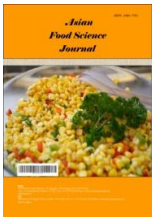

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Isolation and Partial Characterization of 2-Deoxy-D-glucose Resistant *Saccharomyces cerevisiae* Strain from Fruits Harvested in Yamagata, Japan

Takeshi Nagai[1,2,3*], Tomoyuki Nakagawa[4], Norihisa Kai[5], Yasuhiro Tanoue[6] and Nobutaka Suzuki[7]

[1]Graduate School of Agricultural Sciences, Yamagata University, Yamagata 9978555, Japan.
[2]The United Graduate School of Agricultural Sciences, Iwate University, Iwate 0208550, Japan.
[3]Graduate School, Prince of Songkla University, Songkhla 90112, Thailand.
[4]Graduate School of Natural Science and Technology, Gifu University, Gifu 5011193, Japan.
[5]Oita University, Oita 8701192, Japan.
[6]National Fisheries University, Yamaguchi 7596595, Japan.
[7]Nagoya Research Institute, Aichi 4701131, Japan.

Authors' contributions

This work was carried out in collaboration among all authors. Author T. Nagai directed the project, carried out the experiments, analyzed and interpreted the results and wrote the manuscript. Authors T. Nakagawa, NK, YT and NS analyzed and interpreted the results. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study aimed to isolate and characterize 2-deoxy-D-glucose (2-DG) resistant *Saccharomyces cerevisiae* from fruits to establish distinctive bread making technology using wild-type yeasts in the future.
Study Design: The research was conducted experimentally.
Place and Duration of Study: Yamagata University, Yamagata, Japan, from April 2015 to March 2019.
Methodology: Wild-type yeasts with 2-DG resistance were isolated using the following

**Corresponding author: Email: nagatakenagatake@yahoo.co.jp, tnagai@tds1.tr.yamagata-u.ac.jp*

experiments: 1. Separation by yeast nitrogen base-maltose plate medium, 2. Carbon dioxide (CO_2) and ethanol production tests, 3. Leavening ability tests using bread doughs, 4. Sequence analysis. The identified yeast strain was used for freezing and drying torelance tests. Moreover, it tried to improve drying tolerance of yeasts.

Results: Yeasts were separated from twenty varieties of five fruits species. Among them, a yeast strain (YTPR1) isolated from pear *Redbartllet* fruits was identified as *S. cerevisiae*. YTPR1 possessed high fermentation ability and freezing tolerance, however, CO_2 and ethanol production decreased after lyophilization of yeasts. In contrast, the cultivation with trehalose, glycerol, and L-glutamic acid at low concentration enhanced the fermentation ability of YTPR1.

Conclusion: Yeast YTPR1 isolated from pear *Redbartllet* fruits utilized maltose as well as glucose, fructose, and sucrose. To improve drying tolerance of yeast YTPR1, it was useful to incubate with 6% trehalose, 0.1-2.0% glycerol and 1.0% L-glutamic acid.

Keywords: 2-deoxy-D-glucose resistance; freezing and drying resistance; isolation; Saccharomyces cerevisiae.

1. INTRODUCTION

Yeasts for the production of fermented foods, such as breads, wines, and beers, belong to Saccharomycetaceae. Among them, *Saccharomyces cerevisiae* was yeast that was found in nature in the late 19th century and was obtained by pure culture. However, it is known that yeasts as genera *Candida*, *Hanseniaspora*, *Hansenula* and *Pichia* exists in nature, and that these yeasts show the properties differed from *S. cerevisiae*. Generally, dried yeasts (baker's yeasts strain *S. cerevisiae*) are widely used in bread making because of long-term storage ability.

On the other hand, wild-type yeasts inhabit in soil, air, underwater, sap and surface of plants, such as trees, flowers, fruits, leaves, and branches. These yeasts possess the characteristic aroma and assimilative abilities associated with growth environments. Recently, the development of breads using wild-type yeasts is receiving a lot of attention in baking industries. For example, yeasts, such as *Shirakamikodama* yeast [1], *Minowa* yeast [2], *Matebashii* yeast, *Kuchinashi* yeast and *Tokachino* yeast [3], were separated and isolated and were then utilized for bread making. However, most of wild-type yeasts are inferior to commercially available baker's yeasts (commercial dried yeasts) in freezing and drying resistance and in leavening ability of bread dough [2,3].

Sugars, such as glucose, fructose, maltose, and sucrose, need for fermentation of dough in bread making. The amount of sugar added depends on a type of bread. For example, for making sweet bread, a large quantity of sugar (sucrose) approximately 25% (w/w) is added in bread dough. Therefore, yeast cells are exposed to high-osmotic stress condition. In contrast, for making French bread, yeasts need to leaven bread dough using only starch-derived maltose, as sucrose is not added in bread dough. Thus, it is necessary to make high-quality breads to assimilate maltose produced by starch hydrolysis.

Generally, microorganisms repress gene expression encoding enzymes that catabolize sugars, such as maltose and sucrose, in the presence of glucose (glucose repression) [4]. In yeasts, this is a major regulatory mechanism. 2-deoxy-D-glucose (2-DG), non-metabolizable and toxic glucose analogues, is a gratuitous repressor for many kinds of enzymes and transport proteins in yeasts [5]. Thus, mutant strains of yeasts obtained in medium contained 2-DG exhibit repression of maltose uptake in the presence of glucose [6]. It is important to obtain 2-DG resistant mutant strains of yeasts for applications of baking and related industries.

In recent years, frozen-dough technique has been accepted in baking industries for supplying oven-fresh breads and for improving working conditions of bakers. In this case, freeze and subsequent thawing of bread dough causes injury in yeast cells, resulting in lower leavening ability of bread dough. In bread making, yeast cells are exposed to multiple stresses, such as temperature [7-11], freezing [7,12,13], dehydration [12,14,15], desiccation [16,17], hyperosmotic [7,18] and ethanol [19]. Therefore, it is desirable to acquire yeasts with not only freeze tolerance, drying resistance, and osmotic tolerance but also aromatic property and assimilative ability to establish high quality of bread making technology.

