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A Thesis for the Degree of Master of Science

Improvement of sucrose tolerance of baker's yeast, *Saccharomyces cerevisiae* by mating

미생물 교배를 이용한 제빵용 효모 *Saccharomyces cerevisiae* 의 당 내성 형질 개량

By

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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements

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農 學 碩 士 學 位 論 文

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이 論文을 農學碩士學位論文으로 提出함

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2018年 2月

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ABSTRACT

Baker's yeast, *Saccharomyces cerevisiae*, used as a starter in fermentation during a baking process is the main determinant of bread quality. *S. cerevisiae* SPC-SNU 70-1, isolated from *Nuruk* has excellent fermentation characteristics such as good leavening ability, generating volatile organic acids which affect a mild flavor and delaying the aging of bread. *S. cerevisiae* SPC-SNU 70-1 was able to show a good fermentability in lean (0% sucrose) and regular dough (8% sucrose), though, CO_2 production decreased significantly in sweet dough containing 20 % sucrose. It is necessary to improve traits of the SPC-SNU 70-1 strain in high sucrose concentrations for various applications, while maintaining the inherent fermentation characteristics.

Eight novel strains to be mated with SPC-SNU 70-1 were isolated from *Nuruk* and grape. Those strains have high sucrose tolerance but

poor fermentation activity in lean dough. Among 8 strains isolated, N1, N2, N5 and N6 strains with excellent leavening ability in sweet dough were selected for mating, but the N1 strain was excluded because it was impossible to form spores which is the first step of mating. The selected strains underwent sporulation, tetrad dissection and analysis. The isolated spores maintained haploids, thus the genetic sequences of the *HO* gene (*HOmothallic switching endonuclease* gene, encoding HO endonuclease that changes the mating type) were analyzed. All selected strains were found to be heterothallism. The haploid strains for mating were selected by the *MAT* locus PCR, barcode PCR and leavening ability. After mating, the same selection process was repeated to select mated strains.

Total 17 strains were selected and checked for the amount of CO_2 production in lean, regular and sweet doughs. The strains with high industrial capability are designated as S-HW2, S-HW8, S-HW9, S-

HW11 and S-HW16, S-HW17. Among them, the S-HW16 was selected based the fermentation ability in three different doughs. The amount of CO_2 production of the S-HW16 strain increases 1.1-folds in lean dough, 1.3-folds in regular dough and 1.8-folds in sweet dough compared with that of SPC-SNU 70-1. The amount of fragrance components also increases. In sweet dough, the amount of esters which give a sweet flavor increases 10.6-folds and the amount of acetoin which give a buttery flavor increases 2.1-folds. This study demonstrated that mating was successfully used to develop new yeast strains with desired characteristics for baking.

Keywords : Baker's yeast, *Saccharomyces cerevisiae*, industrial strain, novel isolated strain, high sucrose tolerance, leavening ability, sporulation, tetrad analysis, mating, industry applicability

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I. INTRODUCTION

1. Baker's yeast isolated from *Nuruk*

Baker's yeast, *Saccharomyces cerevisiae,* is one of the main factors that determine the bread quality. During dough fermentation, *S. cerevisiae* converts sugar into CO_2 as leavening gas. The yeast also makes secondary products like alcohols, organic acids and esters that impact a flavor in bread. (Birch, Petersen et al. 2013)

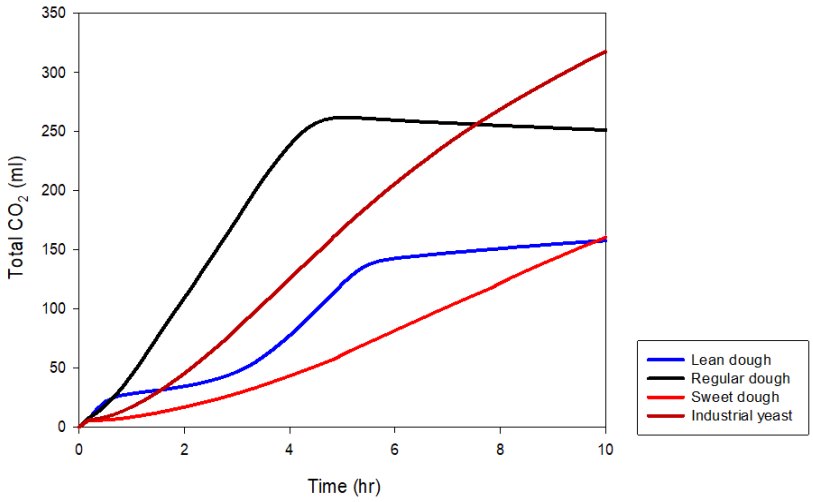
In the previous study, *S. cerevisiae* SPC-SNU 70-1 was isolated from Korean traditional *Nuruk* as a fermentation starter for baking (SPC 2015). The bread fermented by *S. cerevisiae* SPC-SNU 70-1 has a low fermented odor, a mild taste, and good chewy texture. In addition, the SPC-SNU 70-1 strain has a good leavening ability in regular dough containing 8g sucrose per 100g flour, and an effect of delaying the aging of bread. (SPC 2015)

However, in the bread making process, the baker's yeast is put at risk of many environmental stresses such as air drying, freeze thaw and high sucrose concentrations (Attfield 1997). Among them, high sucrose concentrations bring about serious osmotic stress and inhibit the optimal fermentation ability of the baker's yeast (Sasano, Haitani et al. 2012). Yeast strains that have tolerance to high sucrose concentrations are useful for sweet dough fermentations (Attfield 1997). Thus, *S.*

cerevisiae used for sweet bread making must adapt to high sucrose concentrations in dough fermentation processes.

For the SPC-SNU 70-1 strain, have low sucrose tolerance. Figure 1 shows results of the leavening ability of SPC-SNU 70-1 and yeast strain. In that results, the SPC-SNU 70-1 strain is excellent in lean and regular doughs, but not in sweet dough, as it shows inferior to the commercial strain. In this study, *S. cerevisiae* SPC-SNU 70-1 were improved to sucrose tolerance by mating with resistant strains while maintaining the advantages of the SPC-SNU 70-1.

A



B

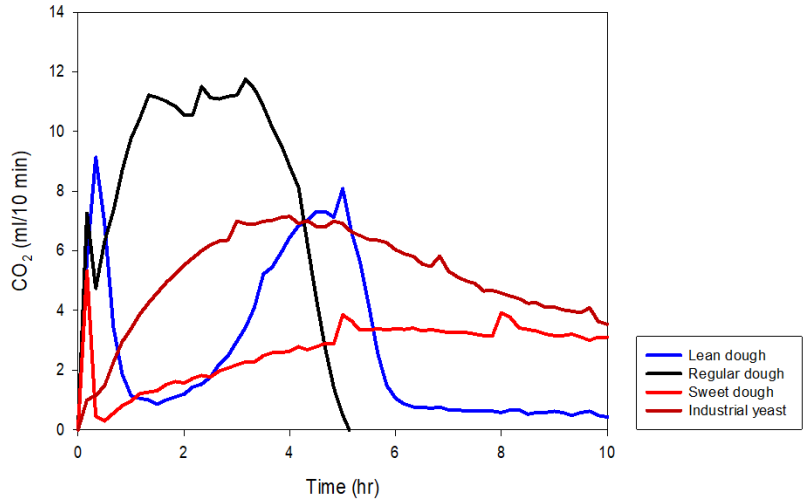


Figure 1. Results of leavening ability of SPC-SNU 70-1 in lean, regular and sweet dough. The wine color line is leavening ability of commercial strain in sweet dough. (A) Cumulative gas generation, (B) gas generation per 10 min.

2. Mating

2.1. *Saccharomyces cerevisiae* life cycle

Yeast cells can exist in haploid, diploid and polyploidy over the triploid state. Haploid cells have a mating type a or alpha (α) and can mate with opposite types. In diploid cells, mating type locus portions are heterozygous. Both haploids and diploids can proliferate asexually by budding under nutrient-rich conditions. But under nutrient-poor conditions, the diploid can progress sporulation. It is meiosis followed by spore formation. As a result, the diploid cell converts four haploid spores, two possessing mating type a and two alpha, that can germinate into vegetative cells when conditions improve. At this time, the haploid derivatives can progress a mating type switch in homothallic strains. The change is caused by an endonuclease encoded by the *HO* gene. In this case, a switched cell can mate with nearby sister cells of the opposite mating type and form diploid cells. On the other hand, in heterothallic strains, the *HO* gene is inactive and haploid derivatives cannot switch a mating type. The mechanism of the mating type switch in homothallic strains is occurred by the *MAT* locus on chromosome III. The *MAT* locus contains both *Hidden MAT Left* and *Right* (*HML* and

HMR). The *HML* and *HMR* have a silenced copy of *MAT*a and *MAT*α. HO endonuclease cleaves DNA specifically at the *MAT* locus, and DNA repair occurs with *HML* or *HMR* as templates. Then a gene conversion event occurs. (Steensels, Snoek et al. 2014)

2.2. Different strain improvement techniques using hybridization

Several strategies can be applied to constructing new yeast strains for industrial applications. The existing natural diversity can be studied by genotyping and phenotyping strains that isolated from wild or yeast collections to select interesting variants. In addition, diversity can be artificially created. Strategies to artificially generate diversity include deriving genetic diversity in one strain or shuffle the genomes from several strains. Strains obtained from these strategies are considered as non-genetically modified yeasts. They can be used for industrial fermentations without GMO issues.

Sexual and asexual hybridization is a strong technique to make artificial diversity. Most techniques start from two selected parent strains with the target phenotype. These hybrid techniques include

direct mating, rare mating, mass mating, cytoduction and protoplast fusion. (Steensels, Snoek et al. 2014)

2.3. Direct mating

Direct mating consists of the crossing of two selected parent strains possessing a target phenotype. In case of yeast strains, spore-to-spore, spore-to-cell, and cell-to-cell mating approaches exist. The choice of which method to use depends on the parent strains.

The following is explanation and summary of the previous researches (Steensels, Snoek et al. 2014). When one or both parent strains are homothallism, no stable haploid spore cells can be obtained. It means a prescreening step is not feasible. In this case, using spore-to-cell mating and spore-to-spore mating. Spores are obtained from homothallic strains and cells are obtained from heterothallic strains. These methods are possible by placing two spores to be hybridized close to one another on an agar surface, and then monitoring and isolating the developed zygotes using a micromanipulator. Zygotes can be formed if the spores have an opposite mating type. It has been used successfully to generate wine yeasts with improved cryotolerance, by

crossing *S. cerevisiae* with cryotolerant species such as *S. kudriavzevii* or *S. bayanus* (Kishimoto 1994, Zambonelli, Passarelli et al. 1997, Pérez-Través, Lopes et al. 2012). Recently, spore-to-cell mating was applied to developing multistress-tolerant *S. cerevisiae* strains (Benjaphokee, Hasegawa et al. 2012).

If both parent strains are heterothallism, best haploid cells can be prescreened for the mating experiment. This technique dates back to 1943, when it was described in a seminal paper, and is called 'cell-to-cell' mating (Lindegren and Lindegren 1943). Hybrids can be isolated by simply mixing cell cultures of two selected stable parents and screening diploid cells. The great advantage of cell-to-cell mating is that both parent haploids can be completely phenotyped before the mating experiment, which increases the probability of obtaining an excellent hybrid. Several wild strains show a stable haploid mating type, due to a mutation in the *HO* endonuclease gene. It means these strains fit well for cell-to-cell mating experiments. If the wild yeast is homothallism, the HO gene can be modified to perform cell-to-cell mating, but in this case recombination of the yeast occurs and classified as a GMO (Genetically Modified Organism), making it unsuitable for industrial uses. Novel hybrids with improved characteristics developed

by cell-to-cell mating have been reported (Hara, Iimura et al. 1981). The paper used this approach to construct cryotolerant wine yeasts able to produce killer toxins. Another paper described the use of cell-to-cell mating to eliminate the unwanted ‘phenolic off-flavor’ phenotype from brewer’s yeast (Russell, Hancock et al. 1983). This approach was also used to construct wine, bread, and beer yeasts with optimal fermentation characteristics (Eschenbruch, Cresswell et al. 1982, Nakagawa and Ouchi 1994, Marullo, Bely et al. 2006). More recently, it was used to combine specific phenotypes of ale and lager yeasts in order to improve stress resistance and fermentation performance (Sanchez, Solodovnikova et al. 2012).

