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# Transcription Analysis of the Acid Tolerance Mechanism of *Pichia kudriavzevii* NBRC1279 and NBRC1664

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**Abstract:** Simultaneous saccharification and fermentation (SSF) has been investigated for the efficient production of ethanol because it has several advantages such as simplifying the manufacturing process, operating easily, and reducing energy input. Previously, using lignocellulosic biomass as source materials, we succeeded in producing ethanol by SSF with *Pichia kudriavzevii* NBRC1279 and NBRC1664. However, various acids that fermentation inhibitors are also produced by the hydrolysis of lignocellulosic biomass, and the extent to which these acids affect the growth and ethanol productivity of the two strains has not yet been investigated. In this study, to better understand the acid tolerance mechanism of the two strains, a spot assay, growth experiment, and transcriptome analysis were carried out using *Saccharomyces cerevisiae* BY4742 as a control. When the three strains were cultured in SCD medium containing 15 mM formic acid, 35 mM sulfuric acid, 60 mM hydrochloric acid, 100 mM acetic acid, or 550 mM lactic acid, only *P. kudriavzevii* NBRC1664 could grow well under all conditions, and it showed the fastest growth rates. The transcriptome analysis showed that "MAPK signaling pathway-yeast" was significantly enriched in *P. kudriavzevii* NBRC1664 cultured with 60 mM hydrochloric acid, and most genes involved in the high osmolarity glycerol (HOG) pathway were up-regulated. Therefore, the up-regulation of the HOG pathway may be important for adapting to acid stress in *P. kudriavzevii*. Moreover, the $\log_2$-transformed fold change value in the expression level of *Gpd1* was 1.3-fold higher in *P. kudriavzevii* NBRC1664 than in *P. kudriavzevii* NBRC1279, indicating that high *Gpd1* expression may be accountable for the higher acid tolerance of *P. kudriavzevii* NBRC1664. The transcriptome analysis performed in this study provides preliminary knowledge of the molecular mechanism of acid stress tolerance in *P. kudriavzevii*. Our data may be useful for future studies on methods to improve the tolerance of *P. kudriavzevii* to acids produced from lignocellulose hydrolysis.

**Keywords:** *Pichia kudriavzevii*; NBRC strain; SSF; acid tolerance; transcriptome; HOG pathway; *Gpd1*



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## 1. Introduction

Since the 2000s, the production of biofuel using biomass as a raw material has been studied because the amount of greenhouse gases in the atmosphere does not increase when biomass is consumed [1]. The fermentation of bioethanol is one of the most well-known examples of the utilization of biomass as a raw material. Bioethanol can be classified as first, second, or third-generation based on differences in the source materials or the production method. First-generation bioethanol is produced from food crops, which are different in the countries. For example, corn, sugarcane, and potato are used as raw materials in the United States, Brazil, and Europe, respectively [1]. Those raw materials are used because the carbon source required for the growth of the microorganisms used in bioethanol fermentation can be easily extracted. Using food crops as raw materials, more than 130 billion liters of ethanol are produced by fermentation worldwide, and the USA and Brazil are the

main suppliers [1]. However, nowadays, the utilization of food crops for fermentation is concerned with increasing global population growth and decreasing arable land area. Thus, the production method of second-generation bioethanol, which does not compete with food crop consumption, has been studied since the 2010s.

Second-generation bioethanol is produced from lignocellulosic biomass through the following process [2,3]: (1) hydrothermal treatment of particles from lignocellulosic biomass; (2) enzymatic hydrolysis of the hydrothermally treated samples to prepare the hydrolysate; and (3) fermentation of ethanol using the hydrolysate. Based on this process, we performed pilot-scale production of ethanol using a xylose-utilizing recombinant *Saccharomyces cerevisiae* MA-R4 [4], in which the *XYL1* and *XYL2* genes encoding xylose reductase and xylitol dehydrogenase from *Scheffersomyces* (*Pichia*) *stipitis*, and *XKS1* gene encoding xylulokinase from *S. cerevisiae* are expressed by chromosomal integration into the flocculent *S. cerevisiae* IR-2 [5], in 50 L scale solid mixer and 70 L scale fermenter [6]. As a result, 53.5 g/L of ethanol was successfully produced from hydrolysate prepared using Japanese eucalyptus particles after 72 h of fermentation, and the ethanol yield (82.2% of the theoretical yield) was comparable to that observed in laboratory-scale production. However, the operation time from the hydrothermal treatment of the Japanese eucalyptus particles to the fermentation of ethanol with the hydrolysate was 8 days, and the operation was complicated, which resulted in high production costs.