Various kinds of fruits are cultivated in Yamagata, Japan, as it is known as a fruit kingdom. Therefore, it is most likely to acquire wild-type yeasts suitable for distinctive bread making. In the present investigation, we aimed to isolate 2-DG resistant *S. cerevisiae* strains from fruits harvested in Yamagata, Japan. Moreover, it was investigated freezing and drying tolerances of yeasts for applications of industrial bread making.

2. MATERIALS AND METHODS

2.1 Samples

Twenty varieties of five fruits species [apple (Crispin, Jonathan, *Orei*, *Orin*, Richard Delicious), cherry (*Jabore*, *Nanyo*, *Napoleon*, Rockport Bigarreau, *Sato Nishiki*, *Seneca*, *Zao Nishiki*), grape (*Aki Queen*, Crimson glory vine, *Kyoho*, *Olympia*), pear (Claude Blanchet, *Silver Bell*, *Redbartllet*) and persimmon (*Hiratanenashi*)] were harvested in the field of Yamagata University, Japan and were used in the study. Commercially available dried yeast (Supercamellia) was obtained from Nissin Foods Inc. (Tokyo, Japan). Commercially available bread flour was purchased from Kobe Bussan Co., Ltd. (Hyogo, Japan). All chemicals were of analytical grade.

2.2 Separation of Yeasts

Whole fruits were grind using a mortar. These were preserved in vial bottles and were pre-cultured at 30°C for 2 days. Broth was inoculated to Yeast Extract Peptone Dextrose (YPD) plate medium (1% yeast extract, 2% polypeptone, 2% D-glucose, and 2% agar) and was cultured at 30°C for 2 days. The colonies obtained were observed using a biological microscope (JTO-1500T, Kenis Ltd., Osaka, Japan) equipped with a digital image processing system (220F-HD, Kenis Ltd., Osaka, Japan). Size of cells was measured using software (Photo Measure Ver.3, Kenis Ltd., Osaka, Japan). Moreover, the yeast-like cells were purified by the inoculation three times with the same methods.

2.3 Isolation of 2-DG Resistant Strains

2-DG resistant strains were isolated as described by Sekiguchi [2] with some modifications. Strains separated were inoculated to Yeast Nitrogen Base-maltose (YNB-M) plate medium (0.67% yeast nitrogen base without amino acids, 2% maltose, and 2% agar) and was then incubated at 30°C for 6 days. The colonies obtained were continuously inoculated to the same plate medium containing 0.02% 2-DG and were then incubated at 30°C for 6 days. Next, the colonies obtained were inoculated to the same plate medium containing 0.04% 2-DG and were incubated under the same condition. Finally, the colonies obtained were inoculated to the same plate medium containing 0.06% 2-DG and were incubated under the same condition. The strains obtained were used as 2-DG resistant stains.

2.4 Carbon Dioxide (CO_2) Production Tests

The CO_2 production tests using Durham's tube was performed as described by Kinoshita and Tanaka [20]. 2-DG resistant strains were inoculated to 5.0 ml of Yeast Extract Malt Peptone (YM) liquid culture medium (0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, and 1% D-glucose) and were then incubated at 30°C for 24 h. A 5.0 ml of 5% sucrose solution was added in test tubes and sterilized Durham's tube set reverse in these tubes. After cooling, a 1.0 ml of yeast inoculate was added in test tubes and was then incubated at 30°C. Gas quantities produced were observed every 12 h for 3 days and were evaluated with comparison to that in commercially available dried yeast. CO_2 productivity was expressed as relative values to that of commercially available dried yeast when reached its stationary growth period. In addition, the same cell concentration of yeast inoculate was used in the experiments.

2.5 Ethanol Production Tests

The strains with high CO_2 production were used for ethanol production tests. Yeast inoculate was prepared by pre-incubation at 30°C for 24 h in YPD liquid culture medium. A 1.0 ml of yeast inoculate was added to 5.0 ml of 5% sterilized each molasses (glucose, fructose, maltose, and sucrose) and was then incubated at 30°C for 7 days. In comparison, it was performed in commercially available dried yeast in the same manner. Ethanol production was investigated with passage of the days using a F-kit ethanol (J.K. International Inc., Tokyo, Japan). Ethanol productivity was expressed as relative values to that of commercially available dried yeast when reached its stationary growth period.

2.6 Leavening Ability Tests Using Bread Doughs

The leavening ability tests of bread doughs were performed as described by the Japan Yeast Industry Association [21] with a slight modification. Three conditions were set for addition of sugar in bread doughs. The formulas of doughs were made as follows: French dough [100 g of bread flour, 4 g of yeast, 2 g of salt and an optimum amount of water (70 g)]; white dough [100 g of bread flour, 4 g of yeast, 2 g of salt, 5 g of sucrose, and water (70 g)]; sweet dough [100 g of bread flour, 4 g of yeast, 0.5 g of salt, 30 g of sucrose, and water (60 g)], respectively. Doughs were prepared using an automatic bread machine (menu: natural yeast dough) (SD-BMS106, Panasonic Corp., Osaka, Japan). After bread doughs obtained was rolled into a cylinder and were fermented at 30°C for 5 h in an incubator (CN-25C, Mitsubishi Electric Engineering Co., Ltd., Tokyo, Japan), the volumes of doughs were determined hourly. Yeasts were incubated with gentle shaking at 120 rpm (2 Hz) at 30°C for 48 h in YPD liquid culture medium and was then centrifuged at 8,000 rpm (9,876 x g) at 20°C for 10 min. Yeasts were washed with sterilized saline. After these were centrifuged under the same condition, yeasts were washed in the same manner. Finally, these treatments were repeated again. Yeasts (the moisture content: approximately 80%) obtained were used for making bread. As a control, test was similarly performed using commercially available dried yeast.

