Kasetsart J. (Nat. Sci.) 38 : 255 - 266 (2004)

Hybridization and Selection of *Saccharomyces cerevisiae* Strains from Industrial Baker's Yeasts

Chakamas Wongkhalaung[1] Yasuo Nakatomi[2] and Hiroyuki Takano[3]

ABSTRACT

Hybridization study of two *Saccharomyces cerevisiae* baker's yeast strains isolated in Thailand, IFRPD 6080 and IFRPD 6081, was carried out. Rare-mating technique has been employed with haploid and diploid mating strains of Japanese baker's yeast. Polyploid (triploid and tetraploid) hybrids produced from rare-mating were selected and induced for sporulation. Screening of segregants obtained from individual spore clones of the hybrids was performed by determination of their characteristics, i.e. yield of yeast after cultivation, total carbohydrate and trehalose contents of obtained yeast, and carbon dioxide (CO_2) production during fermentation with bread dough. Two segregants, 6080xs4D s27 and 6080xs4D s39 derived from the parental strain IFRPD 6080 and one segregant, 6081xs34D s12 from the parental strain IFRPD 6081, with desirable characteristics were obtained as the new strains. These segregants possessed good growth yield and higher maltose fermentation ability than their parents. Carbon dioxide productions in non-sugar, low-sugar and high-sugar dough were also markedly improved. Moreover, the strain 6080xs4D s27 acquired certain freeze-tolerant ability from mating strain and was able to use for frozen dough preparation.

Key words: hybridization, rare-mating, freeze-tolerant, *Saccharomyces cerevisiae*, baker's yeasts

INTRODUCTION

Selection of *Saccharomyces cerevisiae*, baker's yeast strains for industrial application in terms of their leavening ability and other desirable characteristics for bread making has been studied extensively. At present, freeze-tolerant ability of baker's yeast is one of the most favorable characteristics for baking industry.

Oda *et al*. (1986) and Hino *et al.* (1987) screened *S. cerevisiae* strains from nature that showed better freeze-tolerant ability than a commercial strain. Yeast other than *S. cerevisiae*, namely *Kluyveromyces thermotolerans* FRI 501 (Hino *et al*.,1987) and *Torulaspora delbrueckii* (Ohshima *et al.*, 1987; Oda and Tonomura, 1993) have been reported to have freeze-thaw resistance for frozen dough preparation. Hybridization technique based on protoplast fusion has been successfully used in the isolation of the hybrids of *S. cerevisiae* (Spencer *et al*., 1985). They reported that some of segregants obtained from fusion hybrids between *Saccharomyces diastaticus* and *Saccharomyces (Zygosaccharomyces) rouxii* had much greater dough-raising capability in simulation bread-dough and sweet dough than either of the

1 Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand.

2 Oriental Yeast Industry Co., Ltd., Azusawa, Itabashi-ku, Tokyo 174, Japan.

3 National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannon-dai, Tsukuba, Ibaraki, Japan.

Received date : 08/04/04 Accepted date : 21/05/04

original hybrid or a commercial baker's yeast. Rare-mating technique was adapted to get hybrid from industrial yeast strain which have lost the ability to sporulate (Spencer and Spencer, 1977). Improvement method of baker's yeast by rare-mating technique was also reported by Oda and Ouchi (1990a), although the strains constructed did not possess both characteristics of freeze-tolerant and high leavening ability simultaneously.

This study intends to improve the quality of two mating-incompetent baker's yeast strains isolated in Thailand by hybridization with Japanese baker's yeasts showing mating ability. Rare-mating techniques with the aid of respiratory-deficient phenotype and dye plate were employed to effectively screen the hybrids. Characterization of the hybrids and their meiotic segregants as well as fermentation abilities were evaluated accordingly.

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strains IFRPD 6080 and IFRPD 6081 are commercial baker's yeast strains from IFRPD Culture Collection, Thailand. Four yeast strains; two haploids; NM33s4 (**a**) and HF804s34 (α) and two diploids; NM33s4D (**aa**) and HF804s34D ($\alpha\alpha$) provided by Japanese baker's yeast company were used for mating. These mating strains are freeze-tolerant and possess high sugar fermentation abilities (Nakatomi, Y., unpublished data).

Media

YPD (yeast extract 1 %, polypeptone 2 %, glucose 2 %), solidified with 2 % agar if desired, was used as growth and maintenance medium. Sporulation was carried out on Sharman medium (glucose 0.05 %, yeast extract 1 %, potassium acetate 1 %, agar 2 %). Lactate medium (peptone 0.35 %, yeast extract 0.3 %, $MgSO_4.7H_2O$ 0.1 %, $(NH_4)_2SO_4$ 0.1 %, Na-lactate 1 %, agar 2 %) was used to check for respiratory-deficient mutants. Medium used for isolation of hybrids was dye plate prepared by adding 1 ml of dye solution (ponceur 3R 500 mg, aniline blue 250 mg per 100 ml) to 100 ml of basal medium (sucrose 5%, yeast extract 0.3%, KH_2PO_4 0.2% $(NH_4)_2SO_4$ 0.15%, polypeptone 0.1%, $MgSO_4.7H_2O$ 0.1%, citric acid monohydrate 0.1%, tri-potassium citrate monohydrate 0.5%, agar 2%) after autoclaving and final pH of 5.2. The dye plates used for detection of variant colonies were prepared according to Hamada *et al.* (1996). Mass cultivation of yeast cells was carried out in yeast nitrogen base (YNB) sucrose medium (sucrose 2 %, sodium succinate 1 %, yeast extract 0.5 %, yeast nitrogen base, without amino acid, 0.67 %) at pH 4.5.

