

Screening Wild Yeast Strains for Alcohol Fermentation from Various Fruits

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Wild yeasts on the surface of various fruits including grapes were surveyed to obtain yeast strains suitable for fermenting a novel wine with higher alcohol content and supplemented with rice starch. We considered selected characteristics, such as tolerance to alcohol and osmotic pressure, capability of utilizing maltose, and starch hydrolysis. Among 637 putative yeast isolates, 115 strains exhibiting better growth in yeast-peptone-dextrose broth containing 30% dextrose, 7% alcohol, or 2% maltose were selected, as well as five α -amylase producers. Nucleotide sequence analysis of the 26S rDNA gene classified the strains into 13 species belonging to five genera; *Pichia anomala* was the most prevalent (41.7%), followed by *Wickerhamomyces anomalus* (19.2%), *P. guilliermondii* (15%), *Candida* spp. (5.8%), *Kodamaea ohmeri* (2.5%), and *Metschnikowia* spp. (2.5%). All of the α -amylase producers were *Aureobasidium pullulans*. Only one isolate (NK28) was identified as *Saccharomyces cerevisiae*. NK28 had all of the desired properties for the purpose of this study, except α -amylase production, and fermented alcohol better than commercial wine yeasts.

KEYWORDS : Alcohol tolerance, Glucose tolerance, Maltose utilization, Yeasts, Wine fermentation

Wine consumption has been increasing steadily worldwide not only due to its good health function but as a cultural preference. In Korea, wine consumption has increased 20~30%/yr, and the market is expected to expand even further. However, the quality of wine produced in Korea is not competitive with foreign wines. Therefore, the Korean wine market has been seized by imported wines from Europe, USA, Chile, and South Africa.

Wine quality is determined mostly by the raw materials, such as grapes and yeast starter. Fermentation and maturation techniques are also significant. The yeast species, fermentation conditions, and nutrient profiles modulate the production of volatile and non-volatile compounds. Consequently, a wine's flavor, appearance, aroma, and texture are determined by these factors [1-3]. Developing a wine grape cultivar takes much time and labor and is highly dependent on soil and weather. These challenges pose a great hurdle for improving the quality of wine production in Korea. Therefore, additional novel types of wine with added health functions are needed to provide a competitive edge to domestic wine producers. Indeed, domestic producers have incorporated various fruits and powdered grain into grapes, usually Campbell Early, to produce higher alcohol content and a unique taste and flavor [4-6].

Natural wine fermentation combines the activities of several yeasts species, which grow sequentially throughout the fermentation process. The process is initiated by

various species of *Candida*, *Debaryomyces*, *Hanseniaspora*, *Pichia*, *Kloeckera*, *Metschnikowia*, *Schizosaccharomyces*, *Torulospora*, and *Zygosaccharomyces*, which naturally exist on the grape surface. Yeast growth is generally limited to the first 2 or 3 days of fermentation due to osmotic pressure caused by the glucose added. Subsequently, the most strongly fermenting and more ethanol tolerant species of *Saccharomyces* dominates the fermentation [7]. However, modern winemaking is founded on the use of selected commercial *S. cerevisiae* strains for their reliable properties, which contribute to the quality of the resulting product.

Developing wine fortified with unique and beneficial compounds and higher alcohol content has been attempted by our research group by adding Kanghwa-do rice and kudzu powders to wine must prepared using Campbell Early grapes from Daebu-do. Starch hydrolysis with amylolytic enzyme(s) provides the yeast with maltodextrins, maltose, and glucose to ferment at a slightly buffered rate, protects the starter microorganism from osmotic shock [8], and allows for a more prolonged fermentation and higher alcohol content. Maltodextrins of various lengths also help smooth the taste of wine. Kudzu has been reported to contain various functional compounds such as antioxidants and pueraline [9].

Wild yeast strains were screened from various grapes and fruits purchased in Korea. A diversity of yeast strains with tolerance to high alcohol and osmotic pressure caused by high sugar concentrations was isolated along with those that could hydrolyze starch or utilize maltose more effi-

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ciently than commercial yeast strains. After collecting wild yeast strains, we selected a novel wine yeast strain along with many others for other types of alcohol fermentation. The yeast collection would contribute to preserving the genetic resources to develop yeast strains with improved functional traits.

