Appl Microbiol Biotechnol (2013) 97:6589–6600
DOI 10.1007/s00253-013-4997-4

BIOENERGY AND BIOFUELS

Engineering redox cofactor utilization for detoxification of glycolaldehyde, a key inhibitor of bioethanol production, in yeast *Saccharomyces cerevisiae*

Lahiru N. Jayakody · Kenta Horie · Nobuyuki Hayashi · Hiroshi Kitagaki

Received: 27 February 2013 / Revised: 6 May 2013 / Accepted: 14 May 2013 / Published online: 7 June 2013

Abstract Hot-compressed water treatment of lignocellulose liberates numerous inhibitors that prevent ethanol fermentation of yeast *Saccharomyces cerevisiae*. Glycolaldehyde is one of the strongest fermentation inhibitors and we developed a tolerant strain by overexpressing *ADH1* encoding an NADH-dependent reductase; however, its recovery was partial. In this study, to overcome this technical barrier, redox cofactor preference of glycolaldehyde detoxification was investigated. Glycolaldehyde-reducing activity of the *ADH1*-overexpressing strain was NADH-dependent but not NADPH-dependent. Moreover, genes encoding components of the pentose phosphate pathway, which generates intracellular NADPH, was upregulated in response to high concentrations of glycolaldehyde. Mutants defective in pentose phosphate pathways were sensitive to glycolaldehyde. Genome-wide survey identified *GRE2* encoding a NADPH-dependent reductase as the gene that confers tolerance to glycolaldehyde. Overexpression of *GRE2* in addition to *ADH1* further improved the tolerance to glycolaldehyde. NADPH-dependent glycolaldehyde conversion to ethylene glycol and $NADP^+$ content of the strain overexpressing both *ADH1* and *GRE2* were increased at 5 mM glycolaldehyde. Expression of *GRE2* was increased in response to glycolaldehyde. Carbon metabolism of the strain was rerouted from glycerol to ethanol. Thus, it was concluded that the overexpression of *GRE2* together with *ADH1* restores glycolaldehyde tolerance by augmenting the NADPH-dependent reduction pathway in addition to NADH-dependent reduction pathway. The redox cofactor control for detoxification of glycolaldehyde proposed in this study could influence strategies for improving the tolerance of other fermentation inhibitors.

Keywords Bioethanol · Glycolaldehyde · *Saccharomyces cerevisiae* · Gre2 · Redox cofactors

Introduction

Second generation approaches to bioethanol production from lignocellulosic materials such as agricultural and forestry biomass residues have the potential to substitute substantially for petroleum-based transportation fuels (Naik et al. 2010). The annual global production of lignocellulose amounts to approximately 50 billion tons (Claassen et al. 1999), and that volume is quite enough to develop environmentally friendly and sustainable bioethanol production to help meet current transportation fuel demands (Naik et al. 2010; Sims et al. 2010). The key technology to production of bioethanol is the development of an efficient system of degradation of lignocellulosic materials and its fermentation by the industrial workhorse *Saccharomyces cerevisiae*.

Numerous pretreatment technologies have been developed to degrade lignocellulosic materials. These include sulfuric acid treatment, alkaline treatment, steam explosion, and enzymatic degradation (Hu and Ragauskas 2012; Yang and Wyman 2008). However, these methods have several drawbacks. For example, sulfuric acid treatment generates enormous hazardous wastes (Yang and Wyman 2008; Yu et al. 2007). In contrast, hot-compressed water treatment is

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-4997-4) contains supplementary material, which is available to authorized users.

L. N. Jayakody · K. Horie · N. Hayashi · H. Kitagaki (✉)
Department of Environmental Science, Faculty of Agriculture, Saga University, Saga 8408502, Japan
e-mail: ktgkhrs@cc.saga-u.ac.jp

L. N. Jayakody · N. Hayashi · H. Kitagaki
Department of Biochemistry and Applied Biosciences, United Graduate School of Agricultural Sciences Kagoshima, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-8580, Japan

recognized as the one of the novel and advantageous pretreatment technologies (Yu et al. 2007). Water in either a subcritical or a supercritical stage or at temperatures above 150 °C with various pressures (5–22 MPa) is defined as hot-compressed water. It breaks down lignocelluloses into various compounds through pyrolytic cleavage, swelling, and dissolution of lignocellulose (Lu et al. 2009). A key advantage of using hot-compressed water is that its use during pretreatment directly breaks lignocelluloses down to fermentable sugars without generating hazardous wastes (Yu et al. 2007).

However, inhibitory compounds are produced during pretreatment of lignocellulosic materials (Lu et al. 2009), which is one of the greatest bottlenecks of progress in ensuring the efficiency of bioethanol production. So far, substances such as furan derivatives (furfural and 5-hydroxymethylfurfural (5-HMF)), acetic acid, methylglyoxal, and phenolic compounds have been extensively studied as the inhibitory compounds (Almeida et al. 2007; Olsson and Hahn-Hägerdal 1993; Palmqvist and Hahn-Hägerdal. 2000; Van Maris et al. 2006), and glycolaldehyde was not paid attention as an inhibitory compound until our former studies (Jayakody et al. 2011, 2012). Glycolaldehyde is formed through retro-aldol condensation of simple sugars such as glucose, fructose, mannose, and xylose when treated with hot-compressed water (Lu et al. 2009; Yu et al. 2007). The concentration of glycolaldehyde depends largely on the hydrolysis conditions as well as the type of biomass, with concentrations of glycolaldehyde generally ranging between 2 and 28 mM (0.12–1.68 g/l) after treatment of lignocelluloses with hot-compressed water (Ehara and Saka 2002; Lu et al. 2009). We first reported that the inhibitory activity of glycolaldehyde on the growth of *S. cerevisiae* is stronger than those of furfural and 5-HMF (Jayakody et al. 2011, 2012). Furthermore, we showed that the *ADH1*-overexpressing strain shows improved tolerance against glycolaldehyde and enhanced ethanol productivity in the presence of glycolaldehyde (Jayakody et al. 2012). However, growth of the developed *ADH1*-overexpressing strain was not fully recovered in the presence of glycolaldehyde. On the contrary, redox balance is now emerging as an intensive target of metabolic engineering (Celton et al. 2012; Heer et al. 2009; Kitagaki et al. 2009).

Here we report engineering redox cofactor utilization for further improvement of tolerance of engineered yeast to glycolaldehyde. Analyses of changes in the redox cofactors, certain metabolites, and gene expression enabled us to show that increased NADPH-dependent glycolaldehyde reduction activity is responsible for the improved conversion of glycolaldehyde and tolerance to glycolaldehyde of the strain overexpressing both *GRE2* and *ADH1*, especially at 5 mM glycolaldehyde. To our best knowledge, this is the first successful attempt for engineering a yeast strain towards glycolaldehyde by introducing a NADPH-dependent reduction system. These novel findings and insights will likely suggest novel targets to alleviate the toxicities of fermentation inhibitors that hinder the efficient production of economically viable levels of bioethanol.

Materials and methods

Strains

Yeast strains used in the study are listed in Table 1. NEB 5-alpha competent *Escherichia coli* cells, obtained from New England Biolabs (Beverly, MA, USA), were used as the hosts for recombinant DNA manipulation.

Construction of overexpression strains

The *GRE2*-overexpressing plasmid was constructed by following standard methods as previously described (Jayakody et al. 2012). Briefly, the *GRE2* gene fragment including its own promoters was amplified by PCR from the genome of *S. cerevisiae* BY4743 by using forward and reverse primers (Table 2). The purified gene fragment of *GRE2* was cleaved with appropriate restriction enzymes and ligated into pAUR123 plasmid. Success of transformation was confirmed by DNA sequencing. The pAUR123-*GRE2* plasmid was transformed into BY4743 using a high efficiency yeast transformation technique (Gietz and Schiestl 2007). The transformants of pAUR123-*GRE2* was selected using 0.5 μg/ml of aureobasidin. The strain has been deposited to the National BioResource Project of Japan database (http://www.nbrp.jp/report) as BY25955. The strain harboring both pRS426-*ADH1* and pAUR123-*GRE2* plasmids was constructed by transforming the pAUR123-*GRE2* plasmid into BYp*ADH1* strain. The transformed strains were selected in synthetic minimal medium without uracil in the presence of 0.5 μg/ml of aureobasidin. The strain has been deposited to the National BioResource Project of Japan database as BY25956.