Hybridization

Petite mutant induction

Yeast cultures, IFRPD 6080 and IFRPD 6081 were converted to the petite form by using ethidium bromide (Spencer and Spencer, 1988). They were grown without shaking in YPD broth containing 20 μg/ml ethidium bromide at 30 °C for 24 hr. Each individual culture was then spread on YPD agar plate to get well-isolated colonies. Respiratory deficient (RD) mutants, converted from diploid cells were selected from small colonies. They were then tested on lactate medium for inability to grow on non-fermentable substrates prior to mating experiment.

Rare-mating

Each strain of respiratory-deficient mutants obtained from IFRPD 6080 and IFRPD 6081, and **a** or α Japanese baker's yeast strains was grown separately in YPD broth at 30°C for 48 hr. Eight pairs of mating were then performed by transferring a 0.1 ml portion of each strain to another tube of YPD broth, mixed and let stand at 30 °C for 48 hr. Consecutive subculturing of mating mixtures to YPD broth every 24 h as shaking cultures were carried out for 3-5 times to encourage selective

growth of hybrid cells. Mating suspension from each pair was spread on dye plate. Hybrid colonies were differentiated from parent colonies by colony color and were transferred to YPD plates for further study.

Spore isolation

The hybrids were spread on sporulation plate (Sharman medium) and incubated at 30 °C for 5-7 days to induce sporulation. One loopful of cultures from Sharman medium was then suspended in Zymolyase 20T solution containing 3 Unit of enzyme/ml, mixed and incubated for 1-2 hr at 30 °C to fracture the cell wall. A 0.5 ml suspension was transferred to Eppendorf tube and equal amount of liquid paraffin was added. They were mixed vigorously and centrifuged at 12,000 rpm for 60 seconds to separate water and paraffin layers. The paraffin phase was transferred to another tube and washed with 0.5 ml water to remove all vegetative cells. Spores suspension in the paraffin phase were spread on the dye plate, incubated at 30 °C for 2 days and at ambient temperature (about 20 °C) for 3-5 days.

Segregants characteristics

Segregants obtained from spores were grown in 5 ml of YNB-sucrose medium in test tubes with shaking at 30 °C for 40-48 hr. Dry weight and growth yield of each segregant in comparison with those of the parents were analysed. Mating type was verified by mixing one drop of each strain (grown in YPD broth for 24-48 hr) with one drop of type strain, MAT**a** or MAT**α,** and incubated at 30°C for 5 hr. Agglutination occurred if cells were of different mating types. Non-mater (**a**α) strains did not agglutinate with either MAT**a** or MATα. Mass cultivation of 2 selected segregant hybrids (non-mater) and their parent strains was carried out in 5-liter flask with baffles containing 1-liter YNB sucrose medium at 30 °C with rotation speed of 170 rpm for 40-48 hr. Yeast cells were harvested by centrifugation, washed and made up to 200 ml water to get yeast suspension of about 4-5 % yield (w/v). They were used to determine yeast quality and characteristics i.e. maltose fermenting ability, sucrose fermenting ability, total carbohydrate and trehalose contents and gas production in bread doughs (0.5 and 30 % sugar). Pulse field gel electrophoresis using CHEF (Contour-Clamped Homogenous Electric Field) technique (Chu *et al*., 1986) was employed in the separation of intact DNA molecules of the parental strains, IFRPD 6080 and IFRPD 6081, hybrids and selected segregants. DNA samples were prepared according to Carle and Olson (1987). CHEF gel analyses were performed for all chromosomes and low molecular-weight chromosomes (365 kbp and lower).

Yeast quality determination

Fermentation ability

Maltose and sucrose fermentation abilities of selected segregants were determined by Brom Cresol Purple (BCP) method (Nakatomi, Y., unpublished data). For non-sugar dough fermentation, substrate-containing maltose as sole carbon source (maltose 2.4%; yeast extract 0.36 %; polypeptone 0.6% and BCP 0.4% in ethanol, 5ml/600ml medium) was used. For high-sugar dough fermentation, maltose was replaced with 60% sucrose and 0.2% of NaCl was also added. One ml of substrate was mixed with 200 μl of yeast suspension (< 10 mg/ml dry matter) and incubated in water bath at 30°C for 1 hr. After making up to 3 ml-volume, intensity of color change from purple (control substrate) to yellow was recorded as plus/minus or could be measured spectrophotometrically at 590nm (Nakatomi, Y., personal communication).

Total carbohydrate, trehalose and invertase determinations

Total carbohydrate and trehalose contents of yeast were determined by the method of McCready *et al*. (1974) and Trevelyan and Harrison (1956), respectively. Invertase activity was

determined according to Oda and Ouchi (1990b). A 0.5 ml yeast suspension was mixed with 0.5 ml of 6.5 % sucrose in acetate buffer, pH 4.5, in ice bath. Enzyme activity was carried out in water bath at 30 °C for 3 min. Reaction was terminated by cooling in ice bath and 3 ml dinitrosalicylic acid reagent (DNS) was added. The mixture was brought to boiling for 5 min, adjusted to 25 ml and measured at 525 nm. One unit of invertase is defined as mg glucose released at 30 °C per minute per one mg of yeast (dry basis) under the experimental conditions.

