

Contribution of YPRO15C Overexpression to the Resistance of *Saccharomyces cerevisiae* BY4742 Strain to Furfural Inhibitor

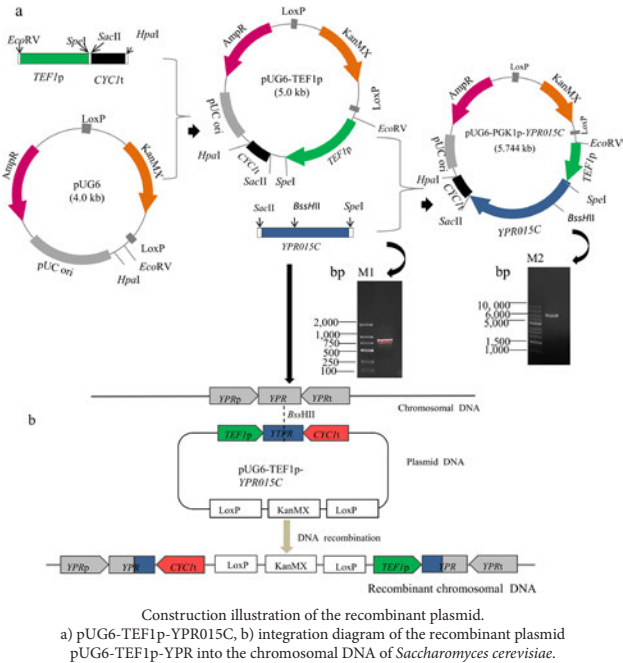
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Abstract

Lignocellulosic biomass is still considered a feasible source of bioethanol production. *Saccharomyces cerevisiae* can adapt to detoxify lignocellulose-derived inhibitors, including furfural. Tolerance of strain performance has been measured by the extent of the lag phase for cell proliferation following the furfural inhibitor challenge. The purpose of this work was to obtain a tolerant yeast strain against furfural through overexpression of *YPR015C* using the *in vivo* homologous recombination method. The physiological observation of the overexpressing yeast strain showed that it was more resistant to furfural than its parental strain. Fluorescence microscopy revealed improved enzyme reductase activity and accumulation of oxygen reactive species due to the harmful effects of furfural inhibitor in contrast to its parental strain. Comparative transcriptomic analysis revealed 79 genes potentially involved in amino acid biosynthesis, oxidative stress, cell wall response, heat shock protein, and mitochondrial-associated protein for the *YPR015C* overexpressing strain associated with stress responses to furfural at the late stage of lag phase growth. Both up- and down-regulated genes involved in diversified functional categories were accountable for tolerance in yeast to survive and adapt to the furfural stress in a time course study during the lag phase growth. This study enlarges our perceptions comprehensively about the physiological and molecular mechanisms implicated in the *YPR015C* overexpressing strain's tolerance under furfural stress.



Key words: *Saccharomyces cerevisiae*, furfural, overexpressed *YPR015C*, tolerance, transcriptomics

Introduction

The production of biofuel and biochemicals using lignocellulose-based techniques has a promising possibility of reducing greenhouse gas emissions, bring-

ing benefits to rural economies, and advancing energy security (De Bhowmick et al. 2018; Patel et al. 2019). Due to its highly effective hexose fermentation ability, the *Saccharomyces cerevisiae* yeast is one of the most well-known host microorganisms in the fermentative

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food sector. It is also the appropriate microorganism for the generation of biofuels. However, the native *S. cerevisiae* strain cannot utilize xylose, the second most abundant carbohydrate in lignocellulosic-derived inhibitor hydrolysate (Ask et al. 2013). The recalcitrant nature of lignocellulosic biomass, on the other hand, poses a technological challenge to release fermentable sugars; consequently, a major bottleneck for the production of renewable from them (De Bhowmick et al. 2018; Singh et al. 2018). They constrain very high inhibition of microbial proliferation and fermentation processes (Singh et al. 2018; Sankaran et al. 2020). Lignocellulosic biomasses are often pretreated before further processing (Kumar et al. 2020; Padmapriya et al. 2021). The numerous commonly used pretreatment methods, including alkaline, ammonia fiber expansion, and dilute acid hydrolysis, usually generate various chemical byproducts' inhibition and inhibitory compounds (Kumar et al. 2020; Padmapriya et al. 2021). Furfural is a commonly encountered inhibitor that affects microbial growth by causing intracellular acidification and reducing enzyme activities and protein and ribonucleic acid (RNA) syntheses, breaks in deoxyribonucleic acid (DNA), and accumulating reactive oxygen species (Ask et al. 2013; Moreno et al. 2019).

Overexpression of target genes in yeast strains from haploid laboratory background has been used to establish molecular-based genetic techniques to improve strain tolerance to lignocellulosic hydrolysate (Gorsich et al. 2006; Park et al. 2011). Transcriptomic characterization of overexpressed target genes of laboratory *S. cerevisiae* strains has demonstrated associated tolerance with improved proliferation under the challenge of furfural inhibitors through NAD(P)H-dependent reductions (Li et al. 2015; Zhao et al. 2015). Transcriptomic analysis of overexpressed diploid and haploid yeast strains of *S. cerevisiae*, known to survive lignocellulose hydrolysates inhibition, revealed elevated levels of expression of *SFA1*, *ADH6*, and *ADH2* genes (Zhu et al. 2020). Additionally, it has been proven that yeast of *S. cerevisiae* clones overexpressing *ADH6*, *ADH7*, and *ADH1* genes can endure in the presence of furfural (Petersson et al. 2006; Laadan et al. 2008; Liu et al. 2008). The stress growth response of the overexpressing yeast strains to furfural was evaluated, and a lag phase length was used to determine the strain tolerance level. It is common for *S. cerevisiae* strains to undergo a lag phase of decreased cell proliferation after challenges with furfural and HMF inhibitors. However, cell growth and fermentation of the overexpressing yeast strains accelerated compared to its parental strain (Zhao et al. 2015).