Table 1. List of previous researches about direct mating

Mating method	Trait	Strains	Reference
Spore-to-spore	Cryotolerance	*S. cerevisiae*	Pérez-Través, L et al. (2012)
		S. kudriavzevii	Zambonelli, C et al. (1997)
		S. bayanus	Kishimoto, M. (1994)
Spore-to-cell	Multistress-tolerance	*S. cerevisiae*	Benjaphokee, S et al. (2012)
Cell-to-cell	Cryotolerance	*S. cerevisiae*	Hara, S et al. (1981) Nakagawa, S et al. (1994
	Eliminating off-flavor	*S. cerevisiae*	Russell, I et al. (1983) Marullo, P et al. (2006)
	fermentation performance	*S. cerevisiae*	Eschenbruch, R et al. (1982)
	Stress resistance and fermentation performance	*S. cerevisiae*	Sanchez, R.G et al. (2012)

3. Research objectives

This study was focused on the improving sucrose tolerance of *S. cerevisiae* SPC-SNU 70-1 while maintaining the inherent advantages. Thus, the SPC-SNU 70-1 strain mated with the isolated sugar tolerance strains. Then improved novel strains are applied to the sweet dough.

The specific objectives of this study are listed as follows.

1) To isolate novel strains resistant to high sucrose from *Nuruk*.

2) To mate *S. cerevisiae* SPC-SNU 70-1 with high sucrose tolerance strains.

3) To apply S-HW strains for sweet dough.

II. Materials and Methods

1. Strains

Five *S. cerevisiae* strains used in this study were listed in Table 1. The main strain in this study was *S. cerevisiae* SPC-SNU 70-1 isolated from Korean traditional *Nuruk* by the SPC Group (Seoul, South Korea) in 2015 (SPC 2015). Six commercial baker's yeast strains were obtained from the SPC Group (Seoul, South Korea). Three laboratory yeast strains were used in this study.

Yeast cells were grown in YPD medium composed of 1% yeast extract (Difco, MI., USA), 2% peptone (Difco, MI., USA) and 2% glucose (Sigma-Aldrich, MO, USA) under the standard conditions (Rose 1990). Yeast strains were stored on YPD medium in a deep freezer at -80°C supplemented with glycerol (15% v/v) until further use.

Table 2. List of the strains tested in this study

Species	Strain designation	Isolation source or application	Ploidy
S. cerevisiae	SPC-SNU 70-1	*Nuruk*	Diploid
S. cerevisiae	CBY	Commercial	Diploid
S. cerevisiae	S288C	Laboratory strain	Haploid (α)
S. cerevisiae	D452-2	Laboratory strain	Haploid (α)
S. cerevisiae	ATCC 208281	Laboratory strain	Haploid (a)

ATCC - American Type Culture Collection, Rockville, Maryland, USA.

2. Isolation of *S. cerevisiae*

2.1. Isolation by maltose subculture

In order to isolate yeasts from the *Nuruk* and grape, 10 g of *Nuruk* was homogenized in 90 ml of 0.85% NaCl solution. At first, the homogenized solution was immediately spread on the YP plate containing 20 g/L of glucose, 50 μg/ml of ampicillin and kanamycin. In case of grape (crimson seedless), grape must (freshly pressed fruit juice) was made and then the flesh and peel were sieved. To increase efficiency, the solution was inoculated into YP medium containing 20 g/L maltose. After incubation for 24 hours, transfer to YP medium again. 7th subcultured cells were spread on the YP plates containing 20 g/L glucose.

2.2. Genomic DNA extraction

S. cerevisiae for genomic DNA extraction was cultured in YPD medium at 30 °C overnight until the cell number reached 10^8 – 10^9. The cultured cells were centrifuged at 13,200 rpm for 2 min and the supernatant was discarded. The harvested cell pellet was washed twice with double-distilled water (DDW). The washed cell pellet was

voltexed with 0.3 g glass beads (0.5 mm), 200 μL PCI solution (Phenol : Chloroform : Isoamyl Alcohol 25:24:1, Sigma-Aldrich, MO, USA) and lysis buffer containing 0.1 M Tris pH 8.0, 1 mM Na_2EDTA, 2% triton X-100 and 1% SDS for final concentration. After vortexing for 4 min, the suspension was resuspended gently with 200 μL TE buffer (10 mM Tris, 0.5 mM EDTA and pH 8.0) and centrifuged at 4°C for 5 min. Approximately 300 μL of the supernatant, avoiding white cell debris and beads, was transferred to 1.5 ml eppen tubes and resuspended with 1 ml absolute ethanol by inversion. The suspension was centrifuged at 13,200 rpm for 2 min to remove the supernatant completely. The DNA pellet was dissolved in 100 μL TE buffer and incubated with 10 mg/ml RNase A at 37 °C for 1 hr. The genomic DNA purity was measured by SPECTROstar Nano (BMG labtech, Germany).

2.3. Strain-specific Polymerase Chain Reaction (PCR)

There have been several reports on PCR identification systems at the species level, based on ribosomal RNA genes and the internal transcribed spacer (ITS) region (Chang, Nam et al. 2007). This is not

an appropriate PCR primer target for the strain level identification because of their high similar sequences. The forward and reverse sequences were added to make the primers longer. The primer sequences are SCDL_F, 5’ AGG AGT GCG GTT CTT TGT AAA GTG 3’; SCDL_R, 5’ TAC TTA CCG AGG CAA GCT ACA TTC C 3’. The PCR product is about 320 bp. It amplified the internal fragment of the *S. cerevisiae* D1/D2 region. All oligonucleotide primers used in this study were synthesized by Bioneer Co. (Daejeon, Korea).

The polymerase chain reaction (PCR) was performed by the GeneAmp PCR System 2400 (*Applied* Biosystems, CA, USA), T100™ Thermal Cycler (Bio-rad, CA, USA). The PCR conditions were pre-denaturation at 95 °C for 10 minutes, followed by 30 cycles of extension at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, annealing at 72 °C for 1 minutes, and final extension at 72 °C for 10 minutes. Electrophoresis was performed on 2% agarose gel in 0.7X TAE buffer at 135 v for 25 min and stained with ethidium bromide (Bioneer, Daejeon, Korea). DNA fragments were visualized by UV illumination and photographed as TIFF format files by an i-MAX™ Gel Image Analysis System (CoreBio, Korea).

2.4. Random amplified polymorphism DNA (RAPD) PCR

RAPD PCR is a method of randomly amplifying DNA using a 10-mer short primer to produce DNA fragments of various sizes. This PCR method is used to distinguish species, strains or substrains. The patterns of these fragments were analyzed using electrophoresis. RAPD primers have more than 10,000 kinds, and the primers were selected by referring to a study in which 8 of primers were used to classify *S. cerevisiae* strains (Echeverrigaray, Paese-Toresan et al. 2000). 20 μl of PCR amplification mixture was composed of TOPsimple™ DyeMIX-Tenuto PCR premix (enzynomics, Daejeon, Korea), 10 pmol of primer, template colony. The PCR conditions were pre-denaturation at 92 °C for 10 minutes, followed by 40 cycles of extension at 92 °C for 45 sec, annealing at 37 °C for 1 min 30 sec, annealing at 72 °C for 2 min, and final extension at 72 °C for 4 min. Electrophoresis was performed on 2% agarose gel in 0.7X TAE buffer at 100 v for 50 to 60 min. In addition, the experiment was further conducted using four types of OPC primers (Pérez, Gallego et al. 2001). Table 2 shows the primer sequences used.

2.5. Ploidy determination by PCR

A mating type of *S. cerevisiae* is regulated by two different alleles of the mating-type (*MAT*) locus (Kostriken, Strathern et al. 1983). The *MAT* locus was analyzed by PCR with the following specific primers designed by Kim Jin-Woo: HML_Yalpha_F, 5' GAAATATTTAAACT CATTTATGGCTTTTAGA G 3' which is located at *MAT*α and *HML*α; HMR_Ya_F 5' GTAATTTGACTAAAGTAGAGCAACATAC 3' which is located at *MAT*a and *HMR*a; MAT_R 5' CAAAAGTCACATCAAG ATCGTTTATGG 3'. When these three oligonucleotides were used in a single PCR, the amplicon size at *MAT*α was 525 bp and the amplicon size at *MAT*a was 757 bp.

The PCR reaction was performed with initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Electrophoresis was performed on 2% agarose gel at 135 v for 25 min.

2.6. SPC-SNU 70-1 strain-specific barcode PCR

SPC-NU 70-1 strain-specific barcode PCR (barcode PCR) primers were designed by Hyo-Jin kim to specifically detect the SPC-SNU 70-1 strain. (Kim, thesis, 2017)

Four Primer sets for multiplex PCR were composed of three stain-specific primer pairs and one species-specific primer pair. The primer sets were as follows: SCDL; CH4_1; CH7_2; CH11_1. (Table 3)

20 μl of PCR amplification mixture was composed of PrimeSTAR® HS PCR PreMix (Takara, Shiga, Japan), 10 pmol of forward and reverse primers, 2 μl of template DNA. The PCR conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 61 °C for 30 sec, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

The amplification products were confirmed by agarose gel electrophoresis at 135V for 25 min.

Table 3. List of RAPD primers used in this study

Primer	Sequence	Primer	Sequence	Primer	Sequence
OPB-01	GTTTCGCTCC	OPX-01	CTGGGCACGA	OPC-01	TTCGAGCCAG
OPB-10	CTGCTGGGAC	OPX-03	TGGCGCAGTG	OPC-03	GGGGGTCTTT
OPB-12	CCTTGACGCA	OPX-06	ACGCCAGAGG	OPC-06	CCGCATCTAC
OPB-14	TCCGCTCTGG	OPX-07	GAGCGAGGCT	OPC-07	TGGACCGGTG

Table 4. List of *S. cerevisiae* SPC-SNU 70-1 specific primers used in this study

Name		Sequence (5' → 3')	Amplicon size [bp]
SCDL	F	AGG AGT GCG GTT CTT TGT AAA GTG	320
	R	TAC TTA CCG AGG CAA GCT ACA TTC C	
CH4_1	F	AACTTGGCTACAAATTATGAACAATGAGGCCGAC	526
	R	GATCCATAAAGCTCATACTGGGGTGTGGTGTAGC	
CH7_2	F	ACTCATGTAAACTGTGATAAACCCGTTATTTCAG	649
	R	CTGTTAAAAATTACCGGCAAAGGTAATATTGCTG	
CH11_1	F	GGATGGAACGCCCGCAAGAGTCTACTAAGCAATC	774
	R	AAAGGCTTACTTACTGATAGTAGATCAACGATCA	

3. Tetrad dissection and mating

3.1. Sporulation

Sporulation is the process of forming the tetrad. Sporulation occurs under certain conditions. Where it does not provide a nitrogen source and glucose but provides acetate as a carbon source. Therefore, 20 g/L potassium acetate medium (Spo) was prepared for sporulation (Freese, Chu et al. 1982, Lo and Hollingsworth 2011) and yeast cells were inoculated. A sufficient amount of tetrad was obtained by culturing for 3 days but it depended on the strain. After sporulation, the cell wall of tetrad must be decomposed before tetrad is separated. In this study, lyticase (Sigma L4025), an enzyme that dissolves the cell wall of yeast, was treated for 3 hours.

3.2. Micromanipulator

Tetrad dissection and mating was performed by a micromanipulator Sporeplay (Singer, Somerset, UK) and AXIOSKOP 40 TETRAD (ZEISS, Oberkochen, Germany). The micromanipulator was used to separate the tetrad. There is a needle in the microscope, and it is a device that can take cells one by one. For tetra dissection, the tetrad is

spread on a 20 g/L glucose YP plate and dried. The dry YP plate is placed upside down on the micro manipulation device. Focus the microscope so that the cells on the medium are clearly visible. The observed tetrad is transferred to a different location on the plate with a needle. The tetrads are separated one by one and moved to four different locations in a spore state. YP plates are incubated at 30 ° C in a static incubator until colonies appear.