CO_2 production measurement

Measurement of CO_2 production from dough was performed using Automatic Measuring System for Dough Testing as described by Hino *et al*. (1988). Each portion of the tested dough, non-sugar, low-sugar and high-sugar, was prepared according to the standard method of Japan Yeast Industry Association (1985) as shown in Table 1. The amount of total gas production and/or gas retention in the dough at 30 °C was automatically measured and recorded every 10 minutes for 2 hr by Fermograph (Atto Co. Ltd., Japan).

Freeze-tolerant ability

Low-sugar bread dough was prepared as described earlier and kept at –20 °C after prefermentation at 30 °C for 120 min. The dough was kept frozen for 1 and 2 weeks before measurement of CO_2 production by Fermograph.

RESULTS AND DISCUSSION

Hybridization

Many industrial strains of *Saccharomyces cerevisiae* are reported to be aneuploid or polyploidy and often sporulate poorly or form nonviable spores (Spencer and Spencer, 1977). Both IFRPD 6080 and IFRPD 6081 were estimated to be heterothallic diploid (**a**α) (data not shown). Apparently, strains IFRPD 6080 and IFRPD 6081 are non-mater with poor sporulation ability. Attempts have been made to isolate the spores and a number of mater segregants (**a** and α), whose cell sizes were small like haploid cell size were obtained. However, most of segregants derived from IFRPD 6080 and IFRPD 6081 exhibited very poor growth rate compared with segregants of Japanese industrial baker's yeast and conventional hybridization procedure was not possible. Therefore, rare-mating technique has been employed for this study.

Rare-mating was the technique based on switching of mating-type in diploid or polyploid cells at low frequency and leading to occasional appearance of mating cells, which will mate and form hybrids with autotrophic strain if cells of

Table 1 Dough formulations for CO_2 measurement.

Ingredient	Amount in gram		
	Non-sugar	Low-sugar	High-sugar
Wheat flour[1/]	20	20	30
Compressed yeast[2/]	0.4	0.4	1.2
Sugar	0	1	9
Salt	0.4	0.4	0.15
Water[3/]	12	12.5	16

1/ Wheat flour contained 12% protein.

2/ Compressed yeast (67% moisture content) was replaced with yeast suspension precalculated to have equivalent amount of yeast.

3/ Water was adjusted accordingly.

another mating type are present (Gunge and Nakatomi, 1972). The diploid strains of *S. cerevisiae*, IFRPD 6080 and IFRPD 6081 were converted to petite form (respiratory-deficient) by ethidium bromide. Hybridization was carried out by mating these RD mutants with mater strain, which has no selective markers to differentiate it from hybrid. Petite (RD) mutants of both IFRPD 6080 and IFRPD 6081 were mated with two respiratory-competent haploids; NM33s4 (**a**) and HF804s34 (α); and their isogenic diploids; NM33s4D (**aa**) and HF804s34D ($\alpha\alpha$).

Galactose assimilation (Oda and Ouchi, 1990b) was also used for isolation of hybrids obtained by rare mating. Takagi *et al.* (1983) reported the use of dye plate to discriminate tetraploid colonies from those of diploids of *S. cerevisiae*. In this report, color of mater strain on the dye plate containing aniline blue and ponceau 3R was grayish blue, while most hybrid colonies were stained dark red. Some dark violet or light red colonies were also hybrids of large cell size.

Strain IFRPD 6080 could not hybridize with either strain HF804s34 or HF804s34D but successfully hybridized with strains NM33s4 and NM33s4D. Strain IFRPD 6081 readily hybridized with all four maters. Many hybrids were obtained but only two hybrids, 6080xs4D from strains IFRPD 6080 and NM33s4D; and 6081xs34D from strains 6081 and HF804s34D were finally selected for spore isolation.

Spore isolation

Cells of the two selected hybrids grown on the YPD plates were inoculated on the sporulation plates and incubated at 25 °C for up to 5 days. The hybrids showed good sporulation after 3 days. They were able to sporulate due to the functional mitochondria derived from the mater strain which are respiratory competent. Mortimer and Hawthorne (1969) stated that tetraploid hybrids of *S. cerevisiae* usually yield spores with high viability (>90%) whereas triploids characteristically have very low spore viability (<10%) presumably due to imbalance occasioned by the aneuploid nature of most of the spores. The hybrids, 6080xs4D and 6081xs34D, seemed to be tetraploid and showed satisfactorily spore viability.

Observed segregation for mating type of the hybrid 6080xs4D was non-mater:**a**:α = 14:24:10. Theoretical ratio of **'aa**α' (non-mater:**a**:α = 16:24:8 ; χ^2 = 0.75, P>0.5). It was likely that the mating type of IFRPD 6080 has changed to 'α' by mitotic chromosome loss, mated with diploid strain NM33s4D (**aa**) and created the hybrid 6080xs4D.