Due to comparative transcriptomic analysis, our previous study discovered an uncharacterized gene, *YPR015C* of *S. cerevisiae*, to be significantly up-regulated under furfural stress condition. Furthermore, BLAST

analysis of the protein encoded by *YPR015C* revealed that it has conserved domains similar to transcription factors from the zinc-finger family. Therefore, we concluded that *YPR015C* is a putative transcription factor of the zinc-finger family. However, it is unknown whether *YPR015C* contributes to furfural inhibitor stress tolerance. Herein, the complete fragment of the coding sequence of *YPR015C* was successfully amplified by PCR. The homologous recombination method was used to establish the *YPR015C* overexpressing strain. We identified differences in phenotypic traits and transcriptional expression between the *YPR015C* overexpressing strain and its parental strain that exhibit different stress tolerance capacities under furfural challenge in time course study during the lag phase growth. Our findings provide information about the molecular mechanisms of *S. cerevisiae* strain tolerance to the lignocellulose-derived furfural inhibitor. This knowledge can be applied to aid engineering efforts to improve tolerant strains.

Experimental

Materials and Methods

Strain, plasmid, and media. The BY4742 yeast genomic DNA (MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0) from Open Biosystems, Inc. (USA) was used for the amplification of *YPR015C* using the primers *YPR015C_F* and *YPR015C_R* (Table SI). *Escherichia coli* DH5 was used as the host for gene cloning and modification. To select recombinant plasmids, competent *E. coli* DH5 cells were cultured on solid Luria-Bertani (LB) medium (w/v, 0.5% yeast extract, 1% peptone, 1% NaCl, pH 7.0, 2% agar) supplemented with 100 mg/l ampicillin. To cultivate yeast strains, yeast peptone dextrose (YPD) medium (w/v, 1% yeast extract, 2% peptone, 20% glucose) medium was used. E.Z.N.A.[®] Yeast DNA Kit (Omega Bio-Tek, Inc., USA) was used for DNA extraction. SanPrep Column Plasmid Mini-Preps Kit and San Prep Column DNA gel extraction kit (Sangon Biotech (Shanghai) Co., Ltd., China) were used for plasmid and gel extraction, respectively. Cycle-Pure Kit was purchased from Omega Bio-Tek, Inc. (USA) for the purification of PCR products. Transformation of *E. coli* was performed using the electroporation method as described (Benatuil et al. 2010), which was also used for the *S. cerevisiae* transformation to overexpress *YPR015C* in the parental *S. cerevisiae* BY4742 strain. The media ingredients, cofactors, aldehydes, kits and reagents utilized in this work were bought from Sangon Biotech (Shanghai) Co., Ltd. (China).

Construction of plasmids and recombinant yeast strain. The complete ORF of the putative transcriptional factor *YPR015C* was amplified by polymerase

chain reaction (PCR) using the extracted genomic DNA from the *S. cerevisiae* BY4742 (MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0) as a template with the primer pairs YPR015C_F and YPR015C_R, containing homologous arms (Table SI). The PCR conditions were: 5 minutes at 95°C, 35 cycles of 1 minutes at 95°C, 30 seconds at 54.1°C, and 3 minutes 27 seconds at 72°C, and a final extension cycle at 72°C for 10 minutes.

The TEF1 promoter and CYC1 terminator were added to the loxP-KanMX-loxP cassette containing plasmid pUG6 as described (Güldener et al. 1996). The linearized recombinant plasmid pUG6-TEF1p-CYCt1 was obtained by digesting the circular vector with SpeI and SacII. The homologous sequence ends of the linearized vector and amplified YPR015C are identical. The linearized plasmid pUG6-TEF1p and purified YPR015C were mixed by in vivo homologous recombination using vazymeCloneExpress II one-step cloning kit and cultured with *Exnase* II at 37°C for 30 minutes for recombination process and to clear the way two linearized DNA cyclization in vitro (Fig. S2a). The designed recombinant plasmid was transformed into a competent *E. coli* DH5 α host cell in LB solid media supplemented with 100 mg/l ampicillin for cloning and isolation of the constructed recombinant plasmid using the electroporation method (Benatuil et al. 2010).