Materials and Methods

Collecting samples to isolate the wild yeast strains. Grape varieties, including *Campbell Early* (from Dae-budo, Wanju, and Sangju), *Gerbong* (Cheonan), Delaware (USA), Thompson Seedless (Chile), *Muscat Bailey A* (Yeongdong, Chile), and *Neo Muscat* (Haman) were purchased from markets and farms around Incheon and Seoul, Korea. Apples, melons, apricots, wild strawberry (Bokbunja) produced in Korea, and kiwi from New Zealand were also purchased in the same area.

Isolation of yeast strains from the samples. Collected samples were diced and placed in 9 mL of yeast-peptone-dextrose (YPD) broth (1% yeast extract, 2% peptone, 2% dextrose, pH 4.5) to make a total volume of 10 mL, which was vortexed vigorously for several minutes. An aliquot (1.5 mL) was centrifuged for 1 min at 12,000 rpm in a microcentrifuge (Hanil Co., Seoul, Korea). The precipitate was resuspended in 200 μ L of lysozyme solution (10 mg/mL) and maintained at room temperature for 5~10 min to lyse the bacteria. Then, the supernatant (20 μ L) was spread on YPD agar plates containing 10% dextrose and chloramphenicol (50 μ g/mL) to inhibit the growth of filamentous fungi and bacteria, respectively. The plates were incubated for 2 days at 30°C, and colonies with yeast-like morphology were streaked onto fresh YPD agar plates. Microscopic observation of the colonies was made using a phase contrast microscope (DE/Axioplan 2; Carl Zeiss, Oberkochen, Germany) at \times 400 magnification to determine their morphology and purity.

Screening of yeast isolates. Wild yeast strains were selected for their tolerance to a high concentration of glucose using YPD agar plates containing 40% dextrose. Strains forming large colonies on plates incubated at 30°C for 48 hr were selected and tested further by culturing in YPD broth containing 30, 40, or 50% dextrose at 30°C for 24 hr with agitation (200 rpm) and compared at OD₆₀₀ using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Upsala, Sweden). The strains were also examined for their tolerance to high alcohol content by culturing them on YPD agar plates containing 15 or 30% alcohol. Those strains that could grow on the alcohol plates were screened further by culturing them in YPD broth containing 5~15% alcohol under the conditions described above and then comparing the growth at OD₆₀₀. The abil-

ity of the isolates to utilize maltose was screened using YP agar or broth containing 2% maltose (YPM). Strains forming larger colonies on YPM plates incubated at 30°C for 48 hr were selected and tested further by culturing them in YPM broth at 30°C for 24 hr with agitation and comparing the growth at OD₆₀₀. Yeast isolates producing α -amylase were screened on YP agar plates supplemented with 1% soluble starch (YPS) and by staining with iodine solution (1% KI, 0.1% KI₂). Two commercial strains of *S. cerevisiae*, Lv001 (Denmark) and La Parisienne (The Netherlands), were used as controls.

Alcohol assay. Yeast strains were cultured in YPD broth containing 10% dextrose at 30°C for 72~96 hr without agitation and centrifuged at 13,000 rpm for 1 min to remove the cells. The supernatant was filtered using a 0.2 μ m syringe filter (Sartorius Stedim Biotech, Goettingen, Germany) and subjected to high performance liquid chromatography (Waters 600S; Meadows Instrumentation Inc., Bristol, WI, USA), using a Rezex ROA-Organic Acid H⁺ column (Phenomenex, Torrance, CA, USA) and water as the solvent.

Identification of yeast isolates. Selected yeast isolates were identified by analyzing their 26S rDNA nucleotide sequences [10]. Yeast isolates were cultured in YPD broth at 30°C for 48 hr and harvested by centrifugation. The genomic DNA was extracted using a Genomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, and a 560 bp DNA fragment of the 26S rDNA gene was amplified by the PCR using primer-NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') as follows: initial denaturation at 94°C for 3 min and 36 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min. Nucleotide sequences of the resulting PCR products were determined (Solgent Co., Daejeon, Korea) using NL1 and NL4 as the primers, and homologous sequences were searched for in the sequences of the National Center for Biotechnology Information (NCBI) database using BLAST.

