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Enhanced tolerance of *Saccharomyces cerevisiae* to multiple lignocellulose-derived inhibitors through modulation of spermidine contents

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ABSTRACT

Fermentation inhibitors present in lignocellulose hydrolysates are inevitable obstacles for achieving economic production of biofuels and biochemicals by industrial microorganisms. Here we show that spermidine (SPD) functions as a chemical elicitor for enhanced tolerance of *Saccharomyces cerevisiae* against major fermentation inhibitors. In addition, the feasibility of constructing an engineered *S. cerevisiae* strain capable of tolerating toxic levels of the major inhibitors without exogenous addition of SPD was explored. Specifically, we altered expression levels of the genes in the SPD biosynthetic pathway. Also, *OAZ1* coding for ornithine decarboxylase (ODC) antizyme and *TPO1* coding for the polyamine transport protein were disrupted to increase intracellular SPD levels through alleviation of feedback inhibition on ODC and prevention of SPD excretion, respectively. Especially, the strain with combination of *OAZ1* and *TPO1* double disruption and overexpression of *SPE3* not only contained spermidine content of 1.1 mg SPD/g cell, which was 171% higher than that of the control strain, but also exhibited 60% and 33% shorter lag-phase period than that of the control strain under the medium containing furan derivatives and acetic acid, respectively. While we observed a positive correlation between intracellular SPD contents and tolerance phenotypes among the engineered strains accumulating different amounts of intracellular SPD, too much SPD accumulation is likely to cause metabolic burden. Therefore, genetic perturbations for intracellular SPD levels should be optimized in terms of metabolic burden and SPD contents to construct inhibitor tolerant yeast strains. We also found that the genes involved in purine biosynthesis and cell wall and chromatin stability were related to the enhanced tolerance phenotypes to furfural. The robust strains constructed in this study can be applied for producing chemicals and advanced biofuels from cellulosic hydrolysates.

1. Introduction

Lignocellulosic biomass including corn stover, sugar cane, wood, straw, and waste residues from agriculture and forestry is a promising resource for producing fuels and chemicals. In contrast to sugar or starch-based resources, lignocellulose-derived fuels might be able to replace substantial portions of the transportation fuels produced from petroleum without competition with increasing food demand (Hahn-Hagerdal et al., 2006). Nevertheless, utilization of lignocellulose for producing fuels and chemicals has been hampered due to the recalcitrant nature of lignocellulose materials (Lynd et al., 2008). In order to release fermentable sugars from lignocellulosic biomass, pretreatment and hydrolysis of lignocellulose are necessary (Lynd et al., 2005). To date, various physicochemical pretreatment processes, such as dilute acid, alkali, steam explosion, and hydro-thermal methods (Agbor et al., 2011; Mosier et al., 2005) have been developed. The dilute acid pretreatment is considered the most simple and economical process and has often been employed for large scale operation (Jung et al., 2013). However, this process generates substantial amounts of fermentation inhibitors at toxic levels in lignocellulose hydrolysates. Furan derivatives (2-furaldehyde (furfural) and 5-hydroxymethyl-2-furfural (HMF)), carboxylic acids (acetic acid and formic acid), and phenolic compounds (Almeida et al., 2007) are major inhibitors and aggravate both growth and fermentation capabilities of fermenting microorganisms. Because the toxic nature of the fermentation inhibitors is one of the roadblocks toward large-scale production of lignocellulosic

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bioethanol (Almeida et al., 2009, 2007; Park et al., 2011), various physical and chemical methods have been developed to remove or reduce these inhibitory compounds in lignocellulose hydrolysate (Yang and Wyman, 2008). Still, these detoxification methods usually require additional steps and are not economical for practical use (Mussatto and Roberto, 2004). Therefore, development of inhibitor-tolerant microorganisms is imperative for cost-effective bioethanol production.

Furfural and HMF, the most investigated inhibitors are known to damage microorganisms in several ways including reducing activities of various oxidoreductases and glycolytic enzymes, lowering intracellular NAD(P)H levels, and inducing oxidative stress to yeast cells (Allen et al., 2010; Banerjee et al., 1981; Gorsich et al., 2006; Modig et al., 2002). *Saccharomyces cerevisiae* can convert furfural and HMF to less toxic alcohols, furfuryl alcohol and furan dimethanol, respectively, by an oxygen independent manner (Liu et al., 2008; Taherzadeh et al., 1999; Taherzadeh et al., 2000). Overexpression of oxidoreductases, such as alcohol dehydrogenases (*ADH1*, *ADH6*, and *ADH7*) (Almeida et al., 2008; Liu et al., 2008; Petersson et al., 2006), aldo-keto reductases (*GRE2*) from *S. cerevisiae* (Moon and Liu, 2012), or xylose reductase (*XYL1*) from *Scheffersomyces stipitis* (Almeida et al., 2009) has been shown to increase the specific furfural and HMF conversion rates and hence decreases the lag-phase of the cell growth in the presence of furfural and HMF. Additionally, the NADPH-producing pentose phosphate pathway (PPP) and oxidative stress response pathways are critical factors involved in tolerance against furan derivatives (Gorsich et al., 2006; Kim and Hahn, 2013). While tolerance or detoxifying mechanisms of furfural and HMF have been intensively studied, metabolic engineering strategies for constructing tolerant strains to carboxylic acids, such as acetic acid and formic acid have been rare (van Maris et al., 2006). Unlike furfural and HMF, generation of acetic acid is inevitable regardless of pretreatment methods as it is formed by the hydrolysis of acetylxylan in hemicelluloses. Moreover, concentrations of acetic acid in cellulosic hydrolysates are higher than furfural and HMF. Therefore, identification of environmental and genetic perturbations eliciting enhanced tolerance of fermenting microorganisms to acetic acid has been anticipated.

While previous studies have focused on *iterative* identification and engineering of cellular pathways responsible for enhanced tolerance to specific inhibitors (Alriksson et al., 2010; Gorsich et al., 2006; Kim and Hahn, 2013; Park et al., 2011; Sanda et al., 2011; Sundstrom et al., 2010), these incremental improvements based on combinations of the identified target pathways might not yield optimal strains tolerant to multiple inhibitors. Moreover, accumulated ethanol by yeast and other minor fermentation inhibitors may cause additional stresses to yeast cells especially when combined with the major fermentation inhibitors (Ma and Liu, 2010b). Therefore, it is necessary to construct engineered *S. cerevisiae* strains exhibiting tolerance to multiple inhibitors (furfual, HMF, and acetic acid) and ethanol for efficient production of cellulosic ethanol. Instead of chasing individual cellular pathways related with enhanced tolerance to each component of fermentation inhibitors, we undertook an approach to identify and exploit a global mediator eliciting the enhanced tolerance to multiple inhibitors simultaneously.

Polyamines including putrescine, spermidine, and spermine are low molecular weight aliphatic nitrogen compounds that are found ubiquitously in microorganisms, plants, and animals (Hamana and Matsuzaki, 1992). In plants, these amines are associated with defense to diverse environmental stresses (Bouchereau et al., 1999), and polyamine-deficient yeast cells are much more sensitive to elevated temperature (Balasundaram et al., 1996), paromomycin (Balasundaram et al., 1999), and incubation in oxygen (Balasundaram et al., 1993). Furthermore, it was reported that spermidine extends lifespan of *S. cerevisiae* through induction of autophagy (Eisenberg et al., 2009). These observations suggest that adequate modulation of polyamine contents in *S. cerevisiae* might improve tolerance to fermentation inhibitors and ethanol. Based on this hypothesis, we examined whether or not supplementation of polyamines into yeast cultures can improve tolerance against fermentation inhibitors. Interestingly, exogenously added spermidine (SPD) was able to enhance tolerance of *S. cerevisiae* against fermentation inhibitors. In order to facilitate the enhanced tolerance without exogenous addition of SPD, we identified genetic perturbations eliciting higher SPD levels in *S. cerevisiae*. The endogenous *SPE1*, *SPE2*, and *SPE3* genes involved in the polyamine biosynthetic pathway were overexpressed to increase polyamine contents (Fig. 1). As polyamine synthesis is controlled by feedback regulation on ornithine decarboxylase (ODC) which is encoded by the *SPE1* gene, high levels of SPD might increase ODC antizyme (Oaz1p) which mediates ubiquitin-independent degradation of ODC by the proteasome