Positive transformants containing recombinant plasmids pUG6-TEF1p-YPR015C were incubated in LB solid media containing 100 mg/l ampicillin. Positive single colonies were picked from a solid LB plate medium and cultured into fresh liquid LB (w/v, 0.5% yeast extract, 1% peptone, 1% NaCl, pH 7.0) supplemented with 100 mg/l ampicillin. The size of the constructed recombinant plasmid was confirmed using the PCR recombinant plasmid product as a template (Fig. S1b). The accurate insertion of the YPR015C small fragment was confirmed by PCR validation with the recombinant plasmid pUG6-TEF1p-YPR015C and the primer pair iTEF1p-YPR015C_Fa located at the TEF1 promoter region and iTEF1p-YPR015C_Ra located at the YPR015C region (Fig. S1c). The recombinant plasmid pUG6-TEF1p-YPR015C was digested by the BssHII enzyme and then transformed into the *S. cerevisiae* strain BY4742 for chromosomal DNA integration via electroporation method (Fig. S2b). Positive single colonies were picked on solid YPD (w/v, 1% yeast extract, 2% peptone, 20% glucose, 2% agar) plates with 200 μ g/ml of G418. Verification of the accurate insertion of the YPR015C small fragment into the chromosome of the *S. cerevisiae* strain BY4742 was confirmed by PCR product of amplified fragment of YPR015C gene (Fig. S1c). The resulting recombinant strain was named BY4742-YPR015C.

Cell growth response. For cell proliferation, the YPR015C overexpressing strain was incubated in liquid

YPD medium (w/v, 2% peptone 1% yeast extract, 20% glucose) at 30°C overnight with shaking of 200 rpm. Furfural was added to the freshly prepared 40 ml of YPD medium at a final concentration of 35 mM after yeast cells OD₆₀₀ reached around 0.09 (time 0 h). According to the pretest, 30, 35, and 40 mM of furfural were used to determine the furfural concentration that would be used in this study. Using a Lambda 35 UV/VIS spectrophotometer (PerkinElmer Inc., USA), absorbance at OD₆₀₀ was measured to track the growth of the yeast strain's cells. Under each experiment, the trials were repeated at least three times.

Enzyme activity assay. A Lambda 35 UV/VIS Spectrophotometer (Perkin Elmer Inc., USA) was used to measure all enzyme activities. All the enzyme activities were measured as a decrease in absorbance at 340 nm using the cofactor nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Liu Z et al. 2008). A total volume of 500 μ l was used to measure the enzyme activity (Liu et al. 2008). A reaction mixture contained 470 μ l of buffer, 10 μ l of substrate, 10 μ l of co-enzyme, and 10 μ l of crude protein. The oxidation of NADH and NADPH was followed by the change in absorbance at 340 nm. All samples were kept on ice until use and PBS (pH = 7.0) was heated in a 30°C water bath before enzyme reaction. The enzyme activity assay was carried out in technical triplicate.

Investigation of cell wall susceptibility. To understand the structural changes of cell walls induced by 35 mM furfural, we tested the lyticase-dependent susceptibility analysis (Teixeira et al. 2009). Lyticase, a β -1,3-glucanase from *Arthobacter luteus*, was purchased from Sigma-Aldrich (USA). 10⁷ cells were isolates from various media containing furfural and washed twice with double-distilled water before resuspending in 2.0 ml PBS (pH 7.0). 60 μ l of a 2 mg/ml lyticase were mixed into the cell suspensions, and the decline in the OD₆₀₀ of each cell suspensions was examined from 0 to 4 h.

Fluorescence microscopy and cellular analysis. The optical microscope (fluorescence microscopy), an Axio Imager A2 microscope (Carl Zeiss AG, Germany) equipped with DIC and using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye was used to evaluate the accumulation of ROS as described previously by (Wang et al. 2020). At each point in time, at least 100 cells on each bright-field image were evaluated to validate the accuracy of the results.

Transcriptome sequencing and analysis. The pre-cultivated cells of YPR015C strain were transferred to YPD mediums containing a furfural concentration of 35 mM. The furfural-treated cells were collected for RNA-Sequencing (RNA-Seq) at 2, 8, and 16 h. RNA-Seq was performed by Shanghai Personal Biotechnology

Co., Ltd. (China) using HiSeq-PE150 (Illumina, Inc., USA). We analyzed the GenesCloud server's raw data based on the reference genome sequence for *S. cerevisiae* BY4742. The fragments per kilobase of the mapped transcript per million (FPKM) approach was used to analyze the gene expression levels (Florea et al. 2013). The DEGseq R package was used to perform a differential expression analysis of six samples with three biological replicates (Anders and Huber 2010). The *p*-values were calculated using Benjamini and Hochberg's methods, and the differential gene screening criteria are $\log_2$ (Fold Change) > 0 and *p*-value < 0.05 .

Genes with the differentially expressed level were annotated and functions were categorized according to The *Saccharomyces* Genome Database (SGD) (<https://www.yeastgenome.org>) (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2004) databases. The sequence data for all the treatment samples used in this work have been deposited at NCBI SRA with accession number: PRJNA904555.

Statistical analysis. All the experiments were carried out in biological triplicates and are represented as mean average $\pm$ SE. Statistical analysis such as one-way analysis of variance (ANOVA) with the significance of *p*-values < 0.05 . Differential expressions were determined using the DESeq2 R package. *p*-Values were calculated using the Benjamini and Hochberg's methods, and the differential gene screening criteria is $\log_2$ (Fold change) > 0 and *p*-value < 0.05 (Dong et al. 2017).

Results and Discussion

Cell growth response under furfural challenge.