Mating was also performed using a micromanipulator. The method used in this study is a cell-to-cell mating method in which two haploid cells are mated directly. A haploid single colony isolated from the tetrad was inoculated into 20 g/L glucose YP broth and cultured at 30 °C, for 12 hours. And cultured cells were spread on 20 g/L glucose YP plate. In mating, two haploid cells that have opposite mating type were placed in one place. Colonies were inoculated on 20 g/L YP broth and incubated at 30 °C for 24 h. The cultured cells were spread again in YP plates of 20 g/L glucose and a single colony was obtained. However, this method was not efficient. As a second method, mating was carried out by resuspension a single colony on the plate, rather than obtaining a cell by inoculating broth with stock.

3.1. Sequencing *HO* gene

There are two cases during the life cycle of the diploid yeast. One is called homothallism when the mating type A and alpha are self-changing and hence self-mating is possible. Conversely, if the mating type does not change and maintains the haploid when dividing the diploid, it is called heterothallism. The most important role in determining this is Ho endonuclease. This enzyme affects the *MAT* locus of chromosome 3. HO endonuclease switches mating type a and alpha genes on *MAT* locus. Therefore, if this enzyme is made properly, yeast is homothallism, otherwise heterothallism.

The sequence of the *HO* gene (*HOmothallic switching endonuclease* gene), which encodes the HO endonuclease enzyme, was analyzed to confirm whether the SPC-SNU 70-1 strain and the selected strains were homothallic or heterothallic correctly. If the *HO* gene matches the reference sequence, it will be homothallism, and if the amino acid sequence changes, it will be a heterothallism strain. The primers for amplifying the *HO* gene were F_HO (GTATTCAATTCCTATTCTAAATGGCTTTTA), R_HO (CAGCAT TCGAGTTAAGAAAAAGTCTAAAAA). The PCR product was purified by using Gel Extraction Kit from Takara (Tokyo, Japan) and

sequenced in SolGent (Daejon, Korea). Sequencing results were analyzed and aligned using the Vector NTI program.

4. Analysis of baking ability of *S. cerevisiae*

4.1. Preparation of yeast cell for dough fermentation

To prepare for the inoculums into LD medium, *S. cerevisiae* stock was cultivated in a 5 ml test tube with YP medium containing 20 g/L sucrose at 30 °C, 250 rpm for overnight. The grown cells were transferred to a 500 ml baffled-flask with 100 ml of YP medium containing 50 g/L sucrose at the initial OD_{600} 0.2 and cultivated at 30 °C, 250 rpm for overnight. Optical density (OD_{600}) was measured by a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea) at 600 nm.

When the cells were inoculated into dough, a larger amount of cells is required than when inoculated into LD medium. So large scale cultivation was required. And the process of culturing cells was different from process for LD medium because it followed the method of the SPC laboratory. Cell stock was cultivated in a 10 ml test tube with YM medium at 30 °C, 250 rpm for 24 hours. The grown cells were all transferred to a 500 ml baffled-flask with 90 ml of YM medium at

30 °C, 250 rpm for 24 hours. The grown 100 ml cells were all transferred again to 2 L baffled-flask with 900 ml of YP containing 20 g/L glucose at 30 °C, 250 rpm for 24 hours.

The cultivated cells were harvested by centrifugation at 4 °C, 8,000 rpm for 10 min and washed with DDW.

4.2. Liquid dough (LD) medium

A liquid dough model system was constructed to analyze more easily than dough. Glucose, maltose are substituted for flour, and sucrose is added according to the conditions of the dough. Sorbitol lowers water activity and the other ingredients provide nitrogen, minerals and vitamins. The LD solution (according to a formula provided by Lesaffre International, Lille, France) was prepared as follows. First, a 5X concentrated nutrient solution, containing 5 g of MgSO4•7H2O, 2 g of KCl, 11.75 g of (NH4)2HPO4, 4 mg of thiamine, 4 mg of pyridoxine, and 40 mg of nicotinic acid in a final volume of 250 ml of 0.75 M citrate buffer (pH 5.5), was prepared. The solution was filter sterilized. 20 ml of the concentrated nutrient solution was added to a bottle containing 0.5 g of yeast extract, 3 g of glucose, 9 g of maltose, 12 g of sorbitol, and sucrose (Panadero, Randez-Gil et al. 2005). Sweet LD

contained 900 g/L of sucrose. Double distilled water was added to a final volume of 100 ml. LD medium is diluted 2 times before use.

4.3. Flour-based dough (FD)

According to AACC Method 89-01 (AACC 1990), lean dough was prepared based on 100 g of flour, 2 g of salt, 3 g of shortening, 65 g of water and no sucrose. And regular dough and sweet dough were prepared based on 100 g flour, 3 g of nonfat dry milk, 2 g of salt, 3 g of shortening, 65 g of water and 8 g of sucrose (in regular dough) or 53.8 g of water and 20 g of sucrose (in sweet dough). 4.06 g, 4.32 g, 4.37 g of yeast pellet was added in each dough for fermenting bread.

4.4. Determination of leavening ability

The leaving ability was determined by measuring CO_2 production in LD medium and FD. In case of LD medium, the harvested cells were inoculated to a 250 ml bottle with 16.7 ml of LD medium at the initial OD_{600} 25 and cultivated at 30 °C, 150 rpm. The amount of CO_2 production was measured in the ANKOM RF Gas Production System (ANKOM Technology, NY, USA). 25 g of FD were incubated at 30 °C, and the amount of CO_2 production was recorded for 10 hr in AF-

1101W Fermograph (ATTO, Tokyo, Japan). The data were processed using Kaluza software.

4.5. Volatile fragrance assay

The volatile fragrance component analysis was carried out with 7890A GC System (Agilent Technologies, CA, USA). The column is DB-WAX (60 m x250 μm x 0.25 μM), 230 °C and the Carrier gas is helium, velocity of flow rate is 1 ml/min. The MS detector is Agilent 5975C MSD (El mode). The dough was made by the same method as those for measuring the leavening ability. After 4 hours of dough fermentation, the dough is measured in 1 g and placed in a GC vial. The fragrance component of the dough was evaporated at 85 ° C for 30 minutes to adsorb onto the needle. The fragrance components adsorbed on the needles are analyzed by GC for 25 minutes.

III. RESULTS AND DISCUSSION

1. Isolation of *S. cerevisiae*

1.1. Identification of new strains

When isolating the strain of desired properties from *Nuruk*, 316 colonies were grown in a YP plate containing antibiotics. Those colonies were analyzed in shape, 188 colonies were similar in appearance to *S. cerevisiae*. And then, seven *S. cerevisiae* were confirmed by *S. cerevisiae* specific PCR. When isolating the strains from the grapes, the grape must was subcultured on a YP medium containing maltose. 30 colonies were picked and confirmed to be *S. cerevisiae*. The names of these colonies were given after strain confirmation.

Among the 12 RAPD PCR primers, two primers revealed differences in substrain. The remaining 10 primers showed little difference. Among 37 *S. cerevisiae* isolated from *Nuruk* and grape, 8 strains were found to be different substrain.

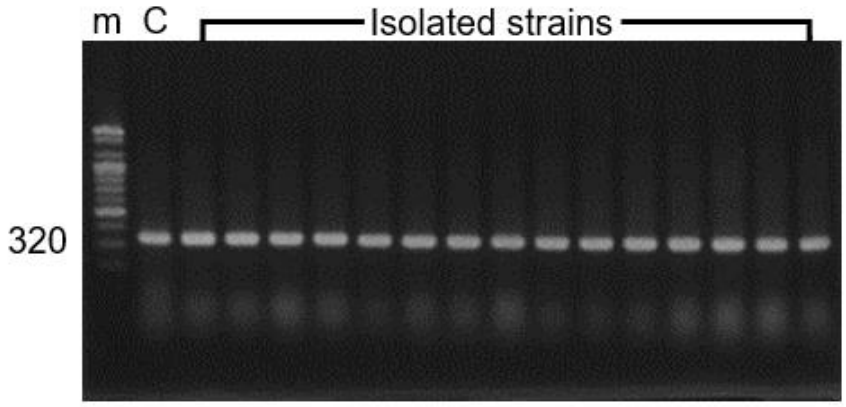
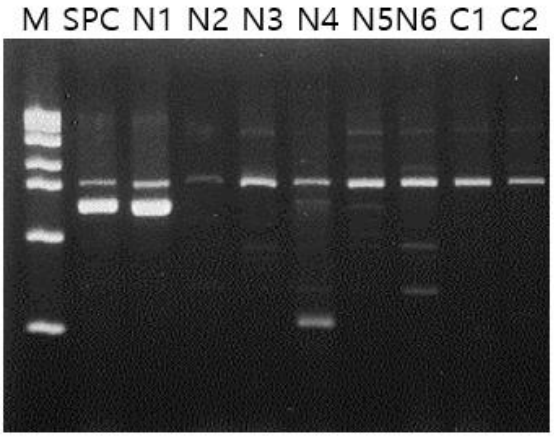


Figure 2. *S. cerevisiae* species-specific PCR, SCDL. PCR product is about 320 bp. Line m, 100bp ladder ; Line C, control strain, *S. cerevisiae* D452-2 ; Line 3 - 17, isolated new strains.

A



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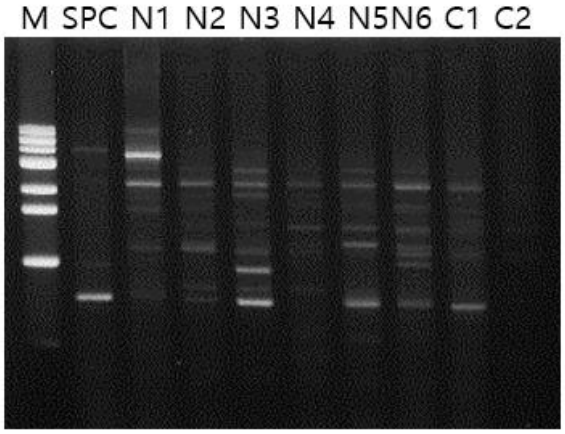


Figure 3. Results of RAPD PCR for isolated *S. cerevisiae* strain distinction. (A) Primer OPB 01, (B) Primer OPC 01. Line M, 1kb ladder ; Line SPC, SPC-SNU 70-1 (SPC) ; Line 3 - 10, isolated new strains.

1.2. Strains analysis by PCR

For mating, strains should be diploid. Therefore, the ploidy was confirmed by the *MAT* locus PCR and the result is shown Figure 4. Both SPC-SNU 70-1 and the new isolates were diploid.

Barcode PCR, will be used to select mating strains, also proceeded. All four bands appeared in SPC-SNU 70-1 strain only. In case of N1, total three bands were shown. Other strains showed only one band that refers to *S. cerevisiae*. So during mating, barcode PCR pattern is useful selection marker for selecting mating strains.

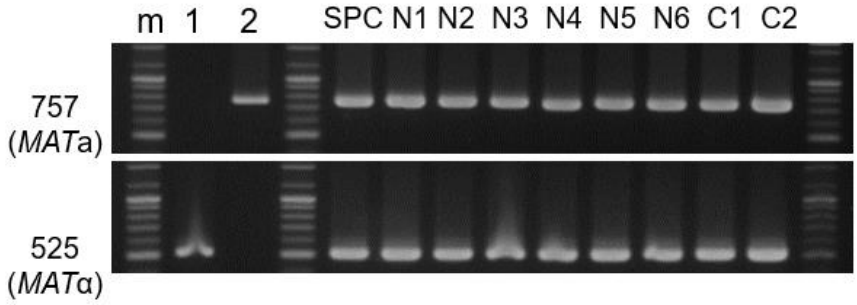


Figure 4. Results of *MAT* locus PCR. Line m, 100bp ladder ; Line 1, *MAT*a control strain, *S. cerevisiae* D452-2 ; Line 2, *MAT*α control strain, *S. cerevisiae* BJ3505 ; Line SPC, SPC-SNU 70-1 ; Line 3 - 10, isolated new strains.