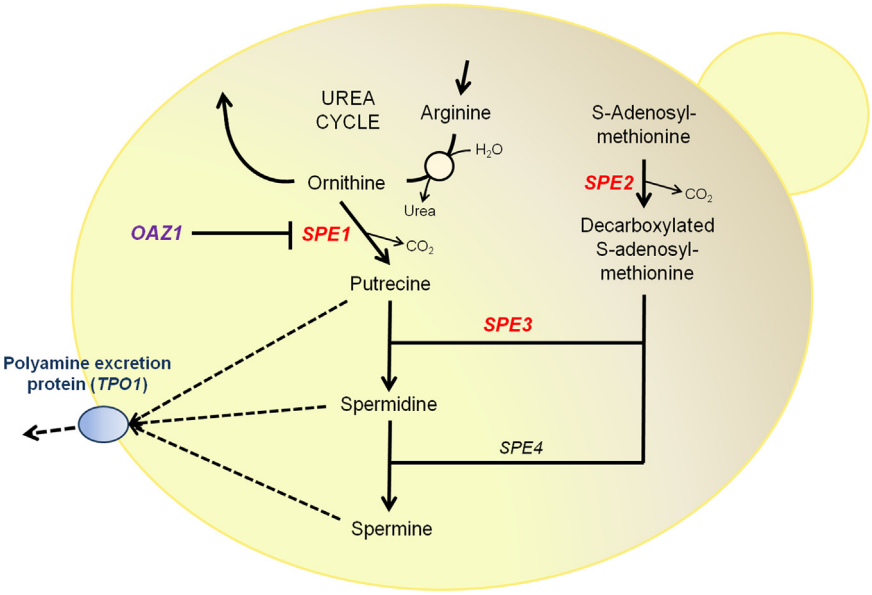

Fig. 1. Strategy for constructing engineered *S. cerevisiae* having high levels of spermidine. Fluxes in the spermidine biosynthetic pathway were amplified by overexpressing ornithine decarboxylase (ODC, *SPE1*), *S*-adenosylmethionine decarboxylase (*SPE2*), and spermidine synthase (*SPE3*). High levels of spermidine upregulate ODC antizyme (*OAZ1*) expression and Oaz1p mediates degradation of ODC. For alleviation of the feedback inhibition on ODC, *OAZ1* coding for ODC antizyme was disrupted. As a polyamine transporter (Tpo1p) located in *S. cerevisiae* plasma membrane excretes intracellular spermidine to the medium, *TPO1* was also disrupted in order to minimize the loss of intracellular spermidine.

(Palanimurugan et al., 2004). Therefore, we disrupted the *OAZ1* gene for alleviating the feedback regulation of ODC by accumulated SPD in yeast. In order to further increase SPD levels in yeast, the *TPO1* gene coding for polyamine transport protein which mainly excretes polyamines to the medium (Uemura et al., 2007) was also disrupted (Fig. 1). Engineered *S. cerevisiae* strains constructed with these genetic perturbations not only showed higher intracellular SPD levels, but also exhibited enhanced tolerance to various fermentation inhibitors. These results suggest that enhanced tolerance to multiple lignocellulose-derived inhibitors and ethanol can be implemented through chemical and genetic perturbations that have not been considered previously.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning and genetic manipulation. *S. cerevisiae* D452-2 (*MATα*, *leu2*, *his3*, *ura3*, and *can1*) was used for constructing the recombinant strains having high levels of SPD contents and tolerance against inhibitors. Strains and plasmids used in this work are described in Supplementary Table 2. The primers used for disruption of the chromosomal *OAZ1, TPO1,* and *ATG7* and cloning of genes coding for enzymes in SPD biosynthetic pathway (*SPE1*, *SPE2*, and *SPE3*) and the genes identified by RNA sequencing (*ADE17*, *PIR3*, and *HTA2*) are listed in Supplementary Table 3.

2.2. Yeast transformation

Transformation of cassettes for overexpressing enzymes in SPD biosynthetic pathway, *ADE17*, *PIR3*, and *HTA2* and disrupting *OAZ1, TPO1,* and *ATG7* was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). pRS403_SPE2, pRS405_SPE1, pRS406_SPE3, pRS403_PIR3, pRS405_ADE17, pRS406_HTA2, pAUR_d_OAZ1, pRS405_d_TPO1, and pAUR_d_ATG7 plasmids were cut with *Msc*I, *Afl*II, *Nco*I, *Nde*I, *EcoR*I, *Nco*I, *Blp*I, *Nco*I, and *Xba*I, respectively, and transformed. Transformants were selected on YSC medium containing 20 g/L glucose. Amino acids and nucleotides were added as necessary. For the selection of *OAZ1* or *ATG7* disrupted strain, YP medium containing 20 g/L glucose and 0.4 mg/L aureobasidin was used.

2.3. Culture conditions and fermentation experiments

LB medium with 50 μg/ml of ampicillin when required was used for *E. coli* culture. Yeast strains were pre-cultured at 30 °C and 250 rpm for 48 h in YP medium (10 g/L yeast extract, 20 g/L bacto peptone) with 20 g/L glucose. A defined YSC medium (6.7 g/L yeast nitrogen without amino acids and 2.0 g/L synthetic complete supplement mixture) containing appropriate amounts of sugars was used for fermentation experiments.

For the tolerance test experiments, pre-cultured cells from YP medium with 20 g/L glucose were harvested and inoculated into main cultures with initial optical density (OD_{600}) of 0.5–1.0. To maintain microaerobic condition and maximize ethanol productivity, flask fermentation experiments were performed at 30 °C and 80 rpm in 50 ml YSC medium containing 48–60 g/L glucose with 2 g/L furfural and HMF, 4 g/L furfural, 1, 2, or 4 g/L of acetate, or 8% (v/v) ethanol. For the fermentation of lignocellulose hydrolysate, corn stover hydrolysate was prepared by National Renewable Energy Laboratory (http://www.nrel.gov/biomass/pdfs/47764.pdf). Briefly, dilute sulfuric acid and heat from steam were used in a two stage pretreatment process. After the pretreatment reaction, a lot of water along with some extents of the acetic acid and furfural were vaporized. The leftover hydrolysate slurry was cooled by dilution water, and its pH was adjusted from 1 to 5–6 by using ammonia. The hydrolysate was mixed with 50 mM sodium citrate buffer (pH 4.8) at 20% (w/v). For enzymatic hydrolysis, 0.1 ml of 2:1 mixture of cellulase (Celluclast 1.5 L, Sigma, USA) and cellobiase (Novozyme 188, Sigma, USA) was added to gram of dry sample weight. The reaction using a 500 ml-scale baffled flask (Nalgene, Rochester, NY, USA) was carried out at 50 °C and 150 rpm for 48 h. After enzymatic hydrolysis, the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was collected and supplemented with concentrated autoclaved YSC medium to result in a hydrolysate mixture with 6.7 g/L yeast nitrogen without amino acids and 2.0 g/L synthetic complete supplement mixture. Initial cell densities were adjusted to OD_{600} of 0.8 and fermentations were performed at 30 °C and 80 rpm in 50 ml working volume.

2.4. Analytical methods

Optical density was measured with a spectrophotometer (RF5301, Shimadzu, Kyoto, Japan) at 600 nm. Glucose, xylose, ethanol, furfural, and HMF concentrations were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) using a REZEX ROA organic acid column (Phenomenex, Torrance, USA). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min at 60 °C, and detection was made with a reflective index detector. Furfuryl alcohol and furan dimethanol concentrations were determined by UV detection at 215 nm using a BIO-RAD Aminex HPX-87H ion exclusion column. The column was eluted with 84% (v/v) 5 mM H_2SO_4 and 16% (v/v) acetonitrile at a flow rate of 0.4 ml/min at 25 °C. In order to measure intracellular concentrations of polyamines, culture equivalents of absorbance 50 were washed two times with sterilized water. After collected cells were resuspended in 1.6 ml of cold 5% TCA solution and incubated on ice for 1 h with vortexing every 15 min, supernatants were neutralized with 100 μl of 2 M K_2HPO_4. 1,7-diaminopentane was used as the internal standard (IS). For the derivatization, 1 ml of dansyl chloride reagent (5 mg/ml) dissolved in acetone and 0.5 ml of saturated $NaHCO_3$ solution were added to a 1 ml of sample solution containing IS. After the reaction mixture was incubated at 40 °C for 1 h with occasional shaking, the solution was dried under vacuum and 1 ml of acetonitrile was added. The concentrations of polyamines were determined using an HPLC system equipped with a CAPCELL PAK C18 MG column (Shiseido, Tokyo, Japan) according to the method described previously (Innocente et al., 2007; Moret and Conte, 1996).