Overexpression of *RPB4*, *PRS3*, and *ZWF1* exhibited tolerance under the treatment of lignocellulose-derived inhibitors compared to the parental strain (Cunha et al. 2015). Furthermore, a yeast tolerance response to furfural was demonstrated, and the lag phase for cell proliferation in response to the inhibitor challenges was used to evaluate stress tolerance manifested at the genome level (Liu 2011). In this study, the *YPR015C* overexpressing- and the parental yeast strains were tested in the presence of 35 mM furfural during the lag phase. Cell growth was decreased under furfural challenge, and a lag phase was detected in the *YPR015C* overexpressing and the parental strains. The *YPR015C* overexpressing strain recovered more rapidly and displayed a lag phase at about 12 h. However, this lag phase was expanded to 24 h in the parental strain (Fig. 1). Notably, the lag phase duration in the *YPR015C* overexpressing strain was much shorter and reached the exponential phase more rapidly than that of its parental strain. This strain showed a much shorter lag phase than those pre-

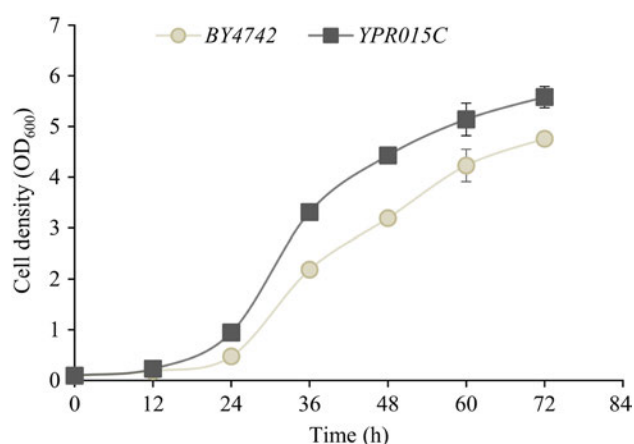


Fig. 1. Cell growth of *Saccharomyces cerevisiae* strains overexpressing *YPR015C* and *BY4742* as measured at OD₆₀₀ on a defined synthetic medium containing 35 mM of furfural.

The prolonged lag phase indicates the inhibitory effect of furfural on the parental strain.

viously reported for the *YNL134C* overexpressing strain (24 h) when challenged by furfural during the lag phase (Zhao et al. 2015; Li et al. 2015).

Aldehyde resistance performance. In *S. cerevisiae*, multiple reductases have activity for reducing furfural (Heer et al. 2009; Wang et al. 2017a). The co-factors NAD(P)H revealed aldehyde reduction activity in both the *YPR015C* overexpressing and the parental strain. Under furfural and HMF stress conditions, the *YPR015C* overexpressing strain performed noticeably better (Fig. 2). NADH co-factor showed that the highest specific activity for furfural reduction was about 9 U/mg, 9.5 U/mg and 8.5 U/mg at 2, 8 and 16 h, respectively (Fig. 2a). When NADPH was used as the co-factor, the *YPR015C* overexpressing strain also showed reduction in furfural enzyme activity at 2.5 U/mg, 4.5 U/mg and 6 U/mg after 2, 8 and 16 h treatments, respectively (Fig. 2c). In summary, *in vitro* enzyme assays indicated that the strain overexpressing *YPR015C* possessed a NAD(P)H-dependent enzyme activity for furfural reduction. In a recent study, overexpression of *YKL071W* showed both co-factors NAD(P)H-dependent enzyme activity for furfural reduction in crude and purified protein during lag phase growth (Heer et al. 2009; Wang et al. 2017a). Similarly, enzyme activity assay in this study indicated that the *YPR015C* overexpressing strain induced reduction in the two co-factors, NADH and NADPH for furfural and HMF inhibitors, respectively (Fig. 2). Furthermore, overexpression of *YPR015C* led to a much better specific activity (9.5 U/mg) when compared to other earlier reports on aldehyde reductases, such as in the overexpression of *Yll056cwp* (0.47 U/mg), *Ykl071wp* (3.38 U/mg), *Ykl107wp* (2.56 U/mg), and *Ymr152wp* (5.05 U/mg) from *S. cerevisiae* for furfural reduction (Wang et al. 2017a; Wang et al. 2017b; Ouyang et al. 2021).