To simplify the production process and ease of operation, and reduce the required energy input, simultaneous saccharification and fermentation (SSF) has been performed for ethanol production using the thermostable yeast strains *Pichia kudriavzevii* NBRC1279 and NBRC1664 with particles from Japanese cedar or eucalyptus [7]. Compared to the above method, the manufacturing process is simpler because enzymatic hydrolysis and fermentation are simultaneously performed in the same vessel. However, the ethanol concentration when produced by SSF is only 21 to 24 g/L, thus the production yield needs to be improved for industrial use. The inhibitory effect of acids on fermentation is thought to be one of the reasons for the decreased production yield. When lignocellulosic biomass is hydrolyzed using cellulase and xylanase, mixed sugars are generated, and several kinds of inhibitors such as aldehydes (furfural and 5-hydroxymethylfurfural) and acids (acetic acid and formic acid) are also generated. In particular, large amounts of acetic acid and formic acid are generated, and they may inhibit the growth of both *P. kudriavzevii* NBRC1279 and NBRC1664 [8]. In ethanol production at the industrial level, hydrochloric acid or sulfuric acid is widely used for acid-based pretreatment methods, and dilute acid is added periodically during fermentation to maintain the optimal pH for ethanol production. Thus, residual acids inhibit fermentation by yeasts, and it is believed that the ethanol production yield can be enhanced by improving the acid tolerance of *P. kudriavzevii* NBRC1279 and NBRC1664, since the strains would be less susceptible to growth inhibition by the organic and inorganic acids. In this regard, we have previously isolated and characterized a novel gene (*GAS1*) encoding glycosylphosphatidylinositol (GPI)-anchored protein that confers low-pH and salt tolerance in *S. cerevisiae* BY4742 by screening a genomic DNA library of *P. kudriavzevii* NBRC1279 [9]. The IoGas1 protein may be involved in maintaining cell wall integrity during environmental stress. Moreover, the tolerance mechanism of acetic acid [10] and lactic acid [11] stresses are investigated by transcriptome analyses, and both tolerances have been reported to convert those organic acids to secondary metabolites by activating various metabolic pathways including the pyruvate metabolic pathway. On the other hand, to date, no detailed comparative analysis has been performed on how *P. kudriavzevii* NBRC1279 and NBRC1664 are acid-tolerant mechanisms to various acids.

In this study, to better understand the acid tolerance mechanism of *P. kudriavzevii* NBRC1279 and NBRC1664, acid tolerance tests and a transcriptome analysis were carried out using *S. cerevisiae* BY4742 as a control. The results of the present study provide useful knowledge for better understanding the acid tolerance mechanism of *P. kudriavzevii* NBRC1279 and NBRC1664, and are expected to be useful in the development of more useful strains for ethanol production by SSF.

## 2. Materials and Methods

### *2.1. Yeast Strains and Media*

To investigate the acid tolerance mechanism in *P. kudriavzevii* NBRC1279 and NBRC1664, these yeast strains and *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) as control strain were used. The two strains of *P. kudriavzevii* (NBRC1279 and NBRC1664) were purchased from the NITE Biological Resource Center (NBRC, Chiba, Japan), and *S. cerevisiae* BY4742 was obtained from Open Biosystems (Huntsville, AL, USA). Three yeast strains were grown in yeast peptone dextrose (YPD) broth or on agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) unless otherwise noted. For spot assay and aerobic growth experiment, these three yeast strains were grown in synthetic complete (SC) minimal medium (6.7 g/L yeast nitrogen base without amino acids) supplemented with the appropriate amino acids and nucleic acids [12], and 20 g/L glucose (SCD medium).

### *2.2. Spot Assay*

The acid sensitivity of *P. kudriavzevii* NBRC1279 and NBRC1664 as well as *S. cerevisiae* BY4742 was determined by the standard drop test technique. Yeast cultures were cultivated in SCD medium until the cells reached an early stationary phase of growth, and then they were diluted with fresh SCD medium to an absorbance at 600 nm ($A_{600}$) of 0.02. Next, 2 µL of each suspension of 3-fold serial dilutions of *P. kudriavzevii* NBRC1279 and NBRC1664, and *S. cerevisiae* BY4742 were spotted onto SCD plates containing different acids (15 mM formic acid, 35 mM sulfuric acid, 60 mM hydrochloric acid, 100 mM acetic acid, or 550 mM lactic acid). The plates were photographed after 2 to 6 days of incubation at 30 °C.

### *2.3. Aerobic Growth Experiment*

The three yeast strains were pre-cultivated aerobically in SCD medium (pH 5.8) at 30 °C for 16 h. The cells were then washed with sterile water, and inoculated into an SCD medium containing the same concentration as a spot assay of acidic reagents (formic acid, sulfuric acid, hydrochloric acid, acetic acid, or lactic acid) in 96-well plates at an initial $A_{600}$ of 0.02. All 96-well microplates were cultivated with mild agitation (150 rpm) at 30 °C, and the absorbance ($A_{600}$) was measured using a HiTS microplate reader (Scinics, Tokyo, Japan) as described previously [13]. Cultivation was repeated three times.

### *2.4. RNA Preparation*

The three yeast strains were cultivated separately in an SCD medium containing 60 mM hydrochloric acid at 30 °C for 12 h. To prepare sufficient amounts of RNA for transcriptome analysis, three yeast strains were grown in 100 mL of the medium. After the cells were washed with sterile water, the total RNA was extracted using Yeast Processing Reagent (Takara Bio, Shiga, Japan) and NucleoSpin RNA (Takara Bio) according to the manufacturer's instructions. The concentration and quality of the resulting RNA were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively.