2.5. Enzyme activity assays

The crude protein extracts were prepared as described previously (Park et al., 2011). To determine reduction activity toward HMF and furfural, the reaction solution was formulated with 100 μl of 100 mM potassium phosphate buffer (pH 7.2), 60 μl of 33 mM furfural or HMF, and 20 μl of the crude enzyme solution. The absorbance change at 30 °C and 340 nm of wavelength was monitored by a 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA) after addition of 20 μl of 1 mM NAD(P)H. One unit of reduction activity was defined as the amount of enzyme oxidizing 1 μmol NAD(P)H per minute under the reaction conditions. Protein concentration was determined by the protein assay kit (Bio-Rad, Richmond, USA).

2.6. RNA-seq analysis by next generation sequencing (NGS)

For the RNA-seq experiment, initial OD_{600} values were adjusted to 1.3, and flask fermentation experiments were performed at 30 °C and 80 rpm in 50 ml YSC medium containing 50 g/L glucose with 4 g/L furfural. The RNA-seq was carried out in the Illumina MiSeq platform. In order to make a messenger RNA (mRNA) library for the NGS, total RNA was extracted by Qiagen RNA extraction kit (Qiagen, Germany). We retrieved mRNAs by magnetic oligo-dT beads (Illumina, San Diego, CA, US) and constructed a cDNA library for next generation sequencing by TruSeq RNA sample preparation kit (Illumina, San

Diego, CA, US). The sequencing of the cDNA library was carried out in the MiSeq machine with the paired-end sequencing reagent kit (version 2.0, 2 × 250 cycles). The number of total reads for 12 libraries (4 samples × 3 replicates) with quality over Q30 was 25,847,149 from the sequencing. The quality of raw reads was inspected by the FastQC program (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and low-quality reads were filtered using the Bioconductor/R package (Gentleman et al., 2004). The 5′and 3′ends of reads were trimmed by the Phred quality score (threshold < Q20) using the ShortRead package in the Bioconductor/R package (Morgan et al., 2009). We used the sequence of *S. cerevisiae* strain S288c as a reference genome for read alignment by the Bowtie2 program (Langmead and Salzberg, 2012) and used the transcript database (TxDb.Scerevisiae.UCSC.sacCer2.sgdGene) in the Annotation package in Bioconductor/R package. Counting reads mapped on coding regions were the Rsamtools package (Gentleman et al., 2004). Differentially expressed genes (DEGs) among different conditions were examined by the edgeR package (Robinson et al., 2010).

3. Results

3.1. Effects of polyamines on tolerance of S. cerevisiae against furan derivatives

To investigate effects of polyamines on tolerance phenotypes of yeast against fermentation inhibitors, we examined whether or not exogenously added spermidine (SPD), spermine (SPM), or putrescine (PUT) can alter tolerance of *S. cerevisiae* against furfural and HMF. While addition of three polyamines into culture media generally increased corresponding polyamine content in *S. cerevisiae* D452-2 (Supplementary Fig. 1), the addition of 2 mM SPD increased intracellular SPD contents substantially whereas the addition of 2 mM of SPM and PUT did not increase corresponding polyamines contents as much as SPD. The highest polyamine content of 2.5 mg SPD/g cell was obtained from the strain supplemented with 2 mM SPD, which was six-fold higher than that of a control strain without polyamine supplementation (Supplementary Fig. 1).

Microaerobic batch fermentations of *S. cerevisiae* D452-2 with and without 2 mM polyamines (SPD, SPM, and PUT) supplementation were carried out using yeast synthetic complete (YSC) medium containing 2 g/L of furfural and 2 g/L of HMF. Although addition of both 2 g/L of furfural and 2 g/L of HMF substantially inhibited cell growth, the D452-2 strain was able to grow after 62 h in the lag phase (Supplementary Fig. 2a). Under the furfural and HMF challenge, the addition of 2 mM SPD or SPM shortened the lag-phase period from 62 h to 25 h (Fig. 2a; Supplementary Fig. 2). However, the addition of PUT to culture medium did not shorten the lag-phase. In all cases with polyamine supplementation, exit times from lag-phases coincided with complete conversion of furfural into furfuryl alcohol even though HMF remained in the culture medium at the exit times. These results confirmed that furfural is a major inhibitor of glucose consumption and ethanol production by yeast (Liu et al., 2008; Park et al., 2011). Because of the shortened lag period with the addition of SPD and SPM, volumetric ethanol productivities in both cases were 88% higher than the culture without SPD and SPM (Fig. 2b). The reason for this improvement by SPD and SPM supplementation is ascribed to the faster detoxification of furfural than the control culture without supplementation. Although both of SPD and SPM function as a chemical elicitor for enhanced tolerance of *S. cerevisiae* against furan derivatives, we chose SPD as a target molecular because the SPM biosynthetic pathway requires an additional enzyme (SPE4) compared to that of SPD. In order to determine an optimum concentration of SPD for eliciting tolerance against furan derivatives, various concentrations (0, 1, 2, and 4 mM) of SPD were applied into the culture medium containing 2 g/L of furfural and 2 g/L of HMF. Intracellular levels of SPD were proportional to the amounts of SPD supplemented (Supplementary Fig. 3). Of four tested conditions, supplementations of 1 and 2 mM SPD improved tolerance whereas 4 mM SPD addition aggravated tolerance against furan derivatives (Supplementary Fig. 4). Because the growth pattern of the strain supplemented with 4 mM SPD was similar to that of the control strain in the YSC medium without furan derivatives, we postulated that an excess amount of SPD (4 mM) might cause detrimental phenotypes against furan derivatives. The addition of 2 mM SPD into culture medium resulted in 19% higher ethanol productivity than the addition of 1 mM SPD (Supplementary Table 1), suggesting that 2 mM SPD seemed to be an optimal concentration to improve tolerance of *S. cerevisiae* D452-2 against furan derivatives. In all cases, specific rates of glucose consumption and ethanol production were almost identical. The positive effects of SPD supplementation on tolerance against furfural and HMF in yeast were also confirmed for other *S. cerevisiae* strains (L2612 and CEN.PK2-1D) (Supplementary Fig. 5).

3.2. Effects of SPD on tolerance of S. cerevisiae to acetic acid and lignocellulose hydrolysates

The concentrations of acetic acid in lignocellulose hydrolysates are often higher than the furan derivatives (Almeida et al., 2007). Acetic acid is toxic to *S. cerevisiae* and strongly inhibits cell growth and fermentation of yeast. Especially, the fermentation of xylose that is the second most abundant sugar in lignocellulose hydrolysates is known

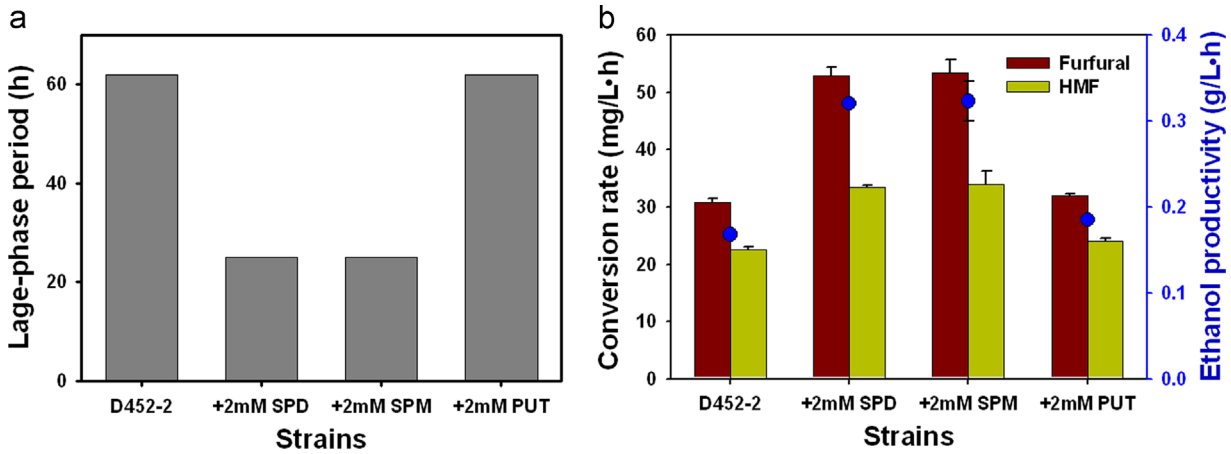

Fig. 2. Comparison of fermentation parameters by *S. cerevisiae* D452-2 and D452-2 supplemented with 2 mM spermidine (SPD), spermine (SPM), or putrescine (PUT) in the presence of 2 g/L furfural and HMF. (a) Lag-phase periods. (b) Furfural and HMF conversion rates and ethanol productivities. Results are the mean of duplicate experiments and error bars indicate s.d.

