Biomass Utilization Technologies to Cope with Future Energy Demand in Asia: Recent Advances in Engineering of a Yeast Strain Resistance to Glycolaldehyde

アジアにおける将来のエネルギー需要増加に対処するため、バイオ燃料を有効に利用する最新技術を提案する。

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Abstract Hot-compressed water treatment is a promising pre-treatment technique to degrade cellulose for cellulosic ethanol production. However, further degradation of glucose, fructose and mannose yields 2-22 mM glycolaldehyde through retro-aldol condensation. We have for the first time reported that glycolaldehyde is the key fermentation inhibitor in hot-compressed water-treated hydrolysate. Moreover, inhibitory effect is greater than those of 5-HMF and furfural. Recently, we elucidated that reduction of glycolaldehyde to less toxic ethylene glycol with yeast Adh1p significantly attenuates the toxicity of glycolaldehyde and remarkably improves the ethanol fermentation in hot-compressed water treated-cellulose hydrolysate. On the other hand, overexpression of *GRE2* improves the yeast resistance to glycolaldehyde but it is lower than compared to the *ADH1*-overexpressing strain. However, the multiple overexpression of *ADH1* and *GRE2* genes in the yeast are shown to be notably hyper-resistant to glycolaldehyde.

Keywords Yeast; Glycolaldehyde; Hot-compressed water; *ADH1*; *GRE2*

Introduction

The rapid rising regional economy of Asia is projected to increase automobile usage by more than two-fold in most of the member countries in 2035. In fact, the number of vehicles in China and India will increase by three- and five-fold, respectively. Hence, it has been estimated that the future transportation fuel demand in Asia will increase by 15-350% at the end of 2030. However, the Asian region lacks natural gas and oil lobbies for production of fuel at least to cater the present transportation fuel demand. Thus, most Asian countries depend on fuel or natural gas importation to meet their own transportation fuel demand. Moreover, the gasoline and diesel exhaust are associated to 5-45% of total air pollution load in the megacities around Asia. Hence, these facts highlight the important of carbon neutral biofuel production to meet the future transportation fuel demand in Asia while mitigating the air pollution in the region.

Among the biofuels, industrialization of bioethanol has been proved to be practical, and annual global bioethanol production has reached 86 billion liters. However, the contribution of Asia accounts for only about 12 billion liters. Moreover, the current production system based on starch or cane sugar is not sufficient to have major impact on petroleum use, because of the raw material limitation. Furthermore, it competes for arable land for food production and

subsequently intensifies the food crisis. Considering all of these factors, cellulosic ethanol production from agricultural and forestry biomass residues has been shown to have a major impact on substituting petroleum fuel in Asia.

The degradation of β1-4 linked D-glucopyranose containing celluloses into fermentable sugar is a key step in cellulosic ethanol production. Hot-compressed water treatment is a novel and promising pre-treatment method to recover sugars for production of bioethanol from cellulose and hemicellulose (Nakata et al. 2006; Kumagai et al. 2004; Adschiri et al. 1993; Bonn et al. 1983) contrast to other available pretreatment methods. It is defined as water in sub-critical or a super-critical stage or the temperature above 150 ^{0}C with various pressures. It breaks down celluloses into various compound basically through pyrolytic cleavage, swelling and dissolution of the glycosidic bond in the cellulose. (Yu et al. 2008; Lu et al. 2009). The degradation of cellulose with hot-compressed water at the temperature range from 230–400 ^{0}C mainly yielded glucose, fructose, erythrose, dihydroxyacetone, pyruvaldehyde, cellobiose, cellotriose, cellopentaose and cellohexaose. Treatment of cellulose with hot-compressed water has several advantages such as no hazardous wastes are produced in the process, the reaction rate is quite fast, and mass scale production is economically feasible (Kumar et al. 2009). One of the major drawbacks of this method is the resultant solution has an inhibitory effect on ethanol fermentation by yeast cells. Because the glucose yielded in hydrolysis process of cellulose further decomposed and forms furfural, 5-HMF, methylglyoxal, it has been believed that those substances are responsible for the inhibition of ethanol fermentation (Klinke 2004). However, during the treatment of celluloses with pressurized hot-water, glycolaldehyde is produced (Fig. 1) at the concentration of 1 mM to 22 mM through retro-aldol condensation of glucose, fruc-