### *2.5. Transcriptome Analysis*

The extracted total RNA samples were subjected to quality tests, including assessments of the RNA concentration, RNA integrity number, as well as contamination by DNA, protein, and salt ions. Subsequently, the mRNA was enriched using magnetic beads with Oligo dT, then an appropriate amount of stop reagent was added to the fragments. A cDNA library was constructed using the mRNA as a template, and was sequenced using the BGISEQ-500 platform.

To obtain high-quality cleaned reads, quality control was carried out on the raw sequencing reads. After the reads containing 5% or more unknown bases, adaptor-polluted reads, and low-quality reads were removed, the remaining reads were used as clean reads for the subsequent experimental studies. De novo assembly was performed by Trinity v2.0.6 (Parameters: --min_contig_length150 --CPU8--min_kmer_cov3--min_glue3--bfly_opts'-V5--

edge-thr=0.1-- stderr') [14] using the cleaned reads. Differentially expressed genes (DEGs) were detected by PossionDis (Parameters: Fold Change $\geq$ 2.00, False discovery rate (FDR) $\leq$ 0.001) [15]. Functional annotation was performed using gene ontology (GO) enrichment (Parameters: default) [16] and Kyoto Encyclopedia of Genes and Genomes (KEGG; Parameters: default) [17] pathways. Based on the results of the functional annotation, DEGs were classified. After calculating the FDR for each *p*-value, the gene was enriched when the FDR was greater than 0.01.

## 3. Results and Discussion

### *3.1. Effect of Different Acids on Growth*

To investigate the acid tolerance of *P. kudriavzevii* NBRC1279 and NBRC1664 as well as *S. cerevisiae* BY4742 (a control strain), these strains were grown on SCD plates containing multiple acids, including formic acid, sulfuric acid, hydrochloric acid, acetic acid, or lactic acid (Figure 1). Among the three strains, only *P. kudriavzevii* NBRC1664 grew well under all conditions, while *P. kudriavzevii* NBRC1279 showed weak growth, and *S. cerevisiae* BY4742 was unable to grow on all plates. These results indicated that the acid tolerance of *P. kudriavzevii* NBRC1664 was stronger than that of *P. kudriavzevii* NBRC1279.

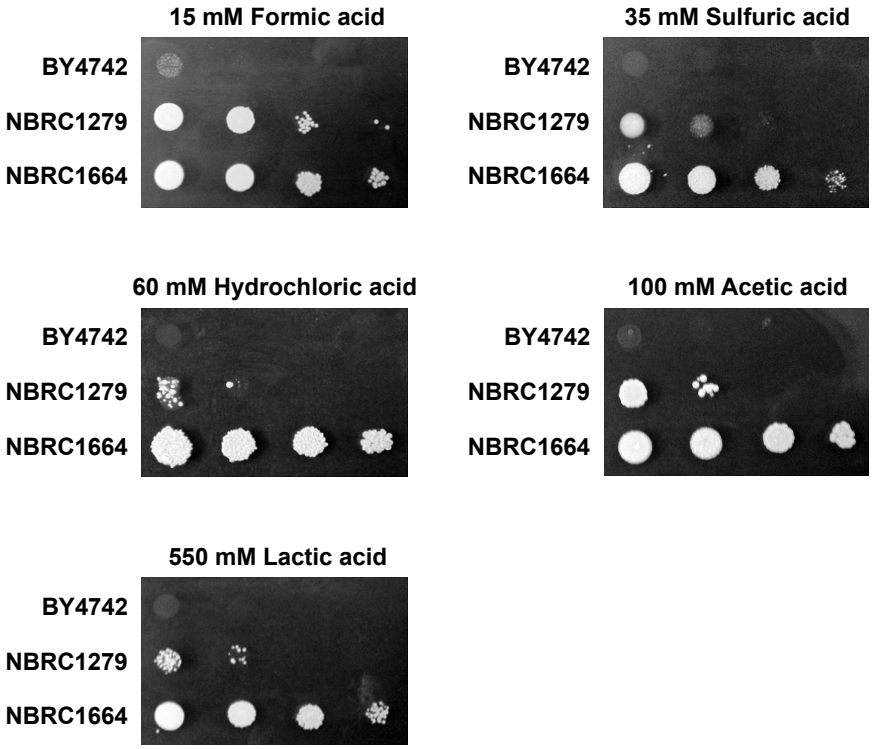


**Figure 1.** Growth phenotypes of the three yeast strains in the presence of different acids. Aliquots (2 mL) of 3-fold serial dilutions (starting from the absorbance of 0.1 at 600 nm) of the wild-type *S. cerevisiae* BY4742 and *P. kudriavzevii* NBRC1279 and NBRC1664 were spotted onto SCD plates containing various acids (15 mM formic acid, 35 mM sulfuric acid, 60 mM hydrochloric acid, 100 mM acetic acid, and 550 mM lactic acid). The various plates then were incubated at 30 °C for 2, 3, 6, 2, or 3 days, respectively.