API 20C AUX kit analysis. The fermentation properties of the wild yeast isolates were characterized using the API 20C kit (Biomérieux, Marcy l'Etoile, France). Yeast strains were cultured on YPD agar plates, and the colonies were resuspended in 0.85% NaCl solution to a final turbidity equal to 2 McFarland units. The yeast suspension (100 μ L) was transferred to an ampoule of API C medium and homogenized gently with a pipette. The strip cupules were filled with the suspension in the ampoule of API C medium and incubated at 30°C for 72 hr. A cupule that was more turbid than the control indicated a positive

reaction, and the results were interpreted using the database (V4.0) and the Apiweb™ software provided by the manufacturer.

Results and Discussion

Isolation of wild yeasts with tolerance to osmotic pressure and alcohol. During wine fermentation, yeast cells are subjected to number of stressors, the most important being osmotic and ethanol stress [11]. Therefore, yeast strains tolerant to high alcohol or glucose concentrations were screened first in this study. From the samples collected, 637 putative yeast strains were isolated based on their colony morphology and microscopic observations. Among them, 70 isolates that grew on YPD agar plates containing 40% dextrose were selected. The capability of the isolates to grow on medium with high sugar concentrations by tolerating high osmotic pressure was examined further by culturing them in YP broth containing 30, 40, or 50% at 30°C for 24 hr and comparing the results at OD₆₀₀. Eighteen isolates showed better growth in YP broth containing 30% dextrose, which is closer to the glucose concentration (24° Brix) for wine fermentation, than the commercial strain (*S. cerevisiae* Lv001), which is widely used for wine fermentation (Fig. 1). The commercial strain grew better than isolates in YP broth containing 40% or more dextrose. Those isolates that achieved better growth in YP broth with 30% dextrose than the commercial yeast

(RG6, RG10, RG11, RG13, RG18, RG32, RG33, RG35, RG42, RG45, RG46, RG55, RG56, RG59, RG61, RG69, RG71, RG73, and NK28) were all isolated from Campbell Early grapes except NK28, which was isolated from kiwi. Isolates with tolerance to high osmotic pressure were identified as various strains of *Wickerhamomyces anomalus* and *Pichia anomala*, and a *S. cerevisiae* strain.

The yeast isolates were examined for their resistance to alcohol on YPD agar plates containing 15 or 30% alcohol in an attempt to isolate strains that could tolerate high alcohol concentrations. Generally, light wines, such as red, white, and sparkling wines, contain less alcohol (10~14%), whereas alcohol and dessert wines (sweet wines usually served with desserts) contain 14~20% alcohol. None of the isolates or the commercial strain grew on YPD agar plates containing 30% alcohol. Twenty-four isolates were selected based on their ability to form colonies on YPD agar plates containing 15% and were tested further for their alcohol tolerance in YPD broth with various alcohol percentages increased at 1% increments from 5 to 15% (Fig. 2). Generally, the isolates did not grow well in YPD broth containing more than 7% alcohol. Isolates with higher alcohol tolerance were selected based on their capability to grow relatively well in YPD broth containing 7% alcohol. They were RG1, RG5, RG11, RG19, RG21, RG24, RG28, RG29, RG30, RG32, RG33, RG34, RG35, RG37, RG40, RG43, RG44, RG67, RG68, KG56 from red grapes; and NK28 from kiwi. Particularly,

