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Engineering the robustness of *Saccharomyces cerevisiae* by introducing bifunctional glutathione synthase gene

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Abstract Robust, high-yielding *Saccharomyces cerevisiae* is highly desirable for cost-effective cellulosic ethanol production. In this study, the bifunctional glutathione (GSH) synthetase genes GCSGS at high copy number was integrated into ribosomal DNA of *S. cerevisiae* by Cre–LoxP system. Threefold higher GSH contents (54.9 μmol/g dry weight) accumulated in the engineered strain BY-G compared to the reference strain. Tolerance of BY-G to H_2O_2 (3 mM), temperature (40 °C), furfural (10 mM), hydroxymethylfurfural (HMF, 10 mM) and 0.5 mM Cd^{2+} increased compared to reference strain. Twofold higher ethanol concentration was obtained by BY-G in simultaneous saccharification and fermentation of corn stover compared to the reference strain. The results showed that intracellular GSH content of *S. cerevisiae* has an influence on robustness. The strategy is used to engineer *S. cerevisiae* strains adaptive to a combination of tolerance to inhibitors and raised temperature that may occur in high solid simultaneous saccharification and fermentation of lignocellulosic feedstocks.

Keywords Bioethanol · GSH · Lignocellulose · *S. cerevisiae* · Robustness

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Introduction

The utilization of biomass for the production of biofuel has received considerable attention in recent years. In particular, ethanol produced from agricultural residues has been suggested to be a promising alternative fuel [11]. However, an important factor for the cost-effective production of ethanol from lignocellulosic feedstock is the high-yield, high-rate fermentation of biomass hydrolysates to ethanol. The demands on *Saccharomyces cerevisiae* that perform these reactions are more complicated than those for conventional ethanol production from single hexose or their disaccharides [11]. Plant hydrolysates contain numerous compounds that reduce ethanol yield and productivity by affecting the yeast performance during the fermentation step [4, 19]. Various detoxification methods such as water washing, overliming, vaporization, ion-exchange absorption, and biodetoxification have been tried. However, these methods resulted in many negative effects and no detoxified materials were used as the feedstock for ethanol production in fermentation practice [4, 10, 18].

One of the barriers for production of biofuels is the development of robust, high-yielding microbes and processes for their production [3]. The yeast *S. cerevisiae* has proved to be more robust than bacteria, both with respect to tolerance to the end product ethanol and to other compounds with inhibitory effects present in hydrolysates [1]. The development of novel yeast strains with increased inhibitor tolerance is highly desirable. *S. cerevisiae* yeast strains with increased tolerance towards hydrolysate-derived inhibitors have been obtained by genetic engineering and evolutionary engineering strategies [5, 13, 16]. The inhibitors impeded several enzymes in glycolysis, decrease specific ethanol production rate, affect cell growth and

survival and induce formation of reactive oxygen species (ROS) in *S. cerevisiae* with DNA and membrane damage as a consequence [2, 8–10].

Glutathione (GSH) is the main antioxidant system in living cells and it is indispensable for, but not limited to, oxidative stress responses [12, 15]. The robustness of *S. cerevisiae* increased when three homologous genes involved in glutathione metabolism were overexpressed [2]. However, the first key enzyme was feedback inhibited by GSH to prevent overaccumulation of the GSH [17]. The bifunctional glutathione synthetase GCSGS from *Streptococcus thermophilus* is neither redox-regulated nor sensitive to feedback inhibition by GSH [7]. An extreme accumulation of GSH in *S. cerevisiae* cells could be obtained and thereby a robust *S. cerevisiae* could be constructed. However, the robustness of *S. cerevisiae* constructed by introducing bifunctional glutathione synthetase genes has not been evaluated. In this study, the *S. cerevisiae* was engineered to modulate the levels of intracellular glutathione and the increased robustness of *S. cerevisiae* under stressful conditions in industrial fermentations was evaluated.