To further investigate the acid tolerance of *P. kudriavzevii* NBRC1279 and NBRC1664 as well as *S. cerevisiae* BY4742, the strains were cultured aerobically in SCD media containing various acids (Figure 2). The reason why the acid concentrations in the liquid media for aerobic cultivation were the same as that in the spot assay was to further verify whether *P. kudriavzevii* NBRC1664 had better acid tolerance than *P. kudriavzevii* NBRC1279 even in liquid media at these acid concentrations. Under all conditions, the growth of *S. cerevisiae* BY4742 was markedly inhibited, which was consistent with the results of the spot

assay (Figure 1). When cultured in SCD medium containing 15 mM formic acid, *P. kudriavzevii* NBRC1279 and NBRC1664 showed no growth inhibition. When cultured in SCD medium containing 100 mM acetic acid or 550 mM lactic acid, *P. kudriavzevii* NBRC1279 and NBRC1664 showed similar growth curves, although the time to reach the stationary phase differed. In contrast, when cultured in SCD medium containing 60 mM hydrochloric acid or 35 mM sulfuric acid, *P. kudriavzevii* NBRC1279 showed slight growths (the $A_{600}$ values increased to about 0.07), while *P. kudriavzevii* NBRC1664 showed more growth in the medium containing 60 mM hydrochloric acid than in that containing 35 mM sulfuric acid. *P. kudriavzevii* NBRC1664 showed faster growth rates than *P. kudriavzevii* NBRC1279, except in the medium with 550 mM lactic acid (Table 1). Notably, the growth rate of *P. kudriavzevii* NBRC1664 was more than 16-fold higher than that of *P. kudriavzevii* NBRC1279 in SCD medium with 60 mM hydrochloric acid. In any event, *P. kudriavzevii* NBRC1664 grew better than *P. kudriavzevii* NBRC1279 on these acidic media.

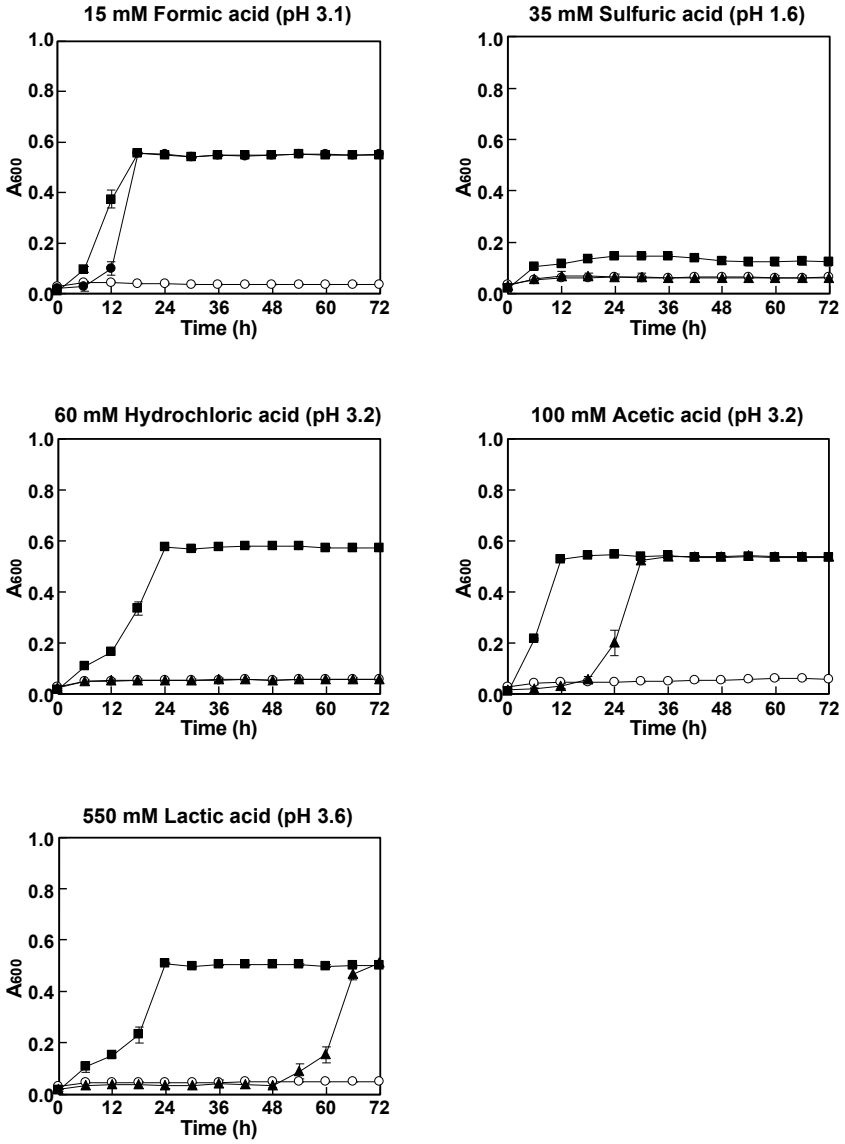


**Figure 2.** Growth curves of the three yeast strains cultured in the presence of various acids. The aerobic growth of *P. kudriavzevii* NBRC1279 (closed triangles) and NBRC1664 (closed squares) as well as *S. cerevisiae* BY4742 (open circles) in SCD medium containing 15 mM formic acid, 35 mM sulfuric acid, 60 mM hydrochloric acid, 100 mM acetic acid, and 550 mM lactic acid was measured over 72 h by assaying the absorbance at 600 nm. Error bars indicate the standard error (n = 3). Values are the means of three independent experiments.

