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Rational synthetic combination genetic devices boosting high temperature ethanol fermentation

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ABSTRACT

The growth and production of yeast in the industrial fermentation are seriously restrained by heat stress and exacerbated by heat induced oxidative stress. In this study, a novel synthetic biology approach was developed to globally boost the viability and production ability of *S. cerevisiae* at high temperature through rationally designing and combing heat shock protein (HSP) and superoxide dismutase (SOD) genetic devices to ultimately synergistically alleviate both heat stress and oxidative stress. HSP and SOD from extremophiles were constructed to be different genetic devices and they were preliminary screened by heat resistant experiments and anti-oxidative experiments, respectively. Then in order to customize and further improve thermotolerance of *S. cerevisiae*, the HSP genetic device and SOD genetic device were rationally combined. The results show the simply assemble of the same function genetic devices to solve heat stress or oxidative stress could not enhance the thermotolerance considerably. Only *S. cerevisiae* with the combination genetic device (FBA1p-*sod-MB4*-FBA1p-*shsp-HB8*) solving both stress showed 250% better thermotolerance than the control and displayed further 55% enhanced cell density compared with the strains with single FBA1p-*sod-MB4* or FBA1p-*shsp-HB8* at 42 °C. Then the most excellent combination genetic device was introduced into lab *S. cerevisiae* and industrial *S. cerevisiae* for ethanol fermentation. The ethanol yields of the two strains were increased by 20.6% and 26.3% compared with the control under high temperature, respectively. These results indicate synergistically defensing both heat stress and oxidative stress is absolutely necessary to enhance the thermotolerance and production of *S. cerevisiae*.

1. Introduction

Microbial fermentation of biomass-derived feedstocks represents an attractive solution for production of clean, affordable and reliable energy [1]. About 100 billion liters of ethanol are transformed annually from mainly sugarcane saccharose and corn starch by *Saccharomyces cerevisiae* [2,3], which is also a growing interest for production of biofuels. However, raw materials of the biofuels are often pretreated through saccharifcation and simultaneous fermentation (SSF) [4,5]. The activity of enzyme in hydrolysis of starch or biomass is highest around 50 °C [4]. Therefore, the production of ethanol or advanced biofuels benefits greatly from fermentations at high temperature (≥40 °C) [6]. High temperature fermentation may reduce cooling costs, help to prevent contamination [4] and enable more efficient hydrolysis of the feedstock, thus improve productivities in simultaneous saccharification and fermentation. Unfortunately, the growth and production of the strains in the fermentation are often hampered by heat stress (≥34 °C for yeast), which may cause cell morphological abnormalities, inhibit cell division and growth, destroy cytoskeletal integrity, and impact metabolic activity [7,8].

However, not all effects are directly caused by heat stress but by synergism of the heat stress and heat caused stresses, like the

Abbreviations: HSP, heat shock protein; SOD, superoxide dismutase; SSF, saccharifcation and simultaneous fermentation; ROS, reactive oxygen species; OE-PCR, overlap extension PCR; IS, industrial *S. cerevisiae*; PBS, phosphate buffered saline; DCFH-DA, 2′,7′-dichlorofluorescin diacetate.

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oxidative stress. The heat-damaged mitochondrial electron transport system will produce the main toxic by-product intracellular reactive oxygen species (ROS), which consequently generates the oxidative stress [9–11]. Oxidative stress can damage a wide variety of cellular components resulting in lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA [12]. Therefore, the optimally thermotolerant phenotype could not arise unless solving the heat shock generating ROS.