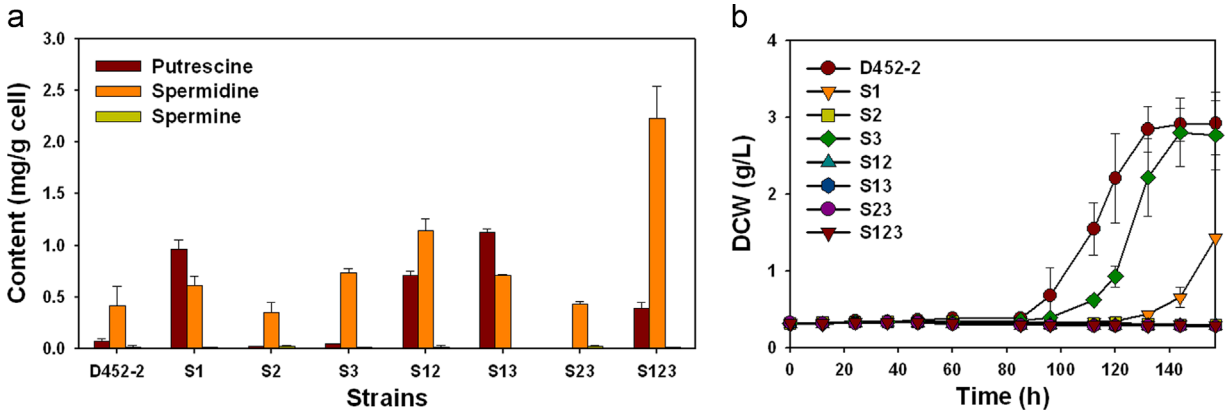

Fig. 3. Effects of amplification of genes coding for enzymes in the SPD biosynthetic pathway on polyamine contents (a) and cell growth (b). (a) Intracellular levels of polyamines in engineered strains. Abbreviations: S1, D452-2 overexpressing *SPE1*; S2, D452-2 overexpressing *SPE2*; S3, D452-2 overexpressing *SPE3*; S12, D452-2 overexpressing *SPE1,2*; S13, D452-2 overexpressing *SPE1,3*; S23, D452-2 overexpressing *SPE2,3*; S123, D452-2 overexpressing *SPE1,2,3*. (b) Cell growth of S1, S2, S3, S12, S13, S23, S123, and control D452-2 strain on a defined synthetic medium in the presence of 2 g/L furfural and HMF. Results are the mean of duplicate experiments and error bars indicate s.d.

to be highly inhibited by acetic acid (Lee et al., 2012; Wei et al., 2013). Therefore, we investigated the effects of SPD addition on tolerance of yeast against acetic acid. While lag-phase periods increased with increasing acetic acid concentrations (Supplementary Fig. 6), addition of 2 mM SPD shortened the lag-phase periods from 12 h to 4 h at 2 g/L of acetate, and 73 h to 36 h at 4 g/L of acetate (Supplementary Fig. 6). The initial pH values of culture media were 3.6 for with SPD supplementation and 3.2 for without SPD supplementation when acetate concentration was 4 g/L. Because low pH increases toxicity of acetic acid by increasing the undissociated form of acetic acid, we investigated whether or not SPD supplementation can improve tolerance of *S. cerevisiae* to acetic acid under identical pH conditions. With 4 g/L acetic acid concentration and the pH value of medium with SPD supplementation adjusted to 3.2, the lag-phase period was 48 h, which was still 34% shorter than that of the control strain (Supplementary Fig. 6b). At the end of fermentation, the pH values of the control strain and 2 mM SPD addition with pH adjustment to 3.2 were identical (pH 2.8). These results indicated that SPD basicity was not a major factor for improved tolerance of *S. cerevisiae* to acetic acid.

In addition, we examined the effects of SPD supplementation on tolerance of yeast against a real lignocellulose hydrolysate that contains numerous fermentation inhibitors in addition to furfural, HMF and acetic acid. Interestingly, we consistently observed the beneficial effects of SPD supplementation on tolerance of yeast even when using corn stover hydrolysate (Supplementary Fig. 7).

3.3. Construction of engineered *S. cerevisiae* stains with high SPD contents

We observed that a chemical perturbation (addition of 2 mM SPD into culture media) enhances tolerance of yeast against major inhibitory compounds (furan derivatives and acetic acid) present in lignocellulose hydrolysates. Nevertheless, this chemical perturbation is not desirable for large-scale fermentations because of additional costs. Therefore, we sought to identify genetic perturbations eliciting high levels of SPD without extracellular addition of SPD. We hypothesized that SPD might be overproduced in *S. cerevisiae* through altering expression levels of the enzymes involved in the SPD biosynthesis pathway. To this end, we selected the following genes as genetic perturbation targets. The key enzymes involved in the SPD biosynthesis include ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (SAMDC), and spermidine synthase (SPDS), which are encoded by *SPE1*, *SPE2*, and *SPE3*, respectively (Fig. 1). We constructed expression cassettes consisting of *SPE1*, *SPE2*, and *SPE3* genes under the control of a strong and constitutive promoter (GPD_P) and integrated these cassettes into the *S. cerevisiae* genome. A set of seven strains overexpressing three genes combinatorially was constructed (Supplementary Table 2) and intracellular levels of SPD, SPM, and PUT in each strain were measured. Among the strains tested, single or double overexpressing strains did not show SPD levels as high as the levels of intracellular SPD in yeast when 2 mM SPD was supplemented (Fig. 3a). When three genes were overexpressed simultaneously (S123), intracellular SPD levels were almost identical to the case of 2 mM SPD supplementation. Growth patterns of the engineered strains were almost identical to that of the control D452-2 strain in the YSC medium without fermentation inhibitors. However, in YSC medium containing 2 g/L furfural and 2 g/L HMF, the engineered *S. cerevisiae* strains (S2, S12, S13, S23, and S123) failed to grow, or showed prolonged lag-phase periods (S1 and S3) compared with the control strain (Fig. 3b). A previous study reported that when furan derivatives were added, the intracellular ATP concentration was 19% lower than that of the control strain (Ask et al., 2013). Because ODC antizyme is also known to cause continuous consumption of ATP when it mediates the degradation of overproduced ODC (Porat et al., 2008), we reasoned that *SPE1* overexpression in the presence of furfural might cause detrimental effects to yeast cells due to ATP depletion. Therefore, to relieve the ATP depletion and improve tolerance, we additionally disrupted *OAZ1* coding for ODC antizyme in the engineered strains. The effects of the *OAZ1* disruption on SPD contents were similar to the *SPE1* overexpression, and a combination of *OAZ1* disruption and *SPE1* overexpression elevated SPD contents further. The highest SPD content of 6.5 mg/g cell was obtained when *OAZ1* disruption and overexpression of *SPE1*, *SPE2*, and *SPE3* were combined (DO+S123). As a result, the intracellular SPD level of the OS123 strain was 15.5-fold higher than that of the control strain (Fig. 4a).