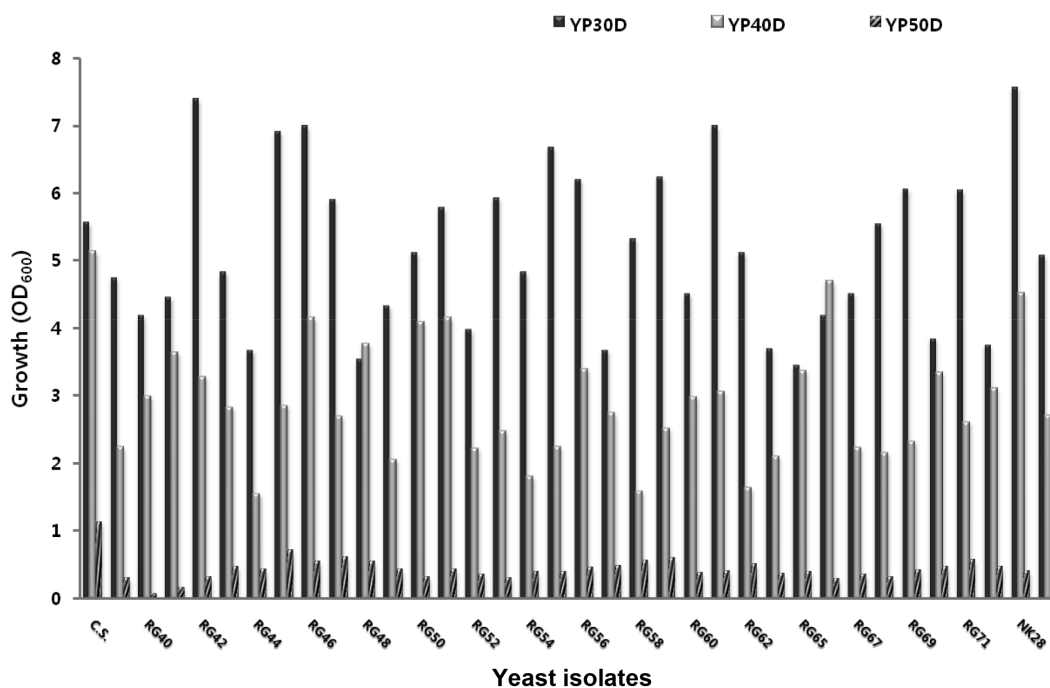


Fig. 1. Tolerance of wild yeast isolates to high glucose concentrations. Tolerance of yeast isolates to glucose was examined by culturing the isolates in yeast-peptone broth containing 30, 40, or 50% dextrose at 30°C for 24 hr, and the growth was compared at OD₆₀₀. Results of only one group of isolates are presented. All experiments were conducted in duplicate, and the averages of two readings are presented.

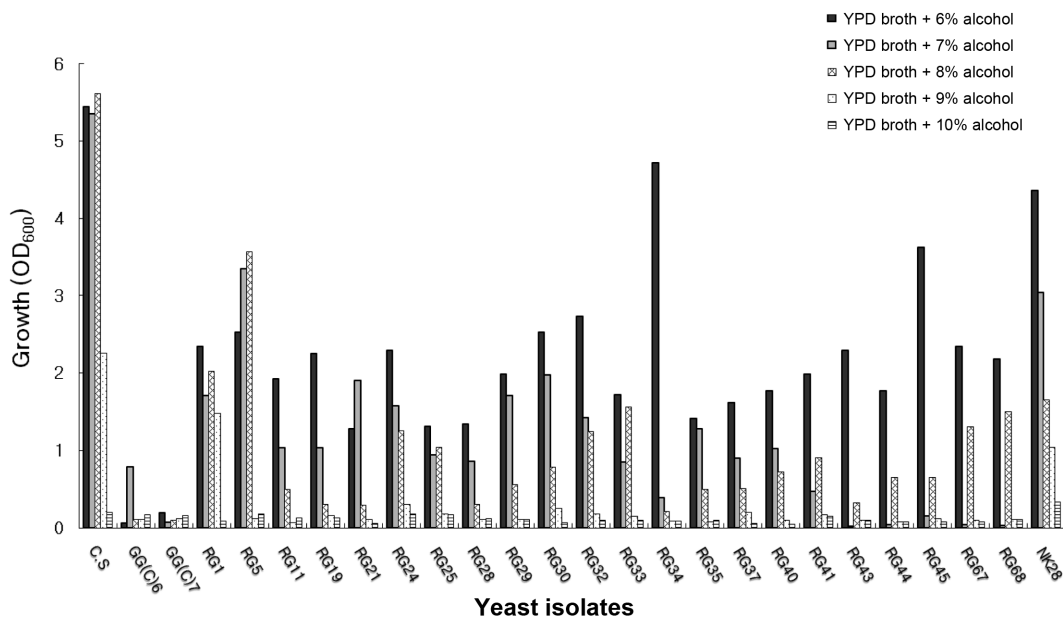


Fig. 2. Tolerance of wild yeast isolates to high alcohol concentrations. Tolerance of the yeast isolates to alcohol was examined by culturing the isolates in yeast-peptone-dextrose broth (YPD broth) containing 6~10% alcohol at 30°C for 24 hr, and the growth was compared at OD_{600} . All experiments were conducted in duplicate, and the averages of two readings are presented. Results of only one group of isolates are presented.