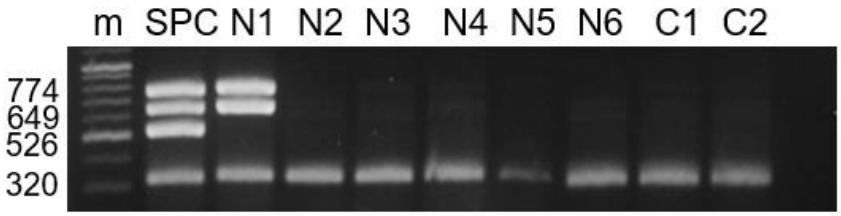


Figure 5. Results of barcode PCR. There are four PCR product about 320bp, 526bp, 649bp and 774bp. Each band is from primer SCDL, CH4_1, CH7_2, CH11_1. Line m, 100bp ladder ; Line SPC, SPC-SNU 70-1 ; Line 3 - 10, isolated new strains.

1.3. Determination of homo- or heterothallism

Depending on whether the strains are homothallism or heterothallism, the mating method is completely different. Therefore, it is necessary to check the type of these strains first. The *HO* gene was sequenced and compared with the homothallic reference sequence. The S288C strain is a heterothallic strain with four nucleotide sequences changed (SGD, *Saccharomyces* Genome Database, www.yeastgenome.org). Figure 6 show the amino acid sequences of the *HO* gene. According to the results, HO endonuclease was not produced by inserting a stop codon in the middle of *HO* gene. The SPC-SNU 70-1 strain and the selected strains N2, N5 and N6 all were heterothallism.

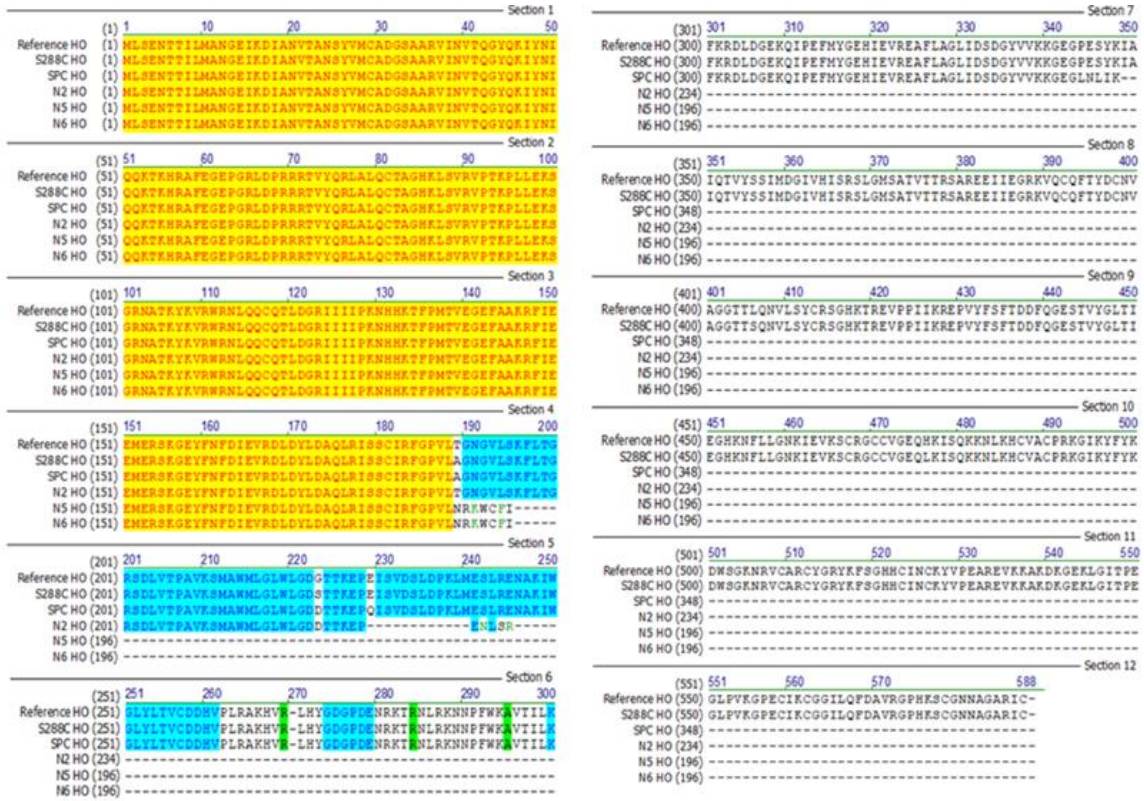


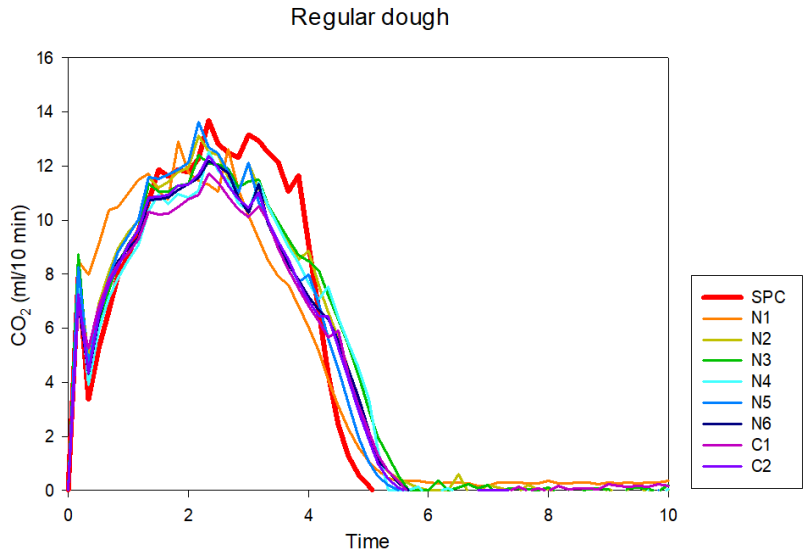
Figure 6. Results of translated *Ho* gene sequence.

1.4. Selection by leavening ability

The amount of CO_2 generated from 25 g of regular and sweet dough was measured for 10 hours. The figure showing CO_2 generated every 10 minutes showed not only the overall yield but also the initial gas amount. It was an important result in the bread industry because dough fermentation usually takes about 4 hours. Figure 7 shows the isolated 8 strains have similar leavening ability to SPC-SNU 70-1 in regular dough, and better than SPC-SNU 70-1 in sweet dough. Therefore, wild type strains isolated from *Nuruk* and grapes were found to be more resistant to high sucrose concentration than SPC-SNU 70-1 strains.

In Figure 8, average of total emissions of CO_2 was compared to the SPC-SNU 70-1 for selection of mating strains. The amount of CO_2 production of SPC-SNU 70-1 was 121.6 ml. And in order of superior leavening ability, the amount of CO_2 production of N1 and N6 were 243.8 ml and 214.3 ml. The amount of CO_2 production of N5, N2 were 201.3 ml, 196.7 ml. And N3, N4, C1 and C2 strain emitted 186.4 ml, 140.7 ml, 149.9 ml and 189.8 ml of CO_2. As a result, N1, N2, N5 and N6 strains with high CO_2 production were selected as mating strains.

A



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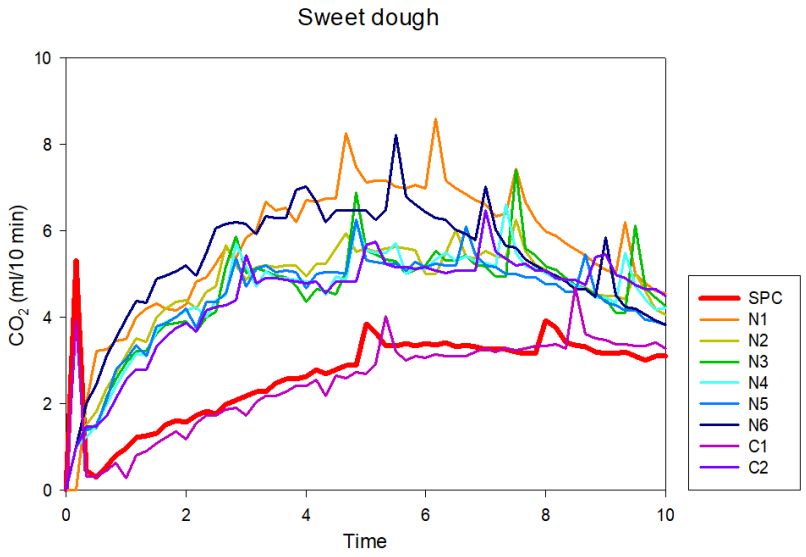


Figure 7. Results of leavening ability of SPC-SNU 70-1 and novel isolated strains in (A) regular dough and (B) sweet dough.

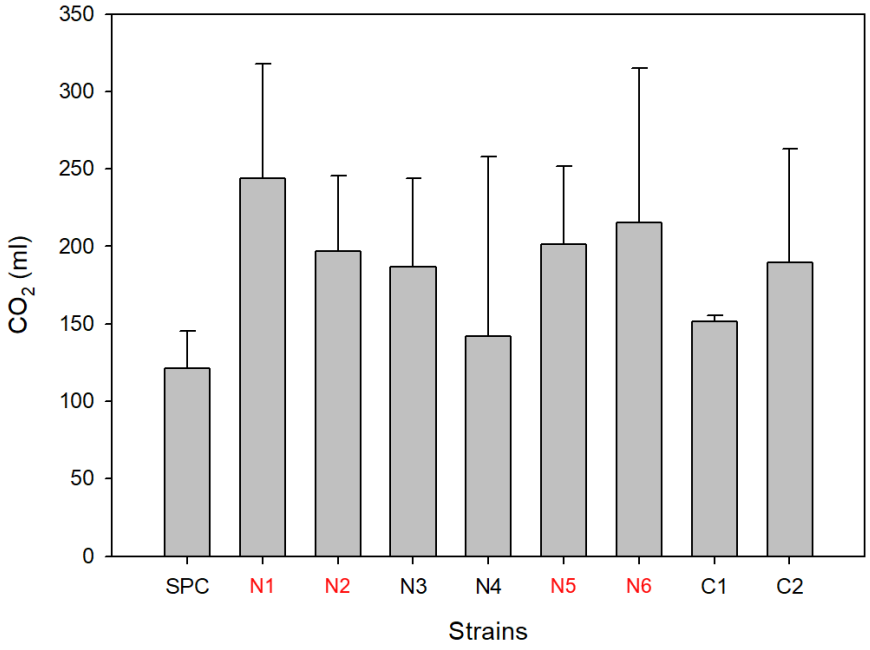


Figure 8. Average of leavening ability in sweet dough for selection.

2. Tetrad dissection

2.1. Sporulation

After 3 hours of sporulation and 30 minutes of lyticase treatment, the cells were observed under a microscope. Sporulation was carried out with SPC-SNU 70-1 and the selected strains N1, N2, N5 and N6. It was confirmed that all strains except for the N1 strain formed a significant amount of tetrad. However, no tetrad was found for the strain N1. The culture time was increased to one week in Spo medium, but tetrad was not formed at all. Therefore, N1 was excluded for mating and mating proceeded only with strains N2, N5 and N6.

2.2. Determination of ploidy

The tetrad was divided into four spores using a micromanipulator. Since the parent strain is heterothallism, each spore should be haploid if the spores are properly split. For SPC-SNU 70-1, a total of 16 spores were obtained by dividing four tetrads. For the N2 strain, total 4 spores were obtained from two tetrads. In N5 and N6, 4 spores and 2 spores were obtained. After confirming ploidy, it was confirmed that all spore cells were haploid. SPC-SNU 70-1 haploids obtained 8 a type and 8

alpha type haploids. In N2, one a type and two alpha type haploids were obtained. In the N5, two a and two alpha type, and in the N6, two a type haploids were obtained.

2.3. Selection by barcode PCR

The characteristics of haploids obtained from SPC-SNU 70-1 were examined by the barcode PCR. As a result, all four haploids isolated from one tetrad showed different band patterns. The 320 bp bands representing *S. cerevisiae* were all present. One showed only the 774 bp band, and another showed all three bands. Two cells showed two bands, 774 and 649 bp, and 774 and 526 bp, respectively. This may be due to the fact that the sequences of the chromosome amplified by the barcode is different in the diploid, and divided into different combinations when the spore is separated. All four haploids are considered tetratype with different genotypes, and thus the phenotype would be different. The pattern shows that the chromosome 11 with the 774 bp band will have the same sequences, and the chromosomes 4 and 7 with the 649 bp and 526 bp bands are expected to have different sequences.