**Table 1.** Growth rates ($h^{-1}$) of the three yeast strains at log phase when cultured in the presence of various acids.

| Yeast | 15 mM Formic Acid | 35 mM Sulfuric Acid | 60 mM Hydrochloric Acid | 100 mM Acetic Acid | 550 mM Lactic Acid |
|---|---|---|---|---|---|
| *S. cerevisiae* BY4742 | 0.0028 | 0.0080 | 0.0040 | 0.0064 | 0.0010 |
| *P. kudriavzevii* NBRC1279 | 0.243 | 0.0049 | 0.00570 | 0.161 | 0.138 |
| *P. kudriavzevii* NBRC1664 | 0.288 | 0.0177 | 0.0945 | 0.520 | 0.132 |

Based on the observed growth phenotypes (Figure 1), growth curves (Figure 2), and growth rates (Table 1), *P. kudriavzevii* NBRC1279 and NBRC1664 were shown to have superior acid tolerance compared to *S. cerevisiae* BY4742. Moreover, it appears that *P. kudriavzevii* NBRC1664 has higher acid tolerance than *P. kudriavzevii* NBRC1279 because *P. kudriavzevii* NBRC1664 showed remarkable growths in the presence of inorganic acids such as hydrochloric acid and sulfuric acid and the time to reach the stationary phase is short in the presence of organic acids. A higher yield of ethanol is expected using *P. kudriavzevii* NBRC1664 than *P. kudriavzevii* NBRC1279 when performing SSF with acid-based pretreatment methods, which is consistent with the results of our previous study that carried out SSF with particles from Japanese cedar or eucalyptus as raw materials [7]. Thus, in the future, we will use the *P. kudriavzevii* NBRC1664 strain to develop SSF with acid-based pretreatment methods.

### 3.2. Transcriptome Analysis of Yeast Strains under Hydrochloric Acid Stress

To investigate the differences in the acid tolerance mechanism under acid stress of *P. kudriavzevii* NBRC1279 and NBRC1664, the three yeast strains cultured in SCD media containing 60 mM hydrochloric acid were used for transcriptome analyses (Figure 3) because hydrochloric acid made the most significant differences in growth phenotypes (Figure 1), growth curves (Figure 2) and growth rates (Table 1) among the acids used in our experiment. Furthermore, hydrochloric acid is used in the pretreatment method of particles from lignocellulosic biomass and as a pH adjustment solution for fermentation of ethanol at the industrial level. Therefore, we thought that it would be advantageous to perform a comprehensive analysis of genes related to acid tolerance between *P. kudriavzevii* NBRC1279 and NBRC1664 using hydrochloric acid as a representative acid in this experiment.

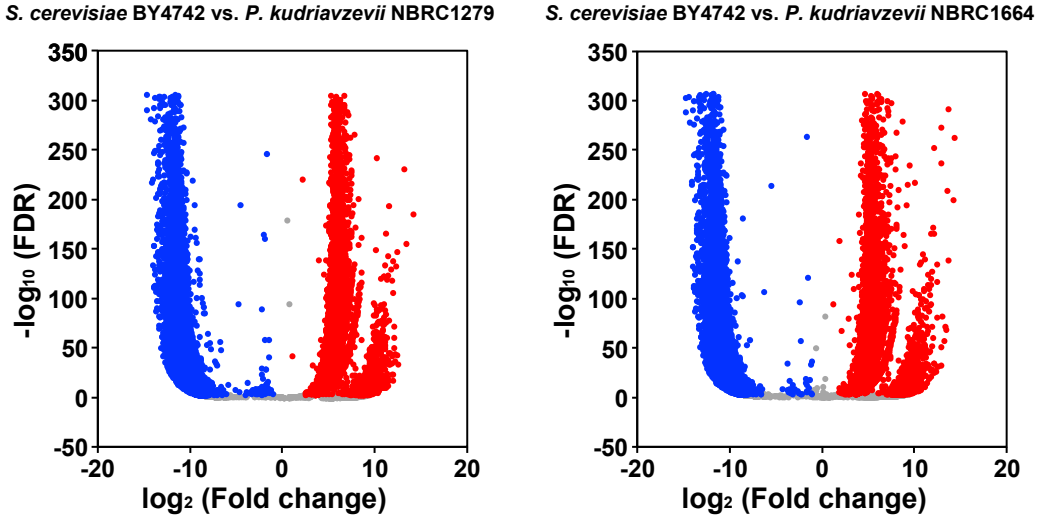


**Figure 3.** Volcano plot of the DEGs. The *y* and *x* axes show the $-\log_{10}$-transformed significance and $\log_2$-transformed fold change, respectively. Red, blue, and gray points represent up-regulated DEGs, down-regulated DEGs, and not significantly differently regulated DEGs, respectively.