Yeast growth assay with glycolaldehyde

Three independent experiments were performed using the respective independent starter cultures to examine the inhibitory effects of glycolaldehyde on the growth of different yeast strains. Yeast cells were grown in 10 ml of synthetic minimal medium containing 2 % (*w*/*v*) glucose in the presence or absence of glycolaldehyde in 15 ml glass test tubes. The initial cell concentration was set at 0.1 OD_{600} (corresponding to 1×10^6 cells/ml), and the growth was monitored by measuring OD_{600} at different time points.

Table 1 Yeast strains used in this study

Strain	Relative genotype	Source/reference
BY4743	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ ura3Δ0/ura3Δ0*	EUROSCARF
BY *Δgdn1*	BY4743 *Δgdn1*	Open Biosystems (Huntsville, AL, USA)
BY *Δsol1*	BY4743 *Δsol1*	Open Biosystems (Huntsville, AL, USA)
BY *Δtkl1*	BY4743 *Δtkl1*	Open Biosystems (Huntsville, AL, USA)
BYp426	BY4743+pRS426	Jayakody et al. (2012)
BYp123	BY4743+pAUR123	This study
BYp425	BY4743+pRS425	This study
BYp*ADH6*	BY4743+pRS425-*ADH6*	This study
BYp*ADH1*	BY4743+pRS426-*ADH1*	Jayakody et al. (2012)
BYp*GRE2*	BY4743+pAUR123-*GRE2*	This study
BYp*ADH1*p123	BY4743+pRS426-*ADH1*+pAUR123	This study
BYp*ADH1*p*GRE2*	BY4743+pRS426-*ADH1*+pAUR123-*GRE2*	This study

Fermentation of hot-compressed water-treated cellulose hydrolysate

The hot-compressed water-treated cellulose hydrolysate was prepared by the method described previously using cellulose microcrystalline (Avicel PH-101) (Jayakody et al. 2012). The generated hydrolysate was supplemented with 790 mg/l of complete supplementary media and 0.67 % (*w*/*v*) of yeast nitrogen base without amino acids and ammonium sulfate prior to cell inoculation. The pH of the media was adjusted at 5.5 with NaOH solution. Two milliliters of the medium in 3 ml glass test tubes was inoculated with the strains and analyzed in terms of growth and fermentation products.

Analysis of fermentation products

To analyze metabolites produced by fermentation, yeast strains were grown in synthetic minimal medium containing 2 % (*w*/*v*) glucose with or without glycolaldehyde. Samples were collected at different time intervals and analyzed in terms of the cell dry weight, as described previously (Jayakody et al. 2011). Concentrations of glucose, ethanol, glycolaldehyde, ethylene glycol, glycerol, and acetic acid were quantitated according to the methods as described previously (Jayakody et al 2012).

Analysis of the glycolaldehyde-dependent NAD(P)H oxidation

Cell lysate was extracted with Y-PER (Thermo Scientific, Rockford, USA) solution by following the manufacturer's protocol. To extract the cell lysate, cells were grown overnight by shaking at 30 °C in 10 ml of synthetic minimal medium containing 2 % (*w*/*v*) glucose. The protein concentration of the extracted lysate was assessed using Bradford reagent (Sigma Aldrich, St. Louis, USA). The glycolaldehyde-dependent NAD(P)H oxidation was measured by adding 2 μg of cell lysate to 1 ml of reaction mixture containing 100 mM potassium phosphate buffer (pH 7.2), 10 mM glycolaldehyde, and 100 μM of either NADH or NADPH. Reaction mixtures were shaken at 25 °C for 3 min, and the OD at 340 nm was assessed to measure the glycolaldehyde-dependent NAD(P)H oxidation (Moon and Liu 2012).

Table 2 Primers used for genes amplification and real-time quantitative RT-PCR analysis

	Gene	Forward primers 5′–3′	Reverse primer 5′–3′
Construction of overexpressing plasmids	*ADH1*	*Xoh*I-CCC CTC GAG ACT GTA GCC CTA GAC TTG ATA	*Eco*RI-CCC GAA TTC GGT AGA GGT GTG GTC AAT AA
	GRE2	*Kpn*I-CCC GGT ACC ATG TCA GTT TTC GTT TCA GG	*Sal*I-CCC GAC CTA CCA TTT TGT GAA TCA A
Real-time quantitative RT-PCR analysis	*GRE2*	GGC AAA AGA AAA CGA TAA GTC	TCC CAA GCT GCT TTT TCA
	ZNF1	TCT CTT GAC TAT GGA AAG ACC	AGA TTT ACC GTA CTG GCC CAA
	GND1	TTT TGA AAT TCG ACG ACG	CAC CAA TCA AAG TAA CTG GCA
	RDN1	CCA TGG TTT CAA CGG GTA ACG G	GCC TTC CTT GGA TGT GGT AGC C

Quantification of intracellular NADH and $NADP^+$ contents

Yeast cells were grown for 24 h at 30 °C in 10 ml of synthetic minimal medium with or without different concentrations of glycolaldehyde. The NADH and $NADP^+$ contents of the extracted cell lysate were analyzed using the Amplite™ Colorimetric NAD/NADH and NADP/NADPH assay kits "Blue color" (AAT Bioquest Inc, Sunnyvalve, CA, USA), respectively, according to the manufacturer's protocols. After 1 h incubation of the assay mixture in the dark, the absorbance was monitored at 545 nm, using the ChroMate plate reader (Awareness Technology, Inc.). To detect the NADH content, samples were heated for 30 min at 60 °C to deactivate the NAD^+, prior to the assay.

Total RNA extraction and real-time quantitative reverse transcriptase (RT)-PCR

Total RNA was extracted using the hot phenol method (Kohrer and Domdey 1991) after 6 h of incubation of yeast strains with or without different concentrations of glycolaldehyde in 10 ml of synthetic minimal medium containing 2 % (*w*/*v*) glucose. The extracted RNA was further purified by using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and was frozen at −80 °C until further analysis. Expression of the *GRE2*, *ZNF1*, and *GND1* genes were analyzed by real-time quantitative RT-PCR using the Roche LightCycler™ (Roche Applied Biosystem) with the one-step SYBR® Prime script™ PLUS RT-PCR KIT (Perfect Real Time) (TaKaRa Biotechnology [Dalian] Co., Ltd), according to the manufacturer's protocol. The purified 50 ng of template total RNA and the respective primers were used for real-time quantitative RT-PCR analysis (Table 2). The *RDN1* gene was used as an internal control for normalization. The RT reaction was carried out at 42 °C for 5 s, followed by heating at 95 °C for 10 s, and the genes were amplified in 40 cycles at 95 °C for 5 s followed by 60 °C for 20 s. The relative gene expressions of *ZWF1*, *GND1*, and *GRE2* genes were calculated by following the method described by Livak and Schmittgen (2001).

Statistical analysis

All experiments were performed independently in triplicate from the respective independent starter cultures. The results were expressed as mean values and SEM. A one-tailed Student's *t* test was adapted for pairwise comparison of the differences between the sample averages of two groups. A one-way analysis of variance (ANOVA) and Tukey's post hoc honest significance difference test were implemented for multiple comparisons.