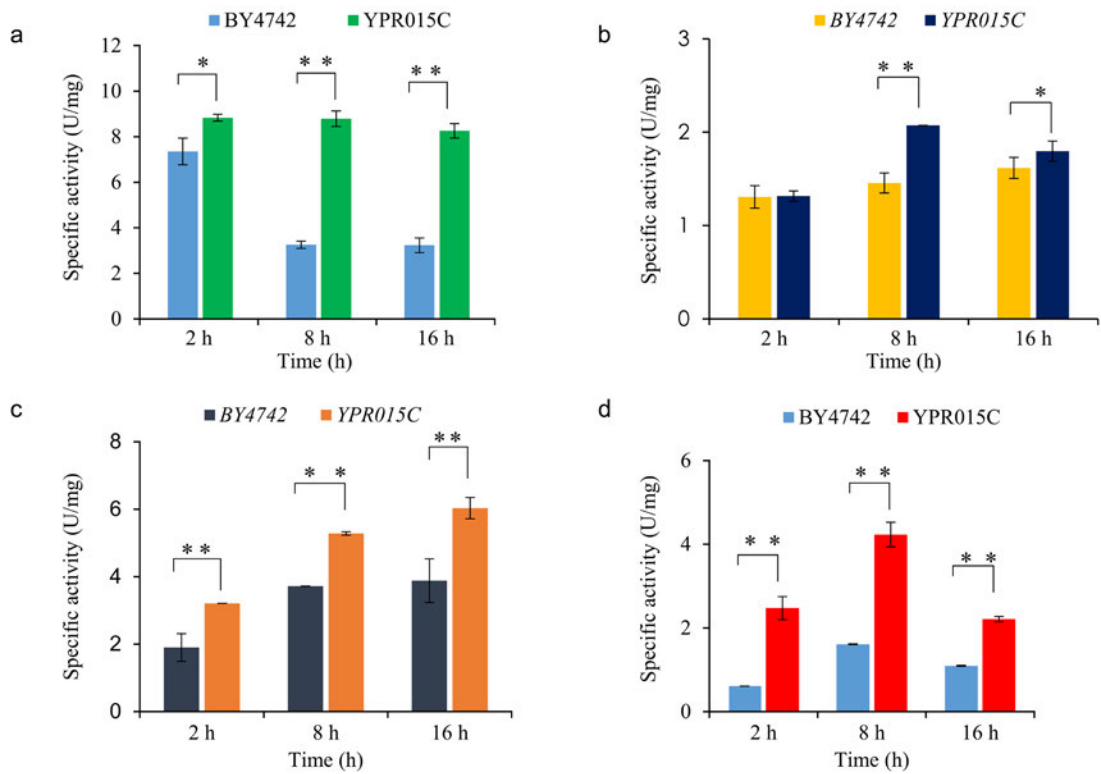


Fig. 2. Furfural and HMF reduction of activities in the crude cell extracts of the parental (BY4742) and YPR015C overexpressing strains at 35 mM of furfural. The activities were measured using NADH or NADPH as cofactors. a) NADH and c) NADPH used for furfural reduction, b) and d) for HMF reduction, respectively. The data represent averages of three experiments. * $p < 0.05$; ** $p < 0.01$ indicates significant differences.

Accumulation of reactive oxygen species. Furfural is one the most well-known lignocellulose-derived inhibitors that cause intracellular oxygen radical species accumulation. The oxidizing environment induced by ROS is known to cause irregularity and inactivity of cell components in *S. cerevisiae* (Almeida et al. 2008; Allen et al. 2010; Liu et al. 2020). It has been demonstrated that furfural is a thiol-reactive electrophile that can lead to oxidative stress (Kim et al. 2013; Liu et al. 2020) by activating transcription factors that mediate *S. cerevisiae*'s response to oxidative stress (Toone et al. 1999). In this study, following 2 h of treatment with 35 mM furfural, 14% and 12% of the cells of the strain overexpressing YPR015C and the parental strain, respectively, exhibited a positive oxygen radical signal (Fig. 3b). After 8 h of treatment, 38 and 34% of cells of the overexpressing YPR015C and the parental strain, respectively, showed positive ROS signals. Furthermore, positive ROS signals were detected in 84% and 70% of cells of the YPR015C overexpressing strain and the parental strain, respectively, after 16 h of treatment. The YPR015C overexpressing strain and its parental strain showed significant differences in the positive ROS signals displayed in the medium supplemented with the same furfural concentration independent of time. Remarkably, the percentage of yeast cells stained positive for ROS signals increased

over the time intervals studied (Fig. 3b). We also found that the percentage of cells that accumulate ROS after 16 h decreased to a lower level in the YPR015C overexpressing strain compared to its parental strain after the treatment with 35 mM furfural. This result implied that ROS accumulation peaked after 16 h of furfural treatment, indicating that the percentage of cells containing ROS increased over time. Excessive ROS accumulation at this time may cause damage to DNA, proteins, and lipids (Rowe et al. 2008).

Cell wall susceptibility analysis. *S. cerevisiae* cell wall and membrane act as the first-line defense barrier to external toxic stimuli under external factors such as temperature. Cell wall analysis in the furfural treated and untreated cells were used to validate the increased furfural stress tolerance in cells that had encountered cell wall improvement using a lytic enzyme, a β -1,3-glucanase from *Arthobacter luteus* (Teixeira et al. 2014). After 4 h of lyticase treatment, the cell density of samples cultured with 35 mM furfural for 2 h was absorbed without substantial differences compared to that of its parental strain, while samples treated with 35 mM furfural decreased steadily after supplementation with lyticase in 8 h of culture, lowering to approximately 43.7% (Fig. 4). The YPR015C overexpressing strain treated with 35 mM furfural revealed a rapid decrease in cell density in the lyticase-supplemented medium after 16 h