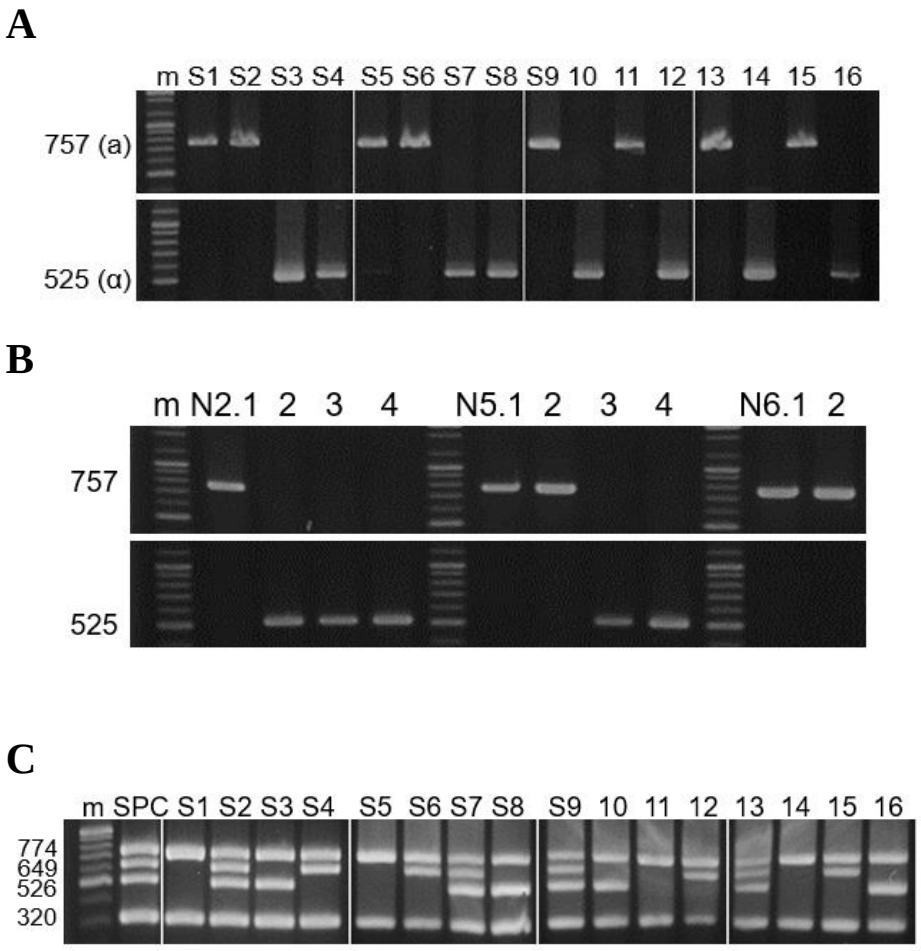


Figure 9. Results of MAT locus PCR and barcode PCR. (A) MAT locus PCR of SPC-SNU 70-1 haploids, (B) MAT locus PCR of N2, N5 and N6 haploids, (C) barcode PCR of SPC-SNU 70-1 haploids. N2, N5 and N6 haploids have no barcode bands.

2.4. Selection by leavening ability

The amount of CO_2 production of each haploid was measured in sweet LD medium. Among them, the amount of CO_2 production was compared in order to select the haploids for mating. Each haploid had higher or lower amount of CO_2 production than the parent strains. If two haploids form a diploid, this diploid is assumed to be the mean value of the haploids. Therefore, it is expected that the CO_2 generation power will be improved by mating two haploids having high fermentation ability.

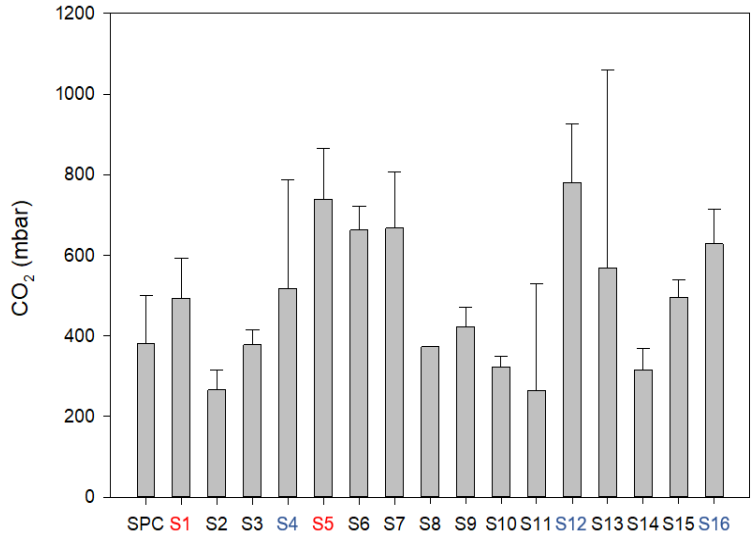
When choosing haploid, mating type and leavening ability were considered. In SPC-SNU 70-1, S1, S4, S5, S12 and S16 strains were selected. In the case of N2 strain, a cell N2.1, alpha cell N2.2 were selected. In N5 and N6, N5.1 and N6.2 was selected.

As a result, S1, S5, N2.1, N5.1, and N6.2 were the a type cells and S4, S12, S16, and N2.2 were the alpha type cells. Those nine haploids were combined to perform mating.

However, even if haploid has better leavening ability than parent strain, it is unsuitable in industry. This is because when the strain is cultured in a large volume with a fermenter, the yield of the cell is only half as high as that of the diploid, and the efficiency is lowered. It is

because the diploid is ellipsoid-shaped with a diameter of 5-6um, while the haploid is more spherical with a diameter of 4um (Sherman 2002). For most environments and generation times the mean cell volume of diploid cells was between 1.52 and 1.83 of the haploid cell volume (Adams 1977).

A



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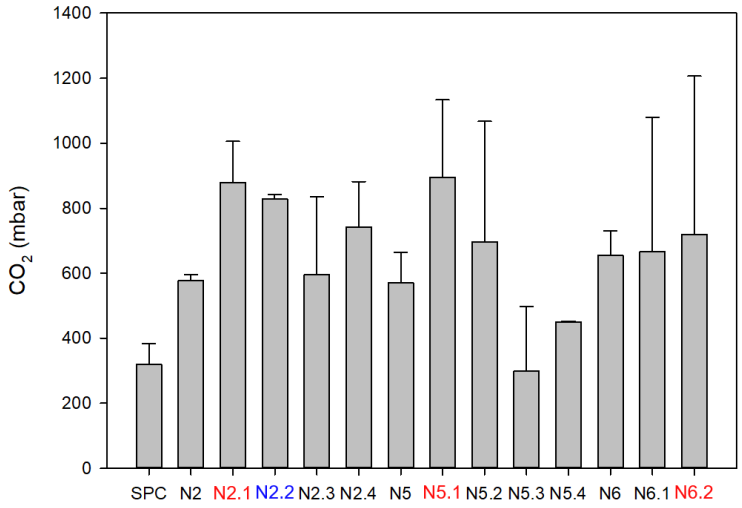


Figure 10. Average of leavening ability in sweet LD medium. (A) Result of SPC-SNU 70-1 haploids, (B) N2, N5 and N6 haploids. Red means selected a type, blue means selected α type haploids.

3. Mating

3.1. Determination of ploidy

10 strains were obtained by mating S4 strain with S5 strain that isolated from SPC-SNU 70-1. When confirmed by the *MAT* locus PCR, diploid cells were observed in five strains, MSPC 1, 3, 4, 5 and 6. MSPC2, 7, and 8 strains showed only one PCR band indicating the alpha type, MSPC9 and 10 strains showed only one PCR band indicating the a type. So it was judged to be a haploid that was not mating properly.

16 strains were obtained by mating the SPC-SNU 70-1 with the N2 strain. The strains identified as diploid by the *MAT* locus PCR were SN2.2, 3, 5, 7, 12, 13, 14, 15, and 16. SN2.8 strains did not show any band, and the remaining 6 strains were identified as haploids. Twelve colonies were obtained from SPC-SNU 70-1 strain and N5, SPC-SNU 70-1 and N6 strain each, and all colonies were diploid.

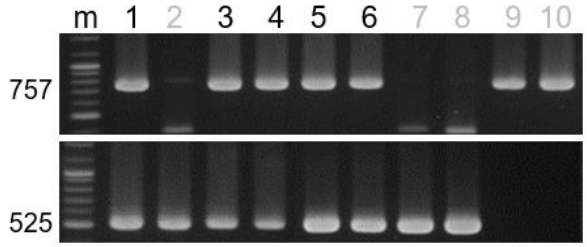
Because the probability of successful mating with the N2 strain was 56%, mating was retried using the second method. In the first mating, strains were not obtained from mating S5 with N2.2 and mating S4 with N2.1. In addition, mating S1 with N2.2, which are good

combinations, were also retried. As a result of second mating, all 12 colonies were diploid.

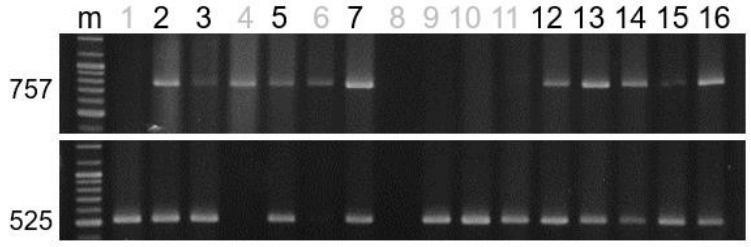
Table 5. List of combination of haploids for mating

a type	α type	a type	α type
S5	S4	N5.1	S4
S1	N2.2		S12
S5			S16
N2.1	S4	N6.2	S4
	S12		S12
	S16		S16

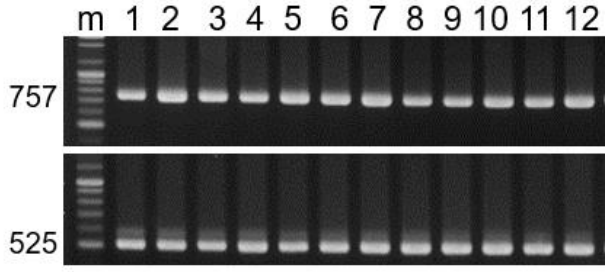
A


m 1 2 3 4 5 6 7 8 9 10
757
525

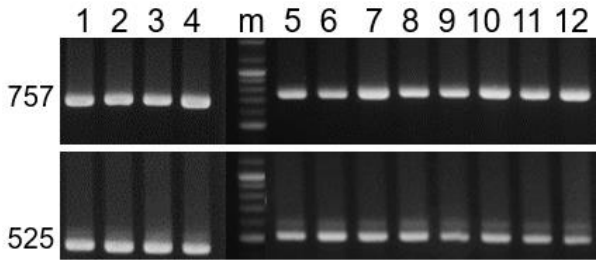
B


m 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
757
525

C


m 1 2 3 4 5 6 7 8 9 10 11 12
757
525

D



E

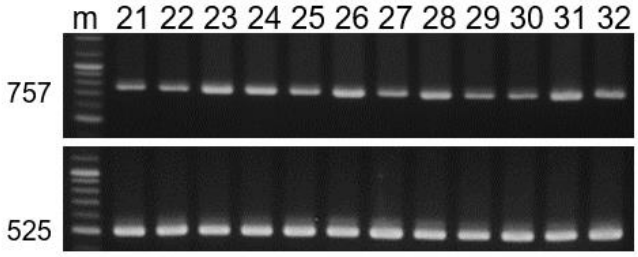


Figure 11. Result of mating by *MAT* locus PCR. (A) MSPC strains, (B) SN2 strains, mating from stock cells, (C) SN5 strains, (D) SN6 strains and (E) secondary SN2 stains, mating from colonies.