In total, 15,644 DEGs with significantly different expression levels were identified between *S. cerevisiae* BY4742 and *P. kudriavzevii* NBRC1279; 5633 were up-regulated DEGs, and 10,011 were down-regulated DEGs. Similarly, 15,338 DEGs were identified between *S. cerevisiae* BY4742 and *P. kudriavzevii* NBRC1644; 5349 were up-regulated DEGs and 9989 were down-regulated DEGs.

Subsequently, to obtain functional information on the DEGs, we performed GO enrichment analysis, which provides three types of descriptions for gene products, namely, biological process (BP), cellular component (CC), and molecular function (MF). Using the GO enrichment analysis data, KEGG pathway analysis was carried out (Figure 4). From the comparisons of *S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1279 and *S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1664, 14 enriched pathways were identified to be in common for both *P. kudriavzevii* NBRC1279 and NBRC1664, namely, pathways related to BP ["basal transcription factors", "biosynthesis of amino acids", "biosynthesis of secondary metabolites", "citrate cycle (TCA cycle)", "glycerophospholipid metabolism", "longevity regulating pathway-multiple species", "mitophagy-yeast", "phagosome", "proteasome", "RNA degradation", and "RNA transport"], CC ("MAPK signaling pathway-yeast", and "protein processing in endoplasmic reticulum"), and MF ("oxidative phosphorylation"). In particular, "MAPK signaling pathway-yeast" was significantly enriched ($p$-value < 0.00045).

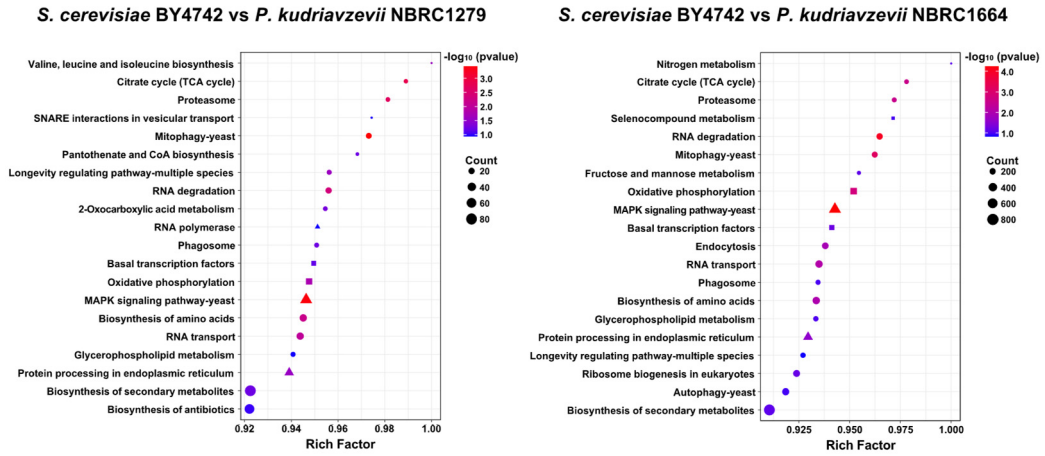


**Figure 4.** KEGG pathway enrichment analysis. The $y$ and $x$ axes show the KEGG pathways and rich factors, respectively. The depth of the colors shows the significance of the enrichment. The pathways related to BP, CC, and MF are indicated as circles, triangles, and squares, respectively.

In yeast cells, the MAPK signaling pathway is activated through a cascade of three sequentially activated kinases when the cells are exposed to environmental stress, resulting in the up-regulation of transcription factors and the expression of specific sets of genes to respond to the stress. For example, in *S. cerevisiae* cells, the MAPK signaling pathway is involved in cell-wall integrity, hyperosmotic adaptation, mating response, pseudohyphal development, and sporulation [18,19]. In the draft DNA genomes of *P. kudriavzevii* NBRC1279 and NBRC1664, the gene sets for the MAPK signaling pathway are conserved, which indicates that both strains have a similar mechanism of acid tolerance when compared to the mechanisms of other known yeasts.

### *3.3. Involvement of the HOG Pathway in Acid Stress Tolerance*

The high osmolarity glycerol (HOG) pathway is one of the most well-studied MAPK pathways [18]. When the dissolved solute concentration in the extracellular medium is higher than the internal osmotic pressure in the cell, in the absence of mechanisms to restore osmotic balance, cell growth becomes severely inhibited, which can lead to cell death. As a means of counteracting extracellular hypertonic stress, yeast cells increase the synthesis of glycerol through the HOG pathway to increase the internal osmotic density. The HOG pathway consists of two upstream osmotic sensors (SLN1 and SHO1), and a downstream

MAPK cascade (SSK2/SSK22 and STE11 MAPKKK, PBS2 MAPKK, and HOG1 MAPK) [19]. In response to osmostress, signals are transduced from Sln1 and Sho1, which are integrated by Pbs2 to activate HOG1. Ultimately, the activated HOG proteins are rapidly translocated into the nucleus, where they promote the expression of osmo-responsive genes through several transcription factors. To examine the differences between *P. kudriavzevii* NBRC1279 and NBRC1664, the expression levels of genes related to the HOG pathway were compared between the two strains (Table 2).