In nature, in order to adapt, proliferate, or survive such adverse conditions, cells have developed lots of fairly sophisticated mechanisms [13]. Most of all, many kinds of heat shock protein (HSP) genes are activated by heat stress to prevent denatured proteins from aggregating, or promote their refolding. Indeed, heat stress can also induce a number of antioxidant genes expression to solve the oxidative stress problems [14,15], such as superoxide dismutase (SOD), catalase and glutathione peroxidase [16]. However, the high level induced expression of these protective genes was temporary [17] and the endogenous protective proteins of yeast may be not so thermostable to protect yeast from the long-time lethal high temperature. Therefore, the constitutive and continuous expression of more thermo-stable HSPs and antioxidants could be needed for rescuing cells from longtime heat stress. There is already a report proving that the constitutive expression of heat shock proteins from *T. tengcongensis* MB4 that could efficiently improve thermotolerance of *S. cerevisiae* [18].

As previously reported, several methods such as physical and chemical mutagenesis, adaptation evolution, protoplast fusion and genetic engineering strategies have been used to improve the thermotolerance of *S. cerevisiae* [19,20]. Although thermotolerant *S. cerevisiae* has been applied in industrial fermentation [21], such strains still cannot meet the industrial requirement of direct application in consolidated bioprocessing and simultaneous saccharification and fermentation. During the long-term natural evolution, extremophiles show superior robustness under harsh conditions owing to their well-adapted stress response genes [22,23]. Recently, identification and introduction of such genes from extremophiles has been proven to be an effective approach for engineering cellular robustness of microbes [7,24–26]. The thermotolerance of *Escherichia coli* is significantly enhanced by overexpressing the GroESL from the *Thermoanaerobacter tengcongensis* [27]. In another study, thermotolerance could be gained through constructing heat-resistant genetic devices in *S. cerevisiae* [18]. The above single heat-resistant gene products are only used to solve the heat stress problems; however, the effect coming from the heat induced oxidative stress is ignored. Meanwhile, using simple or single strategy to improve the thermo-robustness of the complicated biological system, like overexpression of heat shock protein genes, is not sufficient and unrealistic [28,7]. To improve the thermotolerance of microbes, it is not alleviating the symptoms, but effecting a permanent cure. The thermo-stable HSPs and antioxidants should synergistically protect cells from heat stress and heat induced stress, which could efficiently and globally enhance thermotolerance of cells.

Here, we developed a synthetic biology approach to globally boost the viability and production ability of *S. cerevisiae* at high temperature through rationally combing different functional genetic devices to alleviate both heat stress and oxidative stress (Fig. 1). A list of genes involved in defensing heat stress and oxidative stress were mined from sequenced thermophiles' genomes as functional parts and the highly homologous candidates were chosen and constructed as two types of genetic devices, HSP genetic devices and SOD genetic devices. In order to obtain the excellent candidates, the HSP genetic devices and SOD genetic devices were preliminary screened by heat resistant experiments and anti-oxidative experiments, respectively. Then the most excellent HSP genetic device and SOD genetic device were rationally combined to customize and further improve thermotolerance of *S. cerevisiae*. Furthermore, the engineered *S. cerevisiae* with HSP and SOD combination genetic devices were applied to high temperature ethanol fermentation to enhance the ethanol producing ability.

2. Materials and methods

2.1. Strains, vectors, media, and reagents

The strains of *Thermoanaerobacter tengcongensis* MB4 (provided by Dr. Ma, Institute of microbiology Chinese academy of sciences), *Thermus thermophiles* HB8 (China Center of Industrial Culture Collection) *S. cerevisiae* INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52/MATa his3D1 leu2 trp1-289 ura3-52*) (Invitrogen, Carlsbad, CA) and *Escherichia coli* Top10 (Novagen, USA) were genetically manipulated in this study. LB medium (NaCl 10 g/L, yeast extract 5 g/L, tryptone 10 g/L) with 100 mg/L Kanamycin and YPD medium (glucose 20 g/L, tryptone 20 g/L, yeast extract 10 g/L) with 300 mg/L G418 (Invitrogen, Carlsbad, CA) were used to select *E. coli* and *S. cerevisiae* transformants respectively. Plasmid pRS42K was purchased from EUROSCARF, Frankfurt, Germany. Restriction enzymes and DNA polymerase were obtained from Fermentas (Burlington, ON). The primers were synthesized by Sangon Biotech (Shanghai, China).

