Analysis and enhancement of the ethanol resistance of *Pichia kudriavzevii* N77-4, a strain newly isolated from the Korean traditional fermentation starter Nuruk, for improved fermentation performance

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**Abstract.** Nuruk is a traditional fermentation starter used for making starch-based Korean alcoholic drinks. The yeast strain *Pichia kudriavzevii* N77-4 was newly isolated from *nuruk*. Resistance to ethanol stress is a crucial characteristic in alcoholic beverage production, but the ethanol stress-resistance of *P. kudriavzevii* remains to be clarified. In this study, we attempted to analyze the ethanol resistance of *P. kudriavzevii* N77-4, and to improve it by ethyl methanesulfonate (EMS) mutagenesis. After 4 h of cultivation in 5% ethanol, N77-4 showed a growth defect accompanied with accumulation of reactive oxygen species (ROS). In addition, exposure of N77-4 to ethanol stress induced a more than 3-fold increase in the expression of *PkSOD2*, which encodes a superoxide dismutase, indicating that the strain was able to generate *PkSOD2* to decrease ROS. EMS mutagenesis was performed to improve the ethanol resistance of N77-4. An isolated mutant, HER8, exhibited higher ethanol resistance, a 50% decrease in ROS accumulation, and enhanced expressions of *PkGPX2*, which encodes a glutathione peroxidase, and *PkSOD1*. Moreover, HER8 showed 10% greater ethanol production, indicating that up-regulation of these antioxidant genes is important for improving ethanol resistance in *P. kudriavzevii*, and use of the HER8 strain will improve the brewing fermentation.

1. **Introduction**

*Nuruk* has been used as a traditional fermentation starter for making Korean alcoholic drinks such as *Makgeolli* (unfiltered Korean traditional rice wine), *Cheongju* (filtered clear rice wine) and *Soju* (distilled liquor) from ancient times. *Nuruk* is made up of natural grains and the enzymes derived from them, as well as a variety of microorganisms, including wild yeasts and other fungi such as *Rhizopus*, *Aspergillus* and *Lichtheimia* spp. [1]. In order to analyze the mechanisms underlying its role in alcoholic fermentation, various microorganisms were previously isolated from *nuruk* [2]. One of these microorganisms, the yeast strain *Pichia kudriavzevii* N77-4, was found to exhibit better resistance to ethanol than other isolated non-*Saccharomyces* yeasts from *nuruk*, and also high extracellular enzyme
activities that contribute to an increase in the level of volatile components. This strain was thus suggested to play important roles in the production of alcoholic drinks using nuruk.

Resistance to ethanol is one of most important characteristics for the yeasts used in brewing fermentation. This is because ethanol, although produced mainly by yeasts, is also toxic to the growth and fermentation of yeasts and other microorganisms [3]. Ethanol stress increases the fluidity of the plasma membrane and the levels of unsaturated fatty acids, resulting in a decrease in the membrane integrity that is so crucial to maintaining intracellular pH, metabolism and other aspects of cellular homeostasis in the yeast Saccharomyces cerevisiae [4, 5]. Incorporation of glucose in addition to several amino acids is also damaged by ethanol stress [6, 7]. Bulk mRNA transport from the nucleus and translation of the mRNAs in the cytoplasm are inhibited by high concentrations of ethanol [8]. Reactive oxygen species (ROS) are also reported to be generated under ethanol stress conditions and act as a secondary stressor, causing loss of cell viability [9]. ROS inflict damages on a wide range of molecules, including nucleic acids, proteins and lipids. Finally, in yeasts, high ROS concentrations induce apoptosis. In response to ethanol stress, expression of INO1—which encodes a rate-limiting enzyme responsible for inositol biosynthesis—is upregulated [10]. Inositol biosynthesis was previously shown to be important for the growth of S. cerevisiae under ethanol stress conditions [11]. Inositol is a major component of the plasma membrane lipid phosphatidylinositol [12], and high phosphatidylinositol content increases proton pumping activity [11]. Therefore, INO1 expression helps to prevent the reduction of intracellular pH and nutrient incorporation caused by ethanol stress. Recently, we found that expression of PkINO1 was decreased under ethanol stress conditions, but overexpression of PkINO1 mitigated the growth defect of P. kudriavzevii N77-4 under ethanol stress conditions [13]. Transcriptional regulation of the antioxidant defense system plays an important role in the adaptation to ROS stress; such regulation includes the transcription of SOD genes encoding superoxide dismutase, which breaks superoxide anion down to hydrogen peroxide, and of the CTA1 and CTT1 genes, which encode the catalase that decomposes hydrogen peroxide to H₂O and O₂ [14]. Hydrogen peroxide is also reduced to H₂O by the glutathione peroxidase encoded by GPX genes. In this reaction, oxidized Gpx proteins are reduced by glutathione (GSH) synthesized by the function of GSH1 and GSH2 genes, and the resulting glutathione disulfide (oxidized glutathione; GSSG) is reduced and regenerated to GSH by the glutathione reductase encoded by GLR1 using NADPH as a reducing agent.