**Table 2.** Differentially expressed genes related to the HOG pathway in strains cultured with 60 mM hydrochloric acid.

| | | ***S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1279** | | ***S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1664** | |
|---|---|---|---|---|---|
| **Gene** | **Description** | **Regulation** | **$\log_2$ (Fold Change)** | **Regulation** | **$\log_2$ (Fold Change)** |
| *Hkr1* | Signaling mucin HKR1 | Up | 7.27 | Up | 4.73 |
| *Sho1* | Osmosensor SHO1 | Up | 4.75 | Up | 3.39 |
| *Opy2* | Protein OPY2 | Down | −11.5 | Down | −11.5 |
| *Cdc42* | Cell division control protein 42 | Down | −11.3 | Down | −11.3 |
| *Cdc24* | Cell division control protein 24 | Down | −10.3 | Down | −11.2 |
| *Ste20* | p21-Activated kinase1 | Up | 5.53 | Up | 4.97 |
| *Cla4* | Serine/threonine-protein kinase CLA4 | Up | 6.07 | Down | −10.4 |
| *Ste11* | Mitogen-activated protein kinase kinase kinase | Up | 5.85 | Up | 5.01 |
| *Ste50* | Protein STE50 | Up | 5.21 | Up | 4.43 |
| *Pbs2* | Mitogen-activated protein kinase kinase | Up | 10.1 | Up | 9.06 |
| *Hog1* | Mitogen-activated protein kinase HOG1 | Up | 6.55 | Up | 5.97 |
| *Sln1* | Sensor histidine kinase SLN1 | Up | 5.49 | Up | 4.75 |
| *Ypd1* | Phosphorelay intermediate protein YPD1 | Up | 5.54 | Up | 4.60 |
| *Ssk1* | Mitogen-activated protein kinase kinase kinase SSK1 | Up | 7.38 | Up | 6.56 |
| *Ssk2* | Mitogen-activated protein kinase kinase kinase SSK2 | Up | 6.46 | Up | 5.68 |
| *Ssk22* | Mitogen-activated protein kinase kinase kinase SSK22 | Down | −10.3 | Down | −10.3 |
| *Ptp2* | Tyrosine protein phosphatase PTP2 | Down | −13.0 | Down | −13.0 |
| *Ptp3* | Tyrosine protein phosphatase PTP3 | Down | −9.93 | Down | −9.93 |
| *Hot1* | High-osmolarity-induced transcription protein 1 | Down | −10.1 | Down | −10.1 |
| *Smp1* | Transcription factor SMP1 | Up | 6.38 | Up | 5.51 |
| *Gpd1* | $NAD^+$-dependent glycerol-3-phosphate dehydrogenase | Up | 10.4 | Up | 13.1 |

When cultivated in SCD medium containing 60 mM hydrochloric acid, *Hkr1*, *Sho1*, *Ste20*, *Ste11*, *Ste50*, *Pbs2*, *Hog1*, *Sln1*, *Ypd1*, *Ssk1*, *Ssk2*, *Smp1*, and *Gpd1* were up-regulated in both *P. kudriavzevii* NBRC1279 and NBRC1664. This indicated that the acid tolerance of *P. kudriavzevii* NBRC1279 and NBRC1664 may be attributable to the increased expression

levels of the genes involved in the HOG pathway. When the $log_2$-transformed fold change values of the up-regulated genes of both strains, except for *Gpd1*, the values of *P. kudriavzevii* NBRC1279 were 1.1–1.5-fold higher than those of *P. kudriavzevii* NBRC1664. Similarly, the $log_2$-transformed fold change value of *GAS1* of *P. kudriavzevii* NBRC1279 was 1.3-fold higher than that of *P. kudriavzevii* NBRC1664. Those results indicated that *P. kudriavzevii* may show acid resistance through the utilization of the HOG pathway proteins, in addition to the expression of *GAS1*. On the other hand, the phenotypic analyses showed that *P. kudriavzevii* NBRC1664 has a superior acid tolerance compared to *P. kudriavzevii* NBRC1279. Based on those results, we considered that *P. kudriavzevii* NBRC1664 may show superior acid tolerance by the expression of *Gpd1*.

### *3.4. Effect of Gpd1 Expression on Acid Tolerance*

When acetic acid-responsive transcriptional activators, which are encoded as *HAA1* or *PPR1*, are overexpressed in *S. cerevisiae* cells, acetic acid tolerance is enhanced [20–22]. Similarly, ethanol production is improved under acetic acid stress by overexpression of *SET*, which encodes histone methyltransferase [22]. *HAA1*, *PPR1* and *SET* are conserved in both *P. kudriavzevii* NBRC1279 and NBRC1664, but those genes were down-regulated under the hydrochloric acid stress (Table 3). Those results indicated that the acetic acid tolerance mechanisms reported in *S. cerevisiae* may be present in both *P. kudriavzevii* NBRC1279 and NBRC1664, but those mechanisms do not function in both strains under the hydrochloric acid stress.