3.2. Selection by barcode PCR

Among the diploid cells identified by the *MAT* locus PCR, mating strains were selected by the barcode PCR pattern same as SPC-SNU 70-1 spore. Reference 320 bp band always appear because all strains are *S. cerevisiae.*

First, in the case of MSPC strains, two SPC-SNU 70-1 haploids were mated, so the band patterns of two haploids were considered. The PCR pattern of S5 strain was only 774 bp band, and the pattern of S4 strain was 774 bp and 649 bp bands. Therefore, MSPC strains should have 774 bp, 649 bp bands. Results of MSCP1, 3, 4 strains satisfied this qualification.

Total 21 of SN2 strains were obtained. The PCR pattern of S1 and S5 and mating strains was 774 bp band. The PCR pattern of S4 and S12 and mating strains was 774bp, 649 bp bands. In addition, the PCR pattern of S16 and mating strains have 774 bp, 526 bp bands. As a result, SN2.2, 3, 12, 13, 14, 15, 16 and SN2.21 to SN2.32 strains were suitable.

Finally, SN5 and SN6 strains included S4, S12 and S16 strain. In result, just two strains, SN5.1 and SN5.8, not satisfied this barcode pattern. So 10 strains of NS5, 12 strains of SN6 were obtained.

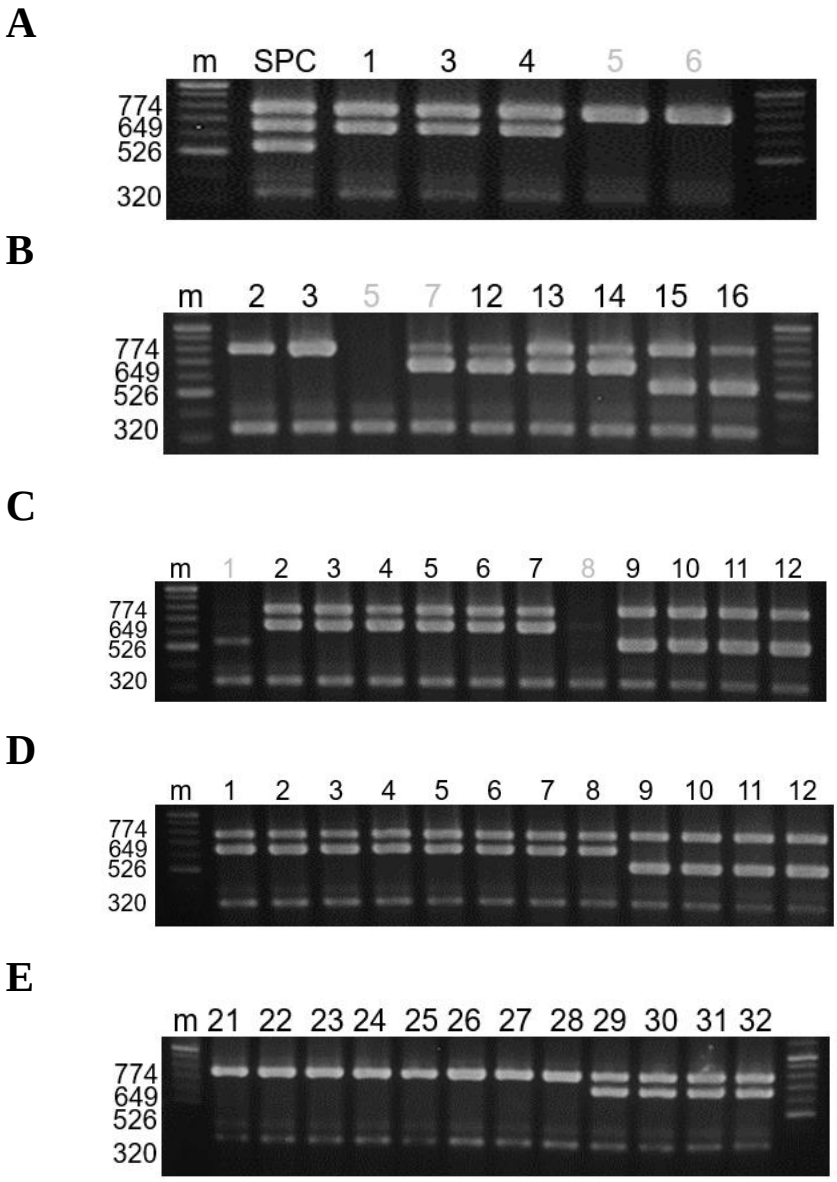


Figure 12. Result of mating by barcode PCR. (A) MSPC strains, (B) SN2 strains, (C) SN5 strains, (D) SN6 strains and (E) secondary SN2 stains.

3.3. Selection by leavening ability

All MSPC strains were higher than the parent strains. However, those strains have low leavening ability when compared with the other strains. Other strains showed basically 600 mbar or more, whereas MSPC strains showed about 500 mbar. As a result, the MSPC strain is not suitable for industrial use.

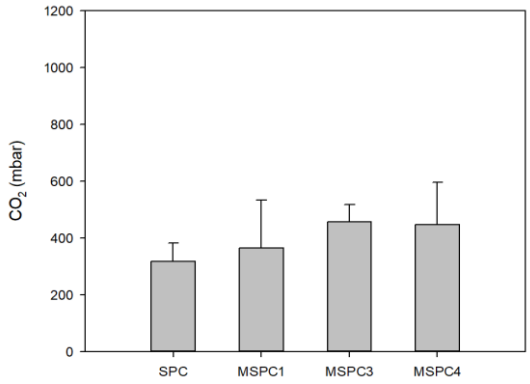
In case of SN2 strains, mating was proceeded 2 times. At first mating, SN2.3, 12, 13, 14, 16, total 7 mating strains showed better leavening ability than the parent strain. Second mating, among 12 mating strains, 8 strains were found to be superior to the parent strains. However, there are a lot of strains, so each strain selection was considerd with combination of haploids. Therefore, SN2.24 strain that mating S1 with N2.2, SN2.25 strain that mating S5 with N2.2, and SN2.31 strain that mating N2.1 with S4 strain were selected.

Eight SN5 strains, and four SN6 strains were excellent in gas generation. SN5 was also selected in the same way because there were a lot of superior strains. SN5.9, 10, and 11, which are exceptionally good in generating gas, were all selected. Therefore SN5.4 strain was selected in the combination of N5.1 and S4 strain, SN5.6 strain was selected among mating strains of S12. In SN6, all four strains with

good leavening ability were selected. Selected SN6.2, 3, 4 in the combination of N6.2 and S4 and SN6.12 in the combination of N6.2 and S16.

Mating combinations and selected strains are listed in Table 5. Of the total 62 strains, 18 strains were not mated and 17 strains were selected by leavening ability. The selected 17 mating strains, are improved SPC-SNU 70-1 strains, are renamed S-HW1 to S-HW17. Table 6 shows the list of new name.

A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CO2 (mbar)
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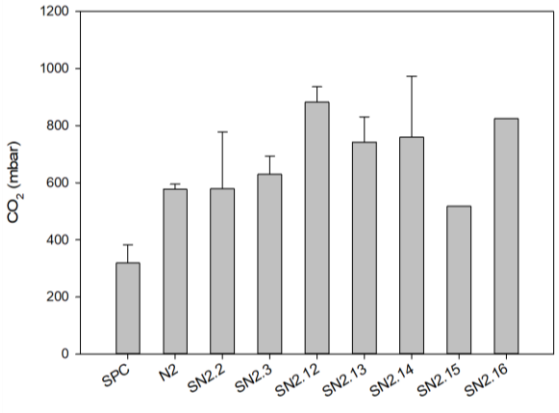
MSPC1
MSPC3
MSPC4

B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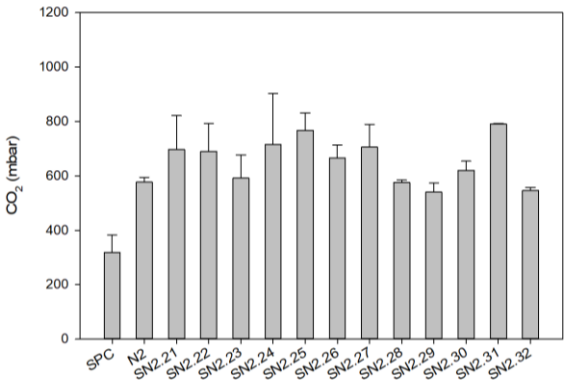
SN2.15
SN2.16

C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SN2.21
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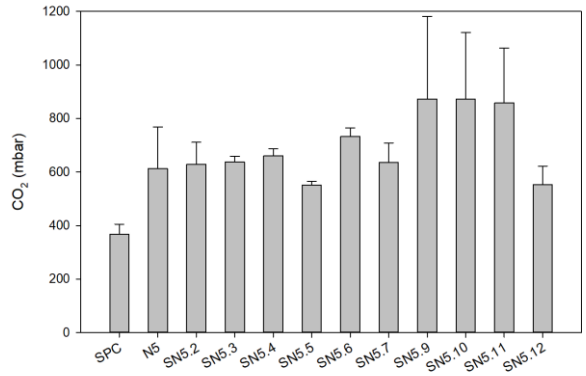
SN2.31
SN2.32

D



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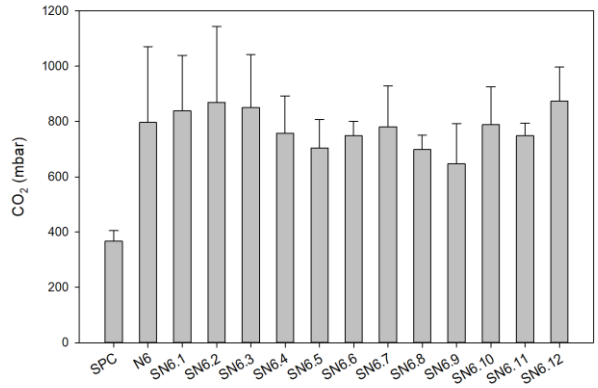


Figure 13. Result of mating by measuring leavening ability. (A) MSPC strains, (B) SN2 strains, (C) SN5 strains, (D) SN6 strains and (E) secondary SN2 stains.

Table 6. List of mating cells, selected cells are highlighted by green

a type	α type	Mating cell
S5	S4	MSPC1
		MSPC2
		MSPC3
		MSPC4
		MSPC5
		MSPC6
		MSPC7
		MSPC8
		MSPC9
		MSPC10
S1	N2.2	SN2.1
		SN2.2
		SN2.3
		SN2.4
		SN2.21
		SN2.22
		SN2.23
		SN2.24
S5		SN2.5
		SN2.6
		SN2.7
		SN2.8
		SN2.25
		SN2.26
		SN2.27
		SN2.28
N2.1	S4	SN2.9
		SN2.10
		SN2.29
		SN2.30
		SN2.31
		SN2.32
N2.1	S12	SN2.11
		SN2.12
		SN2.13
		SN2.14
	S16	SN2.15
		SN2.16
N5.1	S4	SN5.1
		SN5.2
		SN5.3
		SN5.4
	S12	SN5.5
		SN5.6
		SN5.7
		SN5.8
		SN5.9
	S16	SN5.10
		SN5.11
		SN5.12
N6.2	S4	SN6.1
		SN6.2
		SN6.3
		SN6.4
	S12	SN6.5
		SN6.6
		SN6.7
		SN6.8
	S16	SN6.9
		SN6.10
		SN6.11
		SN6.12

Table 7. List of selected mating cells and renamed those cells

Selected cells	New name	Selected cells	New name
SN2.3	S-HW1	SN5.4	S-HW9
SN2.12	S-HW2	SN5.6	S-HW10
SN2.13	S-HW3	SN5.9	S-HW11
SN2.14	S-HW4	SN5.10	S-HW12
SN2.16	S-HW5	SN5.11	S-HW13
SN2.24	S-HW6	SN6.2	S-HW14
SN2.25	S-HW7	SN6.3	S-HW15
SN2.31	S-HW8	SN6.4	S-HW16
		SN6.12	S-HW17

4. Application to baking

4.1. Analysis of dough fermentation characteristics

The 17 strains selected by comparing the gas generating ability in LD medium were tested to check the applicability in the bread industry by inoculating to the actual bread dough. Each strain was inoculated into lean, regular and sweet doughs. Comparison was made with the corresponding parent strains, SPC-SNU 70-1 and N2, N5, N6.