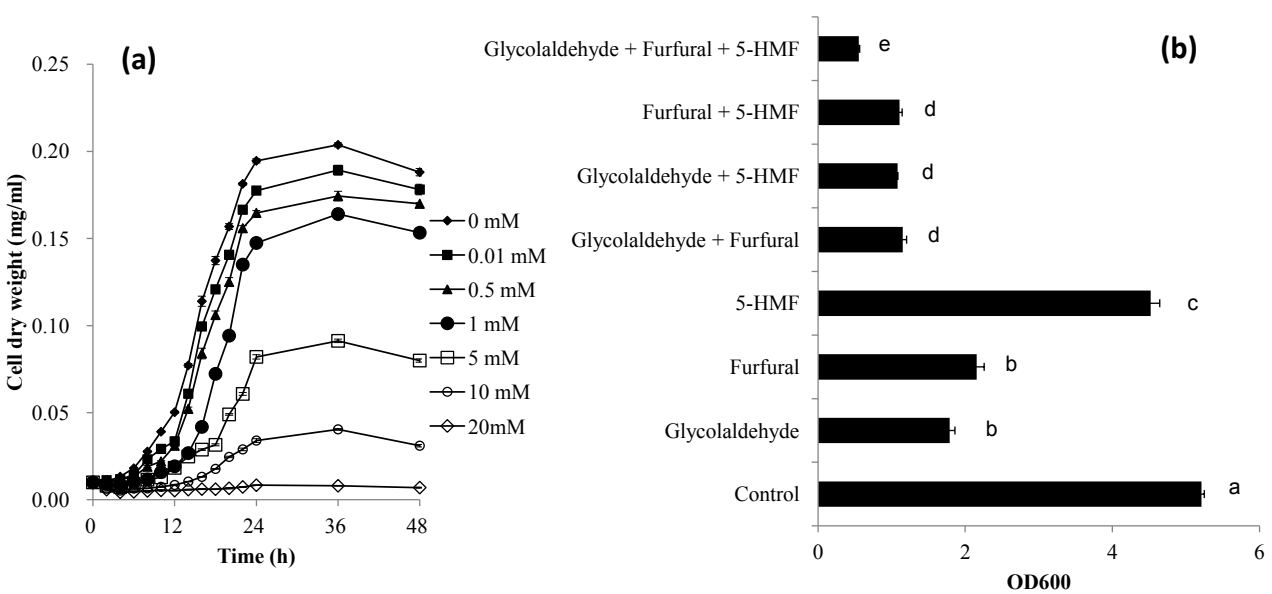

Fig. 1 Glycolaldehyde is a key inhibitor of yeast *Saccharomyces cerevisiae*

(a) *S. cerevisiae* BY4743 cells were grown at 30 °C in 96-well plates containing 100 µl of SC media supplemented with CSM and the different concentration of glycolaldehyde. Growth was monitored at OD_{600} at different time intervals. Cell dry weights were calculated based on OD_{600} of 1 equal 0.45 mg of cell dry weight. **(b)** BY4743 + pRS426 in media containing 2.3 mM glycolaldehyde, 3.3 mM furfural, 3.5 mM 5-HMF and their combinations, OD_{600} were measuured at 24 h. The results are expressed as the mean ± SEM of the independent triplicate experiments from the respective independent starter cultures.

tose and mannose (Katsunobu and Shiro 2002; Lu et al. 2009). For the first time we have identified the significance of glycolaldehyde in the ethanol fermentation (Jayakody et al. 2011). Glycolaldehyde is an α-hydroxyaldehyde, with a hydroxyl bond next to the aldehyde bond, which differentiates this molecule from other general aldehydes, and there have been very few studies of glycolaldehyde in the field of fermentation. Generally, aldehydes are characterized by their polarized π-electron clouds surrounding the carbonyl bonds and low pKa of α-carbon because of inductive effect. However, since α-hydroxyaldehyde bears a hydroxyl bond in the vicinity of their carbonyl bond, it is considered to form Schiff base with amino bases of proteins followed by Amadori rearrangement, conversion to aldoamine, regeneration of carbonyl base and cross-linking of proteins (Acharya and Manning 1983) or followed by formation of carboxymethyllysine and related advanced glycation endproducts (Glomb and Monnier 1995). Glycolaldehyde is peculiar in α-hydroxyaldehydes because it has only 2 carbons, thus generating a drastic molecular characteristic such as the 2109-fold increased activity of Maillard reaction relative to glucose (Hayashi and Namiki 1986).

There has been less information about detoxification mechanism of glycolaldehyde or the metabolic fate of glycolaldehyde in a reductive environment. Hence, in this study, we present the overview of glycoladehyde as the primary fermentation inhibitor in hot-compressed water-treated cellulose hydrolysate and strategy for engineering a yeast strain with improved tolerance to the hot-compressed water-treated cellulose by attenuating the toxicity of the glycolaldehyde present in the hot-compressed water-treated cellulose.

Materials and Methods

Construction of ADH1 and GRE2 overexpressing plasmids

Cloning and restriction enzymes were obtained from Takara Bio (Kyoto, Japan). The *ADH1* and *GRE2* genes fragment was obtained by PCR from the *S. cerevisiae* BY4743 genome with the respective forward and the reverse primers of XhoI $ADH1_{fw}$ (5'-CCC CTC GAG ACT GTA GCC CTA GAC TTG ATA-3') , EcoRI $ADH1_{rv}$ (5'-CCC GAA TTC GGT AGA GGT GTG GTC AAT AA-3') for *ADH1* and KpnI $GRE2_{fw}$ (5'-CCC GGT ACC ATG TCA GTT TTC GTT TCA GG-3'), SalI $GRE2_{rv}$ (5'-CCC GAC CTA CCA TTT TGT GAA TCA A-3') for *GRE2*. The amplified PCR product was purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics GmbH, Mannheim, Germany). The purified gene fragments were cleaved with respective restriction enzymes and ligated into desired plasmids by using the DNA ligase (Takara, Kyoto, Japan) to form pRS426-*ADH1* and pAUR123-*GRE2*. The correct insertion of the PCR fragment into the plasmid was confirmed both by a restriction enzyme treatment and sequencing with Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT, USA). The pRS426-*ADH1* and pAUR123-*GRE2* was transformed into BY4743 by a high efficiency yeast transformation technique (Gietz et al., 2007). The successful transformants of *ADH1* and *GRE2* were selected using minimal media supplemented with complete supplementary media without uracil and minimal media supplemented with complete supplementary media presence of 0.5 μg/ml of aureobasidin respectively. The multiple genes overexpression strain of *ADH1* and *GRE2* were selected in minimal media supplemented with complete supplementary media without uracil presence of 0.5 μg/ml of aureobasidin.

Measurement of concentrations of glucose

The glucose concentration was measured at OD_{505} by using a UV spectrophotometer (UV-1800; Shimadzu Scientific Instruments, Kyoto, Japan) with a Glucose CII-test kit (Wako Diagnostic, Osaka, Japan).