KG56, which was identified as *P. anomala*, grew much better than the commercial strain (*S. cerevisiae* Lv001) in YPD broth containing 15% alcohol ($OD_{600} = 1.35$). These alcohol tolerant yeast strains were identified as various strains of *W. anomalus*, *Candida* spp., and *P. anomala*. Isolation of alcohol and/or sugar tolerant *S. cerevisiae* strains has been reported from nuruk, cashew apple, and soil [12-14]. These strains were capable of growing in a

medium containing 25~50% glucose or 15% alcohol.

Selection of wild yeasts with efficient maltose utilization. Wine yeasts are usually killed during fermentation due to drastic increases in alcohol content by rapid glucose catabolism in wine must. Therefore, supplementing wine with maltose rather than glucose might relieve this problem by retarding yeast growth and alcohol formation,

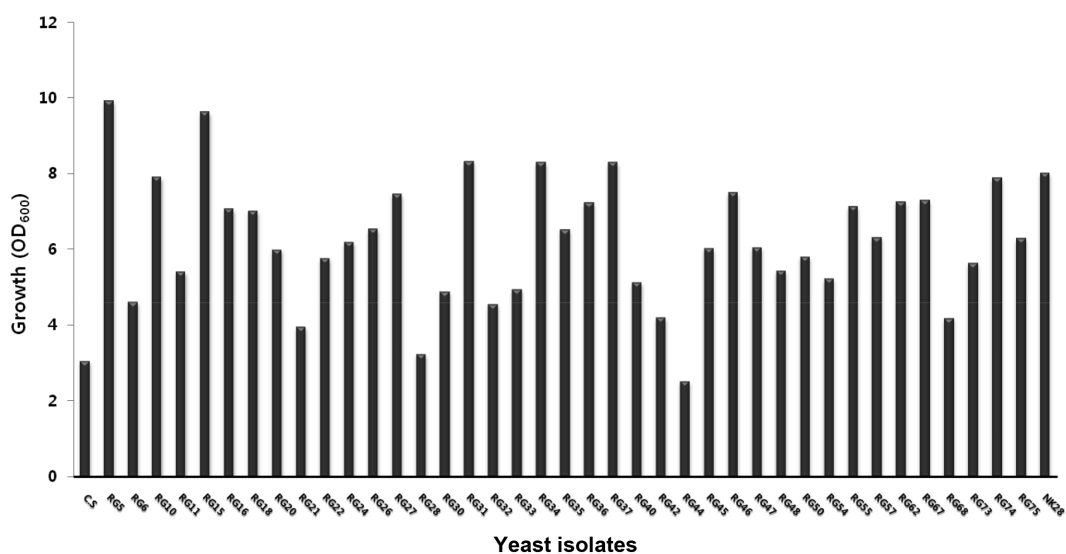


Fig. 3. Wild yeast isolates with efficient maltose utilization. The ability to utilize maltose was examined by culturing the isolates in yeast-peptone broth containing 2% dextrose at 30°C for 24 hr, and the growth was compared at OD_{600} . All experiments were conducted in duplicate, and the averages of two readings are presented. Results of only one group of isolates are presented.