2.7 Sequence Analysis

Using a forward primer (5'-ACCCGCTGAACTTAAGC-3') and a reverse primer (5'-TACTACCACCAAGATCT-3'), 28S rRNA gene of strain was amplified by polymerase chain reaction (PCR). After fragment was sequenced using a sequencer (ABI PRISM 377, Applied Biosystems, USA), sequence obtained was used for homology search study using Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Rockville Pike, USA) to identify as *S. cerevisiae*.

2.8 Freezing and Drying Tolerance Tests

The strains sub-cultured in YPD plate medium were incubated with shaking at 120 rpm (2 Hz) at 30°C for 48 h in YPD liquid culture medium and were then centrifuged at 8,000 rpm (9,876 x g) at 20°C for 10 min. Yeasts were washed with sterilized saline. After these were centrifuged at the same condition, yeasts were washed in the same manner. Finally, these treatments were repeated again. Yeasts (moisture content: approximately 80%) obtained were quickly frozen and were stored at -28°C for 360 days. In contrast, yeasts obtained by centrifugation as described above were lyophilized using a freeze dryer (FDU-12AS, AS ONE Corp., Osaka, Japan). After vacuum packaging in polyethylene bags, these were stored at -20, 4, 15, and 30°C for 30 days. CO_2 and ethanol production were observed as freezing and drying tolerance of cells after storage.

2.9 Statistical Analysis

Each assay was repeated 3 times independently and the mean was reported.

3. RESULTS AND DISCUSSION

3.1 Isolation of Yeasts

The stains of wild-type yeasts were separated from twenty varieties of five species of fruits. 266 strains obtained were separated using a biological microscope. As a result, 200 strains were obtained as *S. cerevisiae*. Moreover, 90 strains were separated in medium containing 0.06% 2-DG. Next, the CO_2 production tests of these strains were performed to select strains with high gas productivity. 55 strains were used for ethanol production tests. As a result, 7 stains were selected from two kinds of fruits [apples (Jonathan and Richard Delicious) and pears (Claude Blanchet and *Redbartllet*)]. These yeasts had globular, elliptical and cylindrical shapes.

3.2 Leavening Abilities in Bread Doughs

The leavening abilities in bread doughs were observed under the conditions for making French breads, white breads, and sweet breads. In each condition, volume of bread dough prepared by commercially available dried yeast linearly increased to approximately 220-265 ml for 5 days (Table 1). In making French bread condition, volumes of doughs prepared by 7 strains increased very slowly for 5 days. Among them, volume of dough prepared by a strain isolated from pear *Redbartllet* increased approximately 70 ml after 5 days, and volumes of doughs prepared by other strains were low in decreasing order: apple (Jonathan), pear (Claude Blanchet),

and apple (Richard Delicious). In condition making white breads, volume of dough prepared by each strain linearly increased for 5 days as follows: pear *Redbartllet* (200 ml), pear Claude Blanchet (115-175 ml), apple Jonathan (100 ml), and apple Richard Delicious (75 ml) (Table 1). In contrast, in condition making sweet breads, volumes of doughs prepared by strains isolated from pears (Claude Blanchet and *Redbartllet*) slightly increased for 5 days, but doughs prepared by other strains were not leavened for 5 days at all (Table 1).

Table 1. Leavening abilities of bread doughs for making French breads, white breads, and sweet breads prepared using the isolated strains

Strain	Days					
	0	1	2	3	4	5
For making French breads						
Pear (Claude Blanchet)-1	0.0	0.0	15.0	20.0	30.0	30.0
Pear (Claude Blanchet)-2	0.0	0.0	0.0	0.0	5.0	5.0
Pear (Claude Blanchet)-3	0.0	0.0	10.0	10.0	15.0	20.0
Pear (*Redbartllet*)-1	0.0	0.0	0.0	0.0	0.0	5.0
Pear (*Redbartllet*)-2	0.0	20.0	40.0	50.0	65.0	70.0
Apple (Jonathan)	0.0	0.0	10.0	20.0	25.0	40.0
Apple (Richard Delicious)	0.0	0.0	0.0	5.0	5.0	10.0
Commercially available dried yeast	0.0	45.0	85.0	145.0	190.0	220.0
For making white breads						
Pear (Claude Blanchet)-1	0.0	10.0	60.0	90.0	125.0	175.0
Pear (Claude Blanchet)-2	0.0	0.0	35.0	60.0	85.0	115.0
Pear (Claude Blanchet)-3	0.0	5.0	45.0	70.0	100.0	130.0
Pear (*Redbartllet*)-1	0.0	0.0	15.0	40.0	60.0	70.0
Pear (*Redbartllet*)-2	0.0	55.0	90.0	125.0	160.0	200.0
Apple (Jonathan)	0.0	10.0	25.0	50.0	75.0	100.0
Apple (Richard Delicious)	0.0	0.0	25.0	45.0	50.0	75.0
Commercially available dried yeast	0.0	75.0	135.0	180.0	215.0	265.0
For making sweet breads						
Pear (Claude Blanchet)-1	0.0	0.0	5.0	10.0	15.0	15.0
Pear (Claude Blanchet)-2	0.0	0.0	0.0	0.0	0.0	0.0
Pear (Claude Blanchet)-3	0.0	0.0	0.0	0.0	5.0	5.0
Pear (*Redbartllet*)-1	0.0	0.0	0.0	0.0	0.0	0.0
Pear (*Redbartllet*)-2	0.0	10.0	15.0	15.0	20.0	20.0
Apple (Jonathan)	0.0	0.0	0.0	5.0	5.0	5.0
Apple (Richard Delicious)	0.0	0.0	0.0	0.0	0.0	0.0
Commercially available dried yeast	0.0	40.0	115.0	160.0	210.0	235.0

Abilities were expressed as increased volumes (ml) of the leavened bread doughs.