As mentioned above, the ethanol-adaptation response has been intensively studied, and some of the ethanol-resistance mechanisms have been revealed in S. cerevisiae, a eukaryotic model organism and the most widely used industrial microorganism. However, the mechanisms of the response and resistance of P. kudriavzevii to ethanol stress remain to be clarified. In the present study, therefore, we investigated the mechanisms underlying the resistance of P. kudriavzevii N77-4 to ethanol stress, and attempted to improve the ethanol-stress resistance of this organism. Our results should increase basic knowledge of the yeast P. kudriavzevii used in brewing fermentation and lead to an enhancement of its fermentation performance.

2. Materials and Methods

2.1. Strains, genes and media

The P. kudriavzevii strain N77-4 was previously isolated from the Korean traditional fermentation starter nuruk [2]. The diploid S. cerevisiae lab strain BY4743 (MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 lys2Δ0/lys2Δ0 ura3Δ0/ura3Δ0) was previously described [15]. BY4743 was obtained from the NBRP Yeast Genetic Resource Center (http://yeast.nig.ac.jp/yeast/top.xhtml). PkSOD1, PkSOD2, PkCTA1, PkGPX2 and PkGLR1 were identified by a BLAST search of the Ensembl Fungi database (http://fungi.ensembl.org/index.html) using the amino acid sequences of S. cerevisiae SOD1, SOD2, CTA1, CTT1, GPX1, GPX2 and GLR1 from the Saccharomyces Genome Database (https://www.yeastgenome.org) as a query. PkSOD1 (JL09_g3534), PkSOD2 (JL09_g422) and PkGLR1 (JL09_1478) shared high amino acid sequence similarities to S. cerevisiae SOD1 (66% identity), SOD2 (53% identity) and GLR1 (65% identity), respectively. Since the BLAST search using the amino acid sequences of S. cerevisiae CTA1 and
resulted in identification of a single gene, JL09_g3375 (53% and 43% amino acid sequence identities, respectively), the gene name PkCTA1 was used as an orthologous gene for S. cerevisiae CTA1 and CTT1 in this study. Similarly, since a BLAST search using the amino acid sequences of S. cerevisiae GPX1 and GPX2 resulted in identification of a single gene, JL09_g3375 (52% and 65% amino acid sequence identities, respectively), the gene name PkGPX2 was used as an orthologous gene for S. cerevisiae GPX1 and GPX2 in this study. Yeast strains were grown at 30°C in YPD medium containing 5% YPD broth (Difco, Detroit, MI) supplemented with 40 mg/L of adenine (Wako Pure Chemical Industries, Tokyo). For solid media, 2% agar (Wako Pure Chemical Industries) was added.

2.2. Ethanol sensitivity and spot tests
Yeast cells grown in YPD medium at 30°C for 16 h were harvested and resuspended in YPD medium containing 0%, 5% or 7% ethanol to give OD$_{600}$ nm = 1.0. Cells were then cultivated for 4 h at 30°C while monitoring the optical density. For spot tests, yeast cells in mid-log phase from 5 mL YPD culture were collected, washed once with sterile water and re-suspended in 100 µL of sterile water. Cell density was measured and the suspension was then serially diluted 10-fold from 0.2×10$^6$ cells/µL to 0.2×10 cells/µL. Next, 5-µL aliquots of cells at each dilution were spotted on YPD plates containing 0%, 9% or 10% ethanol and incubated at 30°C for 5 days.