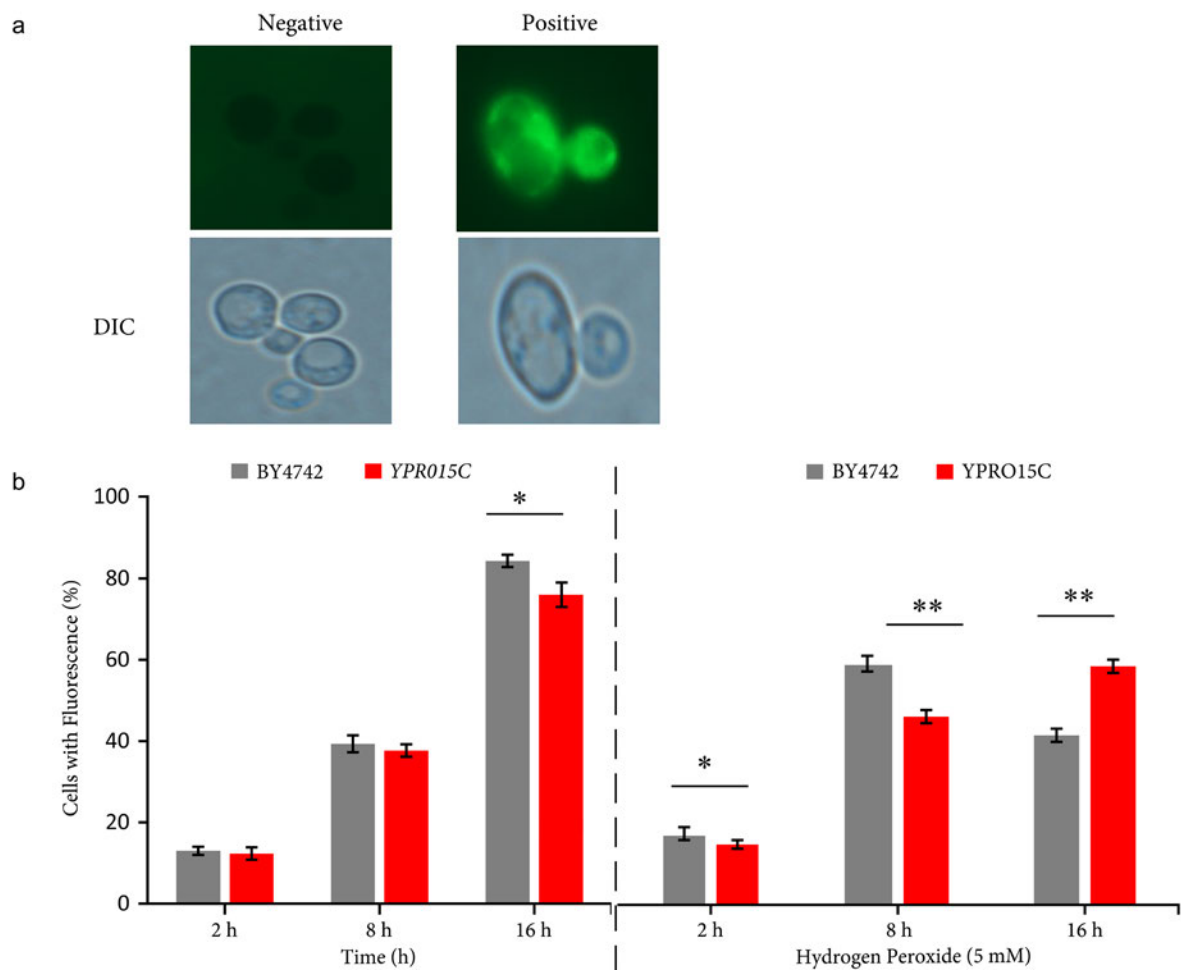


Fig. 3. Accumulation of reactive oxygen species (ROS) caused by furfural. a) Representative images of cells stained with the ROS indicator 2',7'-dichlorofluorescein diacetate, b) percentage of cells at each concentration of furfural and peroxide that stained positive for ROS by 2',7'-dichlorofluorescein diacetate after 2, 8, and 16 h. The data represent averages of three experiments with standard error. * $p < 0.05$; ** $p < 0.01$ indicates significant differences when at least 100 cells were examined on each bright-field image.

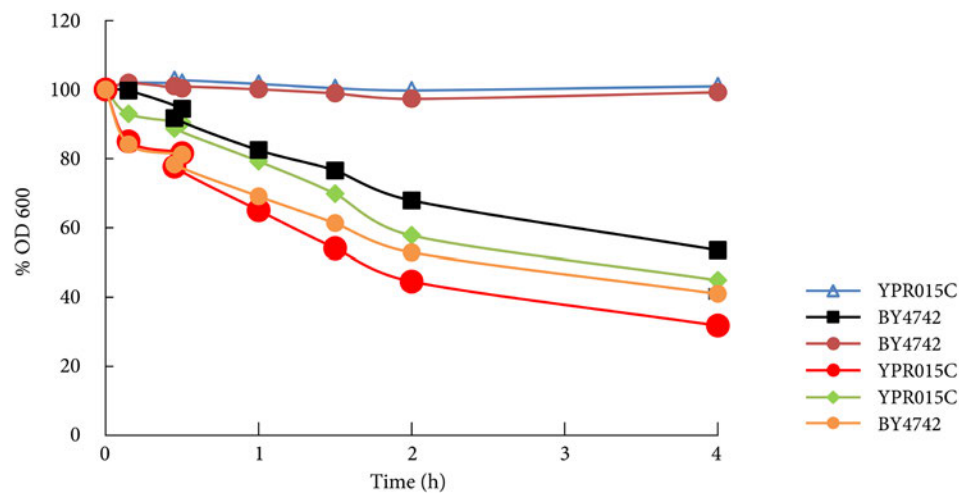


Fig. 4. Changes in cell densities after the treatment with lyticase during 4 h of incubation. *Saccharomyces cerevisiae* strains were treated for 2, 8, and 16 h (from top to bottom) with 35 mM of furfural, respectively. The mean values of relative optical density are presented with vertical error bars, each representing a single standard deviation (n = 3).

transcriptional level under furfural challenge treatment during the late stage of the lag phase. Interestingly, we discovered one *SRX1* extended list of genes involved in the oxidative damage stress response that had increased expression in response to furfural challenge, which had not been reported in earlier studies. In conclusion, our

of treatment; this decrease was more significant than the cell density decrease reported at 2 and 8 h after furfural challenges compared to its parental strain.