Measurement of concentrations of ethanol, ethylene glycol, acetic acid, and glycerol

The ethanol concentration was quantified using a gas chromatograph (GC 17-A, Shimadzu Scientific

Instruments, Kyoto, Japan) equipped with a DB-WAX column (Agilent Technologies, Santa Clara, CA, USA) (length = 30 m, internal diameter = 0.25 mm, film thickness = 0.25 μm) and an FID detector. Acetone (final 3% (v/v) was added to 500 μl of the fermented sample as an internal control. Six microliters of the prepared samples were applied to gas chromatography under the following conditions: initial column temperature: 55 ^{0}C, holding time for the initial temperature: 5 min, final temperature: 170 ^{0}C, rate of temperature incerase: 10 ^{0}C /min, detector and injection temperatures: 200 ^{0}C, split ratio: 50:1, carrier gas: helium, and carrier gas flow rate: 0.78 ml/min. The retention time for the peak of ethanol was identified to be 2.8 min. The standard calibration curve exhibited good reproducibility and linearity with a correlation coefficient of 0.914. The retention time for the peak of ethylene glycol was identified to be 16.7 min as reported previously (Yao and Porter, 1996). The standard calibration curve exhibited good reproducibility and linearity with a correlation coefficient of 0.974. The retention time for the peak of acetic acid was identified to be 13.9 min. The standard calibration curve exhibited good reproducibility and linearity with a correlation coefficient of 0.961. Glycerol concentrations were measured by using Glycerol Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instruction.

Measurement of glycolaldehyde concentrations

The concentrations of glycolaldehyde were quantified using the HPLC method described earlier (Anderson et al. 1997; Paz et al. 1965) with minor modifications. Briefly, the derivative of glycolaldehyde and MBTH was separated by a reverse phase HPLC using an Excelpak SIL-C18 5B column (Yokogawa, Tokyo, Japan) with a gradient consisting of linear segments; solvent A consisted of 5% methanol and 0.1% trifluoroacetic acid, and solvent B consisted of 90% methanol and 0.1% trifluoroacetic acid. Solvents were run through HPLC in the following series: solvent A, 100% over 5 min; solvent B, 0–60% over 5 min; and solvent B, 60–100% over 30 min. The flow rate was 0.7 ml/min and detection was at 598 nm. The retention time of glycolaldehyde was identified to be 19.4 min. The standard calibration curve was calculated with standard glycolaldehyde solutions of 1, 2, 5, 8 and 10 mM. The standard calibration curve exhibited good reproducibility and linearity with a correlation coefficient of 0.992. Samples (20 μl) were applied to the column, and the glycolaldehyde concentrations in the samples were measured and calculated from the obtained standard calibration curve.

Preparation of hot-compressed water-treated cellulose

For treatment of cellulose with hot-compressed water, three independent experiments were carried out and the hydrolysates were applied for further manipulations and analyses. A solution containing 10% (w/v) microcrystalline cellulose (Avicel PH-101) was hydrolyzed in a 3-ml volume reactor vessel with hot-compressed water. The reaction was carried out for 1 min at 280 ^{0}C at an internal pressure of 7 MPa. Then, the reactor vessel was immediately cooled to room temperature using a water bath. The treated sample was centrifuged, and the liquid fraction was used in further experiments as the hot-compressed water-treated cellulose.

Growth experiments

For all growth experiments, three independent experiments were carried out from the respective independent starter cultures. To examine the inhibitory effects on the growth, yeast was grown in 2 ml of minimal media supplemented with 790 mg/l of complete supplementary media (CSM) in the presence of different concentrations of inhibitors in 3 ml glass test tubes. The initial cell concentration was set at 0.05 OD_{600} (corresponding to 0.5×10^6 cells/ml, Fig. 1a) or 0.1 OD_{600} (corresponding to 1×10^6 cells/ml, Fig. 2a, Fig. 4a and Fig. 5), and the growth was monitored by measuring OD_{600} at different intervals. To investigate the growth of the strains

in the hot-compressed water-treated cellulose, the hot-compressed water-treated cellulose was supplemented with 790 mg/l CSM and a 0.67% (w/v) yeast nitrogen base without amino acids and ammonium sulphate. Then, 2 ml of the medium in 3-ml glass test tubes was inoculated with the strains.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to detect the significance of the differences in the effects of inhibitors on the fermentation. Tukey's *post hoc* honest significance difference test was implemented for multiple comparisons. For a pair-wise comparison of the differences between the sample averages of two groups, a one-tailed Student's *t*-test without any known deviations was adopted. All experiments were performed independently in triplicate from the respective independent starter cultures. The results were expressed in mean values and standard errors of means (SEM).

Results and Discussions

Glycolaldehyde is a key growth and fermentation inhibitor of yeast

Glycolaldehyde significantly inhibits cell growth of yeast even at a concentration as low as 0.01 mM (Fig. 1a).The IC_{50} value of glycolaldehyde on *Saccharomyces cerevisiae* is approximately 10 mM. Hence, the concentration of glycolaldehyde contained in the actual pressurized hot-compressed water-treated cellulose hydrolysate is high enough to inhibit yeast growth. Furthermore, growth analysis indicated that glycolaldehyde affects both cell growth rate and the lag phase of cell growth. Moreover, Fig. 1b shows that the inhibitory activity of glycolaldehyde is greater than the major reported inhibitors of 5-HMF and furfaral at 5 mM concentration, in addition the specific growth rate and the cell dry weights of glycolaldehyde-treated cells are smaller than those of 5-HMF and furfural-treated cells. Furthermore, statistically significant greater combinational inhibitory activity of glycolaldehyde exhibits with 5-HMF and furfural at the concentration present in actual hot-compressed water-treated cellulose (Fig. 1b, ANOVA; F value = 13.7, degrees of freedom = 11, 3, p value = 0.00162, Tukey's *post hoc* honest significance difference test: $p < 0.05$).