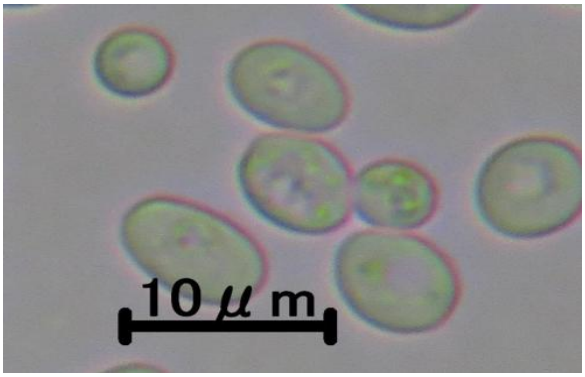

Fig. 1. Micrograph of *Saccharomyces cerevisiae* strain YTPR1 isolated from pear *Redbartllet* fruits

3.3 Morphological Characteristics of Strains

Morphological characteristics of selected strains were observed. All colonies exhibited white color or milky white. Shapes and diameters of these yeasts were as follows: apple Jonathan (spherical and 3-4 μm), apple Richard Delicious (spherical and 3-5 μm), pear Claude Blanchet (spherical and 2-4 μm), and pear *Redbartllet* (spherical or oval and 6-10 μm), respectively. From above tests, a stain from pear *Redbartllet* was finally selected for the following tests.

3.4 Sequence Analysis

To identify a strain as *S. cerevisiae*, nucleotide sequence of amplified fragment was analyzed using custom synthesized primers. As a result, 28S rRNA sequence of a strain isolated from pear *Redbartllet* fruits showed high homology with those of *S. cerevisia*e YJM1078 (99.9%), *S. cerevisiae* YJM993 (99.9%), and *S. cerevisiae* strain NCIM3107 (99.8%), respectively. Thus, a strain from pear *Redbartllet* fruits was identified as *S. cerevisiae*. Sequence was assigned to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank (DDBJ/EMBL/GenBank) Accession Number LC331262. Micrograph of yeast YTPR1 isolated from pear *Redbartllet* fruits is shown in Fig. 1.

3.5 Freezing Tolerance

In general, it is known that yeasts are difficult to store for a long time to decrease freezing and drying tolerance [22]. Yeast YTPR1 (moisture content: approximately 80%) obtained by centrifugation were vacuum-packed and were then stored at -28°C for 360 days to investigate the freezing tolerance. The yeasts were taken out from a freezer after storage and were then used for CO_2 production tests. As a control, it was investigated using commercially available dried yeast. After incubation, rapid CO_2 production (logarithmic growth phase) was observed in commercially available dried yeast, and it reached a stationary state after 24 h. Yeast YTPR1 after frozen storage for 1 and 7 days showed the same pattern as commercially available dried yeast (Fig. 2A). In contrast, yeast YTPR1 after frozen storage for 14 days to 360 days did not produced gas till after incubation for 12 h at all. However, since then it suddenly began to produce gas, and reached stationary states after incubation for 24 h.

Next, yeasts were taken out from a freezer after storage and were then used for ethanol production tests. As a result, it was observed ethanol productivity approximately 90% to stationary growth period after frozen storage for a day in commercially available dried yeast, and then it reached a stationary state after frozen storage for 5 days. In contrast, yeast YTPR1 after frozen storage for 1 and 7 days showed the same ethanol production pattern as commercially available dried yeast (Fig. 2B). Yeasts after frozen storage for 14 days to 360 days drastically produced ethanol the day after start of incubation (ethanol production approximately 80% to stationary state). It reached stationary state after incubation for 5 days in yeast after frozen storage for 14 days to 360 days. In the previous investigations, *S. cerevisiae* AFT [23] and *Torulaspora delbrueckii* D2-4 [24] were separated as high freeze-tolerance baker's yeast. Oda [25] and Oda and Tonomura [26] performed freezing tolerance tests in bread doughs using yeasts *T. pretoriensis* IFO 0022, *T. pretoriensis* YK-1, and a commercially available non-freeze tolerant *S. cerevisiae* FL 2209 after frozen storage at -20°C for 15 days. They reported that fermentative powers on *T. pretoriensis* IFO 0022 and *T. pretoriensis* YK-1 slightly decreased during storage, however, that on *S. cerevisiae* FL 2209 reduced up to approximately 30% after frozen storage for 14 days in comparison to that on non-freeze strain. In contrast, Hahn and Kawai [27] deduced that the strains, which had relatively high freeze-tolerance, belonged to *Torulaspora* spp. From these results, it had become clear that yeast YTPR1 isolated from pear *Redbartllet* fruits possessed an enough fermentability after frozen storage at -28°C for 360 days, suggesting high freezing tolerance. Thus, it was necessary to store yeast YTPR1 at -28°C to maintain high fermentative power for a long time.

3.6 Drying Tolerance

Yeast YTPR1 (moisture content: approximately 80%) was lyophilized. After vacuum packaging, dried yeasts were stored at -20, 4, 15, and 30°C for 30 days to investigate drying tolerance of yeasts. Yeasts were taken out from a freezer or an incubator after storage for 1, 7, 14 and 30 days, and then CO_2 production tests were performed under incubation for 72 h. In comparison, it was investigated using commercially available dried yeast. It showed linear CO_2 productivity in commercially available dried yeast at any storage temperature till 24 h,

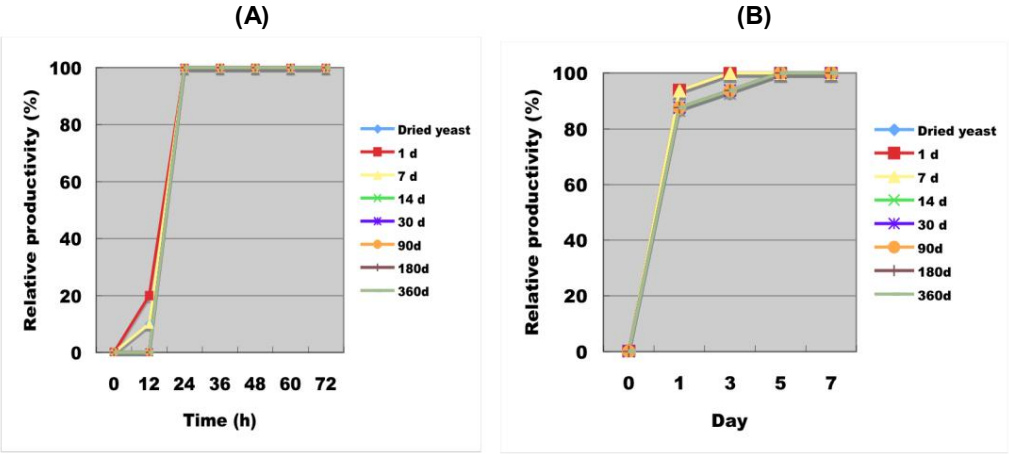

Fig. 2. Carbon dioxide productivity (A) and ethanol productivity (B) of yeast strain YTPR1 after storage at -28°C for 1-360 days