Observed segregation for mating type of the hybrid 6081xs34D was non-mater:**a**:α = 34:5:9. Theoretical ratio of **'aa**$\alpha\alpha$' (non-mater:**a**:α = 32:8:8 ; χ^2 = 1.375, P>0.5). It was likely that mating type of IFRPD 6081 has changed to '**aa**' by mitotic recombination, mated with diploid strain HF804s34D ($\alpha\alpha$) and created the hybrid 6081xs34D.

Segregants characteristics

From analysis of the segregants, primarily characterized by good growth yield and high maltose and sucrose fermentation abilities (data not shown), two segregants, namely 6080xs4D s27 and 6080xs4D s39 obtained from spores of hybrid 6080xs4D were then selected as new strains for further investigation. Likewise, for the parental strain IFRPD 6081, two segregants, namely 6081xs34D s12 and 6081xs34D s16, derived from the hybrid 6081xs34D were chosen. Photographs of yeast cells of the parental strains, IFRPD 6080 and IFRPD 6081, the hybrids and selected segregants are shown in Figure 1.

CHEF gel analysis of intact chromosomal DNA molecules of strains IFRPD 6080 and IFRPD 6081, the hybrids and selected segregants revealed the broad size ranges of DNA molecules, i.e. from 225 to 2,200 Kbp (Figure 2). DNA of *Saccharomyces cerevisiae* with chromosome size range of 0.24-2.2 Mb (Chu *et al*., 1986) was used as standard DNA size markers. Analysis of low

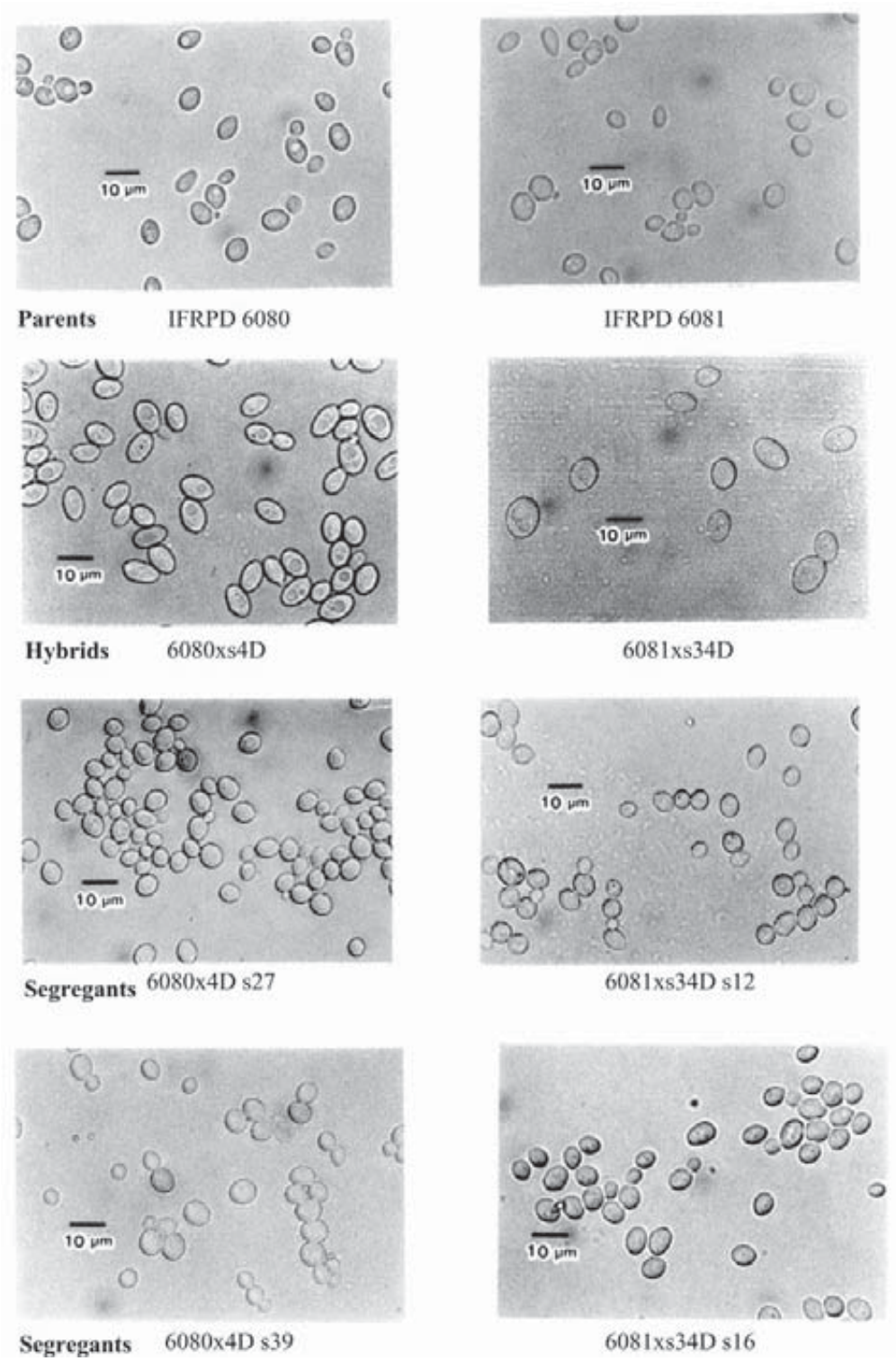

Figure 1 Photographs of yeast cells of parent strains IFRPD 6080 and IFRPD 6081, their hybrids and segregants, taken at the same magnification.