2.3. Measurement of ROS level
Yeast cells were grown in 10 mL of YPD medium from OD$_{600}$ nm = 0.1 to OD$_{600}$ nm = 0.8 at 30°C. Cells were harvested, resuspended in 10 mL of YPD medium containing 5% ethanol and cultivated for 4 h. Fifty µL of 100 µM H$_2$DCFDA (Sigma-Aldrich, St. Louis, MO) was added to 2.5 mL of the culture. After cultivation for 2 h at 30°C, the cells were harvested, washed once with water and dissolved in 1 mL of PBS buffer. They were then stored on ice in the dark prior to measurement of the ROS level. A BD FACSAria™ IIIu cell sorter (BD Biosciences, San Jose, CA) fitted with a 488 nm laser for excitation and a 530/30BP filter with a preceding 502LP filter for detection was used to measure the level of ROS. Signals from 100,000 cells were acquired and analyzed for each sample using FlowJo software (BD Biosciences).

2.4. RNA isolation, cDNA preparation and quantitative real-time PCR
Yeast cells grown in 10 mL of YPD medium at 30°C to mid-log phase were harvested, resuspended in 10 mL of YPD medium containing 5% ethanol and cultivated for 4 h at 30°C. Total RNA was isolated using a RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was prepared using a QuantiTech reverse transcription kit (Qiagen). Quantitative real-time PCR was performed using TB Green Fast qPCR Mix (TakaRa Bio, Tokyo) in triplicate in a Thermal Cycler Dice Real-Time System Lite (TakaRa Bio) according to the manufacturer’s instructions. Relative mRNA levels were normalized to PkACT1 mRNA levels. The primers used for quantitative real-time PCR were as follows: PkACT1-R1 and PkACT1-R2 for PkACT1 mRNA, PkSOD1-R1 and PkSOD1-R2 for PkSOD1 mRNA, PkSOD2-R1 and PkSOD2-R2 for PkSOD2 mRNA, PkCTA1-R1 and PkCTA1-R2 for PkCTA1 mRNA, PkGPX2-R1 and PkGPX2-R2 for PkGPX2 mRNA and PkGLR1-R1 and PkGLR1-R2 for PkGLR1 mRNA (Table 1).

Table 1. Primers used in this study.

| Primer   | Sequence |
|----------|----------|
| PkACT1-R1-R2 | 5'-TCCTGGTGATGGTTACTCAGGTT-3' |
| PkACT1-R1-R2 | 5'-CTCTACCTGTCAAATCTCCCTTA-3' |
| PkSOD1-R1-R2 | 5'-TCTCGGCGAGAACCCTGTTG-3' |
| PkSOD1-R1-R2 | 5'-GAGGTCACGATGTTGAGCCAATTT-3' |
| PkSOD2-R1-R2 | 5'-TACAGAGCTATTGCGACTCATTAG-3' |
| PkSOD2-R1-R2 | 5'-TCGGGTGTCTTGCCGCAATGAA-3' |
| PkCTA1-R1-R2 | 5'-GAGGGCCCAATGTGTTG-3' |
| PkCTA1-R1-R2 | 5'-TTGTAAGCCCGGCAATTAGTT-3' |
2.5. EMS mutagenesis
Ethyl methanesulfonate (EMS) mutagenesis of N77-4 was performed as described in Methods in Yeast Genetics [16]. Treatment of EMS (Wako Pure Chemical Industries) for 45 min (ca. 50% survival rate) was applied in this study.

2.6. Fermentation test
Cells that were pre-cultured in YPD medium at 30°C for 16 h were harvested and inoculated in 50 mL of YPD medium containing 30% glucose, 2% bacto-peptone and 1% bacto-yeast extract at an OD600 nm of 10. Cells were aerobically cultured at 30°C for 42 h at 125 rpm. The ethanol concentration was then measured using gas chromatography on a GC-2014 machine (Shimadzu, Kyoto, Japan). The glucose concentration was analyzed using a Glucose CII Test kit (Wako Pure Chemical Industries).

3. Results and discussion

3.1. Ethanol stress sensitivity and intracellular ROS accumulation in \( P. \) kudriavzevii N77-4
First, to determine the concentration of ethanol that causes modest sensitivity, we measured the growth ability of N77-4 under 0%, 5% and 7% ethanol stress conditions (Figure 1A). The results showed that although 7% ethanol stress completely inhibited the proliferation of N77-4 after 4 h of cultivation, N77-4 displayed slow but consistent growth after 4 h of cultivation under a 5% ethanol condition. Therefore, a 5% ethanol stress condition was adopted as a modest growth inhibitor for the analysis of N77-4 ethanol-resistance in the following experiments.