and then its production reached stationary state (Figs. 3A-D). In contrast, it did not observed gas production in yeast YTPR1 under incubation for 12 h regardless of storage temperature. However, CO_2 production increased with increasing incubation time. Moreover, under incubation for 72 h, yeast produced gas approximately 60% to commercially available dried yeast on stationary growth phase (Figs. 3A-D). Gas productivity of yeast YTPR1 decreased with increasing storage days of yeasts. In particular, yeast YTPR1 stored at 30°C for 30 days came stationary period under incubation for 48 h, and its CO_2 content was only approximately 20% to that of commercially available dried yeast (Fig. 3D).

Next, dried yeasts were stored at -20, 4, 15 and 30°C for 30 days, and ethanol production was investigated under incubation for 7 days. As a result, it was observed quick ethanol production in commercially available dried yeast at any storage temperature, and then the production reached stationary states under incubation after 3 days (Figs. 3E-H). In contrast, the production on yeast YTPR1 gradually increased with increasing incubation days at any storage temperature, and the productivity after incubation for 7 days was the range of approximately 19-56% to that in commercially available dried yeast (Figs. 3E-H). Yeasts simultaneously produce CO_2 and ethanol from glucose during fermentation. It was observed CO_2 and ethanol production on yeast YTPR1 at any storage condition. From these results, it became clear that gassing power on yeast YTPR1 remarkably decreased by freeze drying, suggesting that freeze drying treatment was not suitable for storage of yeast for a long time. It is necessary to develop useful techniques for improving drying tolerance of yeasts.

3.7 Improvement of Freezing and Drying Tolerance of Isolated Yeast YTPR1

It is known that freezing tolerance of yeasts is relevant to the concentration of intracellular compounds. Murakami et al. [24] compared the lipid compositions of freeze-tolerant yeast *T. delbrueckii* D2-4 and its freeze-sensitive mutant 60B3. They elucidated that phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol contents in *T. delbrueckii* D2-4 were higher, and triglyceride contents were lower than those in its mutant 60B3. Hirasawa et al. [28] tried to improve freezing tolerance of baker's yeasts by loading with trehalose. As a result, they reported that intracellular trehalose contents of yeasts increased with increasing of soaking period of yeasts. Moreover, they clarified that freezing tolerance of yeasts increased with increasing of intracellular trehalose content. Shi et al. [29] investigated the cell survival rates and the changes of fermentability in bread doughs after frozen storage using six types of baker's yeasts. They revealed the relationship between freezing tolerance of yeasts and the contents of intracellular trehalose, amino acids, and glycerol. Thus, they elucidated that trehalose, glutamic acid, arginine, proline, asparagic acid, and glycerol in yeasts affected freezing tolerance of the yeasts. Sasano et al. [30] reported that proline accumulation in baker's yeasts enhanced sucrose stress tolerances and fermentation