Materials and methods

Stains and media

Saccharomyces cerevisiae BY4717 and plasmids pBlue-Script II SK(+), JMB125, and p416-MET25 were purchased from Zhuhai Yingping Biotechnology Co., Ltd (Zhuhai, Guangdong, China). Plasmid pETG-10A-GCSGS (containing glutathione synthethase gene (accession number GQ848551)) was friendly provided by Prof. An Zhigang of Northeast Forestry University, Harbin, China.

YPD (Yeast extract 1 %, Peptone 2 %, Glucose 2 %) medium was used to cultivate the *S. cerevisiae*.

Construction of GCSGS expression cassette

The ADH1 promoter was amplified from plasmid JMB125 using ADH1-F [5′-GCCTCGAGTAAAACAAGAAGAGGGTTGAC-3′ (*Xho* I)] and ADH1-R (5′-CTTGGAGTTGATTGTATGC-3′) pairs. The terminator sequences were amplified from plasmid JMB125 using CYC1-F (5′-ATCATGTAATTAGTTATGTC-3′) and CYC1-R (5′-GCGAATTCGCAAATTAAAGCCTTCGAGC-3′ (*Eco*R I)) pairs. The bifunctional glutathione synthase gene GCSGS was amplified from plasmid pETA-10-GCSGS using primer sets (F: 5′-GCATACAATCAACTCCAAGATGACATTAAACCAACTGCT-3′ and R: 5′-CATAACTAATTACATGATTTAAGTTTGACCAGCCACTA-3′). The promoter sequences, GCSGS, and terminator sequences were ligated as one fragments using overlap PCR using primer sets ADH1-F and CYC1-R. The constructs were verified by DNA sequencing. The GCSGS expression cassette was designated as ADH1-GCSGS-CYC1.

Construction of plasmid for recombination

The primers (LoxP-Kan F: 5′-GCGAATTCATAACTTCGTATAATGTATGCTATACGAAGTTATGTTTAGCTTGCCTCGTCC-3′ ((*Eco*R I)); LoxP-Kan R; 5′-GCGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATGTTTTCGACACTGGATGG-3′ (*Bam*H I) containing LoxP segments and restriction loci of *Eco*R I and *Bam*H I were used to amplify the KanMX4 gene from yeast genome DNA. The PCR products LoxP-KanMX-LoxP cassette was digested by *Eco*R I and *Bam*H I and ligated into plasmid pBluescript II SK+by T4-DNA ligase, the constructed plasmids were designated as pBlue-kanMX4. Non-transcriptional space 2 (NTS2) was chosen as chromosomal integration sites. The NTS2 region was added as the homologous arms flanking GCSGS expression cassette. The DNA segments covering NTS2 5′ end (NTS2-5′F: 5′-CACAAGAGGTAGGTCGAAACAGAACATGAAAGTTGGTCGGTAGGTGC-3′; NTS2-5′R: 5′-TCGAGCACCTACCGACCAACTTTCATGTTCTGTTTCGACCTACCTCTTGTGGTAC-3′) and 3′ end (NTS2-3′F: GGCCGCCAGAGGTAGTTTCAAGGTGACAGGTTATGAAGATATGGTGCAAAACCGC-3′; NTS2-3′R: 5′-GGTTTTGCACCATATCTTCATAACCTGTCACCTTGAAACTACCTCTGGC-3′) were annealed together and the segments with sticky ends were subcloned into plasmid pBlue-kanMX4 at the corresponding restriction sites (sites of *Xho*1, *Kpn* 1 for NTS2-5′ ends; sites of *Not* I, *Sac* II for NTS2-3′ ends). Then the gaps were ligated by T4-DNA ligase and the combination plasmids were designated as pBlue-NTS3′-kanMX4-NTS5′.