Glycoaldehyde not only inhibits yeast growth but also greatly reduces the ethanol production, the concentration of glycolaldehyde higher than 1 mM significantly decreases ethanol production. The analysis of glucose and ethanol profile during fermentation with glycolaldehyde further reveals that it's significantly decreases the glucose consumption and ethanol yield by yeast. After 48 h of fermentation at the concentration of 2 mM glycolaldehyde, ethanol concentration is around 1.66±0.13 % (v/v) and glucose consumption is 4.86±0.072% (w/v), where the untreated cells ethanol concentration is 3.42±0.050 % (v/v) and glucose consumption is around 9.2±0.079 % (w/v). Moreover, glycolaldehyde reduces the ethanol yield (g produced ethanol/g consumed glucose), the value of untreated cells was 0.320±0.0039, while that of 2 mM glycolaldehyde-treated cells was 0.254±0.017. The ethanol yield of cells treated with 2 mM glycolaldehyde was significantly lower than that of untreated cells (p = 0.040). As a conclusion glycolaldehyde significantly decreases ethanol production, glucose consumption and ethanol yield by yeast. These results firmly establish that glycolaldehyde is key growth and fermentation inhibitor in hot- compressed water-treated cellulose hydrolysate.

Genome-wide analysis of glycolaldehyde toxicity

Genome-wide screening of genes is powerful biotechnology technique in order to gain insight into the molecular mechanism of yeast fermentation inhibition by glycolaldehyde.

One hundred and seventy genes were identified as genes required for glycolaldehyde tolerance (The mutants which are more than 10% sensitive relative to the wild type and whose sensitivity is significant at $p < 0.05$) by screening the complete mutant collection of *Saccharomyces cerevisiae* BY4743 com-

Table 1 Functional categories those are overrepresented in the sensitive mutants

Category[a]	*p* value[b]	Gene name
GO Cellular Component		
Mitochondrial respiratory chain complex IV	0.00262	*COX9 COX6 COX5B*
Ubiquitin ligase complex	0.00740	*SLX8 BUL2* YNL311c
Polysome	0.00740	*PBP1 CTK1 SSB2*
Elongator holoenzyme complex	0.00821	*ELP2 IKI3*
GO Biological Process		
Response to acid	0.00148	*BCK1 MID2 RLM1*
Golgi to vacuole transport	0.00327	*VPS54 VPS45 APS3 APL2*
Mitochondrial electron transport, cytochrome c to oxygen	0.00334	*COX9 COX6 COX5B*
GO Molecular Function		
Chromatin DNA binding	0.00556	*GON7 RED1*
Phospholipase activity	0.00821	*PLB1* YOR022c

[a] Gene functions were identified by addressing the GO database with the FunSpec statistical evaluation program.
[b] Probability of the functional set occurring as a chance event is shown.

prising of 4848 homozygous diploid deletion strains with 0.01 mM glycolealdehyde (Jayakody et al. 2011).The categories involves in glycolaldehyde resistance obtained by submitting the list of genes that is required for resistance to glycolaldehyde to the GO yeast databases on the FunSpec web-based clustering tool (Robinson et al. 2002).

This study shows that protein cross linking is one of the major targets of the glycolaldehyde toxicity, because mutants defective in ubiquitin ligase complex and polysomes were significantly sensitive to glycolaldehyde. Glycolaldehyde has been reported to cross link proteins through its electrophilic attack towards the lone electron-pair of the nitrogen atom of amino groups of proteins (Glob and Monnier 1995) and thiolate anion of cysteine of proteins (Hayashi and Namiki 1986). Moreover, glycation has been reported to decrease total cellular proteasome activity in human fibroblast and keratinocytes, which is suggested to have a critical role in aging. The observed sensitivity of mutants involved in these systems suggests that glycolaldehyde attacks proteins that are being translated from mRNA and hinder proper folding of proteins, which is alleviated by ubiquitin ligase complex.

Reduction of glycolaldehyde to ethylene glycol

Based on the results of genome-wide analysis and the molecular structure and function of glycolaldehyde, it was suggested that the plus charge of the α-carbon of the glycolaldehyde molecule plays a key role in the inhibition of yeast, because electrophilic attack of the plus charge of carbonyl carbon of glycolaldehyde to negatively charged molecules inside cells is the main cause of the toxicity. Hence, the reduction of plus charge of the carbonyl carbon of glycolaldehyde molecule by NADH was implemented as the principle strategy to develop a resistant strain. Although not detected in the functional categories of glycolaldehyde resistant genes in GO based statistically analysis, a mutant defective in aldehyde dehydrogenases such as *adh1* (0.72 ± 0.0037 fold) was obtained as sensitive genes in the glycolaldehyde screen (Jayakody et al. 2011). This result suggests that these dehydrogenases function to confer glycolaldehyde tolerance, and that glycolaldehyde functions as an aldehyde within cells and the enzymes that reduce the glycolaldehyde to ethylene glycol is effective to mitigate the damage. This result is consistent with the previous study that reported the role of aldehyde dehydrogenase Adh6p against 5-HMF (Petersson et al. 2006). Moreover, ethylene glycol was not toxic to yeast cells when it was administered with the same concentration as glycolaldehyde (Jayakody et al. 2012). Since *adh1* was sensitive to glycolaldehyde and Adh1p is capable of reducing short-chain aldehydes such as acetaldehyde and formaldehyde by using NADH as a cofactor (Leskovac et al. 2002; Grey et al. 1996), it was selected for biochemical reduction of glycolaldehyde into ethylene glycol (Jayakody et al. 2012). This hypothesis was verified by constructing *ADH1*-overexpressing strain.