Next, the level of ROS accumulation in N77-4 was measured under the 5% ethanol stress condition. Since \( P. \) kudriavzevii N77-4 showed lower ethanol resistance than the \( S. \) cerevisiae lab strain BY4743 [13], the ROS accumulation level of \( S. \) cerevisiae BY4743 was also measured (Figure 1B). The results showed that after 4 h cultivation under a 5% ethanol condition, the intracellular ROS levels of both N77-4 and BY4743 increased more than 8-fold compared with that under a 0% ethanol condition. Moreover, we found that the level of ROS in N77-4 increased by 40% compared with that in BY4743 under the 5% ethanol condition, indicating that the difference in ROS accumulation was linked to the difference in ethanol sensitivity between \( P. \) kudriavzevii N77-4 and \( S. \) cerevisiae BY4743.

![Figure 1. Ethanol stress sensitivity and ROS accumulation.](image-url)
(A) Ethanol stress sensitivity of *P. kudriavzevii* N77-4 under 0%, 5% and 7% ethanol stress conditions. The data shown are the means ± SD (n = 3). (B) Accumulation of ROS levels in *P. kudriavzevii* N77-4 and *S. cerevisiae* BY4743 under 0% and 5% ethanol stress conditions. The ROS accumulation levels were expressed as relative ROS values with the values for BY4743 and N77-4 grown under 0% ethanol condition set to 1.0. The data shown are means ± SD (n = 3). Statistical analyses were performed using Student’s *t*-test and a single asterisk indicates a significant difference (*P < 0.05) between BY4743 and N77-4.

### 3.2. Expression analysis of antioxidant genes in *P. kudriavzevii* N77-4 under ethanol stress conditions

Having determined that ethanol stress increased ROS accumulation in *P. kudriavzevii* N77-4, we next examined the expression levels of antioxidant genes—i.e., *PkSOD1* and *PkSOD2*, which encode superoxide dismutases, *PkCTA1*, which encodes a catalase, *PkGPX2*, which encodes a glutathione peroxidase and *PkGLR1*, which encodes a glutathione reductase—under a 5% ethanol stress condition (Figure 2). The results showed that the expressions of *PkSOD1*, *PkCTA1*, *PkGPX2* and *PkGLR1* increased by approximately 20%, 15%, 25% and 45%, respectively, in response to 5% ethanol stress. In addition, *PkSOD2* expression under a 5% ethanol stress condition was enhanced more than 3-fold over that under the 0% ethanol stress condition. These results indicate that *P. kudriavzevii* N77-4 possessed a transcriptional program to induce these antioxidant genes, especially *PkSOD2*, for responding and adapting to the ROS stress generated by ethanol stress.

![Figure 2. Expression of antioxidant genes in N77-4 under ethanol stress conditions.](image)

The expression levels of *PkSOD1*, *PkSOD2*, *PkCTA1*, *PkGPX2* and *PkGLR1* mRNAs in N77-4 cultivated under 0% and 5% ethanol stress conditions were analyzed by quantitative real-time PCR and expressed as relative RNA values (normalized to *PkACT1* expression), with the value for N77-4 grown under 0% ethanol condition set to 1.0. Statistical analyses were performed using Student’s *t*-test, and single and double asterisks indicate significant differences (*P < 0.05 and **P < 0.01) between the 0% and 5% ethanol stress conditions. The data shown are means ± SD (n = 3).

### 3.3. Improvement of ethanol resistance of *P. kudriavzevii* N77-4 by EMS mutagenesis

To study the ethanol-resistance mechanism and also to improve the ethanol resistance of N77-4 for higher ethanol production, EMS mutagenesis was performed and HER (High Ethanol Resistant) mutants were isolated on YPD plates containing 10% ethanol, a condition under which the wild-type N77-4 was completely unable to grow. After screening, 15 HER mutants were isolated. One of them, the HER8 mutant, showed superior ethanol resistance compared with wild-type N77-4 on YPD media containing 9% and 10% ethanol (Figure 3A).

Next, we measured ROS accumulation levels in the HER8 mutant under a 5% ethanol condition (Figure 3B). The results showed that the HER8 mutant displayed a much lower level of intracellular ROS accumulation under both the 0% and 5% ethanol conditions, indicating that lower ROS accumulation contributed to the higher ethanol resistance of HER8.
Figure 3. Ethanol resistance and intracellular ROS accumulation of the HER8 mutant.