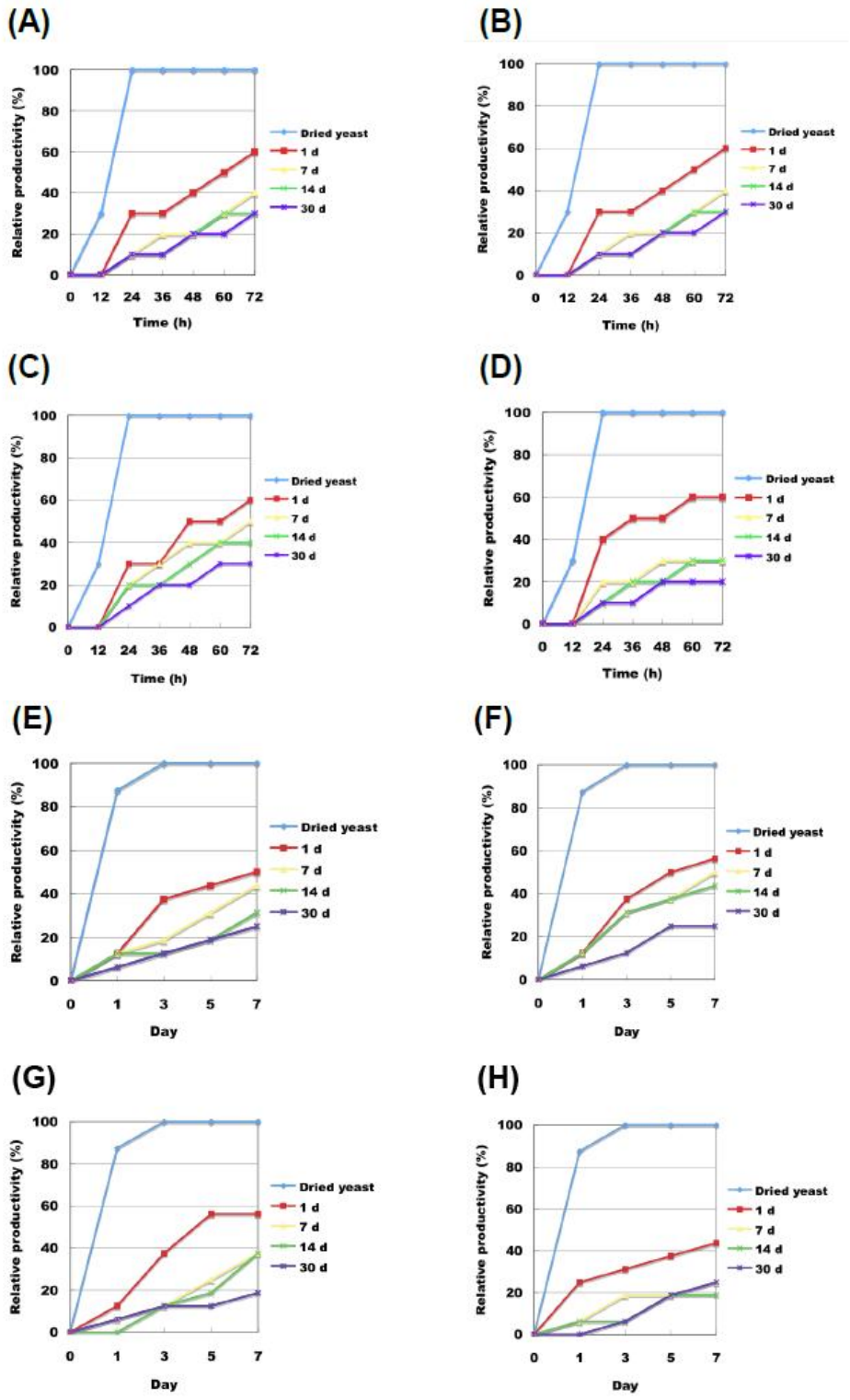

Fig. 3. Carbon dioxide and ethanol productivities of yeast strain YTPR1 after lyophilization and then storage at -20 (A, E), 4 (B, F), 15 (C, G) and 30°C (D, H) for 1, 7, 14 and 30 days. A-D: carbon dioxide productivity, E-H: ethanol productivity

abilities in sweet doughs. They also have shown that simultaneous accumulation of proline and trehalose, which had functions as cryoprotectants in the cells of industrial baker's yeasts, enhanced the fermentation abilities in frozen doughs [31]. Moreover, they clarified that industrial baker's yeasts activated proline and nitric oxide synthetic pathway and then improved fermentation ability under multiple baking-associated stress conditions, such as air-drying and freeze-thaw stresses [32]. Nakagawa and Ouchi [33] tried to improve freezing tolerance of commercial baker's yeasts (non-freeze-tolerant yeasts) in bread doughs. As a result, it found that heat treatment to the fermented doughs at 46°C for 10 min improved freezing tolerance of yeasts, and its treatment was effective for straight method of white dough (for making white breads). Lewis et al. [34] investigated the roles of growth phase and ethanol (0.5 or 2.0 M) and glycerol (2.0 or 5.0 M) at high or low concentrations in freeze-thaw stress resistant *S. cerevisiae*. They reported that ethanol showed significant contribution as one of cryoprotective agent, when yeasts were rapidly frozen at cooling rate approximately 200°C per min. Moreover, they reported that glycerol at low concentration acted as cryosensitizer under step freezing conditions at -20, -70 and -196°C at cooling rate approximately 3°C per min. It seemed that glycerol and trehalose had functions as cryoprotectant [35] and as cryosensitizer of yeast cells under these freezing conditions, respectively.

We tried to improve freezing and drying tolerance on yeast YTPR1 using trehalose, glycerol and L-glutamic acid. The 1-6% trehalose to YPD liquid culture medium was added and then sterilized. The strains sub-cultured were inoculated to the medium and were then cultured at 30°C for 48 h. After centrifugation at 8,000 rpm (9,876 x g) at 20°C for 10 min, yeasts obtained were washed with sterilized saline and then centrifuged under the same condition. Yeasts were washed in the same manner. These treatments were repeated again. Yeasts obtained were then lyophilized. The 0.1-2.0% glycerol and 1.0% L-glutamic acid were treated in the same manner as described above. Lyophilized yeasts were used for CO_2 and ethanol production tests. As a result, yeasts added with 6% trehalose did not produced CO_2 till after incubation for 12 h, however, thereafter suddenly began to produce gas (logarithmic growth phase) and reached stationary state after incubation for 48 h (Table 2). Yeasts added with 3-5% trehalose and those added with 1-2% trehalose did not produced gas till after incubation for 24 h and for 36 h, respectively, however, reached stationary states after 48 h and 60 h, respectively. Gas production in yeasts added with 6% trehalose was superior to those in yeasts after storage above -20°C for 1-30 days. Next, ethanol production tests were performed. Yeasts added with trehalose hardly produced ethanol for a day regardless of trehalose concentration. However, since then the production drastically increased and reached stationary states after 7 days (Table 3). These productivities were superior to those of yeasts after storage above -20°C for 1-30 days.

In the case of addition of glycerol, CO_2 production was not observed till after incubation for 12 h at all, however, thereafter quickly began to produce gas and reached stationary states after 36 h (Table 2). Gas production of yeasts added with glycerol was superior to those of yeasts after storage above -20°C for 1-30 days. Ethanol production tests were performed in the same manner. It was observed the same ethanol production patterns as commercially available dried yeast (Table 3). Production in yeasts added with glycerol was superior to those in yeasts after storage above -20°C for 1-30 days. It suggested that trehalose and glycerol, incorporated into yeast cells from YPD liquid culture medium, acted as one of protectants or stabilizers against freezing and drying of yeast YTPR1.

CO_2 production in yeasts added with 1.0% L-glutamic acid was investigated. Gas production was not observed till after incubation for 12 h at all, however, thereafter quickly began to produce gas and then reached stationary state after 36 h (Table 2). It was also observed the same ethanol production patterns as commercially available dried yeast, although its productivity was inferior to that of commercially available dried yeast after incubation for a day (Table 3).