ADH1-overexpressing strain increases the conversion of glycolaldehyde into ethyleneglycol

It turned out that a strain overexpressing *ADH-1*demonstrated growth that is similar to a strain harboring an empty vector in the absence of glycolaldehyde. In contrast, in the presence of glycolaldehyde, the strain harboring *ADH1*-overexpressing plasmid showed significantly (n = 3, p < 0.01) improved growth in the presence of glycolaldehydes compared to a strain harboring an empty vector (Fig. 2a). The strains harboring *ADH1*-overexpressing plasmid and an empty vector produced similar amounts of ethanol and consumed similar amounts of glucose in the absence of glycolaldehyde. In contrast, in the presence of glycolaldehyde, the strain harboring *ADH1*-overexpressing plasmid produced a significantly (n = 3, p < 0.01) increased amount of ethanol and consumed a significantly (n = 3, p < 0.01) increased amount of glucose relative to the strain harboring an empty vector (Fig.2b and 2c). Further analysis of fermentation profile elucidated that strain harboring *ADH1*-overexpressing plasmid produced statistically significantly (n = 3, p < 0.01) decreased amount of glycerol (Fig.2d), and increased amount of acetic acid (Fig.2e) as compared to the control strain. In addition, extracellular concentration of glycolaldehyde of the medium inoculated with the strain harboring *ADH1*-overexpressing plasmid showed significantly (n = 3, p < 0.05) decreased level as compared to that inoculated with the strain harboring

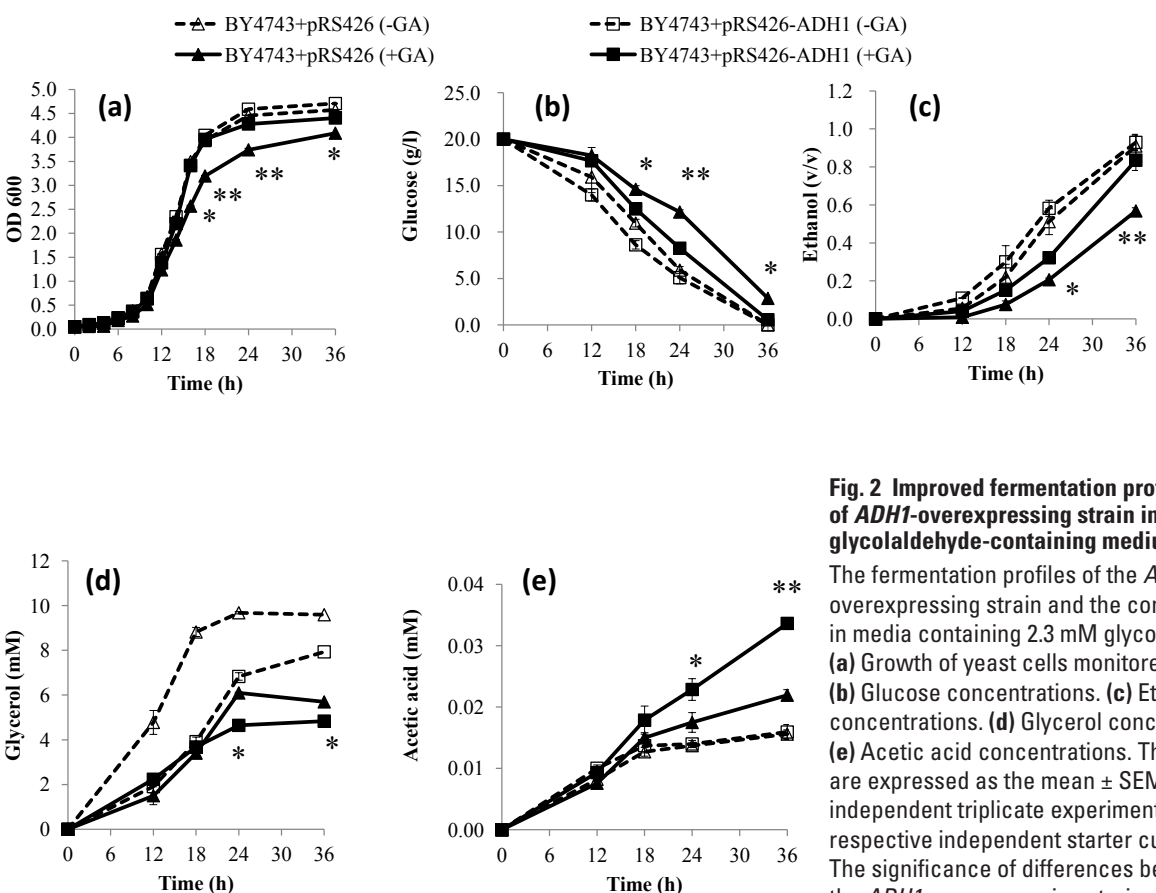

Fig. 2 Improved fermentation profile of *ADH1*-overexpressing strain in a glycolaldehyde-containing medium.

The fermentation profiles of the *ADH1*-overexpressing strain and the control strain in media containing 2.3 mM glycolaldehyde. **(a)** Growth of yeast cells monitored by OD_{600}. **(b)** Glucose concentrations. **(c)** Ethanol concentrations. **(d)** Glycerol concentrations. **(e)** Acetic acid concentrations. The results are expressed as the mean ± SEM of the independent triplicate experiments from the respective independent starter cultures. The significance of differences between the *ADH1*-overexpressing strain and the control strain was evaluated by a one-tailed Student's t-test (n = 3, **; p < 0.01, *; p < 0.05).

an empty vector (Fig. 3a). Furthermore, consistent with the decreased concentrations of extracellular glycolaldehyde in the*ADH1*-overexpressing strain, the extracellular concentration of ethylene glycol, which is the reduced form of glycolaldehyde, was significantly ($n = 3$, $p < 0.01$) increased in the *ADH1*-overexpressing strain (Fig. 3b). The increased ratio of conversion from glycolaldehyde to ethylene glycol at 36 h (*ADH1*-overexpressingstrain: 77±3.6%; control strain: 30±1.9%), statistically significant difference ($n = 3$, $p = 0.0012$) convinced us that *ADH1*-overexpressing strain is highly capable of converting glycolaldehyde to ethylene glycol. Furthermore, the in vivo analysis of cell lysate protein of *ADH1*-overexpressing strain exhibits NADH-dependent higher glycolaldehyde-reducing activity (data not shown). Together, these results support the hypothesis that *ADH1*-overexpressing strain has an improved tolerance to glycolaldehyde by reducing glycolaldehyde into ethylene glycol (Fig. 3c).