The results demonstrated that treatment for 16 h increased the resistance of cells to lyticase, with a maximum effect observed. After 8 hours, cells exposed to 35 mM furfural showed noticeably higher lyticase resistance. However, cells treated for 2 h showed no difference in resistance to lyticase compared with its parental strain. In cell wall susceptibility analysis, we observed that it slowly lowered after lyticase was added to the media after 8 h of furfural treatment, dropping to about 53.5% and 44.9%, respectively (Fig. 4). After 16 h, the samples treated with 35 mM furfural showed a rapid decrease in cell density (31.77%) in the lyticase-supported medium. In comparison to its parental strain, the reduction in cell density in these samples was more significant than the reductions in the samples at 2 h (98.3%), and 8 h (44.9%) following furfural challenge treatment (Fig. 4).

Transcriptome sequencing data analysis. The quality statistics of sequencing data are described in Table SII. Consequently, the RNA sequencing data in this work was reliable in consideration of statistics of RNA sequencing data. The correlation coefficients of samples within and between groups were calculated based on the FPKM values of all the genes in each sample, and a heat maps were constructed (Fig. S3).

Transcriptome differential expressions. Yeast tolerance to furfural inhibitor stress conditions can be shown at the genome level and is most possibly observed during the lag phase (Liu et al. 2004). It is a common practice to use gene expression responses to environmental stimuli to discover tolerant genes under specified conditions (Unrean et al. 2018). *S. cerevisiae* was used to study the genes correlated with stress resistance reaction to single and synergetic inhibitors. About 184 consensus genes were differentially expressed towards lignocellulose-derived inhibitor resistance between *S-C1* and *YC1* strains. Overexpression of *SFP1*, and *ACE2* gene in the stress resistant strain *S-C1* and *YC1* increases bio-ethanol productivity in the existence of furfural (Chen et al. 2016). In this report, we explored how differences in stress tolerance between the *YPR015C* overexpressing and its parental strain were explained by gene expression changes under 35 mM furfural. Seventy-nine up-regulated and down-regulated key genes implicated in various biochemical processes were recognized during the lag phase of the early (2 h), middle (8 h), and late (16 h) stages under furfural challenge. Currently, we identified genes involved in amino acid biosynthesis, oxidative stress response, cell wall and membrane-related response, heat shock protein response, and mitochondrial-associated proteins for the *YPR015C* overexpressing strain (Table SIII). Out of the

genes identified, two genes are implicated in cellular metabolism; *MTD1* and *IMD2* had significantly higher transcription levels in the *YPR015C* overexpressing strain. *MTD1* is a cytoplasmic malate dehydrogenase and monophosphate dehydrogenase.

It has been shown that furfural causes yeast to produce more reactive oxygen species (Allen et al. 2010). Reactive oxygen species, a reduced form of superoxide anion, and hydroxyl radicals, cause high damages to the cellular components such as lipids proteins, and DNA in *S. cerevisiae*. The oxidative damage stress response began as a defensive system against reactive oxidants in *S. cerevisiae*, as in most aerobically developing organisms (Liu and Moon 2009; Liu et al. 2020). In previous studies, four genes essential for oxidative stress response to inhibitory compounds were identified in the transcriptome analysis of an industrial yeast strain (Liu and Ma 2020; Liu et al. 2020). According to a recent proteomic analysis, *Zwf1* consistently showed a high level of enhanced fold changes in response to furfural and HMF treatment (Thompson et al. 2016; Liu and Ma 2020).

In other reports, a comprehensive phenotyping study discovered a tolerable mutant with the trait of overexpressing the thioredoxin-encoding *TRX1* gene (Unrean et al. 2018). Recent studies have shown that thioredoxin and glutaredoxin, two distinct classes of small proteins essential for resistance to oxidative stress, are the key elements linked to several transcriptionally active genes involved in both enzymatic and non-enzymatic systems (Liu and Ma 2020). In this work, the *YPR015C* overexpressing strain showed an enhanced oxidative response with a significantly higher expression activity than its parent strain against the furfural inhibitor challenge. Among the identified genes, three essential genes are included in oxidative stress response. Genes encoding oxidoreductase enzymes are highly expressed, including *AHP1* (thioredoxin peroxidases), *SRX1* (sulfiredoxin), and *GPX2* (phospholipid hydroperoxide glutathione peroxidase). Glutathione and glutaredoxin are implicated in both enzymatic and non-enzymatic defense reactions in the *YPR015C* overexpressing strain against the challenge of furfural inhibitor. It is clear that the two different classes of small proteins glutaredoxin and thioredoxin, which are required for oxidative stress resistance, were the crucial components related to several transcription-active genes involved in different non-enzymatic and enzymatic systems. The specific enzymatic and non-enzymatic defense response system, centered on glutathione, was distributed with these genes. Previous studies have shown that the expression of a few genes encoding the specific oxidative-stress response, including *TRR1*, *ECM4*, *GLR1*, *GRX1*, and *CTT1*, increases under the furfural challenge (Heer et al. 2009; Wang et al. 2017a; Liu et al. 2020). However, in this study, these genes are not expressed at the

work's results were consistent and similar to those of early reported studies.