thereby prolonging the length of fermentation. For this purpose, yeast isolates that could utilize maltose efficiently were selected by culturing them on YPM agar plates containing 2% maltose. A total of 123 isolates, which formed larger colonies on YPM agar plates, were tested further using YPM broth and by comparing growth (Fig. 3). Among them, 14 isolates from Neo Muscat (GG2, GG3, GG7, GG9, GG11, GG14, GG16~18, GG21, GG22, GG28, GG30, and GG36), 27 isolates from Campbell Early (RG5, RG10, RG11, RG15, RG16, RG18, RG20, RG22, RG24, RG26, RG27, RG31, RG34~37, RG46~48, RG50, RG54, RG57, RG62, RG67, RG74, and RG75), one from wild strawberry (WSS76), and one from kiwi (NK28) utilized maltose and grew better than the others including the commercial yeast strain (*S. cerevisiae* Lv001). The isolates were identified as various strains of *W. anomalus*, *Candida* sp., *P. anomala*, *Metschnikowia* aff. *fructicola*, and a *S. cerevisiae* strain. Maltose utilization in yeasts is controlled by catabolite repression in the presence of glucose and is carried out by inducible maltose permease and maltase (α -glucosidase) [15]. However, the diversity of yeasts catalyzing maltose efficiently has not been investigated.

Isolation of yeast strains producing amylase. Rice starch is added to wine to round-out the taste. Rice starch in wine must should be hydrolyzed first into smaller molecules by adding amylolytic enzyme(s) and then alcohol fermentation is performed by the yeast, creating a two-step fermentation process. Oligosaccharides and glycerol produced from carbohydrate in the wine provide the body and roundness to the wine. Yeast strains capable of producing amylolytic enzymes have been screened or developed by various research groups in an attempt to improve process time and cost [16-18]. Some of the strains were identified as *S. diastaticus*, *Saccharomycopsis fibuligera*, and *Sporobolomyces holsaticus*. All isolates obtained in this study were subjected to an amylase test using the

YPS agar plate assay, but the frequency of amylase-positive yeast was very low, and only five strains were positive (Fig. 4). α -Amylase activity was detected clearly after 3 days of cultivation. The strains were isolated from peach and *Rubus coreanus* Miquel (bokbunja), and all were identified as strains of *Aureobasidium pullulans*.

Diversity of the selected isolates. Among 637 isolates of wild yeasts, 120 strains with at least one of the desired properties, i.e., better glucose and alcohol tolerance, superior capability to utilize maltose, or α -amylase production, were selected and identified by analyzing the partial 26S rRNA gene nucleotide sequence. As a result, the isolates were classified into 13 species in seven genera. Twenty-three strains were identified as *W. anomalus*, 50 strains as *P. anomala*, seven strains as *Candida* spp., three strains as *Kodamaea ohmeri*, 18 strains as *P. guilliermondii*, and three strains as *Metschnikowia* spp. (Fig. 5). The sequences of the 10 isolates did not match any of the sequences in the NCBI database. Recently, *P. anomala*, which belongs to the non-*Saccharomyces* wine yeasts [19], has been renamed *W. anomalus* [20], making it the most prevalent yeast (60.8%) with desired properties in fruits. *P. anomala* is one of the selected non-*Saccharomyces* wine yeasts and is used for cofermentation to enhance wine aroma [21]. *W. anomalus* has low virulence and is present in a variety of niches with a wide geographical distribution due to its capability to flourish in harsh environments [22]. Only one strain, NK28, was identified as *S. cerevisiae* with 100% identity for the 565 bp 26S rDNA fragment amplified by PCR and sequenced. All five strains producing α -amylase were identified as *A. pullulans*, which is known to produce pullulan.

Two of the isolates identified as *W. anomalus* TY16 (RG5, RG32), one as *P. anomala* GS80A (RG67), and one as *S. cerevisiae* (NK28), exhibited the three desirable properties regarding glucose and alcohol tolerance as well

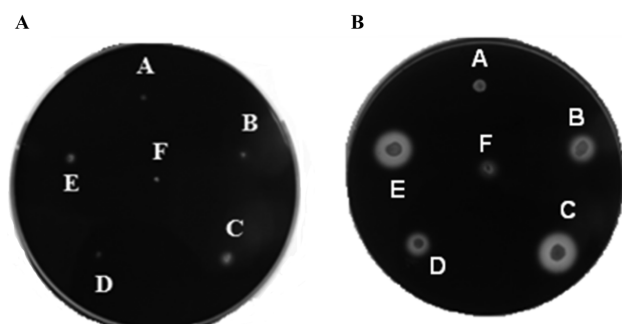


Fig. 4. Starch agar plate assay of wild yeast isolates. The starch hydrolyzing activity of the yeast isolates was examined by culturing on yeast-pectone agar plates containing 1% soluble starch at 30°C for 24 hr (plate A) to 72 hr (plate B). Colony A, *Saccharomyces cerevisiae* Lv001; Colony B~F, Wild yeast isolates.