We also performed fermentation experiments to investigate whether or not the disruption of *OAZ1* and overexpression of three *SPE* genes improves tolerance of yeast in the presence of 2 g/L furfural and 2 g/L HMF. Although the disruption of *OAZ1* in the engineered strains overexpressing two or three *SPE* genes (DO+S12, DO+S13, and DO+S123) resulted in poor growth, the disruption of *OAZ1* in an engineered strain overexpressing *SPE1* only (DO+S1) led to better growth than a control strain under 2 g/L of furfural and 2 g/L of HMF (Fig. 4b). Among these engineered strains, the DO+S3 strain expressing *SPE3* showed the shortest lag-phase period of 72 h, which was 33% shorter than that of the control strain. These results indicate that double or triple overexpression of three *SPE* genes might cause

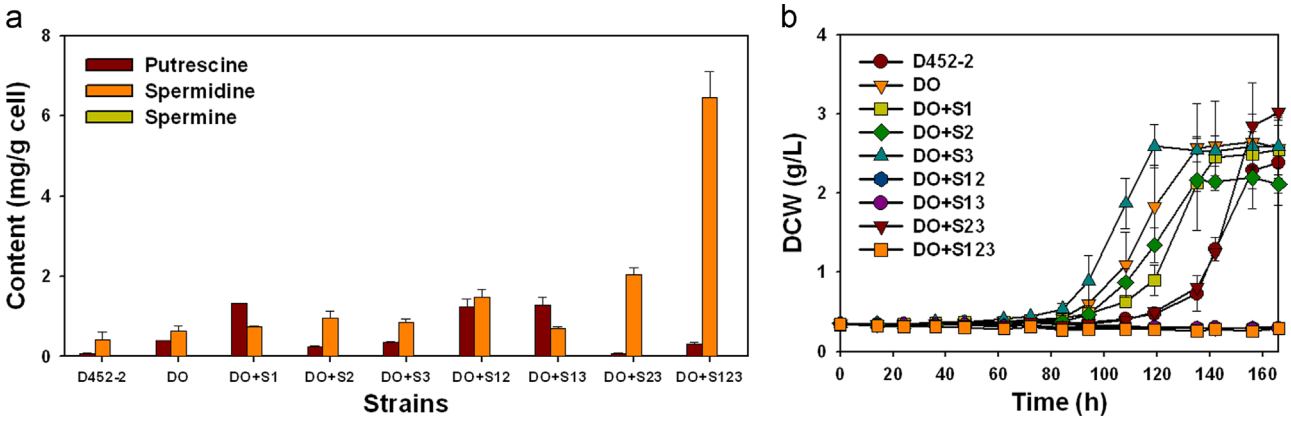

Fig. 4. Effects of alleviation of feedback inhibition on polyamine contents (a), and cell growth (b). (a) Intracellular levels of polyamines in engineered strains. Abbreviations: DO, D452-2 Δ*OAZ1*; DO+S1, DO overexpressing *SPE1*; DO+S2, DO overexpressing *SPE2*; DO+S3, DO overexpressing *SPE3*; DO+S12, DO overexpressing *SPE1,2*; DO+S13, DO overexpressing *SPE1,3*; DO+S23, DO overexpressing *SPE2,3*; DO+S123, DO overexpressing *SPE1,2,3*. (b) Cell growth of DO, DO+S1, DO+S2, DO+S3, DO+S12, DO+S13, DO+S23, DO+S123, and control D452-2 strain on a defined synthetic medium in the presence of 2 g/L furfural and HMF. Results are the mean of duplicate experiments and error bars indicate s.d.

metabolic burden and hence result in a prolonged lag-phase period. In conclusion, genetic perturbations for higher SPD levels should be optimized in terms of metabolic burden and SPD contents to construct inhibitor tolerant yeast strains.

3.4. Effects of disruption of TPO1 coding for polyamine excretion protein on tolerance of S. cerevisiae

In addition to the metabolic fluxes toward the synthesis of SPD, intracellular concentrations of SPD also can change by polyamine excretion. We hypothesized that the strategy of blocking the excretion of SPD would be more effective than that of expanding metabolic fluxes toward the synthesis of SPD in terms of metabolic burden and SPD contents. Therefore, we blocked the excretion of SPD by disrupting *TPO1* coding for polyamine transport protein (Uemura et al., 2007) to increase intracellular polyamine contents and inhibitor tolerance. Disruption of the *TPO1* gene in the D452-2 strain and the engineered strain overexpressing *SPE3* resulted in spermidine content of 0.79 mg SPD/g cell (the DT strain) and 1.1 mg SPD/g cell (the DTO+S3 strain), which were 90% and 171% higher than that of the control strain (Supplementary Fig. 8).

We performed batch fermentations with three engineered *S. cerevisiae* strains (DT, DO+S3, and DTO+S3) exhibiting higher SPD levels and the parental strain (D452-2) in YSC medium containing 2 g/L furfural and 2 g/L HMF. As expected, all three engineered strains (DT, DO+S3, and DTO+S3) exhibited much shorter lag-phase periods (48–60 h vs. 120 h) as compared to the control D452-2 strain (Fig. 5a; Supplementary Fig. 9). Among them, the highest ethanol productivity of 0.24 g/L h was obtained for the DT strain, which was 85% higher than the control strain (Fig. 5b). The reason for this improvement was ascribed to the faster conversion rate of furfural than the control strain. Interestingly, among the engineered strains capable of accumulating higher levels of SPD, only the DTO+S3 strain showed higher tolerance to acetate. The maximum specific growth rates (μ_{MAX}) of the DTO+S3 strain were 16% and 96% higher than the control strain when acetate concentrations were 3 and 4 g/L, respectively. In addition, the DTO+S3 strain showed a shorter lag-phase period (72 h vs. 107 h) than the control strain when acetate concentration was 4 g/L (Supplementary Fig. 10).

3.5. Identification of genes involved in tolerance against furan derivatives

The previous study reported that SPD supplementation upregulated several autophagy related genes (most significantly *ATG7*) and extended lifespan of *S. cerevisiae* through induction of autophagy (Eisenberg et al., 2009). Atg7p is an autophagy related protein which is required for autophagosome formation (Klionsky et al., 2003). The deletion of *ATG7* diminished the lifespan-extending effects of SPD supplementation in yeast (Eisenberg et al., 2009), suggesting that Atg7p might play a pivotal role in extending life-span via autophagy. As such, we hypothesized that the supplementation of SPD might improve tolerance against fermentation inhibitors via autophagy. However, we observed that disruption of *ATG7* did not influence the beneficial effects of SPD supplementation on tolerance against furan derivatives (Supplementary Fig. 11). These results suggest that the SPD supplementation might elicit the improved tolerance independent of autophagy. Therefore, to investigate putative mechanisms involved in the beneficial effects of SPD on detoxification of furan derivatives, we examined if NADH- and NADPH-dependent activities of furfural and HMF reductions can be enhanced by SPD supplementation or overproduction. Crude extracts of *S. cerevisiae* D452-2 with 2 mM SPD supplementation (+ 2 mM SPD), the DT strain capable of accumulating higher SPD without SPD supplementation, and the control strain were prepared and in vitro reduction activities of the crude extracts with furfural and HMF were measured. While specific activity of the crude extract of the+2 mM SPD toward furfural was slightly higher than that of the control and DT strains, specific activities toward HMF were almost identical (Supplementary Fig. 12). These results indicate that the mechanism involved in improved detoxification rates of furan derivatives by high levels of intracellular SPD might not be directly related to the reduction activities of oxidoreductases toward furfural and HMF.

In all fermentation experiments, exit times from lag-phases coincided with complete conversion of furfural into furfuryl alcohol even though HMF remained in the culture medium at the exit times. These results confirmed that furfural is a major inhibitor in lignocellulose hydrolysates. Therefore, we designed to conduct the RNA seq experiment in the medium containing furfural to obtain more concise data. While the control strain failed to grow in the presence of 4 g/L furfural, the +2 mM SPD and DT strain were able to grow after 62–77 h in the lag phase (Supplementary Fig. 13). To identify the genes involved in tolerance against furfural, the transcriptional responses of the control strain (*S. cerevisiae* D452-2), the SPD supplemented control strain (+ 2 mM SPD), and DT strain against furfural were measured at 15 h after inoculation using RNA sequencing (RNA-Seq) (Supplementary Fig. 14). The transcript levels of 94 and 14 genes changed more than four-fold in the +2 mM SPD and DT strains, respectively, as compared to the control strain ($P \leq 0.01$) (Supplementary Fig. 15a,b). Finding genes contributing to the tolerance phenotypes from these enormous