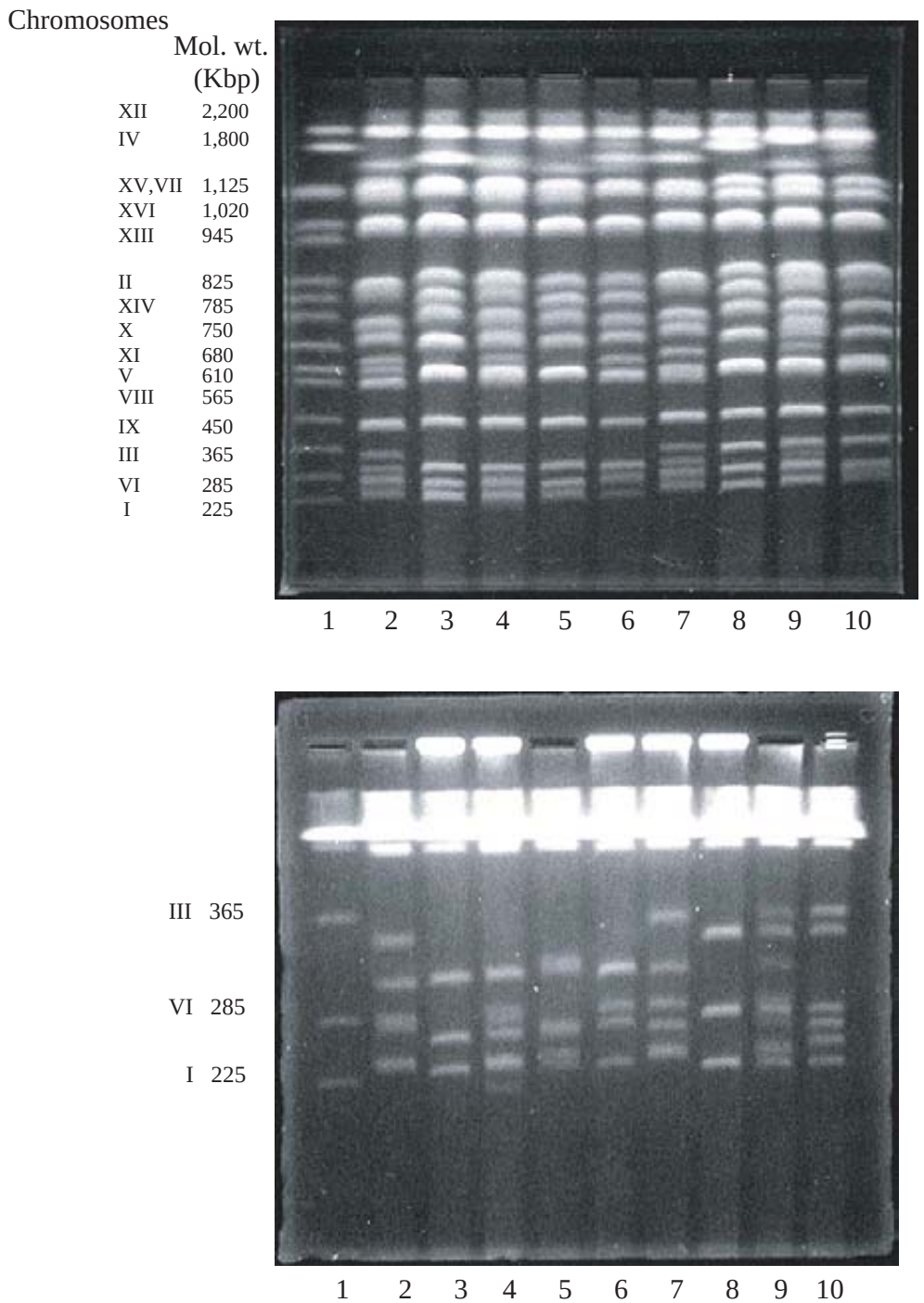

Figure 2 CHEF gel analysis of all DNA chromosome (upper) and low- molecular weight chromosome (lower) of the parental strains, hybrids and segregants.
(1:Standard *S. cerevisiae*, 2: IFRPD 6080, 3: NM33s4D, 4: 6080xs4D, 5: 6080xs4d s27, 6: 6080xs4d s39, 7: IFRPD 6081, 8: HF804s34D, 9: 6081xs34D, 10: 6081xs34D s12)

molecular weight chromosomes was also carried out to get good separation of chromosome bands sizes between 225 and 365 Kbp. It can be seen that, for the low molecular weight chromosomes, both segregant 6080xs4D s27 (lane 5) and 6080xs4D s39 (lane 6) contained 4 DNA bands which some were derived from the parent IFRPD 6080 (lane 2) and some from the mating strain NM 33s4D (lane 3). Size patterns of strain 6080xs4D s27, possess freeze-tolerant ability, was more resembling to the mating strain NM 33s4D than its parent strain.