2.2. Construction of the engineered lab S. cerevisiae and industrial S. cerevisiae

Firstly, the engineered lab *S. cerevisiae* with single HSP genetic devices and SOD genetic devices were constructed. The genes, *shsp-HB8, hsp20-HB8, hsp33-HB8, groes-HB8, groel-HB8, dnak-HB8, dnaj-HB8, grpe-HB8* and *sod-HB8* were cloned from the genome of *T. thermophiles* HB8 while *sod-MB4* was cloned from *T. tengcongensis* MB4 (Primers are listed in Supplementary Table S4). The above genes were assembled with the constitutive promoter FBA1p and terminator SLM5t from the genome of *S. cerevisiae* INVSc1 via overlap extension PCR (OE-PCR). After double-digested with *Eco*R I and *Bam*H I, the OE-PCR products were ligated into linearized pRS42K digested with the same restriction endonuclease, and then transformed into *E. coli* Top10. All of the constructions in this study are listed in Supplementary Table S3. Finally, the recombinant plasmids were extracted and electrotransformed into lab *S. cerevisiae* INVSc1. The positive clones were selected on YPD medium containing 300 mg/L G418 and confirmed via colony PCR.

Then the engineered lab *S. cerevisiae* with rational combination genetic devices was constructed using the DNA assembler [29]. To prepare individual gene expression cassettes, promoter FBA1p and terminator (SLM5t and FBA1t) were cloned from the genome of *S. cerevisiae*. Genes, *sod-HB8*, *shsp-HB8* and *groes-HB8*, were PCR-amplified from the genome of *T. thermophiles*. *sod-MB4* and *gros$_2$-MB4* genes were cloned from genome of *T. tengcongensis* MB4 (Primers are listed in Supplementary Table S5). Each individual gene expression cassettes, FBA1p-*sod-MB4*-SLM5t, FBA1p-*shsp-HB8*-FBA1t, FBA1p-*gros$_2$-MB4*-FBA1t, FBA1p-*sod-HB8*-FBA1t, FBA1p-*groes-HB8*-SLM5t was assembled by OE-PCR. Following electrophoresis, the OE-PCR product was individually gel-purified from a 0.7% agarose gel. Gene cassettes (300 ng) was mixed with the linearized pRS42 K (500 ng) and precipitated with ethanol. The resulting DNA pellet was air-dried and resuspended in 5 μL Milli-Q double deionized water for transformation into the lab *S. cerevisiae*. Meanwhile, the engineered industrial *S. cerevisiae* (IS) was constructed using the same above method. All the strains and genetic devices are listed in Table 1.