Yeast cell wall and membrane-related proteins are involved in biological activities and serve as the first line of defense against external toxic stimuli. The cell wall is made up of several proteins, chitin, β -1,3- and β -1,6-glucans, and other substances that can be cross-linked to create higher-order complexes. Its configuration and degree of cross-linking alter with cell proliferation and in response to different stressors (Wang et al. 2017a). The *S. cerevisiae* cell wall structures were modified to adapt to ethanol stress conditions (Wang et al. 2017a). Glycosylphosphatidylinositol-linked cell wall proteins *SPI1*, *SED1*, and *PIR3*, as well as *DIT1* and *GIP1* for forming spore walls, have recently demonstrated a sustained increase in the expression levels following furfural and HMF treatment at various stages (Liu et al. 2020). In another study, a protein that is hypothesized to be involved in the control of cell walls was produced when up-regulated genes were expressed. This protein was a crucial part of yeast cell membranes and has been connected to resistance to furfural inhibitors (Terashima et al. 2000). In this study, 15 genes involved in the yeast cell wall and membrane-related proteins showed significant expressions in the recombinant strain after furfural treatment compared to its parental strain. Among them *SED1*, *PIR3*, *CWP1*, *YPK2*, *TIP1*, *IZH4*, and *IZH2* were upregulated after the furfural challenge. These genes encode proteins related to cell walls with different functions. For instance, *PIR3* is an O-glycosylated, covalently bonded cell wall protein that is necessary for the stability of the cell wall, *SED1* is a major cell wall glycoprotein that is structurally triggered by stress, *TIP1* – a major cell wall monoprotein, *YPK2* is involved in cell wall integrity signaling pathway, and *CWP1* is a cell wall mannoprotein. Some of the proteins encoded by these genes may not be found in the cell walls, but they encode cell wall-related proteins of various activities. For instance, the *SED1* and *PIR3* genes encode proteins which attach glycosylphosphatidylinositol to the cell wall. In conclusion, our work's results were consistent and similar to those of early reported studies.

Amino acid and fatty acid biosynthesis have been revealed to contribute to the biosynthesis of proteins implicated in stress response and suggested as a component of *S. cerevisiae* tolerance against lignocellulose-derived inhibitors (Okazaki et al. 2007; Liu et al. 2019). Another study found that the *Y31-N* strain could keep the integrity of cell membrane function to resist furfural stress by controlling the fluidity of the cell membrane, modulating the framework of the palmitic acid and stearic acid in the cells (Wang et al. 2023). In this study, at least seven key important genes are implicated in amino acid biosynthesis and fatty acid metabolism

including, *ARG1*, *ARG3*, *IME2*, and *LEU4*. The *LEU4*, *ARG1* and *ARG3* were mainly involved in the metabolism of amino acids (Table SIII). *OLE1* and *FRM2* genes were involved in fatty acid biosynthesis. The findings from earlier studies reveal that *OLE1*, which encodes the Δ -9 fatty acid desaturase, catalyzes the double bonding between carbons 9 and 10 of stearyl CoA and palmitoyl CoA (McDonough et al. 1992). In addition to its activity in fatty acid production, *OLE1* is also essential for the formation and functioning of the mitochondria (Hermann et al. 1998). In this work, the *OLE1* gene showed more than 2.3-fold change and a *p*-value of less than 0.05 in a time course study at the late stage of the lag phase.

Furthermore, in our previous study, we found that phenol tolerance in *Candida tropicalis* affected the expression of genes implicated in fatty acid degradation. These genes were responsible for its tolerance under the stress of phenol (Wang et al. 2020). Four of the seventy-nine genes identified were heat shock proteins and related to amino acid biosynthesis. Among those with known functions, *ARG1* and *ARG3* were involved in arginine biosynthesis and metabolism, while *IME2* is involved in serine/threonine biosynthesis.

The down-regulated genes are primarily involved in ribosome biosynthesis, derivative metabolic processes and amino acid, RNA metabolic processes, and other functional categories. It has recently been discovered that the genes involved in the *S. cerevisiae* survival process and those involved in the response to heat shock stress do not overlap significantly (Gibney et al. 2013). Members of the *HSP70* and *Hsp110* families including *SSA4*, *SSE2*, *SSE2*, and *SSA4*, were significantly repressed, as shown in Table SIII. These repressed genes served to fold proteins either directly or indirectly. Other genes coding for heat shock protein, stress-responsive protein, chaperone, and co-chaperone were repressed in the *YPR015C* overexpressing strain to counteract furfural stress damage to proteins such as *HSP10*, *HSP30*, *HSP78*, and *HSP15*. *HSP12* is involved in the maintenance and organization of the plasma membrane. Other genes mainly engaged in the essential categories of cell wall stability, cell wall protein (*PST1*, *SPI1*), spore wall assembly (*LDS2*), *ARO9* for aromatic aminotransferase, *RTC3* RNA metabolism, and *PHM8* for lysophosphatidic acid hydrolysis were repressed. From this study, we concluded that the accumulation of overexpressed transcriptional genes further shortened the lag phase in response to furfural stress to a certain extent.

Regarding the specific activity of aldehyde reductase, the overexpressed *YPR015C* improves the aldehyde reductase activity dependent on the NADH and NADPH cofactor. The results of comparative transcriptomic profiling identified genes associated with the cell wall and membrane-related biosynthesis, cellular

detoxification, and oxidative stress response. The findings of this research report contribute to our knowledge of the adaptation and tolerance mechanisms of yeast, which will aid in improving yeast tolerance to stress.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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