Overexpression of *RCK1* improves acetic acid tolerance in *Saccharomyces cerevisiae*

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Abbreviations

ROS	reactive oxygen species
MAPK	mitogen-activated protein kinase

Abstract

Mixed sugars derived from lignocellulosic biomass can be converted into biofuels and chemicals by engineered microorganisms, but toxic fermentation inhibitors produced from harsh depolymerization processes of lignocellulosic biomass pose a critical challenge for economic production of biofuels and chemicals. Unlike other fermentation inhibitors generated from sugar degradation, acetic acid is inevitably produced from acetylated hemicellulose, and its concentrations in cellulosic hydrolysates are substantially higher than other fermentation inhibitors. The aim of this study was to identify novel genetic perturbations for improved acetic acid tolerance in *Saccharomyces cerevisiae*. Through a genomic library-based approach, we identified an overexpression gene target *RCK1* coding for a protein kinase involved in oxidative stress. Overexpression of *RCK1* significantly improved glucose and xylose fermentation under acetic acid stress conditions. Specifically, the *RCK1*-overexpressing strain exhibited a two-fold higher specific ethanol productivity than the control strain in glucose fermentation under the presence of acetic acid. Interestingly, the engineered *S. cerevisiae* overexpressing *RCK1* showed 40% lower intracellular reactive oxygen species (ROS) levels as compared to the parental strain when the strains were exposed to acetic acid, suggesting that *RCK1* overexpression might play a role in reducing the oxidative stress caused by acetic acid.

1. Introduction

Biofuel production from lignocellulosic biomass has been studied extensively because lignocellulosic biomass is more sustainable and abundant as compared to agricultural crops, such as corn and sugarcane (Hahn-Hägerdal et al., 2006). Lignocellulosic biomass derived from agricultural and forestry residues contains cellulose, hemicellulose, and lignin. Efficient conversion of cellulose and hemicellulose into fermentable sugars is essential for successful production of value-added chemicals by microbial fermentation at an industrial scale (Alper and Stephanopoulos, 2009). However, the hydrolysis process of cellulose and hemicellulose to release sugars generates unavoidable by-products which are toxic to fermenting microorganisms. Because hemicellulose and lignin contain acetyl groups, treatments of those fractions for obtaining fermentable sugars yield acetic acid ranging from 1 to 15 g/L, which can negatively affect fermentation performances of the fermenting microorganisms (Klinke et al., 2004). *Saccharomyces cerevisiae* is the most widely used microorganism for producing value-added chemicals in industrial fermentation. Therefore, it is important to improve acetic acid tolerance for efficient and economic bioconversion of lignocellulosic sugars by *S. cerevisiae.*

The toxicity mechanisms of acetic acid in *S. cerevisiae* have been intensively studied. At lower pH than the acetic acid pK_a value (4.76, at 25 °C), acetic acid is predominantly the undissociated form, and it can enter into the cell by passive diffusion through plasma membranes (Casal et al., 1996). Once inside the cell, the undissociated form dissociates because of a higher intracellular pH, resulting in free protons and a dissociated form. The expense of ATP to pump out the protons by the plasma

membrane ATPase and the dissociated form accumulated inside the cell impair essential metabolic functions (Pampulha and Loureiro-Dias, 2000). In addition, the cell undergoes a programmed cell death process when it is exposed to lethal concentrations of acetic acid. Specifically, intracellular acidification caused by acetic acid induces reactive oxygen species (ROS) accumulation, cytochrome c release, and caspase-like activity increase with DNA fragmentation, resulting in cell death (Guaragnella et al., 2011). For these reasons, the presence of acetic acid in lignocellulosic hydrolysates can hinder the efficient conversion of fermenting sugars by *S. cerevisiae*.

Various cellular functions are involved in the mechanisms of the yeast response against acetic acid to protect the cell from the acetic acid stress. The chemical genomics-based analysis for identifying determinants in tolerance to acetic acid suggested that metabolism of carbohydrate and amino acid, assembly of cell wall structure, and uptake and biosynthesis of various nutrients were implicated (Mira et al., 2010). Because of the complexity of the acetic acid stress response and the incomplete understanding of interactions in gene regulatory networks, it is challenging to uncover a set of gene targets for efficient sugar fermentation under acidic conditions in *S. cerevisiae* via rational approaches based on the prior knowledge.