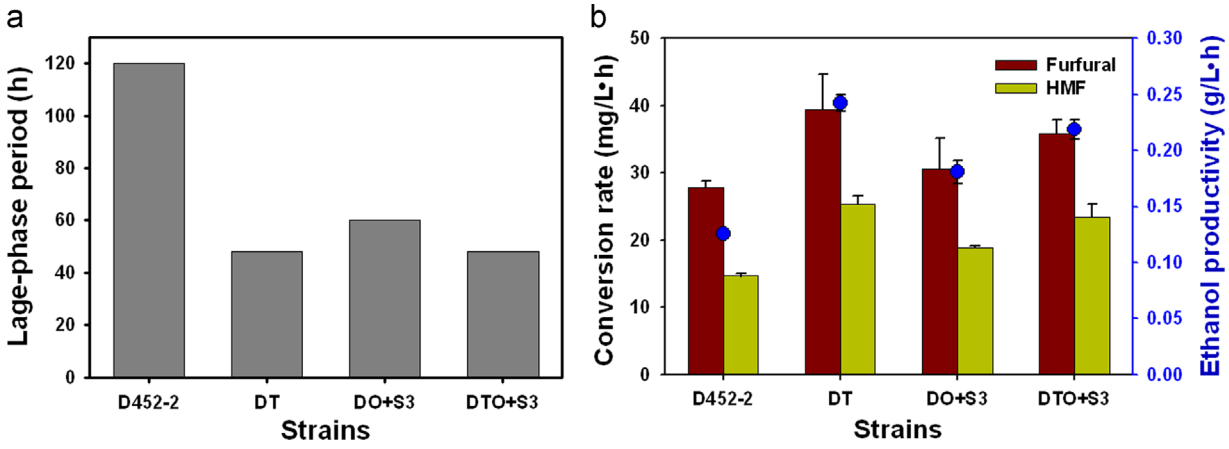

Fig. 5. Comparison of fermentation parameters by *S. cerevisiae* D452-2, DT, DO+S3, and DTO+S3 strains in the presence of 2 g/L furfural and HMF. (a) Lag-phase periods. (b) Furfural and HMF conversion rates and ethanol productivities. Results are the mean of duplicate experiments and error bars indicate s.d.

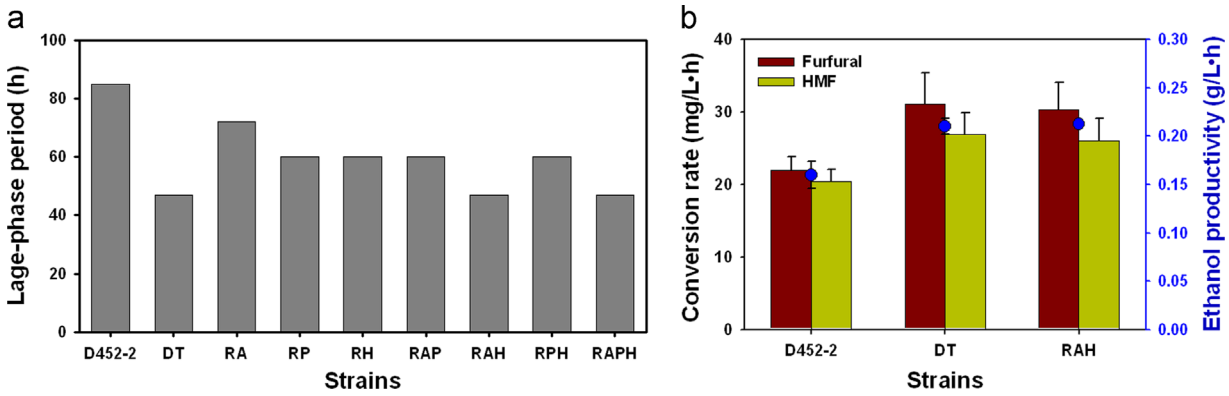

Fig. 6. Comparison of fermentation parameters by *S. cerevisiae* D452-2 and engineered strains in the presence of 2 g/L furfural and HMF. (a) Lag-phase period. Abbreviations: DT, D452-2 Δ*TPO1*; RA, D452-2 overexpressing *ADE17*; RP, D452-2 overexpressing *PIR3*; RH, D452-2 overexpressing *HTA2*; RAP, D452-2 overexpressing *ADE17* and *PIR3*; RAH, D452-2 overexpressing *ADE17* and *HTA2*; RPH, D452-2 overexpressing *PIR3* and *HTA2*; RAPH, D452-2 overexpressing *ADE17*, *PIR3*, and *HTA2*. (b) Higher furfural and HMF conversion rates and ethanol productivities of the engineered strains (DT and RHA). Results are the mean of duplicate experiments and error bars indicate s.d.

transcriptional changes, either individually or in combination, is unfeasible. Among several solutions have been proposed to simplify the detection of biologically relevant changes, we adopted the approach using independent parallel lines and selecting target genes occurring among parallel lines (de Kok et al., 2012; Hong et al., 2011). Therefore, we hypothesized that the genes contributing to the tolerance phenotypes of yeast would be differentially expressed genes both in +2 mM SPD and DT strains. These genes were *ADE17* and *PIR3*, coding for a bifunctional purine biosynthesis protein, and O-glycosylated covalently-bound cell wall protein, respectively. Both *ADE17* and *PIR3* were upregulated by 18.0 fold in the +2 mM SPD, and 5.3 and 6.2 folds in the DT strain as compared to the control strain. As a target for tolerance improvement against furfural, we also selected the genes upregulated in the +2 mM SPD as compared to the DT strain. *HTA2* coding for a histone H2A protein and *YDR034C-A* were upregulated to 6.9 and 45.0 fold in the +2 mM SPD as compared to the DT strain (Supplementary Fig. 15c). Because only *HTA2* was also upregulated in the +2 mM SPD as compared to the control strain, we selected *HTA2* as a target gene. The three genes, *ADE17*, *PIR3*, and *HTA2*, were downregulated to 40.7, 6.1, and 25.9 folds in response to furfural in the control strain, suggesting that furfural might cause lower expression of those genes. Therefore, we hypothesized that overexpression of these genes in *S. cerevisiae* D452-2 might improve tolerance to furfural.

We constructed overexpression cassettes containing *ADE17*, *PIR3*, and *HTA2* genes controlled by the *tHXT7* promoter (Hauf et al., 2000) and integrated these cassettes to the *S. cerevisiae* genome. Engineered strains overexpressing one of the three genes (*ADE17*, *PIR3*, and *HTA2*) indeed exhibited shorter lag-phase periods than that of the control strain (Fig. 6a; Supplementary Fig. 16). Among them, the highest ethanol productivity of 0.21 g/L h was obtained for the D452-2 strain overexpressing *ADE17* and *HTA2* (RAH). The ethanol productivity and conversion rates of furan derivatives by the RAH strain were comparable to that by the DT strain under the same conditions (Fig. 6b). We also investigated whether or not the engineered strains showed an improvement in tolerance to ethanol. The lag-phase periods and maximum specific growth rates of the engineered strains were identical to those of the control D452-2 strain in the YSC medium containing 8% (v/v) ethanol. However, the DTO+S3 and RAH strains increased maximum dry cell weight (DCW) under the ethanol stress condition (Supplementary Fig. 17). Especially, the maximum DCW of the DTO+S3 strain was 26% higher than that of the control strain (Supplementary Fig. 17).

4. Discussion

Fermentation inhibitors present in lignocellulose hydrolysates are among major hindrances for the commercialization of biofuel production. Among them, furfural, HMF, and acetic acid are major inhibitors which negatively affect microbial growth, metabolism, and ethanol production. We identified chemical perturbations eliciting improved tolerance against the major inhibitors, and constructed engineered yeast strains exhibiting improved tolerance without chemical perturbations. Moreover, we captured differentially expressed genes by chemical and genetic perturbations under furfural conditions and

demonstrated that overexpression of the downregulated genes under furfural conditions can lead to improved tolerance to furfural. This study not only identified new gene targets for understanding endogenous tolerance mechanisms against fermentation inhibitors, but also constructed numerous engineered strains showing various tolerance phenotypes.

These engineered strains exhibiting distinct improved phenotypes against fermentation inhibitors can be served as customized host strains for fermenting cellulosic hydrolysates containing different amounts of fermentation inhibitors. In the case of utilizing lignocellulose hydrolysates containing high levels of furfural, the DT strain exhibiting the highest tolerance against furfural could be used. Tpo1p which functions as drug/toxin transporter and multidrug efflux pump is one of the significant elements for yeast survival and adaptation under inhibitory HMF concentrations (Ma and Liu, 2010a). However, disruption of *TPO1* improved tolerance against furfural without affecting tolerance against HMF. On the other hand, in the case of utilizing lignocellulose hydrolysates containing high levels of acetic acid, the DTO+S3 strain showing improved tolerance to both furan derivatives and acetic acid is applicable. In conclusion, we successfully constructed not only the DT strain exhibiting substantial tolerance to furfural but also the DTO+S3 strain exhibiting tolerance to multiple inhibitors including furan derivatives and acetic acid.