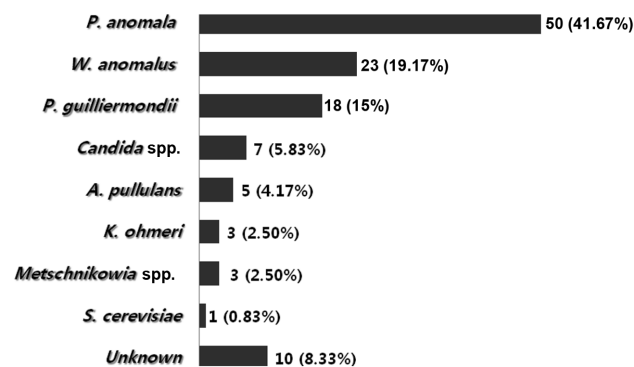


Fig. 5. Diversity and frequency of wild yeast strains isolated from various fruits. *P. anomala*, *Pichia anomala*; *W. anomalus*, *Wickerhamomyces anomalus*; *A. pullulans*, *Aureobasidium pullulans*; *K. ohmeri*, *Kodamaea ohmeri*; *S. cerevisiae*, *Saccharomyces cerevisiae*.

as an ability to efficiently utilize maltose. Generally, the ability to efficiently use maltose was strongly correlated with tolerance to osmotic pressure but not to alcohol tolerance. The results indicated a diversity in the yeasts with tolerance to high glucose and alcohol; those with efficient maltose utilization or those with α -amylase were very limited on fruits. Based on the results, only one *S. cerevisiae* (NK28) strain was obtained and its characteristics were analyzed further to examine its utilization as a selected wine yeast.

Kloeckera apiculata is the most frequently isolated native species from undamaged grapes and may account for over 50% of the total yeast flora from fruits [23]. *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* have also been reported in lesser numbers. The populations of *Saccharomyces* are often less than 50 colony forming unit/mL on grape musts. Therefore, the diversity of yeasts surveyed in this study represents mostly those with selective characteristics of glucose and alcohol tolerance, efficient maltose utilization, and starch hydrolysis.

Properties of the novel *S. cerevisiae* isolate. The only *S. cerevisiae* strain obtained from kiwi, NK28, was compared with other commercial *S. cerevisiae* strains, Lv001 and Parisienne, for tolerance to sugar and maltose utilization. Lv001 is a commercial yeast strain that has been most widely used for wine fermentation. La Parisienne was developed as a commercial baker's yeast but recently began to be used for wine fermentation, as wine fermented by La Parisienne has lower acidity and increased roundness [24]. NK28 showed the strongest glucose tolerance in YPD broth containing 30% dextrose and could utilize maltose as a carbon source better than Lv001. NK28 showed a weaker tolerance to alcohol than Lv001 at low concentrations (6–7%) but similar or better at higher concentrations (8% or above). NK28 could produce 14% and 18% more alcohol than Lv001 and La Parisienne, respectively, in 72 hr of glucose fermentation.

From the results, NK28 seemed to be more closely related to La Parisienne than to Lv001. The characteristics of these *S. cerevisiae* strains were compared using API 20 C AUX, which analyzes the capabilities of yeast strains utilizing 19 different carbon sources, as shown in Table 1. NK28 was different from Lv001 in the utilization of two carbon sources. NK28 could not utilize glycerol but Lv001 could; NK28 could utilize methyl- α -D-glucopyranoside, but Lv001 could not. NK28 showed exactly the same properties as those of La Parisienne for all carbon sources tested. Previously, La Parisienne was reported to produce more alcohol than various wine yeasts [24]. These two strains were different only in the flocculation of cells during liquid culture. The NK28 cells did not flocculate with each other, whereas La Parisienne floccu-