ADH1-overexpressing strain Improves fermentation profile in hot-compressed water-treated cellulose

On the basis of the aforementioned results, we hypothesized that this strain would exhibit an improved tolerance to hot-compressed water-treated cellulose. To verify this hypothesis, hot-compressed water-treated cellulose hydrolysate (cellulose treated at 280 ^{0}C, 5 MPa for 1 min) was inoculated with the *ADH1*-overexpressing strain and the control strain as the model substance. The glucose concentration

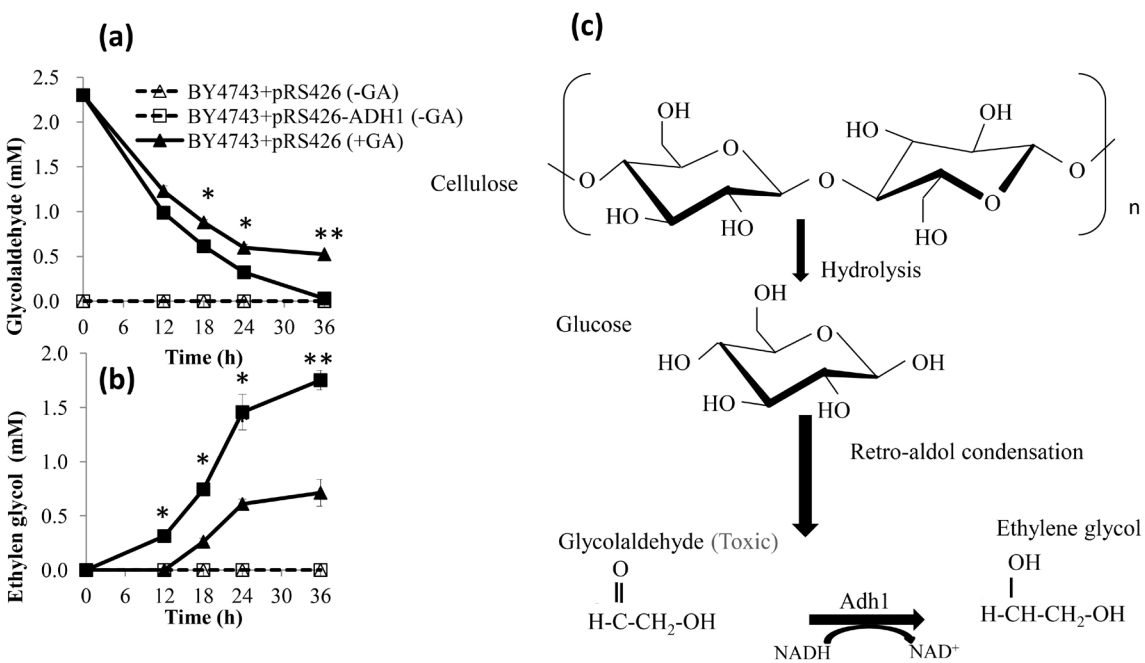

Fig. 3 Novel strategy to reduce glycolaldehyde toxicity by reducing glycolaldehyde into ethylene glycol
The glycolaldehyde-reducing activity of the *ADH1*-overexpressing strain and the control strain was measured in media containing 2.3 mM glycolaldehyde. **(a)** Glycolaldehyde concentrations. **(b)** Ethylene glycol concentrations. **(c)** Principle pathway of glycolaldehyde reduction. The results are expressed as the mean ± SEM of the independent triplicate experiments from the respective independent starter cultures. The significance of differences between the *ADH1*-overexpressing strain and the control strain was evaluated by a one-tailed Student's t-test ($n = 3$, **; $p < 0.01$, *; $p < 0.05$).

of the hot-compressed water-treated cellulose was 24±1.1 g/l (n = 3), which was sufficient to perform ethanol fermentation. The glycolaldehyde concentration in the hot-compressed water treated cellulose was 8.9±0.34 mM (n = 3), which was sufficient for exhibiting an inhibitory effect on fermentation. In media containing the hot-compressed water-treated cellulose, the *ADH1*-overexpressing strain exhibited a statistically significantly improved growth (Fig. 4a), glucose consumption (Fig. 4b), ethanol production (Fig. 4c), glycolaldehyde consumption (Fig. 4d) and ethylene glycol production (Fig. 4e) as compared to the control strain. The ratio of conversion of glycolaldehyde to ethylene glycol was 72±1.7% in the case of the *ADH1*-overexpressing strain, while it was 33±0.85% in the case of the control strain. These results clearly support our hypothesis that the *ADH1*-overexpressing strain exhibits an improved fermentation profile in a medium containing the hot-compressed water-treated cellulose by reducing glycolaldehyde to ethylene glycol.

Development of glycolaldehyde hyper resistance strain by engineering redox cofactor for glycolaldehyde-reducing reaction.