In this study, we employed an inverse metabolic engineering to improve acetic acid tolerance in *S. cerevisiae*. The introduction of the libraries for genetic perturbations into platform microorganisms allows a variety of phenotypes, and the obtained genes responsible for improving the phenotypes are transferable to other strains (Santos and Stephanopoulos, 2008). Through this approach, we identified the *RCK1* gene, and its

overexpression significantly improved acetic acid tolerance under glucose and xylose conditions.

2. Materials and methods

Materials and methods section is available in the online supporting information.

3. Results and discussion

3.1. Yeast genomic library screening under toxic levels of acetic acid

After transforming a yeast genomic library into *S. cerevisiae* CEN.PK2-1D, the transformants were spread on the selective yeast synthetic complete (SC) medium agar plates containing 35 g/L of glucose and 3 g/L of acetic acid with pH adjusted at 4. Then thirty fast-growing transformants were isolated from the plates and further examined in liquid SC medium with acetic acid to confirm the acetic acid tolerance phenotype (Fig. S1A). Among the thirty transformants, the best three fast-growing ones (numbers 22, 24, and 28) were selected for further characterization. Plasmids from the three selected transformants were isolated and then re-transformed into the parental strain (*S. cerevisiae* CEN.PK2-1D) to test whether or not the plasmids harboring gene targets enabled the superior tolerance against acetic acid. As a result, only one plasmid (number 22) confirmed to enable improved cell growth under acetic acid stress in the parental strain (Fig. S1B). The other two plasmids (numbers 24 and 28) did not lead to improved acetic acid tolerance in the re-transformed strains, indicating that unknown beneficial mutations in the genome of the original transformants might enhance the

observed acetic acid tolerance. Sequence analysis of the plasmid 22 revealed that it contained a complete sequence of the gene *RCK1*, which encodes a protein kinase involved in oxidative stress response (Bilsland et al., 2004; Dahlkvist and Sunnerhagen, 1994).

3.2. Improved acetic acid tolerance through *RCK1* overexpression

To examine the effect of *RCK1* on improving acetic acid tolerance in yeast as a transferable gene target, we constructed *RCK1*-overexpressing multi-copy plasmids. The open reading frame (ORF) of the *RCK1* was PCR amplified from the genomic DNA of *S. cerevisiae* D452-2 and cloned into the pRS series of vectors (pRS423 and pRS424) containing *TDH3* promoter and *CYC1* terminator using the restriction enzyme sites *BamH*I and *Xho*I. The resulting plasmids expressing *RCK1* and empty plasmids were transformed using the high-efficiency yeast transformation (Table S1) (Gietz and Schiestl, 2007). First, we confirmed that both the *S. cerevisiae* D452-2 strain overexpressing *RCK1* (D423R) and a control strain (D423C) showed no difference in phenotypes during fermentation of glucose in the absence of acetic acid (Fig. S2). Then, both strains were cultured in yeast extract / peptone (YP) medium containing 80 g/L of glucose and 5 g/L of acetic acid under oxygen-limited conditions (Fig. 1A and B). Without any adjustment, the initial pH of the medium containing 5 g/L of acetic acid was 4.0, which is lower than the p*Ka* of acetic acid (4.76, at 25 °C), resulting in intracellular acidification. The D423R strain consumed 80 g/L of glucose in 48 hours, whereas the control strain (D423C) consumed only 13 g/L of glucose. The specific glucose consumption rate of the D423C strain was 0.468 ± 0.037 g glucose/g cell·h, and that of

the D423R strain was 0.865 ± 0.008 g glucose/g cell·h. Also, the specific ethanol productivity from glucose by the D423R strain (0.332 ± 0.004 g ethanol/g cell·h) was approximately two-fold higher than that by the D423C strain (0.153 ± 0.003 g ethanol/g cell·h).