(A) Growth assay (spot test) of the N77-4 and HER8 strains at 30°C under 9% and 10% ethanol stress conditions. Photographs were taken after incubation for 5 days. (B) Accumulation of ROS levels in the N77-4 and HER8 strains under 0% and 5% ethanol stress conditions. The ROS accumulation levels were expressed as relative ROS values with the value for N77-4 set to 1.0. The data shown are means ± SD (n = 3). Statistical analyses were performed using Student’s t-test, and a double asterisk indicates a significant difference (**P < 0.01) between the N77-4 and HER8 strains.

3.4. Analysis of the expression of antioxidant genes in the HER8 mutant

To investigate the superior ethanol-resistance mechanism of HER8 in more detail, we checked the expression level of antioxidant genes including *PkSOD1*, *PkSOD2*, *PkCTA1*, *PkGPX2* and *PkGLR1* under both the 0% and 5% ethanol stress conditions (Figure 4). The results showed that under the 0% ethanol stress condition, the expressions of *PkSOD1*, *PkSOD2*, *PkCTA1* and *PkGPX2* in HER8 increased by approximately 75%, 30%, 50% and 240%, respectively, compared with those in N77-4 (Figure 4A). Moreover, HER8 showed approximately 50%, 70% and 20% higher expressions of *PkSOD1*, *PkGPX2* and *PkGLR1*, respectively, than N77-4 under a 5% ethanol condition (Figure 4B), indicating that enhanced expression of *PkSOD1*, which is involved in the conversion of superoxide anion to hydrogen peroxide, and *PkGPX2*, which is involved in the conversion of hydrogen peroxide into H₂O, played important roles in the superior antioxidation-mediated ethanol resistance of the HER8 strain.

Figure 4. Expression of antioxidant genes in the HER8 mutant.
The expression levels of \textit{PkSOD1}, \textit{PkSOD2}, \textit{PkCTA1}, \textit{PkGPX2} and \textit{PkGLR1} mRNAs in the N77-4 and HER8 strains cultivated under 0\% (A) and 5\% (B) ethanol stress conditions were analyzed by quantitative real-time PCR and expressed as relative RNA values (normalized to \textit{PkACT1} expression), with the value for N77-4 set to 1.0. Statistical analyses were performed using Student’s \emph{t}-test, and a double asterisk indicates a significant differences (**\textit{P} < 0.01) between the N77-4 and HER8 strains. The data shown are means ± SD (\textit{n} = 3).

3.5. Improvement of ethanol production by the HER8 mutant

Finally, we performed a fermentation test of the HER8 strain under a high concentration of 300 g/L glucose YPD medium, since the HER8 strain showed a much higher ethanol resistance than the N77-4 strain (Figure 5). The results showed that HER8 displayed better growth than N77-4 under a high glucose condition (Figure 5A). Further, we found that HER8 exhibited higher glucose consumption and ethanol production compared with N77-4 (Figure 5B). Finally, the HER8 mutant improved ethanol production by more than 10\% compared with N77-4 after 36 h cultivation.

![Figure 5](image)

(A) Growth curve of the N77-4 and HER8 strains under a high concentration of 300 g/L glucose YPD medium. (B) Glucose consumption and ethanol production of the N77-4 and HER8 strains under a high concentration of 300 g/L glucose YPD medium. Statistical analyses were performed using Student’s \emph{t}-test, and single and double asterisks indicate significant differences (*\textit{P} < 0.05 and **\textit{P} < 0.01) between the N77-4 and HER8 strains. The data shown are means ± SD (\textit{n} = 3).

4. Conclusion

In this study, increased ROS accumulation was observed in \textit{P. kudriavzevii} N77-4 under an ethanol stress condition. We also found that, in order to overcome the increase in ROS, N77-4 invoked a transcriptional program to induce a variety of antioxidant genes, especially \textit{PkSOD2}, as a defense mechanism against ethanol stress. Next, we isolated an N77-4 mutant with superior ethanol-resistance, HER8, and showed that it exhibited a much lower level of ROS accumulation and much higher expression of antioxidant genes, especially \textit{PkSOD1} and \textit{PkGPX2}. Moreover, ethanol production was improved by using HER8. These results indicated that enhancement of the expression of antioxidant genes is very important for improving ethanol resistance in \textit{P. kudriavzevii}, and HER8 will thus contribute to an improvement of brewing fermentation.

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