cells per 1,000 liters of saline was applied to the dehydrator. The time for the pretreatment period with saline that was required for the cells to facilitate filtration was also measured. In Table 1 it is shown that SANK 50268 cells required 4.5 h for saline pretreatment, while YL3 cells required only 1 h. With respect to dehydration, YL3 cells were processed 2.5 times faster than SANK 50268 cells. Moreover, YL3 did not require a frequent change of the starch filter on the dehydrator drum as did SANK 50268. Thus, the dehydration process was greatly improved with YL3 with regard to labor time and economy, such as the amount of electricity needed.

TABLE 3. Fermentation and baking tests after storage of dough with yeast of the parent and diploids at −20°C

Day and strain[a]	Ht of dough fermented (cm)[b]	Ht of dough baked (cm)[b]	Bread vol (ml)	Bread wt (g)	Specific vol[c]
Day 8					
SANK 50268	4.0	5.0	2,275	384	5.9
YL3	4.1	5.6	2,388	384	6.2
F31	4.2	5.1	2,250	385	5.8
Day 19					
SANK 50268	3.0	4.5	2,062	386	5.3
YL3	3.3	5.3	2,175	390	5.6
F31	3.3	4.4	2,075	390	5.3

[a] Frozen doughs with yeast were thawed on days 8 and 19.
[b] Heights of doughs expanded above the upper surface of the metal box were measured in centimeters after the second fermentation and after baking.
[c] Specific volume is obtained from bread volume divided by bread weight.

TABLE 4. Decay of yeast activity of the parent and diploid YL3 during storage of dough at −20°C

Strain	Bread vol (ml) on:			Sweet bread vol (ml) on day 0 (unfrozen)
	Day 0 (unfrozen)[a]	Day 7[b]	Day 21[b]	
SANK 50268	1,722 ± 85	2,104 ± 158	1,726 ± 76	1,787 ± 60
YL3	1,736 ± 64	2,205 ± 120	1,858 ± 65	1,835 ± 57

[a] The unfrozen dough contained a one-third the amount of yeast cells (2 g) in the material, as described in the text.
[b] The dough that received freeze-thawing treatments contained 6 g of yeast cells. The dough with yeast was baked directly after the second fermentation or after frozen storage at −20°C. The frozen dough was thawed and then fermented (second fermentation) on the indicated day. The bread volume is the mean ± standard deviation of experiments repeated 10 times for unfrozen dough and 6 times for dough stored frozen.

Endurance time of compressed yeast cakes on storage at 30°C. The time for compressed yeast cakes to soften, which brings about deterioration in quality, was measured. In Table 2 it is shown that the diploid strain YL3 was superior in endurance than the parent strain SANK 50268. Diploid strain F31 was inferior to the other two strains. Namely, F31 cells began to soften about 3 days earlier than YL3 cells.

Tolerance of yeast cell activity in dough to freeze-thawing. The diploid strains YL3 and F31 were tested with SANK 50268 as a control for tolerance to freeze-thawing or residual fermentation activity after storage at −20°C. F31 was the best fusion product for dough fermentation based on serveral criteria, including tolerance tests. It was screened from over 100 fusion products between two parents carrying the auxotrophic mutation (2). The two parents (haploid) were selected from 62 mutant strains, as described above. F31 was an elite strain among fusion products; the screening of it required much time and computer-aided analysis of many parameters. On the other hand, YL3 was the strain that was selected from only a small population among diploids that were induced by direct diploidization. Such strains showed almost equal potency, and large-scale screening was not necessary. The diploid strain YL3 inherited the excellent properties of the parent, such as tolerance to freeze-thawing and good fermentation capability, except that its cell size was large due to diploidy. Nonetheless, baking tests revealed that YL3 was not inferior to F31 after the storage of yeasted dough at −20°C (Table 3). Comparison of the fermentation activity of parent SANK 50268 and YL3 was carried out in more detail (Table 4). YL3 was somewhat superior to the parent.

Among panelists there was no statistically significant difference in the taste and flavor of bread baked by using SANK 50268 or YL3 (data not shown). These facts indicate that the diploids obtained through perturbed protoplast regeneration express their genetic characteristics perfectly.

Thus, new methods (2) for the induction of artificial diploids through direct diploidization are useful for the genetic improvement of an industrial yeast strain, *T. delbrueckii*. Indeed, one such diploid is now on the market instead of the parental haploid.

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