Because xylose is the second most abundant sugar in lignocellulosic hydrolysates (Kim et al., 2012), efficient xylose utilization in the presence of acetic acid is essential for the successful microbial fermentation. Therefore, next, we tested the effects of the overexpression of *RCK1* on acetic acid tolerance during xylose fermentation. The engineered xylose-fermenting *S. cerevisiae*, SR8, was used as the host strain for the overexpression of *RCK1* (Table S1). When fermenting 40 g/L of xylose with 5 g/L acetic acid, the phenotypic differences between the *RCK1*-overexpressing strain (SR8R) and the control strain (SR8C) were obvious. The SR8R strain consumed 13 g/L of xylose within 96 hours, with a xylose consumption rate of 0.139 ± 0.001 g xylose/L·h, while the SR8C strain could not consume xylose at all (Fig. 1C and D). Although the overexpression of *RCK1* improved acetic acid tolerance in the xylose fermentation, the improvement was not significant when compared to the glucose fermentation by the D423R strain, indicating that xylose fermentation was more sensitive to acetic acid than glucose fermentation. Taken together, the overexpression of *RCK1* in engineered *S. cerevisiae* enhanced acetic acid tolerance under both glucose and xylose conditions.

3.3. Effects of *RCK1* overexpression on intracellular levels of ROS

When *S. cerevisiae* was exposed to acetic acid stress, accumulation of ROS occurs in the cell, resulting in acetic acid-induced programmed cell death (Guaragnella et al., 2007). In previous studies, levels of intracellular ROS in the acetic acid-tolerant strains were significantly lower than those in the control strains, indicating that increased acetic acid tolerance might be closely related to the cellular capacities to reduce levels of intracellular ROS (An et al., 2015; Zheng et al., 2011). Also, zinc ion supplementation triggering an oxidative stress response resulted in up-regulation of *RCK1*, conferring tolerance to acetic acid stress (Ismail et al., 2014; Pagani et al., 2007). As such, we hypothesized that *RCK1* overexpression might increase tolerance to acetic acid by reducing the level of the intracellular ROS. The effect of *RCK1* overexpression on improving capacity to reduce the level of intracellular ROS was demonstrated using the dichlorofluorescein assay (Wang and Joseph, 1999). The amount of intracellular ROS was evaluated under both glucose and xylose conditions by fluorescence intensity per cell when the cell density was adjusted to an optical density at 600nm (OD_{600}) of 5 (Fig. 2A and B). When cultured on glucose without the treatment of H_2O_2, the *RCK1*-overexpressing strain (D423R) and control strain (D423C) showed similar levels of ROS accumulation, suggesting that the overexpression of *RCK1* did not affect accumulation of intracellular ROS under normal conditions. In contrast, we observed substantial increases in ROS accumulation in both strains after adding H_2O_2 or acetic acid to the medium. However, the D423R strain exhibited a 43% lower accumulation of ROS than the D423C strain in the case of H_2O_2 treatment, indicating that overexpression of *RCK1* activated cellular mechanisms for ROS removal and improved the ability to reduce the intracellular ROS. Notably, similar results were obtained in the experiment with acetic

acid treatment. When the strains were exposed to acetic acid, the *RCK1*-overexpressing strain showed 40% lower intracellular ROS intensity compared to the control strain (Fig. 2A). Moreover, when cultured on xylose with H_2O_2 or acetic acid, the *RCK1*-overexpressing strain (SR8R) exhibited significantly reduced intracellular ROS intensity compared to the control strain (SR8C) (Fig. 2B). These results suggested that *RCK1* overexpression might play a critical role in reducing the intracellular ROS, resulting in improved acetic acid tolerance. In previous studies, Chang *et al*. reported that Rck1 up-regulated the hog1 pathway by down-regulating Ptp2, a negative regulator of Hog1 (Chang et al., 2013). The *HOG1* gene encodes a mitogen-activated protein kinase (MAPK), and the active Hog1 as the terminal MAPK of kinase cascade conferred acetic acid tolerance. Also, Rck1 interacted with Yap2, a transcription factor required for oxidative stress resistance (Bilsland et al., 2004; Mollapour and Piper, 2006). Based on previous studies and our observations, the acetic acid tolerance obtained by the overexpression of *RCK1* might be associated with the activation of the Hog1 MAPK and the Yap transcription factor, resulting in induction of a protective response to acetic acid stress. We are currently investigating the underlying mechanisms of the *RCK1* overexpression in engineered *S. cerevisiae*.