First, the results of SPC-SNU 70-1 strain, 157.8 ml of gas production in lean dough, 251.3 ml of gas production in regular dough, and 160.7 ml of gas in sweet dough for 10 hours.

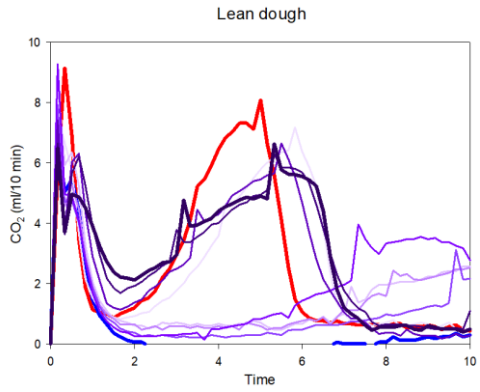
N2 strain produced CO_2 gas 27.8 ml in lean dough, 249.2 ml in regular dough and 231.0 ml in sweet dough. It means N2 strain has no fermentation activity in lean dough. But among SN2 strains, in lean dough, the amount of CO_2 production of S-HW8 strain was 178.2 ml. This result shows the advantage of SPC-SNU 70-1 strain is maintained. And the amount of CO_2 production of S-HW8 strain was 321.5 ml in regular dough. In Sweet dough, the amount of CO_2 production of S-HW2 strain was 284.9 ml. Those results show only the maximum value among SN2 strains. In conclusion, S-HW2 strain and S-HW8 strain are

excellent improved strains.

N5 strain produced 28.1 ml, 267.7 ml, 297.9 ml of CO_2 gas in lean, regular and sweet doughs. Among the SN5 strains, the amount of CO_2 production of S-HW11 strain was 164.3 ml in lean dough, 307.5 ml in regular dough. And the amount of CO_2 production of S-HW9 strain was 309.9 ml in Sweet dough. Those results also show the maximum value among SN5 strains. In SN5, the industrialization potential of S-HW9 and S-HW11 was highly evaluated.

N6 strain produced CO_2 gas 60.0 ml in lean dough, 247.9 ml in regular dough, 235.7 ml in sweet dough. In SN6, the amount of CO_2 production of S-HW17 strain was 171.0 ml in lean dough, 287.4 ml in sweet dough. The amount of CO_2 production of S-HW16 strain was 328.6 ml in regular dough. Therefore, S-HW16 and S-HW17 are superior strains.

A



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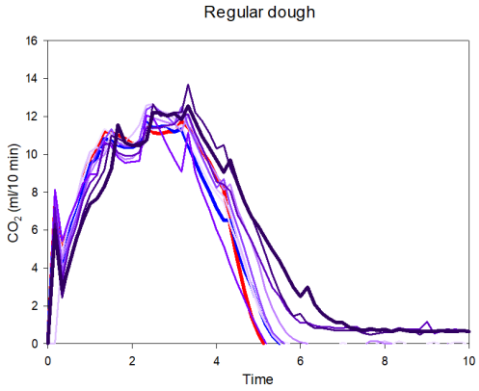

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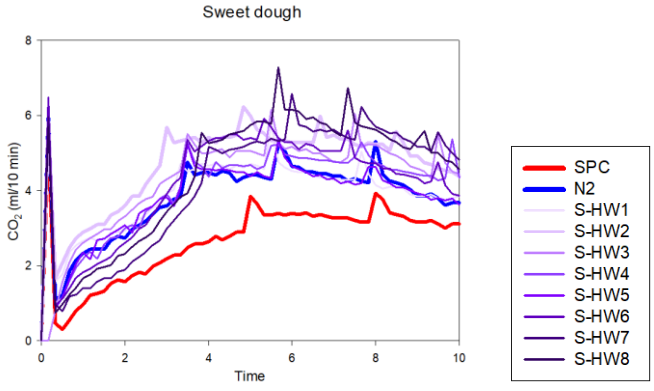
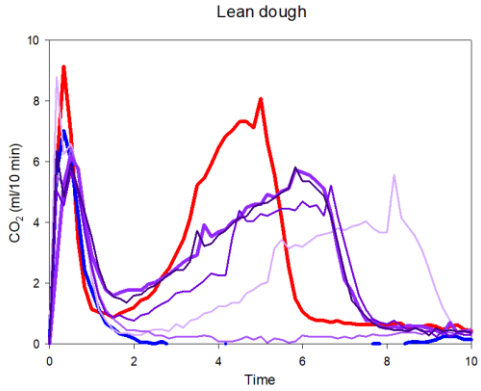


Figure 14. CO_2 production of SN2 strains under (A) lean dough, (B) regular dough and (C) sweet dough.

Table 8. The amount of CO_2 production (ml) of SN2 strain for 10 hours

	SPC	**N2**	**S-HW1**	**S-HW2**	**S-HW3**	**S-HW4**	**S-HW5**	**S-HW6**	**S-HW7**	**S-HW8**
Lean dough	157.8	27.75	158.1	95.06	86.65	64.05	115.5	159.0	177.9	178.2
Regular dough	251.3	249.2	263.1	262	275.6	262.2	203	301.2	316.0	321.5
Sweet dough	160.7	231	238.1	284.9	257.4	247.2	232.9	254	255.1	269.7

A



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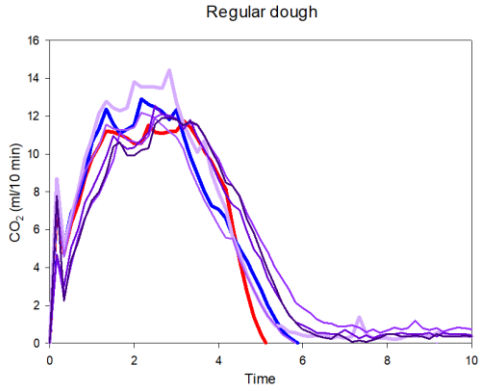

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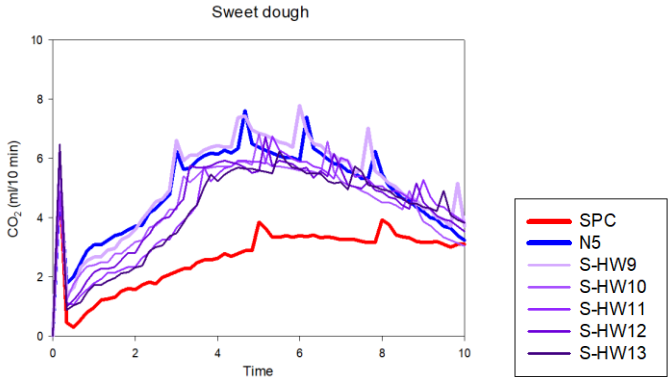
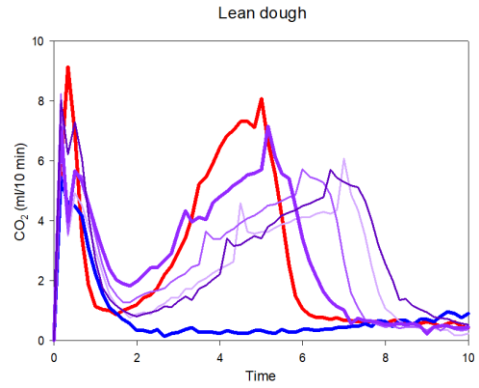


Figure 15. CO_2 production of SN5 strains under (A) lean dough, (B) regular dough and (C) sweet dough.

Table 9. The amount of CO_2 production (ml) of SN5 strain for 10 hours

	SPC	**N5**	**S-HW9**	**S-HW10**	**S-HW11**	**S-HW12**	**S-HW13**
Lean dough	157.8	28.1	144.6	47.3	164.3	139.7	163.4
Regular dough	251.3	267.7	307.1	253.4	307.5	289.3	286.9
Sweet dough	160.7	297.9	309.9	268.5	265.4	270.7	255.7

A



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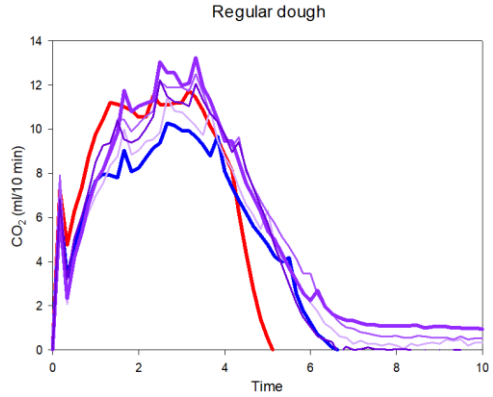

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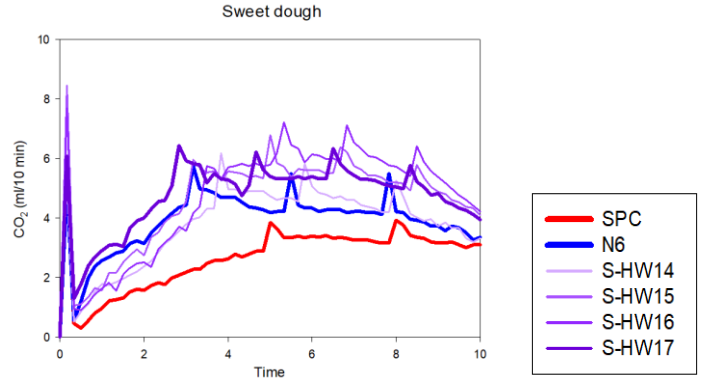

Figure 16. CO_2 production of SN6 strains under (A) lean dough, (B) regular dough and (C) sweet dough.

Table 10. The amount of CO_2 production (ml) of SN6 strain for 10 hours

	SPC	**N6**	**S-HW14**	**S-HW15**	**S-HW16**	**S-HW17**
Lean dough	157.83	50.96	147.62	159.98	169.78	170.98
Regular dough	251.34	247.92	275.65	322.67	328.59	290.05
Sweet dough	160.67	235.71	229.49	280.59	285.61	287.42

4.2. Analysis of fragrance components in dough fermentation

The fragrance components are mainly analyzed for alcohols, esters, aldehydes and ketones. Among them, alcohols are known to give a fermentation odor, esters have a sweet flavor, and ketones have a buttery flavor. However, it should be considered that aroma is complex and difficult to judge accurately because it depends on individual preferences. In this study, the alcohols include ethanol, isobutanol, isoamylalcohol, and 2-phenylethanol, the esters include ethyl acetate, isoamyl acetate, and ethyl octanoate, and the ketones include acetoin.

In case of SPC-SNU 70-1, a total amount of the fragrance component was small, because the fermentation does not occur well in sweet dough. On the other hand, other parent strains, N2, N5, N6, have more of total amount of fragrance components.

Among the mating strains, S-HW2, S-HW9 and S-HW16 strains were superior. Compared with SPC-SNU 70-1, alcohols content of S-HW2 strain increased by 1.8-folds, but esters and acetoin increased by 6.7 and 5.1-folds. Next, the S-HW9 strain increased by 3.5, 9.9 and 2.8-folds for the alcohols, esters and acetoin, and the S-HW16 strain

increased by 3.4, 10.6 and 2.1-folds, respectively.

Finally, S-HW16 strain was selected by considering the leavening ability and fragrance components at the same time. The S-HW16 strain had the highest industrial availability. The amount of CO_2 production of S-HW16 was 1.1-folds, 1.3-folds and 1.8-folds higher than the amount of CO_2 production of SPC-SNU 70-1 in lean, regular and sweet doughs. The amount of fragrance components of S-HW16 was also increased. In addition, lean dough activity and initial gas generation power, which are important in the bread industry, were also increased.