Results

ADH1-overexpressing strain has an increased NADH-dependent, but not NADPH-dependent, glycolaldehyde reduction activity

First, in order to investigate the cause of the incomplete recovery of the *ADH1*-overexpressing strain in the presence of glycolaldehyde, the capacities of cell lysates of strains for the glycolaldehyde-dependent in vitro oxidation of NADH and NADPH were assessed. Results in Fig. 1a show that cell lysates of *ADH1*-overexpressing strains (BYp*ADH1*) had significantly higher glycolaldehyde-reducing activity using NADH as a cofactor than the control strain (BYp426) ($p<0.01$). On the other hand, the glycolaldehyde-reducing activity using NADPH was not significantly different between the two strains ($p>0.05$, Fig. 1b). These results indicated that glycolaldehyde reduction in the *ADH1*-overexpressing strain mainly occurs with NADH rather than NADPH as a cofactor. This result prompted us to hypothesize that the reduced fermentative capacity of the *ADH1*-overexpressing strain in the presence of a high concentration of glycolaldehyde (≥5 mM) could be restored through activation of a NADPH-dependent reduction system.

Pentose phosphate pathway plays an important role in glycolaldehyde tolerance

To further confirm the NADPH-dependent recovery hypothesis, expressions of genes involved in generating intracellular NADPH challenged with different concentrations of glycolaldehyde were investigated. The pentose phosphate pathway is the major intracellular source of NADPH and pentoses, which includes components such as Zwf1, Gnd1, Gnd1, Sol3, and Tkl1. Hence, we analyzed the relative gene expressions of *ZWF1* and *GND1* by real-time quantitative RT-PCR. Zwf1 encodes glucose-6-phosphate dehydrogenase and Gnd1 encodes 6-phosphogluconate dehydrogenase, both of which consume $NADP^+$ and generate NADPH. As a result, the expressions of these genes were significantly increased in the presence of 5 mM glycolaldehyde than when grown in the absence or in the presence of 2 mM glycolaldehyde ($p<0.05$, Fig. 2a, b). This result suggests that NADPH is the key to tolerance to high concentrations of glycolaldehyde (5 mM) and cells are upregulating components involved in NADPH production. To further confirm the NADPH-dependent recovery hypothesis, we analyzed the glycolaldehyde sensitivities of mutants deficient in the pentose phosphate pathway enzymes encoded by the *GND1*, *SOL3*, and *TKL1* genes. As a result, all of these mutants were significantly more sensitive to glycolaldehyde than their parent strain at 5 mM glycolaldehyde ($p<0.05$, Fig. 2c). Altogether, these results are consistent with our hypothesis that activation of the pentose

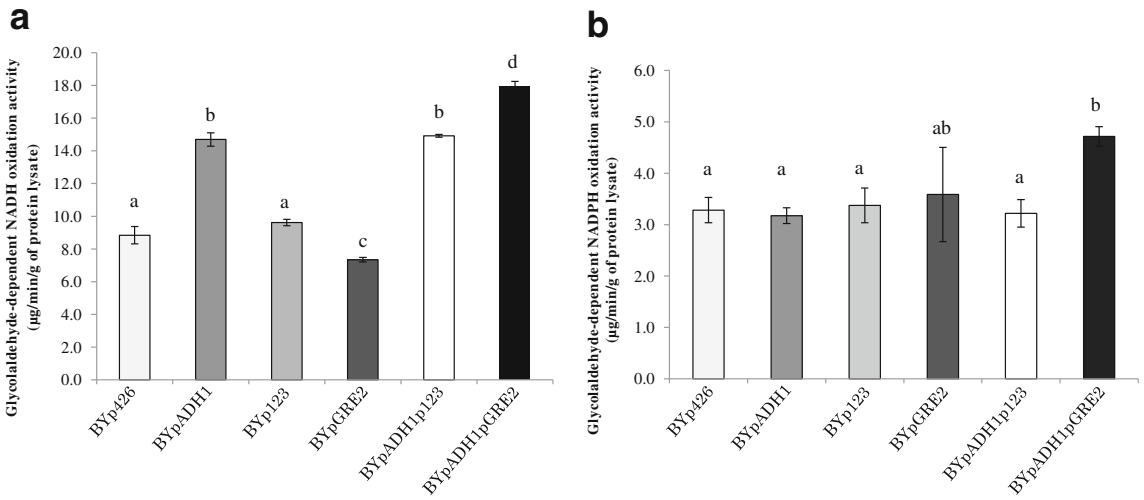

Fig. 1 Glycolaldehyde-dependent NAD(P)H oxidation activities of cell lysate of engineered strains. Ten millimolar solution of glycolaldehyde was added to the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.2) and 100 μM NADH or NADPH. The reaction was started by adding 2 μg of cell lysate and shaken at 25 °C for 3 min. The optical density at 340 nm was assessed to measure NADH- and NADPH-dependent reduction activities. **a** NADH oxidation activity. **b** NADPH oxidation activity. The results are expressed as the mean±SEM of the independent triplicate experiments from the respective independent starter cultures. Bars labeled with different letters indicate statistically significant values ($p<0.05$; one-way ANOVA followed by Tukey's post hoc honest significance difference test)

phosphate pathway, which causes NADPH or pentose production, is the key phenomena for tolerance to a high concentration of glycolaldehyde.

A *GRE2*-overexpressing strain shows partially improved glycolaldehyde tolerance

In our former study, acetic acid production was increased by the addition of glycolaldehyde (Jayakody et al. 2012). Together with the above results and the fact that production of acetic acid is catalyzed by Ald6 by using $NADP^+$ in the cytosol as a cofactor (Saint-Prix et al. 2004), it was considered that intracellular NADPH is consumed to attenuate the toxicity of glycolaldehyde. In order to verify the hypothesis to relieve glycolaldehyde toxicity by augmenting NADPH-dependent reduction pathway, we searched for a NADPH-dependent oxidoreductase that could reduce glycolaldehyde. Overexpression of *ADH6*, which encodes a NADPH-dependent oxidoreductase, has been reported to improve tolerance against furfural and 5-HMF (Almeida et al. 2008; Liu and Slininger 2006; Liu 2011). Therefore, the *ADH6*-overexpressing strain was constructed and its tolerance to glycolaldehyde was investigated. Although the strain showed tolerance to furfural and 5-HMF as reported earlier (Almeida et al. 2007, 2008; Petersson et al. 2006), it did not show significantly different tolerance to glycolaldehyde relative to the control strain ($p>0.05$, Supplementary Fig. S1). Therefore, we shifted to a genome-wide survey of genes that confer tolerance to glycolaldehyde. Our former study of a genome-wide survey of gene deletion mutants sensitive to glycolaldehyde showed that the *Δgre2* mutant is one of the most sensitive mutants to glycolaldehyde (Jayakody et al. 2011). This result suggested that Gre2, a NADPH-dependent reductase (Chen et al. 2003), could confer tolerance to glycolaldehyde. Moreover, Gre2 has been reported to reduce aldehyde inhibitors such as fufural, 5-HMF (Liu et al. 2005; Liu and Slininger 2006), and carbonyl compounds (Katz et al. 2003; Rodrigues-Pousada et al. 2010). Therefore, the *GRE2*-overexpressing strain was constructed and its fermentation profile in the presence of glycolaldehyde was analyzed. The *GRE2*-overexpressing strain (BYp*GRE2*) grew significantly faster than the control strain (BYp123) in the presence of glycolaldehyde ($p<0.05$, Fig. 3a, Table 3). These results indicate that the *GRE2*-overexpressing strain has improved growth ability in the presence of glycolaldehyde. In order to understand the physiology of the *GRE2*-overexpressing strain in detail, we next analyzed the fermentation products produced by the strains in the presence of glycolaldehyde. The results shown in Table 4 revealed that the *GRE2*-overexpressing strain had slightly higher rates of ethanol production and glucose consumption rate than the control strain, although the differences were not statistically significant. The *GRE2*-overexpressing strain had a significantly higher rate of acetic acid production ($p<0.01$) and a lower rate of glycerol production ($p<0.01$) than the control strain (Table 4). Production of ethylene glycol of the *GRE2*-overexpressing strain was significantly higher than that of the control strain at 48 h ($p<0.05$, Fig. 3c). The rates of ethylene glycol production by the *GRE2*-overexpressing strain and the control strain were 11.5±3.2 and 5.9±1.0 μM/g/h, respectively, which were significantly different ($p<0.05$). These results are consistent with the improved growth profiles of the *GRE2*-overexpressing strain in the