There are many factors that affect tolerances of yeasts as follows: temperature [7-11], freezing [7,12,13], dehydration [12,14,15], desiccation [16,17], hyperosmotic [7,18] and ethanol [19]. In the previous papers, it have been reported that there was a relationship between stress tolerances and trehalose rates in yeasts. In general, it is known that trehalose rates in yeasts is approximately 15-20% on dry weight basis, and the trehalose approximately 10% is considered as critical threshold for resistance to some stresses [13]. Lewis et al. [22] have clarified that freezing tolerance of yeasts has

Table 2. Carbon dioxide productivity of yeast strain YTPR1 obtained after cultivation with trehalose, glycerol and L-glutamic acid

	Time (h)						
	0	**12**	**24**	**36**	**48**	**60**	**72**
Trehalose 1%	0	0	0	0	70	100	100
Trehalose 2%	0	0	0	0	80	100	100
Trehalose 3%	0	0	0	40	80	100	100
Trehalose 4%	0	0	0	40	100	100	100
Trehalose 5%	0	0	0	60	100	100	100
Trehalose 6%	0	0	20	80	100	100	100
Glycerol 0.1%	0	0	10	100	100	100	100
Glycerol 0.5%	0	0	20	100	100	100	100
Glycerol 1.0%	0	0	40	100	100	100	100
Glycerol 2.0%	0	0	50	100	100	100	100
L-Glutamic acid 1.0%	0	0	90	100	100	100	100
Commercially available dried yeast	0	10.0	100	100	100	100	100

Carbon dioxide productivity (%) was expressed as relative values in commercially available baker's dried yeast when reached its stationary state.

Table 3. Ethanol productivity of yeast strain YTPR1 obtained after cultivation with trehalose, glycerol and L-glutamic acid

	Days				
	0	**1**	**3**	**5**	**7**
Trehalose 1%	0	6.3	43.8	81.3	100
Trehalose 2%	0	6.3	62.5	93.8	100
Trehalose 3%	0	6.3	68.8	93.8	100
Trehalose 4%	0	6.3	81.3	100	100
Trehalose 5%	0	6.3	100	100	100
Trehalose 6%	0	6.3	93.8	100	100
Glycerol 0.1%	0	68.8	100	100	100
Glycerol 0.5%	0	87.5	100	100	100
Glycerol 1.0%	0	75.0	100	100	100
Glycerol 2.0%	0	87.5	100	100	100
L-Glutamic acid 1.0%	0	81.3	100	100	100
Commercially available dried yeast	0	93.8	100	100	100

Ethanol productivity (%) was expressed as relative values to that in commercially available baker's dried yeast when reached its stationary state.

been associated with not trehalose rates in yeasts but slow freezing, salt, acetic acid, and hydrogen peroxide. Among them, there was significant correlation between stress resistance of yeasts and acetic acid. It needs to acquire yeasts with high-stress resistance against freezing and drying to apply for bread making. In our present study, it was not clear that how these intracellular compounds originally existed in yeast YTPR1, and how these compounds added in medium were taken into yeast cells. Further study is in progress to determine the concentration of intracellular compounds in yeast YTPR1 before and after incubation with these compounds and to investigate the relationship between concentration of these compounds and stress tolerance of yeast YTPR1.

4. CONCLUSION

A strain isolated from pear *Redbartllet* fruits was identified as *S. cerevisiae* by 28S rRNA sequence analysis. Yeast YTPR1 utilized maltose as well as glucose, fructose, and sucrose. In fact, bread dough was leavened in all of conditions not only for making French breads and for making white breads but also for making sweet breads, suggesting that yeast YTPR1 could metabolize these fermentable sugars including maltose produced by starch hydrolysis into CO_2 and ethanol. Yeast YTPR1 after storage at -28°C for 360 days satisfactorily produced the same CO_2 and ethanol as commercially available dried yeast. However, once freeze-dried the yeasts, gassing power and ethanol productivity of

the yeasts significantly reduced. It was useful techniques to prevent these damages in yeasts and to improve the drying tolerance of yeasts to incubate yeasts added with 6% trehalose, 0.1-2.0% glycerol, and 1.0% L-glutamic acid.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Shimosaka C. Preparation method and puffing behavior of bread with *Shirakamikodama* yeast. Journal of Cookery Science of Japan. 2011;44:223-230 (in Japanese).
Available:https://doi.org/10.11402/cookeryscience.44.223
2. Sekiguchi A. Studies on improvement of “Minowa” yeast. Reports of Gunma Industrial Technology Center. 2008;9-12 (in Japanese).
3. Oda Y, Yamauchi H, Tamura M. Development of baker’s yeast “Tokachino” through collaboration of industry, Academia and Government. Journal of the Japanese Society for Food Science and Technology. 2012;59:1-5 (in Japanese).
Available:https://doi.org/10.3136/nskkk.59.1
4. Gancedo JM, Gancedo C. Catabolite repression mutants in yeasts. FEMS Microbiology Reviews. 1986;32:179-187.
https://doi.org/10.1016/0378-1097(86)90289-2
5. Heredia MF, Heredia CF. *Saccharomyces cerevisiae* acquires resistance to 2-deoxyglycose at a very high frequency. Journal of Bacteriology. 1988;170:2870-2872.
Available:https://doi.org/10.1128/jb.170.6.2870-2872.
1988
6. Novak S, D’Amore T, Russell I, Stewart, GG. Sugar uptake in a 2-deoxy-D-glucose resistant mutant of *Saccharomyces cerevisiae*. Journal of Industrial Microbiology. 1991;7:35-40.
Available:https://doi.org/10.1007/BF01575600
7. D’Amore T, Crumplen R, Stewart GG. The involvement of trehalose in yeast stress tolerance. Journal of Industrial Microbiology. 1991;7:191-196.
Available:https://doi.org/10.1007/BF01575882
8. De Virgilio C, Hottiger T, Dominguez J, Boller T, Wiemken A. The role of trehalose systhesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. European Journal of Biochemistry. 1994;219:179-186.
Available:https://doi.org/10.1111/j.1432-1033.1994. tb19928.x
9. Eleutherio ECA, Araujo PS, Panek AD. Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*. Cryobiology. 1993;30:591-596.
Available:https://doi.org/10.1006/cryo.1993.1061
10. Panek AC, Vania JJM, Paschoalin MF, Panek D. Regulation of trehalose metabolism in *Saccharomyces cerevisiae* mutants during temperature shifts. Biochimie. 1990;72:77-79.
Available:https://doi.org/10.1016/0300-9084(90)9017 6-H
11. Slaughter JC, Nomura T. Intracellular glycogen and trehalose contents as predictors of yeast viability. Enzyme and Microbial Technology. 1992;14:64-67.
Available:https://doi.org/10.1016/0141-0229(92)9002
8-M
12. Coutinho C, Bernardes E, Felix D, Panek AD. Trehalose as a cryoprotectant for preservation of yeast strains. Journal of Bacteriology. 1988;7:23-32.
Available:https://doi.org/10.1016/0168-1656(88)9003
2-6
13. Gélinas P, Fiset G, Leduy A, Goulet J. Effect of growth conditions and trehalose content on cryotolerance of baker’s yeast in frozen dough. Applied and Environmental Microbiology. 1989; 55:2453-2459.
14. Gadd GM, Chalmers K, Reed RH. The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. FEMS Microbiology Letters. 1987;48:249-254.
Available:https://doi.org/10.1111/j.1574-6968.1987.t
b02551.x