In summary, *RCK1* was identified as an overexpression target for enhancing acetic acid tolerance through the inverse metabolic engineering approach. The overexpression of *RCK1* in various host strains (*S. cerevisiae* CEN.PK2-1D, D452-2, and SR8) consistently improved acetic acid tolerance under both glucose and xylose conditions. Also, the *RCK1*-overexpressing strain accumulated less intracellular ROS against acetic acid stress, suggesting that the resulting strain attenuated acetic acid

stress by reducing intracellular ROS levels. To our knowledge, this is the first report that shows the effects of *RCK1* overexpression on improving acetic acid tolerance in *S. cerevisiae*. We envision that the strategy demonstrated here can be extended to identify genetic perturbations for improving other fermentation inhibitors such as HMF and furfural.

Acknowledgments

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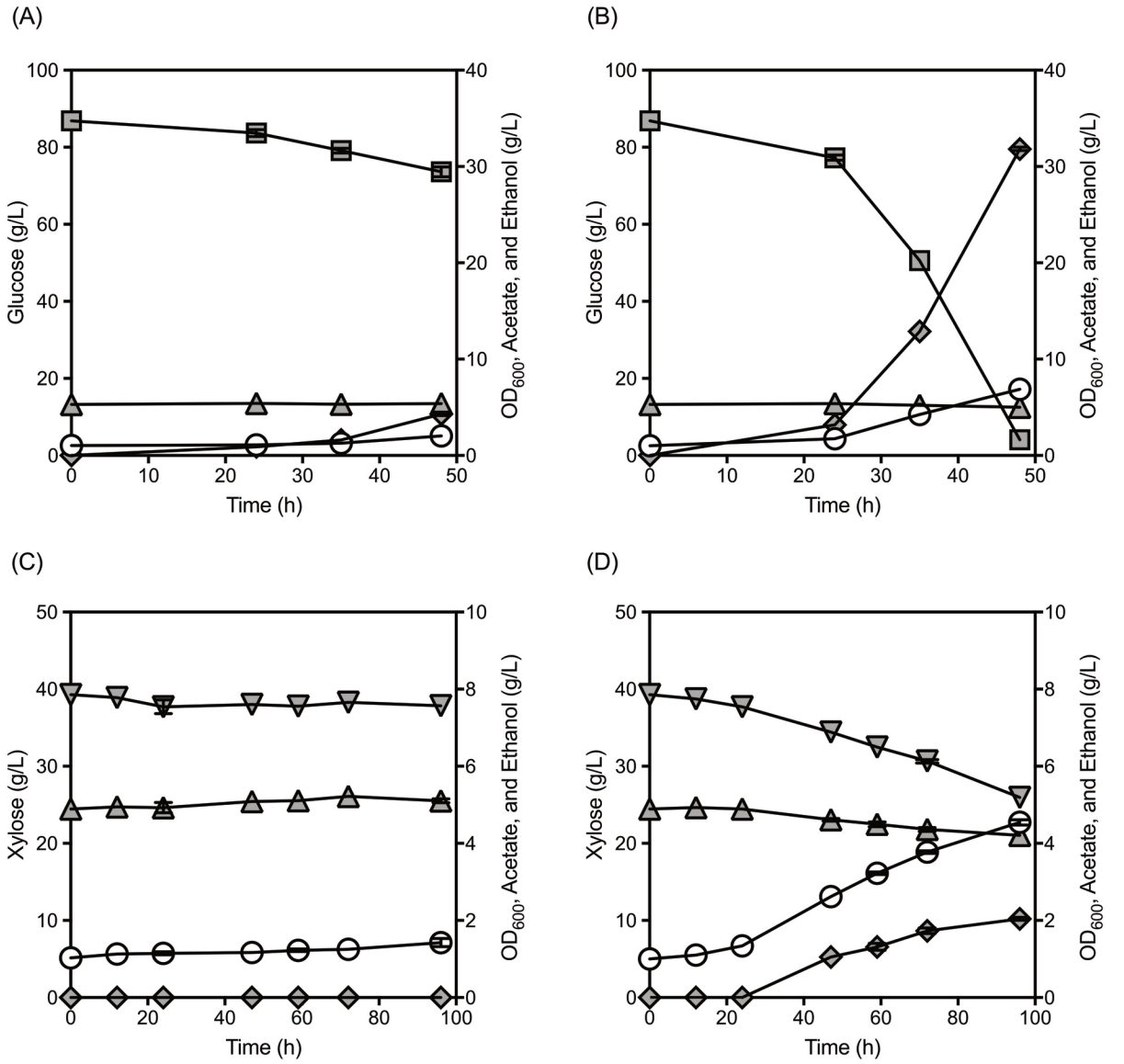
Figure legends