The plasmid pBlue-NTS3′-kanMX4-NTS5′ and GCSGS expression cassette ADH1-GCSGS-CYC1 were digested with *Eco*R I and *Xho* I, respectively, and ligated to construct the expressing plasmid pBlue-NTS3′-kanMX4-NTS5′-GCSGS. The plasmids were transformed into *E. coli* DH5α for preservation of the plasmids. The plasmids were transformed into *S. cerevisiae* BY4717 by the lithium acetate method [2]. The transformants were screened on YPD plates supplemented with G418 (200 μg ml^{-1}) and designated as *S. cerevisiae* BY4717-GCSGS. The plasmid p416-MET25 containing Cre gene was further introduced into *S. cerevisiae* BY4717-GCSGS cells to knock out the kanMX4 genes. The GCSGS and KanMX4 genes were further amplified to determine whether GCSGS gene was introduced and KanMX4 gene was knocked out. The yeast transformants without KanMX4 gene were designated as *S. cerevisiae* BY-G. The expression of GCSGS was detected by Western blotting.

Characterization of engineered *S. cerevisiae* BY-G

The GSH yield of engineered *S. cerevisiae* BY-G in 100 ml YPD media were determined after 12, 24, 36, 48, 60, 72 h growth. The reference strain *S. cerevisiae* BY4717 was used as control. The effects of H_2O_2 (3 mM), growth temperatures (30, 35, 40, and 45 °C), furfural (0, 5, 10, 20 mM), HMF (0, 5, 10, 20 mM), and Cd^{2+} (0, 0.1, 0.5, 1.0 mM) were used to determine growth of *S. cerevisiae* BY-G and compared with biomass formation of the reference strain *S. cerevisiae* BY4717 under the same conditions. The biomass (cell concentration) was determined from absorbance measurements at 600 nm on samples diluted to give an optical density (OD) below 0.7 (Spectrophotometer WFZ UV-2000, Unico, Shanghai, China). The OD_{600} was measured at 12, 24, 36, 48, 60, 72, 84, and 96 h after inoculation to determine the duration of the lag phase and the specific growth rate for each condition.

Simultaneous saccharification and fermentation (SSF)

Corn stover was harvested in fall of 2012 from the Henan Province, China. The materials were milled by a beater pulverizer and screened through mesh (1 × 1 cm). Milled corn stover (500 g) was presoaked with diluted sulfuric acid solution (5.0 % sulfuric acid) with the solid to liquid ratio of 2:1 (wt/wt), the materials were further autoclaved at 121 °C for 10 min. The SSF was carried out at 150 g pretreated materials at 30 % (wt/wt) water insoluble solids content. In the prehydrolysis stage, Accellerase 1000 (Genencor International, Rochester, USA) was inoculated into the reactor at the dosage of 15.0 units of filter paper cellulase (FPU)/g dry matter (DM) within 12 h at 50 °C. The slurry was supplemented with yeast extract (1 %) and peptone (2 %). When the temperature decreased below 37 °C, the *S. cerevisiae* BY-G and BY4741 seeds were inoculated into the fermentation medium at the ratio of 10 % (v/v), respectively. The cultivation was performed at 35 °C and pH 5.0. The fermentation continued for 48 h, and samples were withdrawn regularly throughout the fermentation.

Analytical methods

Yeast biomass was measured as the OD_{600} after cultivation for the appropriate time. The intracellular GSH of *S. cerevisiae* BY-G were determined by the 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method [14] with reduced GSH as a standard. The samples were centrifuged at 14,000*g* for 2 min and filtered through 0.2 μM filter before analysis. The glucose, ethanol, furfural and 5-hydroxymethylfurfural (HMF) were analyzed using HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, USA) at the column temperature of 65 °C. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 ml/min.