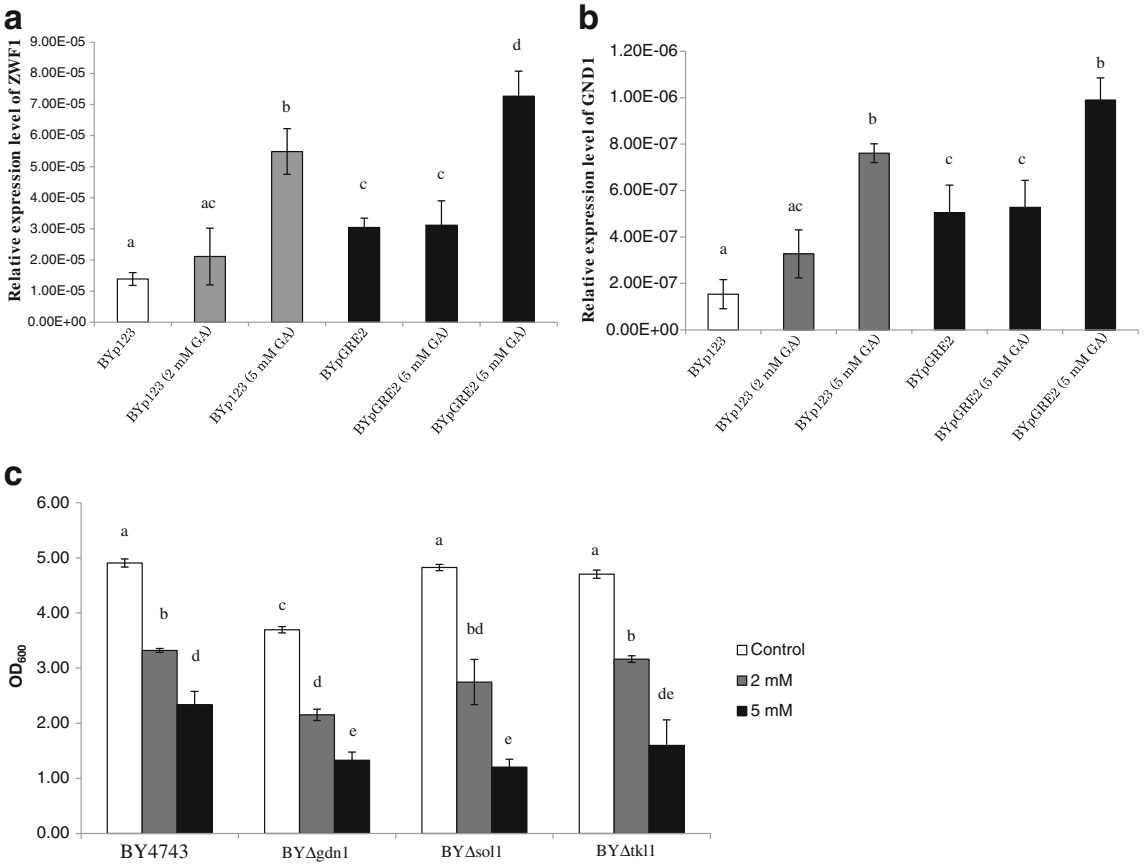

Fig. 2 Involvement of the pentose phosphate pathway in response to glycolaldehyde. Relative expression levels of **a** *ZWF1* and **b** *GND1* of cells incubated with 2 and 5 mM glycolaldehyde (GA) were analyzed by real-time quantitative RT-PCR. **c** Glycolaldehyde sensitivity of mutants defective in pentose phosphate pathway. Mutant strains and their parental strain were grown for 24 h at 30 °C in 2 ml of synthetic minimal medium with different concentrations of glycolaldehyde. The results are expressed as mean values±SEM of independent triplicate experiments from the respective independent starter cultures. Bars labeled with different letters indicate statistically significant values ($p<0.05$; one-way ANOVA followed by Tukey's post hoc honest significance difference test)

presence of glycolaldehyde (Fig. 3a and Table 3). Moreover, the *GRE2*-overexpressing strain showed higher growth in the actual hot-compressed water-treated cellulose than the control strain ($p<0.05$, Fig. 3d). However, the growth parameters of specific cell growth rate and maximum cell growth of the *GRE2*-overexpressing strain were lower than those of the *ADH1*-overexpressing strain (Table 3) (Jayakody et al. 2012). These results indicate that the *GRE2*-overexpressing strain shows an improved growth and tolerance in the presence of glycolaldehyde and hot-compressed water-treated cellulose, although not as effective as the *ADH1*-overexpressing strain.

Simultaneous overexpression of *ADH1* and *GRE2* confers hyper-resistance to glycolaldehyde

Based on the above results, we speculated that overexpression of both *ADH1* and *GRE2* might substantially enhance the ability to eliminate glycolaldehyde toxicity of yeast cells. In order to verify this hypothesis, we developed a strain that overexpresses *ADH1* and *GRE2* under their own promoters (BYp*ADH1*p*GRE2*). Analysis of growth in the presence of glycolaldehyde revealed that the developed strain exhibits improved resistance to glycolaldehyde relative to the *ADH1*-overexpressing strain (BYp*ADH1*p123), especially after 36 h ($p<0.05$, Fig. 4a). The strain overexpressing both *ADH1* and *GRE2* showed significantly higher rates of ethanol production ($p<0.05$, Fig. 4b), glucose consumption ($p<0.05$, Fig. 4c), acetic acid production (Fig. 4d), and ethylene glycol production ($p<0.05$, Fig. 4g) than the *ADH1*-overexpressing strain. It should be noted that especially at the period of vigorous growth (12 to 24 h), the concentration of glycolaldehyde of the strain overexpressing both *ADH1* and *GRE2* was significantly lower than that of the *ADH1*-overexpressing strain ($p<0.05$, Fig. 4f). Glycerol production was not significantly different between the two strains ($p>0.05$, Fig. 4e). The rates of glycolaldehyde conversion of the strain overexpressing both *ADH1* and *GRE2* and the control strain were 101.0±11.2 and 90.3±15.0 μM/g/h, respectively, which were significantly different ($p<0.05$). The conversion rates of glycolaldehyde into ethylene glycol were 75.5±2.5 and 69.4±5.7 % for the