Five and 3 DNA bands of molecular weight below 365 Kbp were observed in the parent strain IFRPD 6081 (lane 7) and the mating strain HF804s34D (lane 8), respectively. Segregant 6081xs34D s12 (lane 10) contained 6 distinct bands, which derived from either its parent or the mating strain (Figure 2).

Yeast quality determination

Fermentation ability

Fermentation ability of parent strains IFRPD 6080 and IFRPD 6081 and new strains (6080xs4D s27, 6080xs4D s39, 6081xs34D s12 and 6081xs34D s16) developed from the parental strains was examined by measurement of CO_2 production in non-sugar, low-sugar and high-sugar bread dough, recorded by Fermograph. Non-sugar (maltose) fermentation ability of parent strains IFRPD 6080 and IFRPD 6081 are relatively low and not appropriate for non-sugar dough preparation such as French bread. Yeast with high non-sugar (maltose) fermentability is desirable for non-sugar dough preparation, which liberated maltose from damaged starch in the flour during fermentation. Maltose is the main fermentable sugar for non-sugar bread dough. It was clearly seen that high maltose fermentation ability was inherited from mater strains. Both segregants from IFRPD 6080 i.e. 6080xs4D s27 and 6080xs4D s39, were better than their parents in terms of CO_2 production for all types of dough (Figure 3). Likewise, the obtained strain 6081xs34D s12 showed improved leavening ability in non-sugar dough than the parent IFRPD 6081 and the strain 6081xs34D s16 (Figure 4).

Total carbohydrate, trehalose and invertase determination

Parent strains IFRPD 6080 and IFRPD 6081, are originally active dried yeast which are mainly used for high sugar and low sugar bread dough, respectively. Thus, both of the parent strains contain relatively high carbohydrate and trehalose contents; i.e. 39.2 and 13.4 %, for strain 6080 and

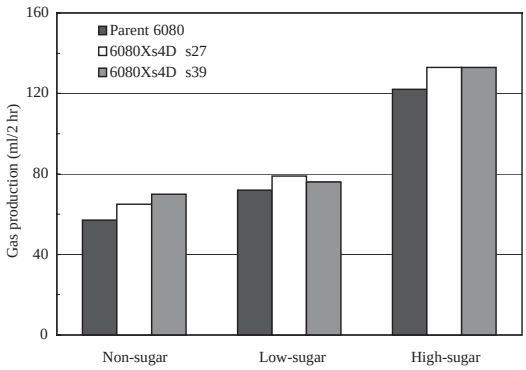

Figure 3 Gas production of the new strains compared with their parent strain IFRPD 6080 in various bread dough.

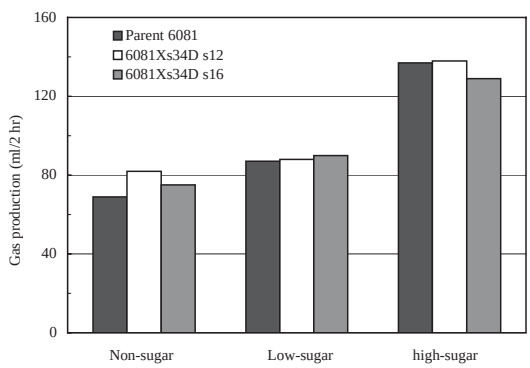

Figure 4 Gas production of the new strains compared with their parent strain IFRPD 6081 in various bread dough.

34.2 and 12.4 % for strain 6081, respectively (Table 2). Trehalose has been reported by many investigators to have certain roles in heat and desiccation resistance and cryoresistance in frozen bread dough method (Hino *et al*., 1990; Meric *et al*., 1995). Diploid strain of *S. cerevisiae* with >5 % trehalose was shown to be more tolerant to heat and freeze-thaw stress than strains that produced <4 % trehalose (Attfield *et al.*, 1992). Resistance to dehydration of *S. cerevisiae* containing high trehalose content was also increased if high level of intracellular trehalose was accumulated in stationary-phase cells or cells incubated in the absence of nitrogen source (Gadd *et al.*, 1987). This characteristic is necessary to be registered in the hybrid as well as freeze-tolerant ability which is also expected to be inherited from mating strain. Thus, hybrids were selected on the basis of trehalose and carbohydrate contents in addition to their cell yields, sucrose and maltose fermentation abilities and CO_2 production in various bread dough.

Segregants 6080xs4D s27 and 6080xs4D s39, resulted from parent IFRPD 6080; contained comparative amounts of carbohydrate and trehalose as their parents but better fermentation activities of maltose as shown in Table 2. On the contrary, segregants 6081xs34D s12 and 6081xs34D s16, from parent IFRPD 6081, possessed higher carbohydrate and trehalose contents than the parent and also much higher activity towards maltose fermentation (Table 2).

Invertase activities of the segregants 6080xs4D s27 (131 U/mg) and 6080xs4D s39 (244 U/mg) reduced markedly compared to their parent strain IFRPD 6080 (315 U/mg). Segregant 6081xs34D s12 also had lower invertase activity (252 U/mg) than the parent IFRPD 6081 (387 U/mg). Since baker's yeast is highly sensitive to high osmotic pressure created by sugar or salt, or both, in bread dough, yeast with low invertase helps to prevent adverse effect of high osmotic pressure (Oura *et al*., 1982). Thus, lower invertase activity of these new strains resulted in higher leavening ability in low- and high-sugar bread dough.