15. Peace DA, Rose AH, Wright IP. Trehalose and trehalase in active dried yeast. Yeast. 1989;5:S453-S457.
Available:https://doi.org/10.1002/yea.320050707

16. Hottiger T, Boller T, Wiemken A. Rapid changes of heat and desiccation tolerance with changes of trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. FEBS Letters. 1987;220:113-115.
Available:https://doi.org/10.1016/0014-5793(87)80886-4

17. Takagi H, Sakai K, Morida K, Nakamori S. Proline accumulation by mutation or disruption of the proline oxidase gene improves resistance to freezing and desiccation stresses in *Saccharomyces cerevisiae*. FEMS Microbiology Letters. 2000;184:103-108.
Available:https://doi.org/10.1111/j.1574-6968.2000.tb08998.x

18. Mackenzie KF, Singh KK, Brown AD. Water stress plating hypersensitivity of yeasts: Protective role of trehalose in *Saccharomyces cerevisiae*. Journal of General Microbiology. 1988;134:1661-1666.
Available:https://doi.org/10.1099/00221287-134-6-16 61

19. Takagi H, Takaoka M, Kawaguchi A, Kubo Y. Effect of L-proline on sake brewing and ethanol stress in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. 2005;71:8656-8662.
Available:https://doi.org/10.1128/AEM.71.12.8656-8662.2005

20. Kinoshita T, Tanaka M. Isolation and wild yeasts from flowers and fruits for bread. Hyogo University Journal. 2007;12:71-82.

21. Japan Yeast Industry Association. Evaluation methods for Bakers' yeast. Tokyo, Japan: Japan Yeast Industry Association; 1996.

22. Lewis JG, Learmonth RP, Attfield PV, Watson K. Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*. Journal of Industrial Microbiology and Biotechnology. 1997;18:30-36.

23. Meng J, Yelian M, Chen JY, You Y, Liu F, Xu L. Growth characteristics of freeze tolerant baker's yeast *Saccharomyces cerevisiae* AFY in aerobic batch culture. SpringerPlus. 2016;5:503.
Available:https://doi.org/10.1186/s40064-016-2151-3

24. Murakami Y, Yokoigawa K, Kawai H. Lipid composition of a freeze-tolerant yeast, *Torulaspora delbureckii* and its freeze-sensitive mutant. Applied Microbiology and Biotechnology. 1995;44: 167-171.
Available:https://doi.org/10.1007/BF00164497

25. Oda Y. Utilization of the yeast *Torulaspora pretoriensis* as a baking strain. Journal of the Japanese Society for Food Science and Technology. 1996;43:1243-1248 (in Japanese).
Available:https://doi.org/10.3136/nskkk.43.1243

26. Oda Y, Tonomura K. Leavening ability and freeze-thaw resistance of the yeast *Torulaspora pretoriensis* IFO-0022 in dough. Journal of the Japanese Society for Food Science and Technology. 1996;43:185-187 (in Japanese).
Available:https://doi.org/10.3136/nskkk.43.185

27. Hahn YS, Kawai H. Screening of freeze-tolerant yeasts and their bread dough fermentative properties. Journal of Home Economics of Japan. 1990;41:115-121.
https://doi.org/10.11428/jhej1987.41.115

28. Hirasawa R, Yokoigawa K, Isobe Y, Kawai H. Improving the freeze tolerance of bakers' yeast by loading with trehalose. Bioscience, Biotechnology, and Biochemistry. 2001;65:522-526.
Available:https://doi.org/10.1271/bbb.65.522

29. Shi X, Miao Y, Chen JY, Chen J, Li W, He X, Wang J. The relationship of freeze tolerance with intracellular compounds in baker's yeasts. Applied Biochemistry and Biotechnology. 2014; 172:3042-3053.
https://doi.org/10.1007/s12010-014-0744-2

30. Sasano Y, Haitani Y, Ohtsu I, Shima J, Takagi H. Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough. International Journal of Food Microbiology. 2012;152:40-43.
Available:https://doi.org/10.1016/j.ijfoodmicro.2011.10.004

31. Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H. Simultaneous accumulation of proline and trehalose in

industrial baker's yeast enhances fermentation ability in frozen dough. Journal of Bioscience and Bioengineering. 2012;113:592-595.
Available:https://doi.org/10.1016/j.jbiosc.2011.12.018

32. Sasano Y, Haitani Y, Hashida K, Ohtsu I, Shima J, Takagi H. Enhancement of the proline and nitric oxide synthetic pathway improves fermentation ability under multiple baking-associated stress conditions in industrial baker's yeast. Microbial Cell Factories. 2012; 11:40.
Available:https://doi.org/10.1186/1475-2859-11-40

33. Nakagawa S, Ouchi K. Improvement of freeze tolerance of commercial baker's yeasts in dough by heat treatment before freezing. Bioscience, Biotechnology and Biochemistry. 1994;58: 2077-2079.
Available:https://doi.org/10.1271/bbb.58.2077

34. Lewis JG, Learmonth RP, Watson K. Role of growth phase and ethanol in freeze-thaw stress resistance of *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. 1993;59: 1065-1071.

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

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