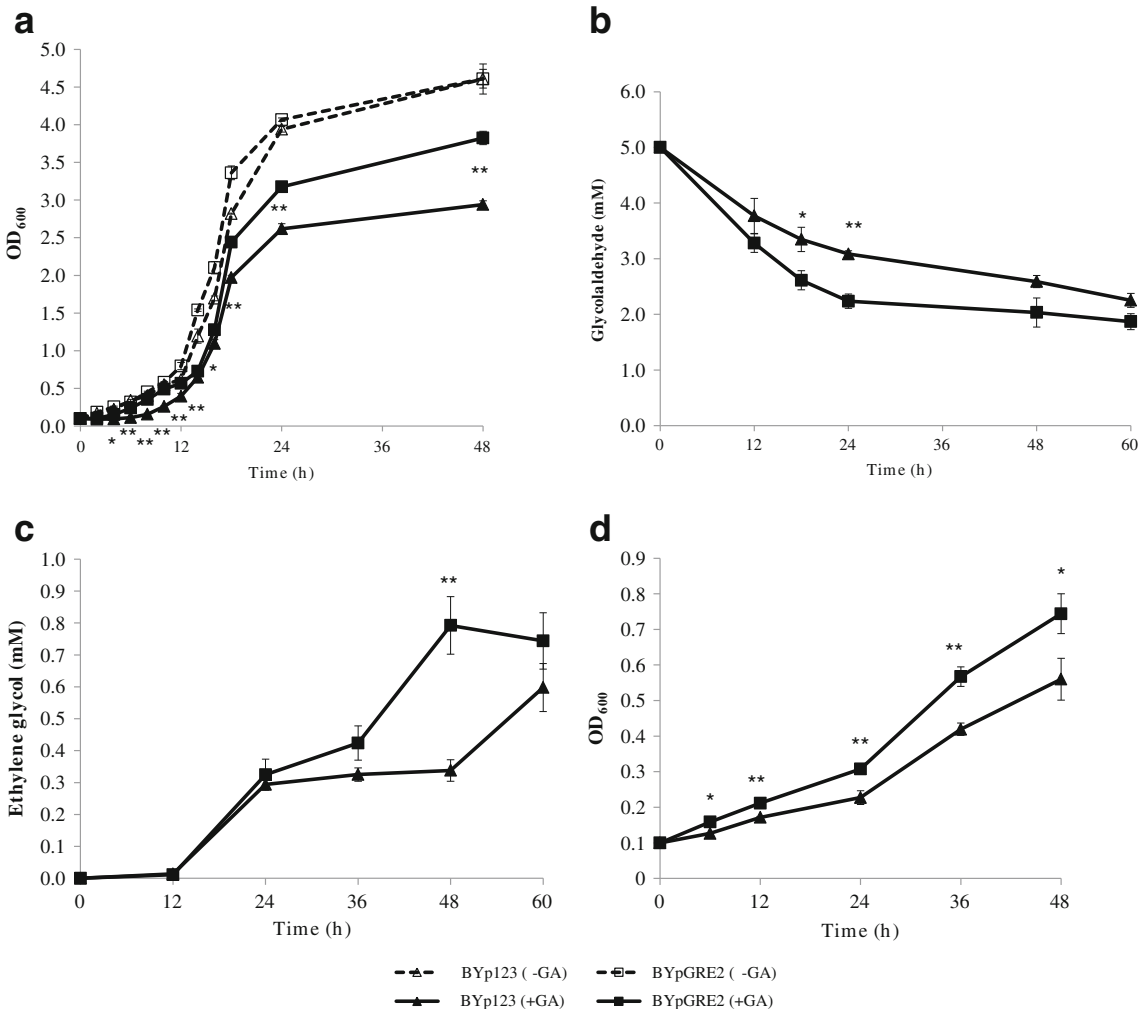

Fig. 3 *GRE2*-overexpressing strain shows improved fermentation profiles in glycolaldehyde- and hot-compressed water-treated cellulose-containing media. The growth and glycolaldehyde reduction profiles of the *GRE2*-overexpressing strain and the control strain were assessed in a medium containing 5 mM glycolaldehyde (GA) (**a**–**c**). **a** Growth profile. **b** Glycolaldehyde concentration. **c** Ethylene glycol concentration. **d** Growth of yeast cells in cellulose hydrolysate treated with hot-compressed water. *Empty triangles*, BYp123 (nontreated); *empty squares*, BYp*GRE2* (nontreated); *filled triangles*, BYp123 (glycolaldehyde-treated); and *filled squares*, BYp*GRE2* (glycolaldehyde-treated). The results are expressed as mean values±SEM of independent triplicate experiments from the respective independent starter cultures. Statistically significant levels of the difference of two strains are indicated (*$p<0.05$, **$p<0.01$)

strain that overexpressed both *ADH1* and *GRE2* and the control strain, respectively.

To better understand the mechanism that enables glycolaldehyde conversion in these strains, we analyzed the glycolaldehyde-dependent NADH and NADPH oxidation activities of the cell lysates extracted from the strain overexpressing both *ADH1* and *GRE2* and the control strain. The results indicated that the strain overexpressing both *ADH1* and *GRE2* has significantly higher conversion activities of glycolaldehyde using NADH and NADPH as cofactors

Table 3 Specific growth rate of strains in the presence of 5 mM glycolaldehyde

	Specific growth rate (g/g/h)		Maximum cell dry weight (g/l)	
	Without glycolaldehyde	With glycolaldehyde	Without glycolaldehyde	With glycolaldehyde
BYp123	0.153±0.001	0.070±0.002	2.070±0.089	1.323±0.022
BYp*GRE2*	0.154±0.002	0.072±0.000*	2.075±0.0565	1.721±0.040*
BYp*ADH1*p123	0.153±0.002	0.095±0.003	2.122±0.008	1.907±0.036
BYp*ADH1*p*GRE2*	0.155±0.001	0.122±0.002	2.112±0.025	2.129±0.031*

*$p<0.05$ indicates statistically significantly different values compared to the control strain

Table 4 Production rates of metabolites per biomass of strains in the presence of 5 mM glycolaldehyde

	Ethanol production (mM/g/h)		Glucose consumption (mM/g/h)		Acetic acid production (mM/g/h)		Glycerol production (mM/g/h)	
	Without glycolaldehyde	With glycolaldehyde	Without glycolaldehyde	With glycolaldehyde	Without glycolaldehyde	With glycolaldehyde	Without glycolaldehyde	With glycolaldehyde
BYp123	1.682±0.391	1.121±0.182	1.152±0.012	0.933±0.001	0.276±0.010	0.286±0.015	0.056±0.000	0.051±0.001
BYp*GRE2*	1.631±0.042	1.313±0.354	1.148±0.014	1.114±0.015	0.275±0.013	0.380±0.003*	0.055±0.000	0.041±0.002*
BYp*ADH1*p123	1.886±0.156	1.438±0.093	1.090±0.001	1.029±0.002	0.128±0.045	0.278±0.011	0.100±0.006	0.035±0.000
BYp*ADH1*p*GRE2*	1.885±0.171	1.614±0.017*	1.090±0.003	1.076±0.007*	0.160±0.002	0.300±0.013*	0.097±0.007	0.028±0.000*

*Indicate statistically significantly different values ($p<0.05$, $n=3$) compared to the control strain

than those of the control strain ($p<0.05$, Fig. 1a, b). These results indicate that simultaneous overexpression of *ADH1* and *GRE2* confers an increased NADH- and NADPH-dependent reduction activity of glycolaldehyde.

Decrease of NADH and increase of $NADP^+$ in the strain overexpressing both *ADH1* and *GRE2*

In order to obtain insight into the redox cofactor preference of the glycolaldehyde-reducing activity of these strains, we analyzed the intracellular redox profiles of the cells by monitoring the intracellular NADH and $NADP^+$ levels. In all of the strains studied, NADH decreased in proportion to the concentration of glycolaldehyde (Fig. 5a). The intracellular level of NADH decreased in the *ADH1*-overexpressing strain treated with 5 mM glycolaldehyde relative to the control strain ($p<0.05$, Fig. 5a), which is consistent with the Adh1-catalyzed oxidation of glycolaldehyde using NADH as a cofactor (Fig. 1a). The intracellular level of NADH further decreased in the strain overexpressing both *ADH1* and *GRE2* treated with 5 mM glycolaldehyde ($p<0.05$, Fig. 5a), suggesting a role of Gre2 in NADH-mediated glycolaldehyde reduction. $NADP^+$ was increased in the *GRE2*-overexpressing strain and the strain overexpressing both *ADH1* and *GRE2* treated with 5 mM glycolaldehyde relative to the control strain ($p<0.05$, Fig. 5b), suggesting Gre2-catalyzed glycolaldehyde reduction using NADPH as a cofactor at 5 mM glycolaldehyde. In contrast, at 2 mM glycolaldehyde, $NADP^+$ was not significantly increased in the *GRE2*-overexpressing strain and the strain overexpressing both *ADH1* and *GRE2* relative to the control strains ($p>0.05$, Fig. 5b), suggesting that glycolaldehyde reduction at 2 mM glycolaldehyde does not predominantly occur by using NADPH as a cofactor.