Results

Construction of engineered *S. cerevisiae* BY-G

The ADH1 promoter (1,500 bp) and Cyc1 terminator (300 bp) were amplified from plasmid JMB125. The GCSGS gene (2,300 bp) was amplified from pETG-10A-GCSGS. The ADH1-GCSGS-CYC1 expression cassette (4.1 kb) was ligated by overlap-PCR and the sequence was determined by sequencing. The kanMX4 gene (1,500 bp) were introduced into vector pBlueScript II SK(+) by restriction enzyme *Eco*R I and *Bam*H I. The ADH1-GCSGS-CYC1 expression cassette was introduced into vector pBlue-NTS3′-kanMX4-NTS5′ by *Eco*R I and *Xho* I. The yeast transformants were screened from colonies on YEPD with 200 μg ml^{-1} G418. When the plasmid p416-MET25 with Cre gene was introduced into *S. cerevisiae* BY4717-GCSGS, the GCSGS of 2300 bp was amplified from yeast BY-G and the kanMX4 was not amplified from BY-G. The antibiotic-resistant gene kanMX4 was knocked out from engineered strains BY-G. Total proteins analysis by SDS-PAGE and Western blotting indicated that the glutathione synthetase of 89 KD was expressed in *S. cerevisiae* BY-G. The intracellular GSH content in BY-G is higher than that in reference strain BY4741 after incubation of 24 h. The intracellular GSH increased quickly prior to 48 h. The highest GSH content of 54.9 μmol/g dry weight (DW) was reached at 60 h. Under the same conditions, the intracellular GSH content of BY4741 is 11.7 μmol/g DW (Fig. 1). A threefold higher GSH contents was obtained by introducing bifunctional glutathione synthetase gene GCSGS into *S. cerevisiae* 4741.

Tolerance of BY-G to stress conditions

The biomass of BY-G is similar to that of BY4741 in YEPD medium without H_2O_2. The growth of BY4741 was repressed by 3 mM H_2O_2, however, the engineered BY-G showed more tolerance to 3 mM H_2O_2. The growth rate and biomass of BY4741 is similar in YEPD with and without H_2O_2 (Fig. 2). At an incubation temperature below 35 °C, there was no influence on the growth of BY4741 and BY-G. At a temperature of 40 °C, the growth of BY4741 was retarded, however, BY-G was more tolerant and could grow at 40 °C. The growth rate of BY-G at 40 °C up to 12 h is similar to that of BY4741 at 35 °C (Fig. 3). Furfural inhibited the growth of BY-G and BY4741 at a concentration of 5 mM. When the furfural concentration was increased to 10 mM, the growth rates and biomass of BY-G is similar to those of BY4741 at the concentration of

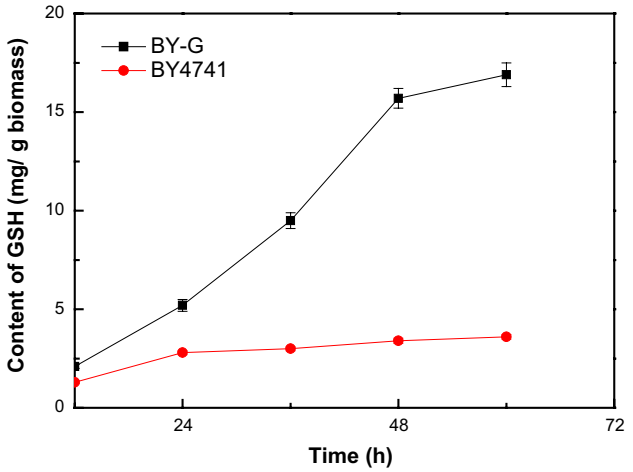

Fig. 1 GSH contents of the engineered strain BY-G and the reference strain BY4741 at different incubation times

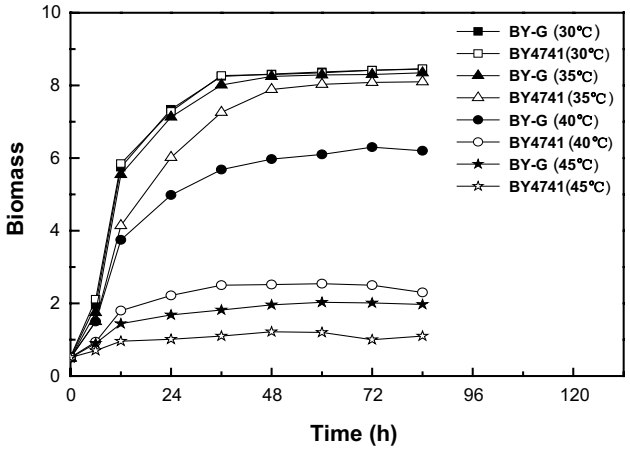

Fig. 3 Effects of different temperature (30, 35, 40, 45 °C) on the growth of the engineered strain BY-G and the reference strain BY4741