Freeze-tolerant ability

Freeze-tolerant abilities of new strains derived from IFRPD 6080 and IFRPD 6081 were evaluated by Fermograph as shown in Figures 5 and 6, respectively. Low-sugar bread dough was prefermented for 120 min before frozen storage for 1 and 2 weeks at -20 °C. Segregant 6080xs4D s27 from the parent IFRPD 6080 maintained better fermentation ability than the others in the group

Table 2 Some characteristics of hybrids and selected segregants compared with their parent strains.

Strain	Growth yield (% w/v)	Total carbohydrate (%)	Trehalose (%)	Invertase activity (Unit/mg)	$^{1/}A_{590}$ maltose	$^{1/}A_{590}$ sucrose
IFRPD 6080	4.58	39.2	13.4	315	0.9	0.08
6080xs4D	4.65	32.7	9.3	344	0.26	0.08
6080xs4D s27	4.68	38.8	13.3	131	0.65	0.09
6080xs4D s39	4.43	38.0	12.1	244	0.68	0.09
IFRPD 6081	4.95	34.2	12.4	387	0.68	0.08
6081xs34D	5.03	30.4	9.3	265	0.13	0.11
6081xs34D s12	4.40	34.8	13.0	252	0.12	0.10
6081xs34D s16	5.15	36.7	13.9	466	0.20	0.09

$^{1/}$ The absorbance value lower than 0.2 (yellow range) was considered high fermentation activity.

with about 15 % decrease in CO_2 production after 1-week storage. For strain 6080xs4D s39, there was not much difference in CO_2 production of dough prefermented for 120 min after 1 week frozen-storage compared with the non-frozen one, but with extended reduction after 2 weeks (Figure 5). Thus, strains 6080xs4D s27 and 6080xs4D s39 was considered to have freeze-tolerant ability in low-sugar (5%) bread dough when prefermented for 120 min before freezing up to 1 week storage at -20 °C.

New strains developed from the parent IFRPD 6081 had lower freeze-tolerant abilities than those from IFRPD 6080 as demonstrated in Figure 6. Stain 6081xs34D s12 was better than the parent strain 6081, strain 6080xs4D and strain 6081xs34D s16 after 120 min-prefermentation and 1 and 2-week frozen storage. However, the amount of CO_2 production was considerably low (about 30 % decrease) after 1 week at -20 °C which made it unsuitable for frozen dough preparation.

CONCLUSION

Two *Saccharomyces cerevisiae* baker's yeast strains isolated in Thailand, IFRPD 6080 and IFRPD 6081, were hybridized with haploid and diploid mating strains of Japanese baker's yeast by rare-mating technique. Characterization and selection of segregants derived from individual spore clones of the hybrids was performed according to their properties i.e. yield of growth, total carbohydrate, trehalose contents, and carbon dioxide production. Two segregants, 6080xs4D s27 and 6080xs4D s39 from the parental strain IFRPD 6080 and one segregant, 6081xs34D s12 from the parental strain IFRPD 6081, with desirable characteristics were finally obtained. These segregants possessed good growth yield and higher maltose fermentation ability than their parents. Carbon dioxide productions in non-sugar, low-sugar and high-sugar dough were also markedly improved. Moreover, freeze-tolerant ability was observed in one segregant, strain 6080xs4D s27, and was able to use for frozen dough preparation.

ACKNOWLEDGEMENTS

We thank STA Fellowship and Japan International Science and Technology Exchange Center (JISTEC) for supporting this work.

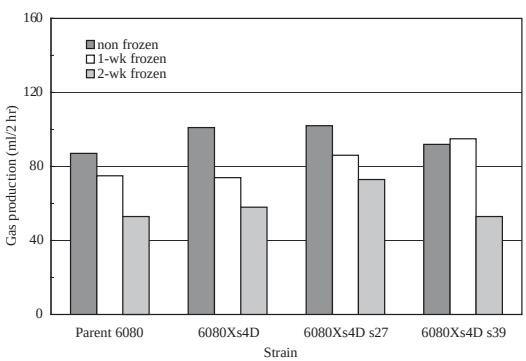

Figure 5 Freeze-tolerant abilities upon 1- and 2-week storage at –20 °C of parent strain IFRPD 6080 and new strains after 120-minute prefermentation of low-sugar bread dough.

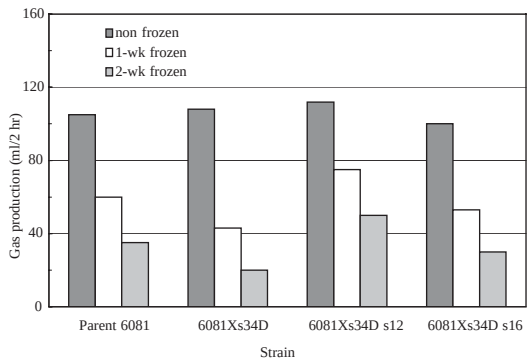

Figure 6 Freeze-tolerant abilities upon 1 and 2-week storage at –20 °C of parent strain IFRPD 6081 and new strains after 120-minute prefermentation of low-sugar bread dough.