Together, these results indicated that at a lower concentration (2 mM) of glycolaldehyde, most of the conversion of glycolaldehyde was mediated by using NADH as a cofactor. In contrast, at 5 mM glycolaldehyde, the relative contribution of NADPH in the glycolaldehyde-reducing system significantly increased relative to 2 mM in all strains ($p<0.05$, Fig. 5b). These results indicate that the conversion of glycolaldehyde to ethylene glycol occurs mainly using NADH at 2 mM glycolaldehyde and mainly using NADPH at 5 mM glycolaldehyde.

The levels of expressions of *GRE2* of yeast cells challenged with different concentrations of glycolaldehyde were investigated by using real-time quantitative RT-PCR (Fig. 6). As a result, the expression of *GRE2* significantly increased in the presence of 5 mM glycolaldehyde relative to when grown in the absence or in the presence of 2 mM glycolaldehyde ($p<0.05$, Fig. 6). As we expected, the strain overexpressing *GRE2* under its own promoter also increased the expression of *GRE2* in response to glycolaldehyde ($p<0.05$, Fig. 6). Together, these results strongly indicate that both *GRE2* expression

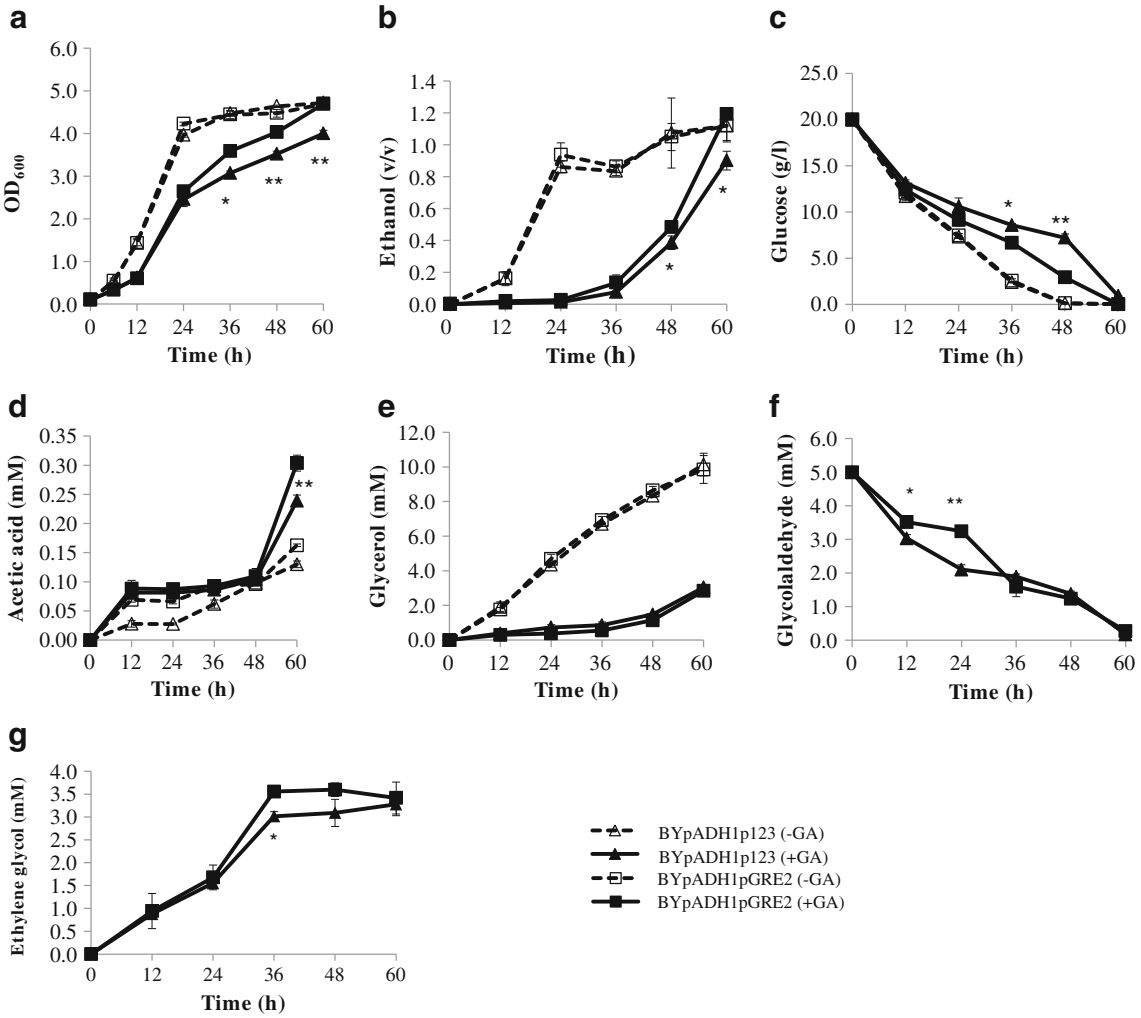

Fig. 4 The strain that overexpresses both *ADH1* and *GRE2* shows hyper-resistance and enhanced fermentation profile in the presence of glycolaldehyde. The growth and fermentation profiles of the strains BYp*ADH1*p123 and BYp*ADH1*p*GRE2* with or without glycolaldehyde (GA). **a** Growth profile of the strains at 10 mM glycolaldehyde. **b–g** Fermentation profiles of the strains at 5 mM glycolaldehyde. **b** Ethanol concentration. **c** Glucose concentration. **d** Acetic acid concentration. **e** Glycerol concentration. **f** Glycolaldehyde concentration. **g** Ethylene glycol concentration. *Empty triangles*, BYp*ADH1*p123 (nontreated); *empty squares*, BYp*ADH1*p*GRE2* (nontreated); *filled triangles*, BYp*ADH1*p123 (glycolaldehyde-treated); and *filled squares*, BYp*ADH1*p*GRE2* (glycolaldehyde-treated). The results are expressed as mean values±SEM of independent triplicate experiments from the respective independent starter cultures. Statistically significant levels of the difference of two strains are indicated (*$p<0.05$, **$p<0.01$)

and the NADPH-dependent glycolaldehyde reduction by Gre2 are increased at 5 mM glycolaldehyde.

Discussion

In this study, we have improved the glycolaldehyde tolerance of the *ADH1*-overexpressing strain, which we developed in an earlier study (Jayakody et al. 2012), by engineering redox cofactor utilization. This is the first report to confer tolerance to glycolaldehyde by introducing a NADPH-dependent reducing mechanism. Moreover, although it has been reported that the reducing activities of furfural and 5-HMF are dependent on NADH and NADPH (Heer et al. 2009), this is the first report that artificially augmented both NADH- and NADPH-dependent reducing mechanisms to confer tolerance to glycolaldehyde.

Expression of *GRE2* is reported to be upregulated under multiple stress conditions, such as osmotic stress, heat shock, oxidative stress, and heavy metal stress (Krantz et al. 2004; Liu and Slininger 2006; Liu and Moon 2009; Liu 2011; Rep et al. 2001). Furthermore, Moon and Liu (2012) have engineered Gre2 to confer tolerance to furfural and 5-HMF using NADH as a cofactor. The metabolic role of glycolaldehyde reduction by Gre2 elucidated in this study will provide insights into the knowledge of aldehyde reductase activities obtained in the former studies on Gre2.