**Table 3.** Differentially expressed genes related to acid tolerance mechanism in strains cultured with 60 mM hydrochloric acid.

| | | ***S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1279** | | ***S. cerevisiae* BY4742 vs. *P. kudriavzevii* NBRC1664** | |
|---|---|---|---|---|---|
| **Gene** | **Description** | **Regulation** | **$log_2$ (Fold Change)** | **Regulation** | **$log_2$ (Fold Change)** |
| *HAA1* | Transcriptional activator | Down | −11.9 | Down | −11.9 |
| *PPR1* | Transcriptional activator | Down | −10.3 | Down | −9.1 |
| *SET1* | Histone methyltransferase | Down | −10.2 | Down | −10.1 |

To further examine the mechanism for the higher acid tolerance of *P. kudriavzevii* NBRC1664, we focused on *Gpd1*. *Gpd1* encodes $NAD^+$-dependent glycerol-3-phosphate dehydrogenase, which catalyzes the production of glycerol-3-phosphate as the precursor of glycerol [23]. Glycerol is an important metabolite of alcoholic fermentation, and it is involved in the maintenance of the intracellular osmotic balance needed for growth under acid stress. According to Remize et al., in the *S. cerevisiae* cells, overexpression of *Gpd1* decreases ethanol production and increases glycerol production [24]. Overexpression of *Gpd1* may alter cell numbers in response to the acetaldehyde concentration during the growth phase, which promotes glycerol production. A similar character is confirmed in Δ*Ald6* mutants of *S. cerevisiae*, the glycerol concentration was enhanced by overexpression of *Gpd1* [25]. On the other hand, when the native strain and Δ*Gpd1* mutants of *S. cerevisiae* were cultured in YNB media containing 100 g/L glucose as a carbon source, the glycerol concentration of Δ*Gpd1* mutants was 1.8-fold lower than that of the native strain, whereas the ethanol concentration of Δ*Gpd1* mutants was 1.1-fold higher than that of native strain [26]. Thus, an increase in *Gdp1*-expression levels is considered one of the means to increase intracellular glycerol levels. Comparison of the up-regulated DEGs between *P. kudriavzevii* NBRC1279 and NBRC1664 showed that the $log_2$-transformed fold change in the expression level of *Gpd1* was 1.3-fold higher in *P. kudriavzevii* NBRC1664 than in *P. kudriavzevii* NBRC1279 (Table 1). However, the amino acid sequence of $NAD^+$-dependent glycerol-3-phosphate dehydrogenase is the same in *P. kudriavzevii* NBRC1279

and NBRC1664, which indicated that the catalytic capacity of both enzymes is the same. Thus, we considered that the superior acid tolerance of *P. kudriavzevii* NBRC1664 is attributed to the increase in *Gdp1*-expression levels. Moreover, *P. kudriavzevii* NBRC1664 may have higher promoter activity than *P. kudriavzevii* NBRC1279 for the expression of *Gpd1*. To identify the promoter of *P. kudriavzevii* NBRC1664, we are now planning to perform a reporter analysis of the promoter region by the methods of Fujii et al. [27]. In this method, the promoter sequence can be identified by measuring the enzyme activity using each promoter sequence prepared to any length. We consider that a metabolic flux analysis is also needed. Based on a metabolic flux analysis, it may be possible to increase glycerol production without decreasing ethanol production by reducing the balance of carbon influx into other metabolic pathways [28]. These results will be described elsewhere in the future.

## 4. Conclusions

In this study, using the acids that are produced when lignocellulosic biomass is hydrolyzed, we performed a spot assay and growth experiment to examine the acid tolerance of *P. kudriavzevii* NBRC1279 and NBRC1664 as well as *S. cerevisiae* BY4742. Among the three strains, only *P. kudriavzevii* NBRC1664 could grow well under all conditions, and it showed the fastest growth rates. In particular, *P. kudriavzevii* NBRC1664 showed the fastest growth rate in SCD medium containing 60 mM hydrochloric acid or 35 mM sulfuric acid. To examine the differences in the acid tolerance mechanism of *P. kudriavzevii* NBRC1279 and NBRC1664, a transcriptome analysis was carried out using cells cultivated under acid stress with 60 mM hydrochloric acid, and the results revealed that "MAPK signaling pathway-yeast" was significantly enriched in both strains. Among the MAPK signaling pathways, we found that the expression levels of most genes involved in the HOG pathway were up-regulated, and that the $\log_2$-transformed fold change in the expression level of *Gpd1* was 1.3-fold higher in *P. kudriavzevii* NBRC1664 than in *P. kudriavzevii* NBRC1279 (Table 1), which may be among the main reasons why *P. kudriavzevii* NBRC1664 showed higher tolerance to various acids.

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