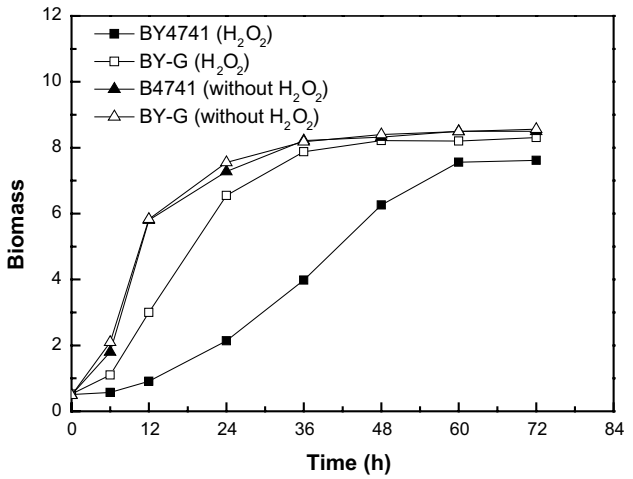

Fig. 2 Effects of 3 mM H_2O_2 on the growth of the engineered strain BY-G and the reference strain BY4741

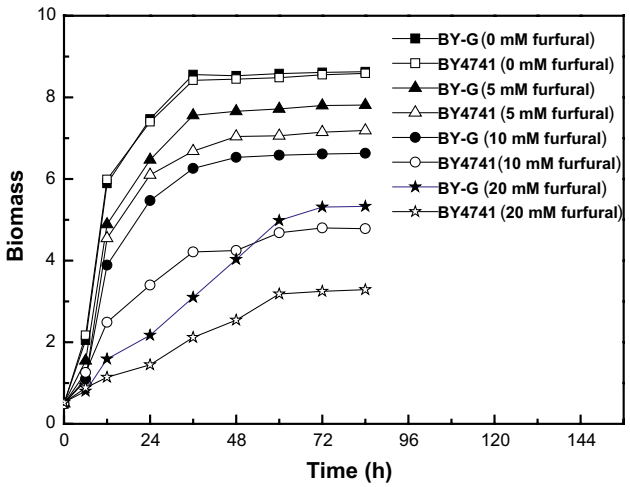

Fig. 4 Effects of furfural (5, 10, 20 mM) on the growth of the engineered strain BY-G and the reference strain BY4741

5 mM. At 20 mM of furfural, the growth rate and biomass of BY-G was similar to those of BY4741 at 10 mM furfural (Fig. 4). Similar growth parameters were observed at 10 and 20 mM HMF (Fig. 5) The tolerance of reference strain BY4741 to furfural and HMF was increased from 5 to 10 mM by introducing bifunctional glutathione synthetase genes. Similarly, the tolerance of the reference strain to Cd was increased from 0.1 to 0.5 mM when higher intracellular glutathione was synthesized (Fig. 6).

Evaluation of engineered strains in simultaneous saccharification and fermentation

The initial concentration of glucose, furfural, and HMF was 48.8 g/L, 8.3, and 9.5 mM, respectively, in the liquid fraction of pretreated corn stover. The time course for accumulation of ethanol during the SSF is shown in Fig. 7. Engineered strains BY-G and reference strain BY4741 started to consume glucose and produce ethanol after 1 h of incubation. All glucose were not metabolized at 18 h for the reference strain BY4741, whereas, the engineered strain BY-G sustained to metabolize quickly glucose to produce ethanol at 18 h. The ethanol production rate of strain BY-G reached 0.89 g/L/h at 12 h and decreased to 0.73 g/L/h at 24 h. After 30 h, the ethanol concentration of 20.6 g/L was reached. The ethanol yield is 0.44 g/g glucose. At 30 h, the remaining glucose concentration was 2.4 and 25.2 g/L for engineered strain BY-G and reference strain BY4741, respectively. The remaining furfural and HMF concentration for engineered strain BY-G decreased