We identified three genes (*ADE17*, *PIR3*, and *HTA2*) involved in tolerance against furfural in yeast through comparing the transcriptomes of the D452-2 strain with and without chemical and genetic perturbations eliciting improved tolerance to furfural. Furfural decreased transcript levels of several genes including *ADE17*, *PIR3*, and *HTA2*. However, *S. cerevisiae* strains with improved tolerance to furfural (the +2 mM SPD and DT strains) exhibited higher transcript levels of *ADH17*, *PIR3*, and *HTA2* as compared to the control strain. Under zinc starvation and oxidative stress, a bifunctional purine biosynthesis protein encoded by *ADE17* has been reported to present increased expression levels for adaptation in yeast cells (Grossklaus et al., 2013; Parente et al., 2013). These results are in agreement with our results showing that overexpression of *ADE17* improve tolerance of *S. cerevisiae* against furfural. Although a previous study reported that damage to the cell wall caused by furfural was undetected by the current scanning electron microscope (SEM) analysis (Allen et al., 2010), yeast cells overexpressing *PIR3* which is known to be upregulated in case of cell wall stress (Jung and Levin, 1999; Terashima et al., 2000) and required for cell wall stability (Klis et al., 2002), exhibited improved tolerance to furfural. In addition, a histone H2A protein encoded by *HTA2* required for chromatin assembly and efficient DNA double-strand break repair (Downs et al., 2000) was identified as an overexpression target in this study. Because furfural causes an accumulation of reactive oxygen species (ROS) and damage to nuclear chromatin (Allen et al., 2010), it is likely that overexpression of *HTA2* alleviated damage to chromatin caused by furfural. Interestingly, although the engineered strains (DT, DO+S3, and DTO+S3) showed increased tolerance to furan derivatives, only the DTO+S3 strain also exhibited faster recovery of growth than the control strain under acetate stress conditions. Finally, we aim to construct an engineered strain having inhibitor tolerance identical to the case of SPD supplementation without extracellular addition through optimization of expression levels of the identified genes. We observed that genetic perturbations for higher SPD levels need to be optimized in terms of metabolic burden and SPD contents. These results are consistent with the previous study, which presented that tolerance phenotypes of *E. coli* against furfural were affected by both specific activity of FucO, an NADH dependent propanediol (and furfural) oxidoreductase, and metabolic burden caused by target protein overexpression (Wang et al., 2013). Therefore, future optimization of expression levels of the enzymes involved in SPD biosynthesis might further improve tolerance against inhibitors.

The undissociated form of acetic acid can penetrate into the plasma membrane from the fermentation medium and then dissociates due to high intracellular pH. The dissociation of acetic acid decreases the intracellular pH, and cells consume ATP to neutralize pH by pumping protons out of the cells (Pampulha and Loureirodias, 1989). This ATP deficiency causes inhibition of cell growth. Moreover, the presence of acetic acid slows down the flux of the non-oxidative pentose phosphate pathway (PPP) and hence overexpression of a gene encoding a PPP-related enzyme, transaldolase or transketolase, improved the tolerance of *S. cerevisiae* to acetic acid (Hasunuma et al., 2011). Additional experiments are under way to elucidate the key genes involved in tolerance of *S. cerevisiae* to acetic acid including genes coding for proton pumps and PPP-related enzymes.

Previous studies have reported that *S. cerevisiae* mutants incapable of synthesizing SPD require supplementation of SPD for optimal growth (Balasundaram et al., 1991; Cohn et al., 1978; Hamasaki-Katagiri et al., 1998) and that one of the important roles for SPD is to activate the eukaryotic translation initiation factor 5A (eIF5A) through hypusine biosynthesis (Chattopadhyay et al., 2008). However, there remains the unanswered question why *S. cerevisiae* cells have a large excess of intracellular SPD and hypusinated eIF5A, because wild-type *S. cerevisiae* cells have 1000-fold and 20-fold higher intracellular SPD and hypusinated eIF5A than those needed for optimal growth (Chattopadhyay et al., 2008). One possible explanation could be that high levels of SPD in baker's yeast might be important for adaptation and survival in diverse environmental stresses.

In this study, we have demonstrated a unique strategy to shorten the extended lag-phase period caused by lignocellulose-derived inhibitors. We found that the chemical perturbation eliciting higher SPD levels improved tolerance of *S. cerevisiae* to furan derivatives and acetic acid, and intracellular SPD contents should be well balanced with metabolic burden caused by genetic perturbations to construct inhibitor tolerant *S. cerevisiae* strains. By combining the amplification of genes coding for enzymes in the SPD biosynthetic pathway with disruption of the genes involved in feedback inhibition (*OAZ1*) and SPD excretion (*TPO1*), we constructed engineered strains with improved tolerance against multiple inhibitors (furfual, HMF, and acetic acid) and ethanol. The strains developed in this study can have broad applications not only to produce ethanol but also many other biochemicals and biofuels from lignocellulosic biomass.

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Appendix A. Supporting information

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References

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: fundamentals toward application. Biotechnol. Adv. 29, 675–685.
Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 2.
Almeida, J.R., Bertilsson, M., Gorwa-Grauslund, M.F., Gorsich, S., Liden, G., 2009. Metabolic effects of furaldehydes and impacts on biotechnological processes. Appl. Microbiol. Biotechnol. 82, 625–638.
Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in

lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82, 340–349.
Almeida, J.R.M., Roder, A., Modig, T., Laadan, B., Liden, G., Gorwa-Grauslund, M.F., 2008. NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 78, 939–945.
Alriksson, B., Horvath, I.S., Jonsson, L.J., 2010. Overexpression of *Saccharomyces cerevisiae* transcription factor and multidrug resistance genes conveys enhanced resistance to lignocellulose-derived fermentation inhibitors. Process Biochem. 45, 264–271.
Ask, M., Bettiga, M., Mapelli, V., Olsson, L., 2013. The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*. Biotechnol. Biofuels 6, 22.
Balasundaram, D., Tabor, C.W., Tabor, H., 1991. Spermidine or spermine is essential for the aerobic growth of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 88, 5872–5876.
Balasundaram, D., Tabor, C.W., Tabor, H., 1993. Oxygen toxicity in a polyamine-depleted *spe2*$^{\Delta}$ mutant of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 90, 4693–4697.
Balasundaram, D., Tabor, C.W., Tabor, H., 1996. Sensitivity of polyamine-deficient *Saccharomyces cerevisiae* to elevated temperatures. J. Bacteriol. 178, 2721–2724.
Balasundaram, D., Tabor, C.W., Tabor, H., 1999. Sensitivity of spermidine-deficient *Saccharomyces cerevisiae* to paromomycin. Antimicrob. Agents Chemother. 43, 1314–1316.
Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. 11, 226–228.
Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J., 1999. Polyamines and environmental challenges: recent development. Plant Sci. 140, 103–125.
Chattopadhyay, M.K., Park, M.H., Tabor, H., 2008. Hypusine modification for growth is the major function of spermidine in *Saccharomyces cerevisiae* polyamine auxotrophs grown in limiting spermidine. Proc. Natl. Acad. Sci. USA 105, 6554–6559.
Cohn, M.S., Tabor, C.W., Tabor, H., 1978. Isolation and characterization of *Saccharomyces cerevisiae* mutants deficient in S-adenosylmethionine decarboxylase, spermidine, and spermine. J. Bacteriol. 134, 208–213.
de Kok, S., Nijkamp, J.F., Oud, B., Roque, F.C., de Ridder, D., Daran, J.M., Pronk, J.T., van Maris, A.J.A., 2012. Laboratory evolution of new lactate transporter genes in a *jen1*Δ mutant of *Saccharomyces cerevisiae* and their identification as *ADY2* alleles by whole-genome resequencing and transcriptome analysis. FEMS Yeast Res. 12, 359–374.
Downs, J.A., Lowndes, N.F., Jackson, S.P., 2000. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. Nature 408, 1001–1004.
Eisenberg, T., Knauer, H., Schauer, A., Buttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., Ring, J., Schroeder, S., Magnes, C., Antonacci, L., Fussi, H., Deszcz, L., Hartl, R., Schraml, E., Criollo, A., Megalou, E., Weiskopf, D., Laun, P., Heeren, G., Breitenbach, M., Grubeck-Loebenstein, B., Herker, E., Fahrenkrog, B., Frohlich, K.U., Sinner, F., Tavernarakis, N., Minois, N., Kroemer, G., Madeo, F., 2009. Induction of autophagy by spermidine promotes longevity. Nat. Cell Biol. 11, 1305–1314.
Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.
Gorsich, S.W., Dien, B.S., Nichols, N.N., Slininger, P.J., Liu, Z.L., Skory, C.D., 2006. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes *ZWF1*, *GND1*, *RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 71, 339–349.
Grossklaus, D.D., Bailao, A.M., Rezende, T.C.V., Borges, C.L., de Oliveira, M.A.P., Parente, J.A., Soares, C.M.D., 2013. Response to oxidative stress in Paracoccidioides yeast cells as determined by proteomic analysis. Microbes Infect. 15, 347–364.
Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Liden, G., Zacchi, G., 2006. Bio-ethanol—the fuel of tomorrow from the residues of today. Trends Biotechnol. 24, 549–556.
Hamana, K., Matsuzaki, S., 1992. Polyamines as a chemotaxonomic marker in bacterial systematics. Crit. Rev. Microbiol. 18, 261–283.
Hamasaki-Katagiri, N., Katagiri, Y., Tabor, C.W., Tabor, H., 1998. Spermine is not essential for growth of *Saccharomyces cerevisiae*: identification of the *SPE4* gene (spermine synthase) and characterization of a *spe4* deletion mutant. Gene 210, 195–201.
Hasunuma, T., Sanda, T., Yamada, R., Yoshimura, K., Ishii, J., Kondo, A., 2011. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. Microb. Cell Factories 10, 2.
Hauf, J., Zimmermann, F.K., Muller, S., 2000. Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 26, 688–698.
Hong, K.K., Vongsangnak, W., Vemuri, G.N., Nielsen, J., 2011. Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. Proc. Natl. Acad. Sci. USA 108, 12179–12184.
Innocente, N., Biasutti, A., Padovese, A., Moret, S., 2007. Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. Food Chem. 101, 1285–1289.
Jung, U.S., Levin, D.E., 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol. Microbiol. 34, 1049–1057.
Jung, Y.H., Kim, I.J., Kim, H.K., Kim, K.H., 2013. Dilute acid pretreatment of lignocellulose for whole slurry ethanol fermentation. Bioresour. Technol. 132, 109–114.
Kim, D., Hahn, J.S., 2013. Roles of the Yap1 transcription factor and antioxidants in *Saccharomyces cerevisiae*'s tolerance to furfural and 5-hydroxymethylfurfural, which function as thiol-reactive electrophiles generating oxidative stress. Appl. Environ. Microbiol. 79, 5069–5077.
Klionsky, D.J., Cregg, J.M., Dunn, W.A., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., Ohsumi, Y., 2003. A unified nomenclature for yeast autophagy-related genes. Dev. Cell 5, 539–545.
Klis, F.M., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 26, 239–256.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.
Lee, S.H., Kodaki, T., Park, Y.C., Seo, J.H., 2012. Effects of NADH-preferring xylose reductase expression on ethanol production from xylose in xylose-metabolizing recombinant *Saccharomyces cerevisiae*. J. Biotechnol. 158, 184–191.
Liu, Z.L., Moon, J., Andersh, B.J., Slininger, P.J., Weber, S., 2008. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 81, 743–753.
Lynd, L.R., Laser, M.S., Brandsby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat. Biotechnol. 26, 169–172.
Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. 16, 577–583.
Ma, M.G., Liu, Z.L., 2010a. Comparative transcriptome profiling analyses during the lag phase uncover *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 11, 660.
Ma, M.G., Liu, Z.L., 2010b. Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 87, 829–845.
Modig, T., Liden, G., Taherzadeh, M.J., 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem. J. 363, 769–776.
Moon, J., Liu, Z.L., 2012. Engineered NADH-dependent GRE2 from *Saccharomyces cerevisiae* by directed enzyme evolution enhances HMF reduction using additional cofactor NADPH. Enzym. Microb. Technol. 50, 115–120.
Moret, S., Conte, L.S., 1996. High-performance liquid chromatographic evaluation of biogenic amines in foods—an analysis of different methods of sample preparation in relation to food characteristics. J. Chromatogr. A 729, 363–369.
Morgan, M., Anders, S., Lawrence, M., Aboyoun, P., Pages, H., Gentleman, R., 2009. ShortRead: a bioconductor package for input, quality assessment and exploration of high-throughput sequence data. Bioinformatics 25, 2607–2608.
Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673–686.
Mussatto, S.I., Roberto, I.C., 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresour. Technol. 93, 1–10.
Palanimurugan, R., Scheel, H., Hofmann, K., Dohmen, R.J., 2004. Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. EMBO J. 23, 4857–4867.
Pampulha, M.E., Loureirodias, M.C., 1989. Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. Appl. Microbiol. Biotechnol. 31, 547–550.
Parente, A.F.A., De Rezende, T.C.V., De Castro, K.P., Bailao, A.M., Parente, J.A., Borges, C.L., Silva, L.P., Soares, C.M.D., 2013. A proteomic view of the response of *Paracoccidioides* yeast cells to zinc deprivation. Fungal. Biol. 117, 399–410.
Park, S.E., Koo, H.M., Park, Y.K., Park, S.M., Park, J.C., Lee, O.K., Park, Y.C., Seo, J.H., 2011. Expression of aldehyde dehydrogenase 6 reduces inhibitory effect of furan derivatives on cell growth and ethanol production in *Saccharomyces cerevisiae*. Bioresour. Technol. 102, 6033–6038.
Petersson, A., Almeida, J.R.M., Modig, T., Karhumaa, K., Hahn-Hagerdal, B., Gorwa-Grauslund, M.F., Liden, G., 2006. A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. Yeast 23, 455–464.
Porat, Z., Landau, G., Bercovich, Z., Krutauz, D., Glickman, M., Kahana, C., 2008. Yeast antizyme mediates degradation of yeast ornithine decarboxylase by yeast but not by mammalian proteasome: new insights on yeast antizyme. J. Biol. Chem. 283, 4528–4534.
Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.
Sanda, T., Hasunuma, T., Matsuda, F., Kondo, A., 2011. Repeated-batch fermentation of lignocellulosic hydrolysate to ethanol using a hybrid *Saccharomyces cerevisiae* strain metabolically engineered for tolerance to acetic and formic acids. Bioresour. Technol. 102, 7917–7924.
Sundstrom, L., Larsson, S., Jonsson, L.J., 2010. Identification of *Saccharomyces cerevisiae* genes involved in the resistance to phenolic fermentation inhibitors. Appl. Biochem. Biotechnol. 161, 106–115.
Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Liden, G., 1999. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 87, 169–174.
Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Liden, G., 2000. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 53, 701–708.
Terashima, H., Yabuki, N., Arisawa, M., Hamada, K., Kitada, K., 2000. Up-regulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in

response to cell wall damage caused by disruption of *FKS1* in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 264, 64–74.
Uemura, T., Kashiwagi, K., Igarashi, K., 2007. Polyamine uptake by DUR3 and SAM3 in *Saccharomyces cerevisiae*. J. Biol. Chem. 282, 7733–7741.
van Maris, A.J.A., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A.H., Wisselink, H.W., Scheffers, W.A., van Dijken, J.P., Pronk, J.T., 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Leeuwenhoek 90, 391–418.
Wang, X., Yomano, L.P., Lee, J.Y., York, S.W., Zheng, H.B., Mullinnix, M.T., Shanmugam, K.T., Ingram, L.O., 2013. Engineering furfural tolerance in *Escherichia coli* improves the fermentation of lignocellulosic sugars into renewable chemicals. Proc. Natl. Acad. Sci. USA 110, 4021–4026.
Wei, N., Quarterman, J., Kim, S.R., Cate, J.H.D., Jin, Y.S., 2013. Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. Nat. Commun. 4, 2580.
Yang, B., Wyman, C.E., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod. Biorefining 2, 26–40.