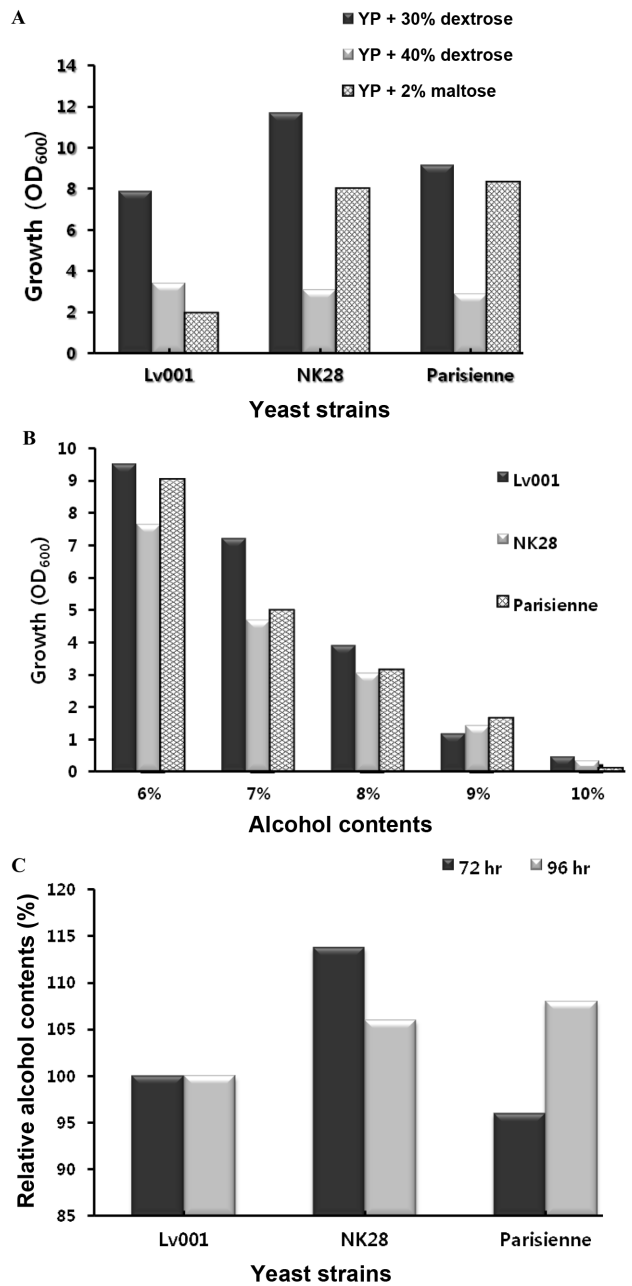


Fig. 6. Characteristics of NK28 compared with those of two commercial wine yeasts. A, Tolerance to high glucose concentration and the ability to utilize maltose; B, Tolerance to high alcohol concentration; C, Alcohol production of three *Saccharomyces cerevisiae* strains. All experiments were conducted in triplicate, and the averages of three readings are presented. YP, yeast-peptone.

lated in liquid culture. These properties of NK28 would make it suitable for the fermentation of a novel wine supplemented with hydrolyzed rice starch. The variety of the yeast collection established in this study could provide select yeast strains for various types of alcohol fermentation or genetic sources to improve conventional strains.

Table 1. Comparison of 3 *S. cerevisiae* strains regarding the characteristics analyzed by API 20C AUX

Yeasts	Substrate																			
	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	FLO
NK28	○	×	×	×	×	×	×	○	×	×	○	×	×	×	○	○	○	○	○	×
Lv001	○	○	×	×	×	×	×	○	×	×	×	×	×	×	○	○	○	○	○	×
La Parisienne	○	×	×	×	×	×	×	○	×	×	○	×	×	×	○	○	○	○	○	○

GLU, glucose; GLY, glycerol; 2KG, 2-keto-D-gluconate; ARA, arabinose; XYL, xylose; ADO, adonitol; XLT, xylitol; GAL, galactose; INO, inositol; SOR, sorbitol; MDG, methyl-D-glucoside; NAG, N-acetyl- glucosamine; CEL, cellobiose; LAC, lactose; MAL, maltose; SAC, saccharose; TRE, trehalose; MLZ, melezitose; RAF, raffinose; FLO, flocculation.

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