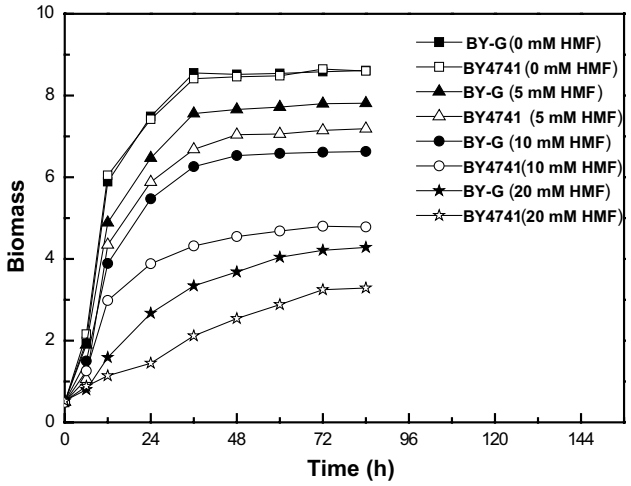

Fig. 5 Effects of HMF (5, 10, 20 mM) on the growth of the engineered strain BY-G and the reference strain BY4741

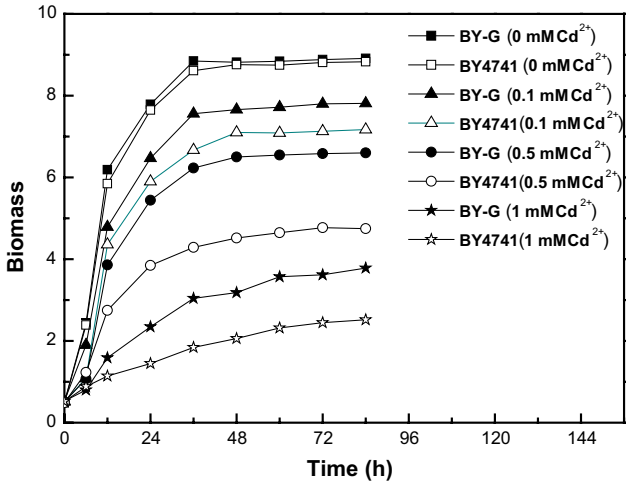

Fig. 6 Effects of Cd^{2+} (0.1, 0.5, 1.0 mM) on the growth of the engineered strain BY-G and the reference strain BY4741

to 0.1 and 0.7 g/L, respectively. The remaining furfural and HMF concentration for reference strain BY4741 is 0.6 and 0.9 g/L, respectively. The results indicate that the engineered strain BY-G could detoxify furfural and HMF and efficiently metabolized glucose from nondetoxified hydrolysates of corn stovers in simultaneous saccharification and fermentation process.

Discussion

Pretreatment is a requisite step in cellulosic ethanol production, however, various lignocellulose derived toxins including furan derivatives are generated during pretreatment processing. Various detoxification methods may result in high