Figure 1. Effects of *RCK1* overexpression on sugar fermentation under toxic levels of acetic acid. For glucose fermentation, tests were conducted using the *S. cerevisiae* D452-2 strains harboring an empty plasmid (A), and a multi-copy *RCK1*-overexpressing plasmid (B). For xylose fermentation, the *S. cerevisiae* SR8 strains containing an empty plasmid (C), and a multi-copy *RCK1* overexpressing plasmid (D) were compared. To examine acetic acid tolerance, YP medium containing 80 g/L of glucose or 40 g/L of xylose was used with 5 g/L of acetic acid added under oxygen-limited conditions. The initial cell density was adjusted to 0.29 g/L (OD_{600} = 1). The symbols are the mean of duplicate experiments; the error bars indicate the standard deviations and are not visible when smaller than the symbol size. Symbols: cell growth (open circle), glucose (solid square), xylose (solid triangle down), acetic acid (solid triangle up), and ethanol (solid diamond).

Figure 2. Effects of *RCK1* overexpression on the accumulation of intracellular ROS. The control (D423C and SR8C) and *RCK1*-overexpressing (D423R and SR8R) strains were tested using a dichlorofluorescein assay. The levels of intracellular ROS were measured after incubating the cells with no addition, H_2O_2, or acetic acid for 32 hours under glucose condition (A) or 65 hours under xylose condition (B). The obtained fluorescence values were divided by cell densities (OD_{600}), representing relative ROS intensity. All fluorescence experiments were performed in triplicate, and error bars represent standard deviation. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Shapiro-Wilk test and Student's t-test.


(A)
(B)
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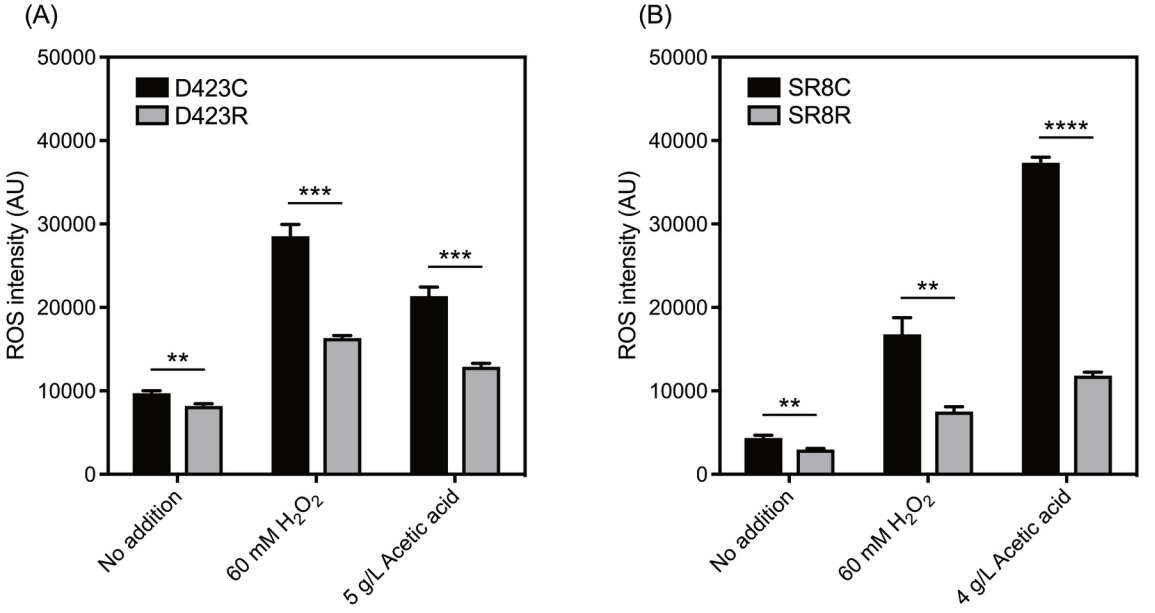
Glucose (g/L)
Xylose (g/L)
OD600, Acetate, and Ethanol (g/L)
Time (h)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