The developed glycoldehyde tolerant strain by overexpressing *ADH1* encoding an NADH-dependent reductase shows partial recovery in high concentration glycolaldehyde containing medium. To overcome this technical barrier, we investigated redox cofactor preference of glycolaldehyde detoxification reaction. Glycolaldehyde-reducing activity of

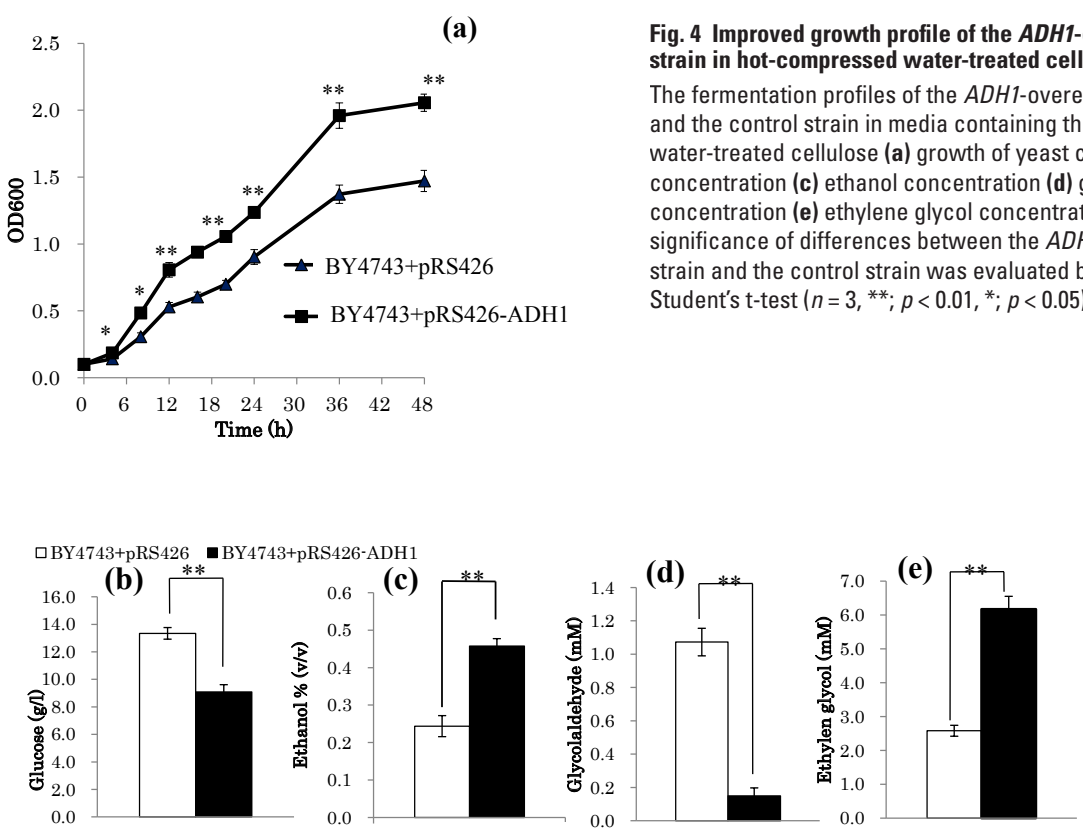

Fig. 4 Improved growth profile of the *ADH1*-overexpressing strain in hot-compressed water-treated cellulose.

The fermentation profiles of the *ADH1*-overexpressing strain and the control strain in media containing the hot-compressed water-treated cellulose **(a)** growth of yeast cells **(b)** Glucose concentration **(c)** ethanol concentration **(d)** glycolaldehyde concentration **(e)** ethylene glycol concentration. The significance of differences between the *ADH1*-overexpressing strain and the control strain was evaluated by a one-tailed Student's t-test (n = 3, **; $p < 0.01$, *; $p < 0.05$).

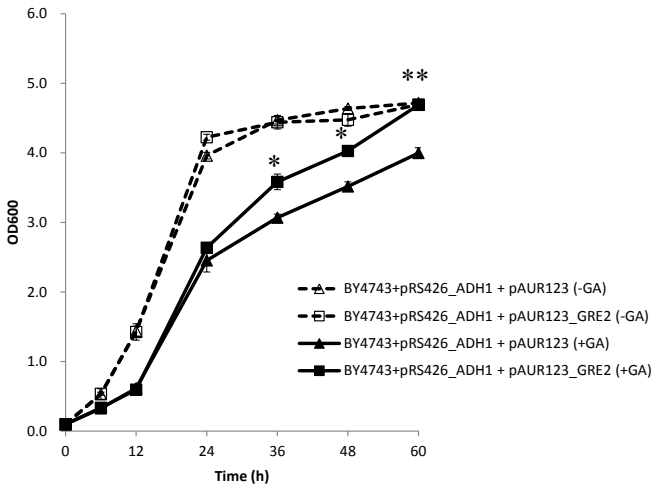

Fig. 5 Strain overexpressing both *ADH1* and GRE2 shows remarkable resistance to glycolaldehyde

The growth profiles of strain overexpressing both *ADH1* and *GRE2* and the control strains were constructed in the SC media containing 10 mM glycolaldehyde. Growth of yeast cells monitored at OD_{600}. The results are expressed as the mean ± SEM of the independent triplicate experiments from the respective independent starter cultures. The significance of differences between the strain overexpressing both *ADH1* and *GRE2* and the control strain was evaluated by a one-tailed Student's t-test ($n = 3$, **; $p < 0.01$, *; $p < 0.05$).