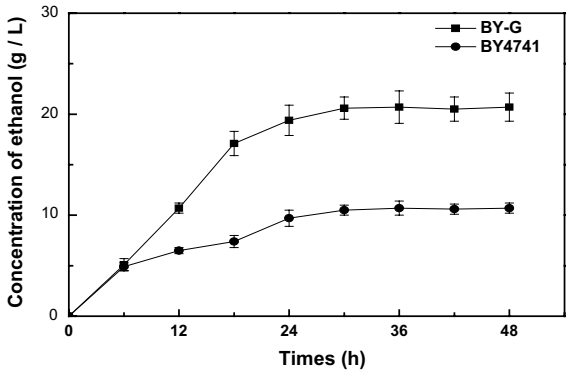

Fig. 7 Ethanol yields produced by the engineered strain BY-G and the reference strain BY4741 during simultaneous saccharification and fermentation (SSF) of corn stovers at 35 °C with 30 % substrate concentration after acid and heat pretreatment

energy cost, massive freshwater usage and wastewater generation, loss of the fine lignocellulose particles and fermentative sugars, and incomplete removal of inhibitors [18]. *S. cerevisiae* has proved to be more robust than bacteria both with respect to the tolerance to ethanol and toxins present in hydrolysates [1], the development of novel yeast strains with increased inhibitor tolerance is highly desirable [4].

The ability of yeasts to adapt to and transform furfural and HMF offers the potential for in situ detoxification and development of highly tolerant strains for ethanol production [8]. The detoxification of furfural and HMF by *S. cerevisiae* involves genes mediated NAD(P)H-dependent aldehyde reduction [9]. Glutathione is the main antioxidant system in living cells. The *S. cerevisiae* strains engineered for higher intracellular glutathione levels have superior robustness under process-like conditions in a simultaneous saccharification and fermentation process [2]. However, the yeast glutathione synthetase gene GSH1 is inhibited by excess glutathione, the GSH1 cloned into the integrative plasmid YIplac211 could not accumulate excess glutathione. The intracellular GSH contents of the engineered strains was below 12.2 μmol/g DW, 40 % of concentration increased compared to reference strain [2]. In this study, the bifunctional glutathione synthetase gene GCSGS derived from *Streptococcus thermophilus* was integrated at a high copy number into the ribosomal DNA of *S. cerevisiae*. In the final step, the antibiotic-resistant gene kanMX4 was removed using the Cre–LoxP system. The bifunctional enzyme is neither redox-regulated nor sensitive to feedback inhibition by GSH [7]. A strain with a high yield of GSH (54.9 μmol/g DW) was constructed. Compared with expression of the GSH1 gene by plasmid YIplac211 in *S. cerevisiae*, the engineered strain in this study can accumulate higher contents of GSH and showed more tolerance to inhibitors in simultaneous saccharification and fermentation process (SSF).

The tolerance of the engineered strain to 3 mM H_2O_2, 40 °C,10 mM furfural, HMF and 0.5 mM Cd^{2+} is much better compared to the reference strain as a result of introducing the bifunctional glutathione synthetase genes. The current compromise between the optimal fermentation (30–35 °C) and saccharification temperatures (55 °C) significantly limits the rate of enzymatic hydrolysis. Use of more thermotolerant strains would enable a reduction of operational costs working at higher temperatures, which is valid especially in tropical countries [16]. Yeasts with higher thermotolerance are needed in a simultaneous saccharification and fermentation process. An engineered strain with increased thermotolerance and tolerance to HMF and furfural would result in a more efficient production of ethanol via simultaneous saccharification and fermentation as well as reduced cooling and detoxification costs. Furthermore, high yield of GSH can be extracted from yeast biomass after the ethanol fermentation process finished. GSH is widely used as a pharmaceutical compound and has the potential to be used in food additives and in the cosmetic industries [6]. Low-price GSH can be obtained from the ethanol fermentation, so the economic profit of ethanol fermentation could be improved.

Conclusions

In this study, the robustness of *S. cerevisiae* BY-G was engineered by integrating bifunctional glutathione synthetase genes (GCSGS) into ribosomal DNA of *S. cerevisiae* by the Cre-LoxP systems. The tolerance of BY-G to H_2O_2, high temperature (40 °C), furfural, HMF and Cd^{2+} improved compared to the reference strain. The engineered strain could accumulate threefold higher GSH contents and twofold higher ethanol concentration. The strategy could be used to construct *S. cerevisiae* strains for simultaneous saccharification and fermentation of cellulosic mass to produce ethanol.

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