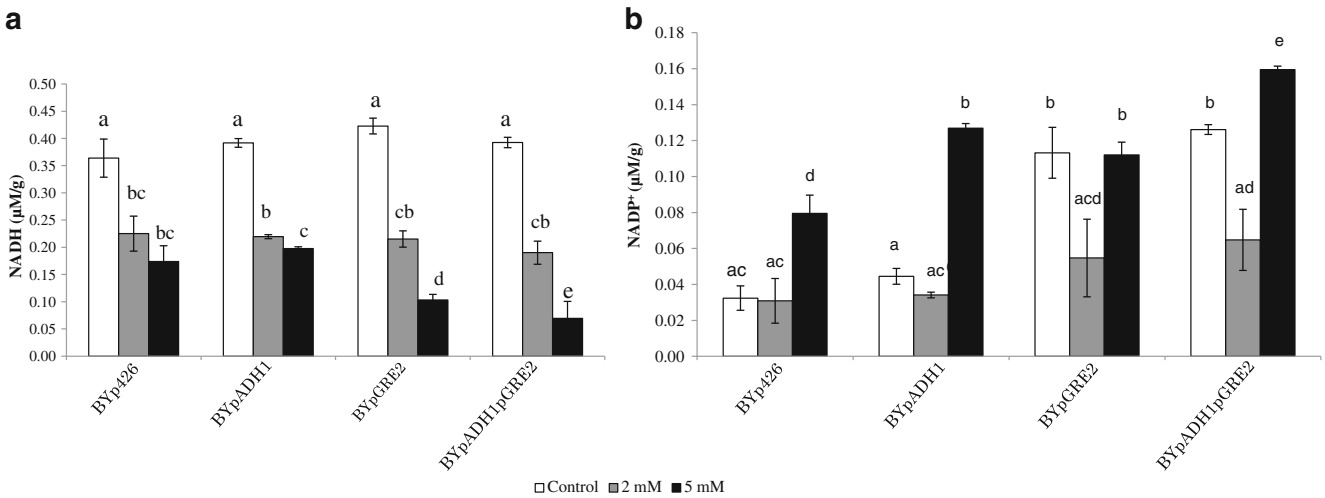

Fig. 5 Intracellular NADH and $NADP^+$ levels in *ADH1*- and *GRE2*-overexpressing strains and strain overexpressing both *ADH1* and *GRE2* treated with or without glycolaldehyde. The intracellular NADH and $NADP^+$ concentrations of extracted cell lysate of yeast cells grown at 30 °C for 24 h in 10 ml of synthetic minimal media with or without different concentrations of glycolaldehyde were analyzed. **a** Intracellular NADH content. **b** Intracellular $NADP^+$ content. The results are expressed as mean values±SEM of independent triplicate experiments from the respective independent starter cultures. Bars labeled with different letters indicate statistically significant values ($p<0.05$; one-way ANOVA followed by Tukey's post hoc honest significance difference test)

A shift in the cofactor preferences of yeast cells for the reduction of aldehydes according to their concentrations has been reported in several other aldehyde-reducing reactions, including those involving the conversions of furfural and 5-HMF (Almeida et al. 2008; Heer et al. 2009; Liu 2011). The reduction of furfural into less toxic furan methanol at a concentration less than 6 mM in yeast cells involves the use of NADH as cofactor (Horvath et al. 2001). In contrast, at a higher concentration (>15 mM) of furfural, the cofactor preference of the furfural-reducing reaction shifts to NADPH, owing to an insufficient supply of NADH (Heer et al. 2009). Celton et al. (2012) have demonstrated that a similar shift in cofactor preference occurs in the acetoin-reducing reaction. However, the shift in cofactor preference occurred in our study at a lower concentration (2 to 5 mM) in the case of glycolaldehyde than the other aldehydes, possibly as a result of its strong toxicity (Jayakody et al. 2011).

Given that *S. cerevisiae* lacks pyridine nucleotide transhydrogenase-like activities (Bruinenberg et al. 1983), the excess NADH generated under anaerobic conditions is primarily balanced by the formation of glycerol, which is a major by-product of yeast fermentation (Zaldivar et al. 2001).

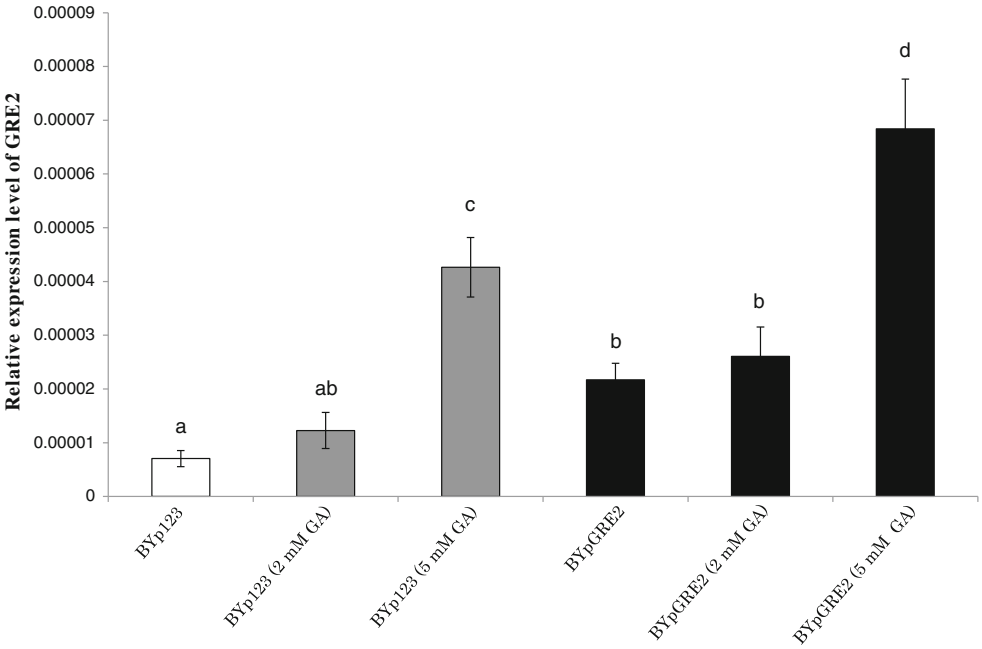

Fig. 6 Expression of *GRE2* with different concentrations of glycolaldehyde. Relative expression levels of *GRE2* incubated with 2 and 5 mM glycolaldehyde (GA) were analyzed by real-time quantitative RT-PCR. The results are expressed as mean values±SEM of independent triplicate experiments from the respective independent starter cultures. Bars labeled with different letters indicate statistically significant values ($p<0.05$; one-way ANOVA followed by Tukey's post hoc honest significance difference test)

Accordingly, a decrease in the glycerol concentration reflects a shortage of NADH within cells. At the same time, it has been reported that reducing the rate of glycerol production increases the ethanol yield (Nissen et al. 2000). The low glycerol production has been reported to be achieved by ablation of the *GDH1* gene and overexpression of the *GDH2* gene (Roca et al. 2003) and recombination of the phosphoketolase pathway (Sonderegger et al. 2004). The results reported in this study indicate that in the presence of glycolaldehyde, the strain overexpressing both *ADH1* and *GRE2* predominantly oxidizes surplus cytosolic NADH and generates NAD^+, which substantially reduces glycerol formation and increases ethanol formation. Thus, the strain overexpressing both *ADH1* and *GRE2* decreased the glycerol yield by 70 % and increased the ethanol yield by around 7 % relative to the *ADH1*-overexpressing strain. This is also similar to the decrease of glycerol by overexpression of a water-forming NADH oxidase (Vemuri et al. 2007).

It has been reported that the increase in acetic acid is interconnected to the NADPH demand of the cells (Grabowska and Chelstowska 2003). A surplus of $NADP^+$ increases acetic acid production (Van Dijken and Scheffers 1986). NADPH is provided mainly through the pentose phosphate pathway (Verduyn et al. 1990). In glycolaldehyde-treated cells, increases in the levels of NADPH are considered to be fueled by activation of the pentose phosphate pathway and to have been provided to the glycolaldehyde-reducing reaction using Gre2. This hypothesis is substantiated by the increased expression of *ZWF1* and *GND1*, encoding components of the pentose phosphate pathway, at a high concentration of glycolaldehyde (≥5 mM). Moreover, the glycerol–dihydroxyacetone cycle, which has been reported to be activated by elevated levels of $NADP^+$, converts NADH into NADPH (Celton et al. 2012; Costenoble et al. 2000). The lower glycerol content in the *GRE2*-overexpressing strain in glycolaldehyde-reducing condition (Table 4) suggests that the glycerol–dihydroxyacetone cycle might be activated in the *GRE2*-overexpressing strains. Many of the other genes have been reported to be involved in the reduction and detoxification of aldehyde inhibitors using NAD(P)H as a cofactor. Genes encoding aldehyde dehydrogenases *ADH1*, *ADH6*, *ADH7*, and *SFA1*; aldehyde reductases *ALD4*, *ALD6*, and *ARI1*; methylglyoxal reductases *GRE2* and *GRE3*; and uncharacterized ORF *YKL071W* have been identified as genes encoding furfural and 5-HMF-reducing enzymes (Heer et al. 2009; Liu 2011). Therefore, the genes other than the *ADH1* and *GRE2* are candidate genes of overexpression to improve tolerance to glycolaldehyde, except *ADH6*, whose overexpression did not confer tolerance to glycolaldehyde in the present study (Supplementary Fig. S1).