LITERATURE CITED

Attfield, P. V., A. RamÛn and C. J. Northcott. 1992. Construction of *Saccharomyces cerevisiae* strains that accumulate relatively low concentrations of trehalose, and their application in testing the contribution of the disaccharide to stress tolerance. **FEMS Microbiol. Lett**. 94: 271-276.

Carle, G.F. and M.V. Olson. 1987. Orthogonal-field-alternation gel electrophoresis, pp. 468-482 *In* **Methods in Enzymology** Vol. 155.

Chu, G., D. Vollrath and R.W. Davis. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. **Science** 234: 1582-1586.

Gadd, G. M., K. Chalmers and R. H. Reed. 1987. The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. **FEMS Microbiol. Lett**. 48: 249-254.

Gunge, N. and Y. Nakatomi. 1972. Genetic mechanisms of rare matings of the yeast *Saccharomyces cerevisiae* heterozygous for mating type. **Genetics**. 70: 41-58.

Hamada, K., Y. Nakatomi. M. Osumi and S. Shimida. 1996. Direct induction of homozygous diploidization in the fission yeast *Shizosaccharomyces pombe* by pressure stress. **FEMS Microbiology Lett.** 136(1996): 257-262.

Hino, A., H. Takano and Y. Tanaka. 1987. New freeze-tolerant yeast for frozen dough preparations. **Cereal Chem**. 64: 269-275.

Hino, A., H. Takano, N. Kitabayashi, F. Nitta, T. Ohishi and Y. Tanaka. 1988. Automatic measuring system for dough testing; design, construction and reproducibility. **J. Food Sci. Technol.** 35: 344-351.

Hino, A., K. Mihara, K. Nakashima and H. Takano. 1990. Trehalose levels and survival ratio of freeze-tolerant versus freeze-sensitive yeasts. **Appl. Environ. Microbiol**. 56(5): 1386-1391.

Japan Yeast Industry Association. 1985. **Evaluation Method of Bakers' Yeast**. Revised ed. pp. 8,16.

McCready R. M., E.D. Ducay and M.A. Gauger. 1974. Automated analyses of sugar, starch, and amylose in potatoes by measuring sugar-dinitrosalicylate and amylose-iodine color reactions. **J. of Assoc. Off. Anal. Chem**. 57(2): 336-340.

Meric, L., S. Lambert-Guilois, O. Neyreneuf and D. Richard-Molard. 1995. Cryoresistance of baker's yeast *Saccharomyces cerevisiae* in frozen dough : Contribution of cellular trehalose. **Cereal Chem.** 72(6): 609-615.

Mortimer, R. K. and D. C. Hawthorne. 1969. Yeast genetics, pp. 386-460. *In*. A. H. Rose and J. S. Harrison (eds.). **The Yeasts**. Academic Press. London and New York.

Oda, Y. and K. Ouchi. 1990a. Hybridization of bakers' yeast by rare-mating method to improve leavening ability in dough. **Enzyme Microb. Technol**. 12(12): 989-993.

Oda, Y. and K. Ouchi. 1990b. Effects of invertase activity on the leavening ability of yeast in sweet dough. **Food Microbiology** 7: 241-248.

Oda, Y. and K. Tonomura. 1993. Selection of a novel baking strain from the *Torulaspora* yeasts. **Biosci. Biotech. Biochem**. 57(8): 1320-1322.

Oda, Y., K. Uno and K. Ouchi. 1986. Selection of yeasts for breadmaking by the frozen dough method. **Appl. Environ. Microbiol**. 52: 941-943

Ohshima, Y., T. Sugaura, M. Horita and T. Sasaki. 1987. Industrial application of Artificially Induced Diploid Strains of *Torulaspora delbrueckii.* **Appl. Environ. Microbiol**. 53: 1512-1514.

Oura, E., H. Suomalainen and R. Viskari. 1982. Breadmaking, pp. 86-141. *In* A.H. Rose (ed.). **Economic Microbiology** Vol. 7. Academic Press.

Spencer, J. F. T. and D. M. Spencer. 1977.

Hybridization of non-sporulating and weakly sporulating strains of brewer's and distiller's yeast. **J. Inst. Brewing** 83: 287-289.

Spencer, J. F. T. and D. M. Spencer. 1988. Yeast genetics, pp. 65-103. *In* I. Campbell and J.H. Duffus (eds.), **Yeast: A Practical Approach**. IRL Press, Oxford, Washington D.C.

Spencer, J. F. T., C. Bizeau, N. Reynolds and D. M. Spencer. 1985. The use of mitochondrial mutants in hybridization of industrial yeast strains. VI Characterization of the hybrid, *Saccharomyces diastaticus* x *Saccharomyces rouxii*, obtained by protoplast fusion, and its behavior in simulated dough-raising tests. **Current Genetics** 9: 649-652.

Takagi, A., S. Harashima and Y. Oshima. 1983. Construction and characterization of isogenic series of *Saccharomyces cerevisiae* polyploid strains. **Appl. Environ. Microbiol.** 45(3): 1034-1040.

Travelyan, W. E. and J. S. Harrison. 1956. Studies on Yeast Metabolism. 1. The trehalose content of Baker's yeast during anaerobic fermentation. **Biochem. J**. 62: 177-183.