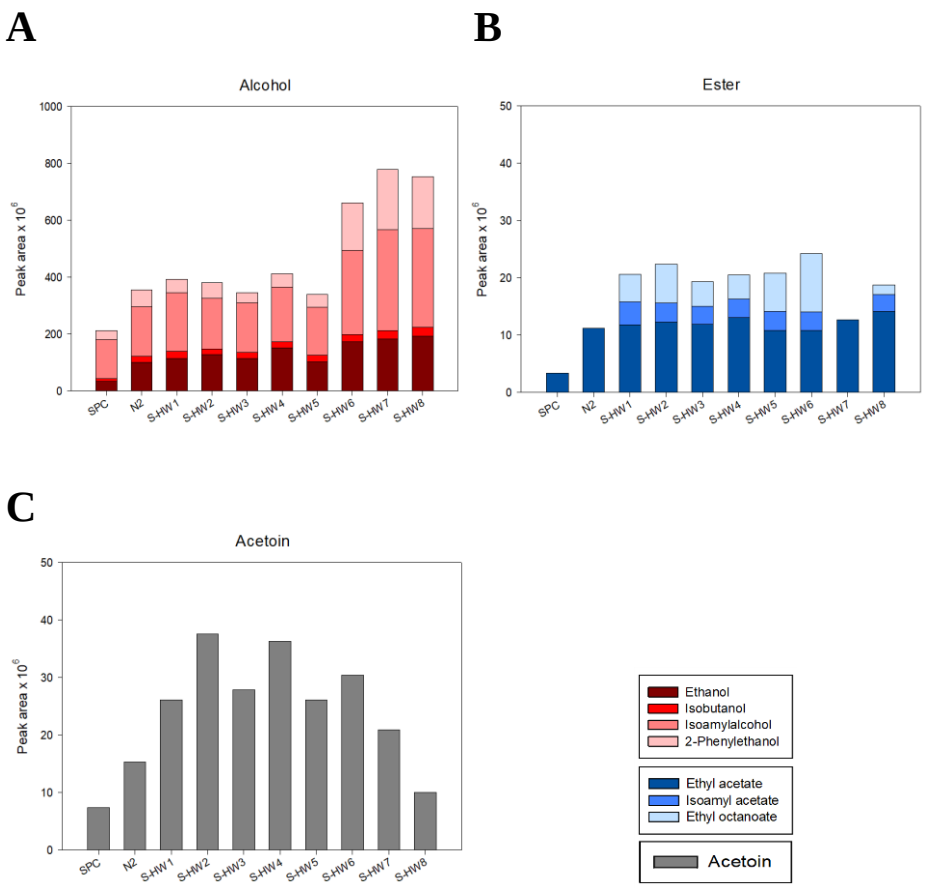

Figure 17. Results of fragrance components analysis of SN2 strains in sweet dough. Amounts of (A) alcohols, (B) esters and (C) acetoin.

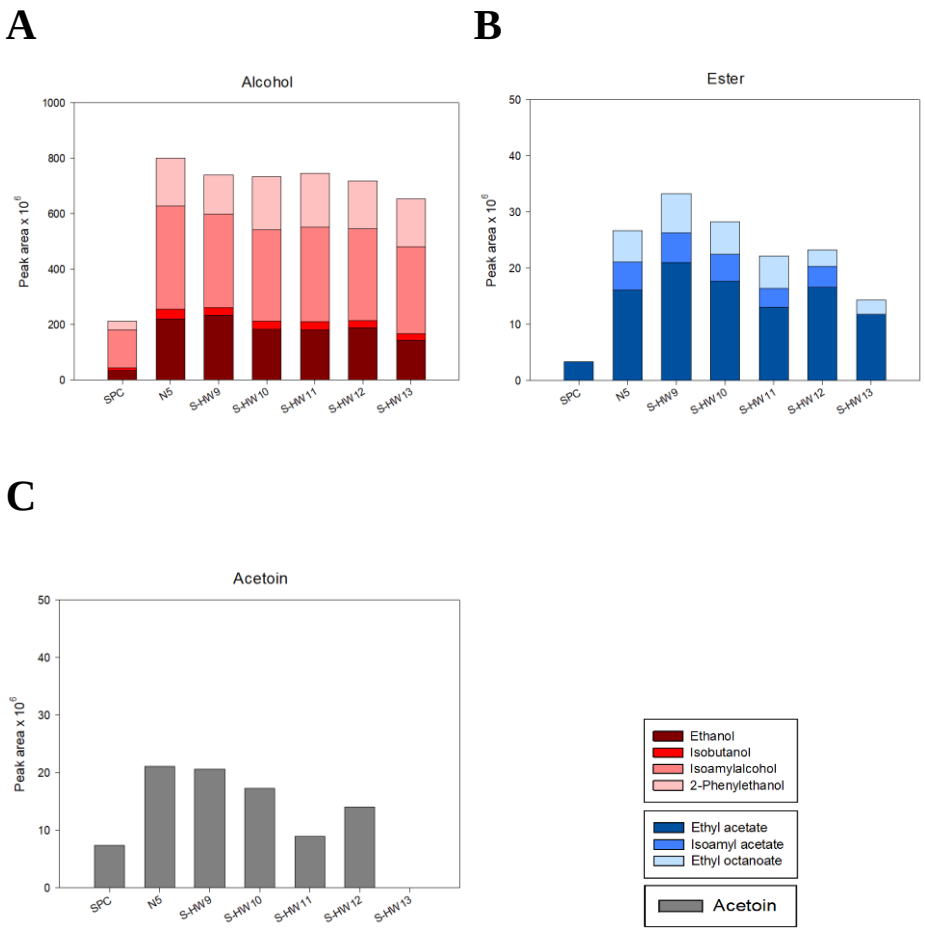

Figure 18. Results of fragrance components analysis of SN5 strains in sweet dough. Amounts of (A) alcohols, (B) esters and (C) acetoin.

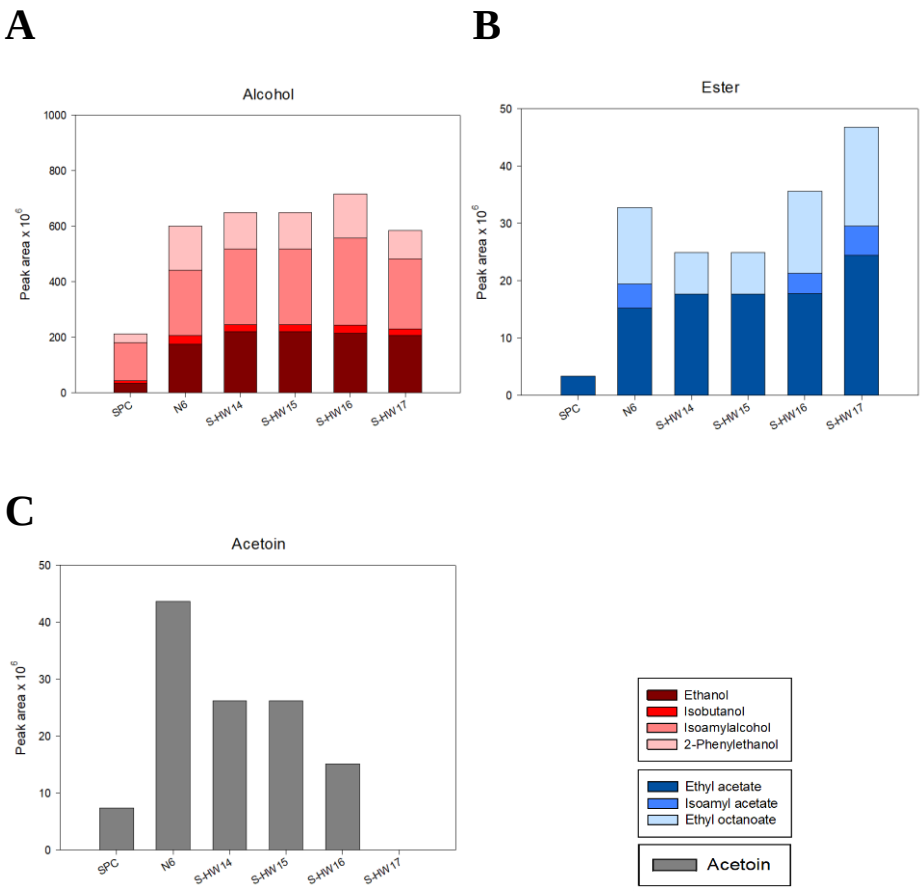

Figure 19. Results of fragrance components analysis of SN6 strains in sweet dough. Amounts of (A) alcohols, (B) esters and (C) acetoin.

IV. CONCLUSIONS

This thesis can draw the following conclusions.

(1) Eight new *S. cerevisiae* strains resistant to high sucrose concentration were isolated from *Nuruk* and grapes.

(2) Total 17 of S-HW strains are obtained by mating that are confirmed by ploidy.

(3) The S-HW strains improve tolerance to high sucrose concentration and show maximum of 1.9-folds increased gas production in sweet dough while maintaining the inherent fermentation characteristics.

(4) S-HW16, mating SPC-SNU 70-1 with N6 strain, show the best result that increases CO_2 production 1.1-folds in lean dough, 1.3-folds in regular dough and 1.8-folds in sweet dough.

(5) The mating method is effective for improving the baking ability of industrial yeast strains.

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국 문 초 록

제빵과정에서 발효의 스타터로 사용되는 효모는 빵의 품질을 결정하는 주요 인자이다. 본 연구에서 사용한 *Saccharomyces cerevisiae* SPC-SNU 70-1은 누룩으로부터 분리한 신규의 효모로서, 발효취가 적고 담백한 풍미와 더불어 부드러운 식감을 내는 등 제빵 특성이 우수하다. 그러나 반죽에 설탕함량이 적은 당 0%, 당 8% 반죽에서는 가스 발생량이 충분하지만, 설탕함량이 많은 당 20% 반죽에서는 그렇지 못하다. 그렇기 때문에 SPC-SNU 70-1 균주의 다양한 활용을 위해 고농도 당 발효 조건에서의 발효능을 개선할 필요가 있다.

제빵용 효모는 빵과 함께 직접 섭취하기 때문에 유전자 조작기술이 아닌 자연적인 균주 개량이 요구된다. 따라서 효모의 특성을 이용한 교배 (mating) 방법을 통해 균주 개량을 시도하였

다. SPC-SNU 70-1 균주와 교배할 신규 균주를 누룩과 포도에서 분리, 동정하였고 총 8종의 당 내성 신규 균주를 얻을 수 있었다. 중합 효소 연쇄 반응을 통해 기존 균주를 포함한 9종의 균주가 모두 이배체 (diploid)임을 확인하였다. 신규 균주 중 당 20% 반죽에서 가스 발생력이 우수한 N1, N2, N5, N6 균주를 교배할 균주로 선택하였지만 N1 균주는 교배의 첫 단계인 포자형성이 불가능하여 제외하였다. SPC-SNU 70-1 균주와 N2, N5, N6 균주의 포자형성 단계와 사분자 분리, 분석 단계를 진행하였다. *HO* 유전자의 서열을 분석하여 위 균주들이 heterothallism임을 확인하였다. 분리된 반수체 (haploid)를 배양, 접종하여 가스 발생량을 측정해 교배에 사용할 반수체를 선별하였다. 교배 후 별도의 선별 표지 (selection marker) 없이 균주가 이배체가 되었다면 교배 성공으로 판단할 수 있다. 교배 균주는 균주를 구분하는 중

합 효소 연쇄 반응과 가스 발생량 측정을 통해 최종적으로 선택되었다.

선택된 균주는 총 17종이며 당 0%, 8%, 20% 반죽에서 가스 발생량을 확인해본 결과 산업화 가능성이 높은 균주는 S-HW2, S-HW8, S-HW9, S-HW11, S-HW16 그리고 S-HW17 균주였다. 그 중에서도 가장 우수한 균주는 S-HW16 이다. 이 균주는 SPC-SNU 70-1에 비해 당 0% 반죽에서 1.1배, 당 8% 반죽에서 1.3배, 당 20% 반죽에서 1.8배 높은 발효력을 보였다. 또한 당 20% 반죽에서 달콤한 향을 주는 에스터류의 양이 10.6배, 버터향을 내는 아세토인의 양이 2.1배 증가하였다. 본 연구는 효모의 교배가 모균주의 특성을 유지하면서 고농도 당에서의 내성이 향상된 개량 균주를 개발하여 산업에서 적용이 가능함을 확인하였다.

주요어 : 제빵 효모, *Saccharomyces cerevisiae*, 산업 균주, 신규 분리, 고당 내성, 가스 발생력, 포자형성, 사분자 분석, 효모 교배, 산업 적용 가능성

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