In conclusion, for the first time, to the best of our knowledge, we have engineered a yeast strain that is hyper-resistant to glycolaldehyde by introducing a NADPH-dependent reduction pathway in addition to the NADH-dependent pathway. The strain overexpressing both *GRE2* and *ADH1* converted glycolaldehyde to ethylene glycol and detoxified it at 5 mM glycolaldehyde dependent on both NADH and NADPH. The redox cofactor utilization strategy for glycolaldehyde detoxification proposed in this study has the potential to influence strategies to improve tolerance of other fermentation inhibitors.

Acknowledgments We would like to acknowledge the Analytical Research Center for Experimental Sciences of Saga University for DNA sequencing. Part of this study was supported by JSPS KAKENHI (24580117) to HK.

References

Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biot 82:340–349

Almeida JRM, Röder A, Modig T, Laadan B, Lidén G, Gorwa-Grauslund MF (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

Bruinenberg PM, Van Dijken JP, Scheffers WA (1983) A theoretical analysis of NADPH production and consumption in yeasts. J Gen Microbiol 129:953–964

Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52:741–755

Celton M, Goelzer A, Camarasa C, Fromion V, Dequin S (2012) A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in *Saccharomyces cerevisiae*. Metab Eng 14:366–379

Chen CN, Porubleva L, Shearer G, Svrakic M, Holden LG, Dover JL, Johnston M, Chitnis PR, Kohl DH (2003) Associating protein activities with their genes: rapid identification of a gene encoding a methylglyoxal reductase in the yeast *Saccharomyces cerevisiae*. Yeast 20:545–554

Costenoble R, Valadi H, Gustafsson L, Niklasson C, Franzén CJ (2000) Micro aerobic glycerol formation in *Saccharomyces cerevisiae*. Yeast 16:483–495

Gietz RD, Schiestl RH (2007) High-efficient yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2:31–34

Grabowska D, Chelstowska A (2003) The *ALD6* gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. J Biol Chem 278:13984–13988

Heer D, Heine D, Sauer U (2009) Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxidoreductases. Appl Environ Microbiol 75:7631–7638

Horvath IS, Taherzadeh MJ, Niklasson C, Liden G (2001) Effects of furfural on anaerobic continuous cultivation of *Saccharomyces cerevisiae*. Biotechnol Bioeng 75:540–549

Hu F, Ragauskas A (2012) Pretreatment and lignocellulosic chemistry. Bioenergy Res 5:1043–1066

Jayakody LN, Hayashi N, Kitagaki H (2011) Identification of glycolaldehyde as the key inhibitor of bioethanol fermentation

by yeast and genome-wide analysis of its toxicity. Biotechnol Lett 33:285–292

Jayakody LN, Horie K, Hayashi N, Kitagaki H (2012) Improvement of *Saccharomyces cerevisiae* to hot-compressed water treated cellulose by expression of *ADH1*. Appl Microbiol Biotechnol 94:273–283

Ehara K, Saka S (2002) A comparative study on chemical conversion of cellulose between the batch-type and flow-type systems in supercritical water. Cellulose 9:301–311

Katz M, Frejd T, Hahn-Hagerdal B, Gorwa-Grauslund MF (2003) Efficient anaerobic whole cell stereoselective bioreduction with recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 84:573–582

Kitagaki H, Cowart LA, Matmati N, Montefusco D, Gandy J, de Avalos SV, Novgorodov SA, Zheng J, Obeid LM, Hannun YA (2009) *ISC1*-dependent metabolic adaptation reveals an indispensable role for mitochondria in induction of nuclear genes during the diauxic shift in *Saccharomyces cerevisiae*. J Biol Chem 284:10818–10830

Kohrer K, Domdey H (1991) Preparation of high molecular weight RNA. Methods Enzymol 194:398–405

Krantz M, Nordlander B, Valadi H, Johansson M, Gustafsson L, Hohmann S (2004) Anaerobicity prepares *Saccharomyces cerevisiae* cells for faster adaptation to osmotic shock. Eukaryot Cell 3:1381–1390

Liu ZL (2011) Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. Appl Microbiol Biotechnol 90:809–825

Liu ZL, Moon JA (2009) novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 446:1–10

Liu ZL, Slininger PJ (2006) Transcriptome dynamics of ethanologenic yeast in response to 5-hydroxymethylfurfural stress related to biomass conversion to ethanol. In: Mendez-Vilas A (ed) Recent research developments in multidisciplinary applied microbiology: understanding and exploiting microbes and their interactions-biological, physical, chemical and engineering aspects. Wiley, New York, pp 679–668

Liu ZL, Slininger PJ, Gorsich S (2005) Enhanced biotransformation of furfural and 5-hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl Biochem Biotechnol 121–124:451–460

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C_T$ method. Methods 25:402–408

Lu X, Yamauchi K, Phaiiboonsilpa N, Saka S (2009) Two-step hydrolysis of Japanese beech as treated by semi-flow hot-compressed water. J Wood Sci 55:367–375

Moon J, Liu ZL (2012) Engineered NADH-dependent *GRE2* from *Saccharomyces cerevisiae* by directed enzyme evolution enhances hmf reduction using additional cofactor NADPH. Enzyme Microbial Technol 50:115–120

Naik SN, Vaibhav VG, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sust Energ Rev 14:578–597

Nissen TL, Kielland-Brandt MC, Nielsen J, Villadsen J (2000) Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab Eng 2:69–77

Olsson L, Hahn-Hägerdal B (1993) Fermentative performance of bacteria and yeasts in lignocellulosic hydrolysates. Process Biochem 28:249–257

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technol 74:25–33

Petersson A, Almeida JRM, Modig T, Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF, Lidén G (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. Yeast 6:455–464

Rep M, Proft M, Remize F, Tamas M, Serrano R, Thevelein JM (2001) The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. Mol Microbiol 40:1067–1083

Roca C, Nielsen J, Olsson L (2003) Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl Environ Microbiol 69:4732–4736

Rodrigues-Pousada C, Menezes RA, Pimentel C (2010) The Yap family and its role in stress response. Yeast 27:245–258

Saint-Prix F, Bönquist L, Dequin S (2004) Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the $NADP^+$-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. Microbiology 150:2209–2220

Sims REH, Mabee W, Saddler JN, Taylor M (2010) An overview of second generation biofuel technologies. Bioresource Technol 110:1570–1580

Sonderegger M, Schumperli M, Sauer U (2004) Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*. Appl Environ Microbiol 70:2892–2897

Van Maris AJA, Abbott DA, Bellissimi E, Van Den Brink J, Kuyper M, Luttik MAH, Wisselink HW, Scheffers WA, Van Dijken JP, Pronk JT (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie van Leeuwenhoek 90:391–418

Van Dijken JP, Scheffers WA (1986) Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol Lett 32:199–224

Vemuri GN, Eiteman MA, McEwen JE, Olsson L, Nielsen J (2007) Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 104:2402–2407

Verduyn C, Postma E, Scheffers WA, Dijken JP (1990) Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J Gen Microbiol 136:395–403

Yang B, Wyman CE (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Biorefin 2:26–40

Yu Y, Lou X, Wu H (2007) Some recent advances in hydrolysis of biomass in hot compressed water and its comparison with other hydrolysis methods. Energy Fuels 22:46–60

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34