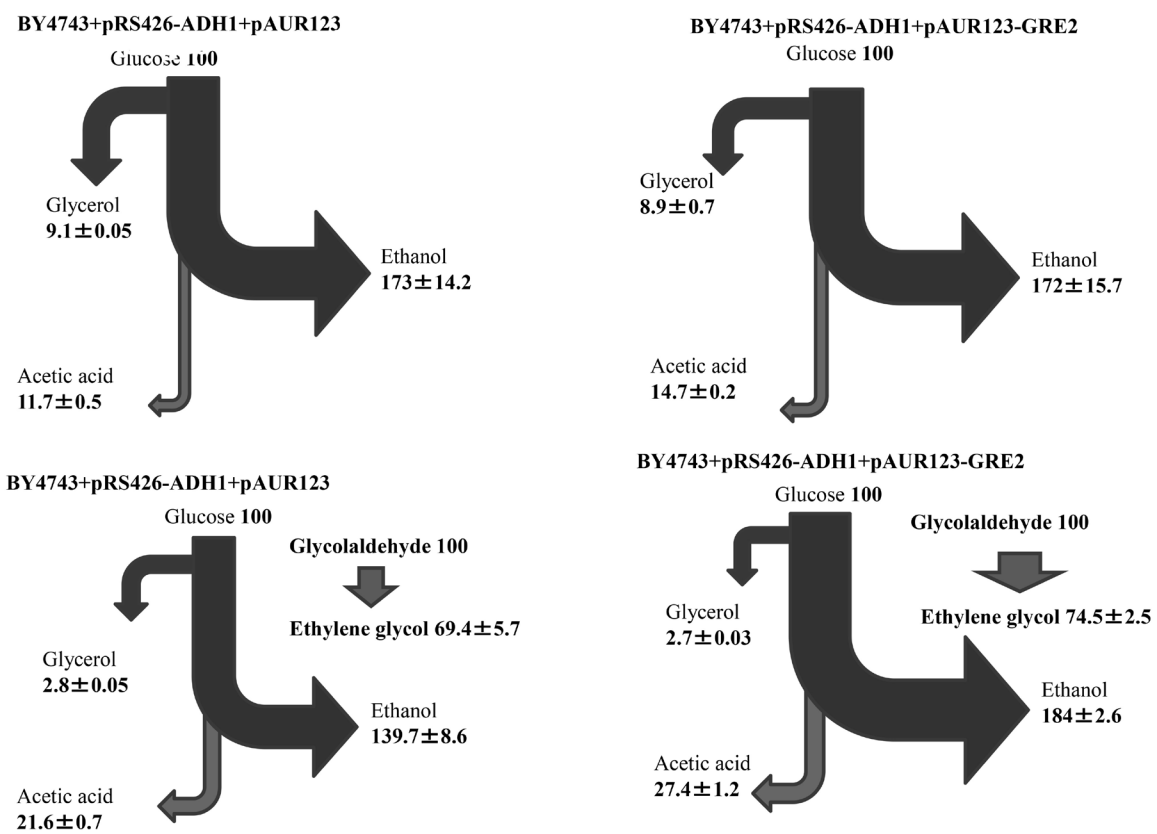

Fig. 6 Change of fermentation metabolic flux and its product s distribution due to glycolaldehyde—reducing reaction

Fermentation product distribution calculated for 100 mol of consumed glucose with or without glycolaldehyde.

ADH1-overexpressing strain was NADH-dependent but not NADPH-dependent (Jayakody et al. 2013). Moreover, genes encoding components of 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 (Jayakody et al. 2013). Genome-wide survey identified *GRE2* encoding an NADPH-dependent reductase as the gene that confers tolerance to glycolaldehyde (Jayakody et al. 2011). Overexpression of *GRE2* in addition to *ADH1* further improved the tolerance to glycolaldehyde (Fig. 5). NADPH-dependent glycolaldehyde conversion to ethylene glycol and $NADP^+$ content of the strain overexpressing both *ADH1* and *GRE2* were increased at 5 mM glycolaldehyde (Jayakody et al. 2013). 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.

Glycolaldehyde causes metabolic shift in glycolysis pathway

The analysis of the fermentation metabolic products revealed that the cells regulated there metabolic carbon fluxes may be adapted to the changing redox status (Fig. 6). Glycerol production by the reduction of dihydroxyacetone phosphate is reported to utilize NADH and competes with the production of ethanol by the reduction of acetaldehyde (Cordier et al. 2007). Therefore, the decrease in the glycerol production in glycolaldehyde-treated cells and *ADH1*-overexpressing strain is explained by the competition of the reaction from dihydroxyacetone phosphate to glycerol-3-phosphate with the reaction of glycolaldehyde to ethylene glycol for the reductive potential of NADH. This result is also consistent with the decrease in glycerol by the mutated *ADH1*-overexpressing strain (Almeida et al. 2009). In contrast, it has been reported that acetic acid is produced by the oxidation of acetaldehyde by using mainly $NADP^+$ and partially NAD^+ as cofactors (Saint-Prix et al. 2004; Wang et al. 1998). The increase in acetic acid in glycolaldehyde-treated cells can be explained by the accumulated acetaldehyde because of the competition between acetaldehyde and glycoaldehyde for Adh1p and NADH. The increase of acetic acid in the glycolaldehyde-treated *ADH1*-overexpressing strain can be explained by the increase in NAD^+ because of the increased reaction of Adh1p-catalyzed reduction of acetaldehyde and glycolaldehyde coupled with oxidation of NADH. This hypothesis is further supported by several other reports which observed an increase of acetic acid in cells overexpressing H_2O-forming NADH oxidase (Heux et al. 2006) or NADH-dependent *GPD1* (Remize et al. 1999; Michnick et al. 1997).

Conclusions

In preceding research on the ethanol fermentation of cellulose and hemicellulose after a hot-compressed water treatment, only 5-HMF and furfural have been focused upon as fermentation inhibitors. Glycolaldehyde was not considered in these studies, although it was generated in the hydrolysate and exhibited inhibitory effects. However, the recent findings are highlighted the glycolaldehyde as the key toxic compound in bioethanol fermentation. Therefore, the toxicity of glycolaldehyde and its detoxification mechanism is highly encouraged the further researches in this field. Furthermore, the novel strategy of reducing glycolaldehyde to ethylene glycol proposed in this study is a promising strategy to decrease the toxicity of hot-compressed water-treated cellulose hydrolysate. This novel information will be certainly valuable to develop biocatalyst for sustainable cellulosic ethanol production system with hot- compressed water treatment to cater the future biofuel demand in Asia.

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