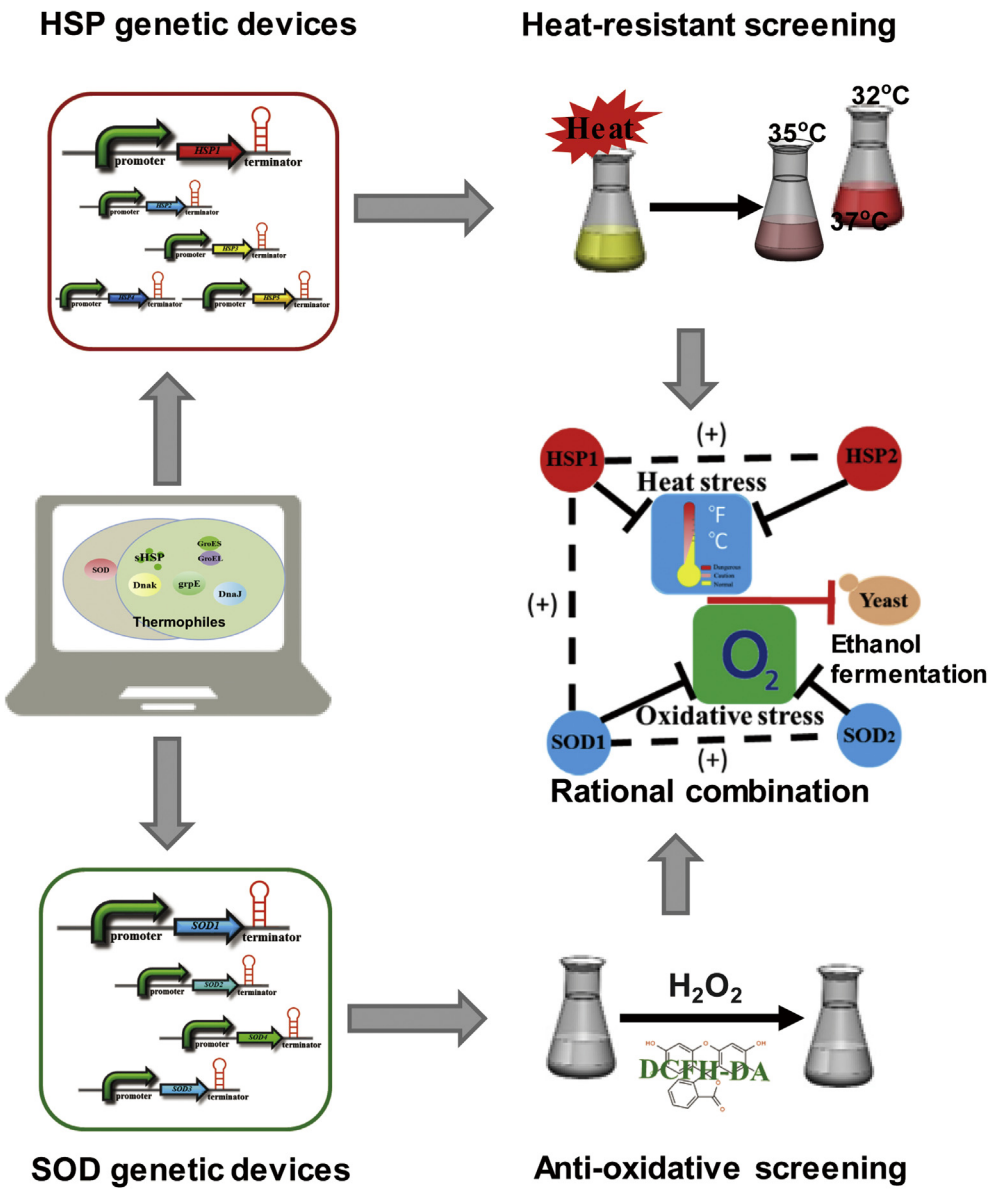

Fig. 1. Schematic illustration of rationally genetic devices combination for boosting high temperature ethanol fermentation.

Table 1
Cell growth of the engineered *S. cerevisiae* under gradually enhanced temperature.

Strains	OD_{660}		
	24 h	48 h	72 h
Control-30 °C	8.6 ± 0.42	10.7 ± 0.61	12.7 ± 0.52
Control- (35–43 °C)	5.79 ± 0.86	8.0 ± 0.78	9.4 ± 0.83
sHSP-*HB8*	6.93 ± 0.58	10.2 ± 0.53	12.8 ± 0.65
HSP20-*HB8*	5.61 ± 0.46	6.4 ± 0.32	8.04 ± 0.56
hslO-*HB8*	6.75 ± 0.43	7.9 ± 0.39	10.9 ± 0.65
GroES-*HB8*	7.32 ± 0.62	9.75 ± 0.38	11.70 ± 0.53
GroEL-*HB8*	7.2 ± 0.83	8.4 ± 0.83	9.4 ± 0.82
DnaJ-*HB8*	5.7 ± 0.21	7.8 ± 0.54	9.0 ± 0.87
DnaK-*HB8*	6.25 ± 0.39	8.0 ± 0.61	9.6 ± 0.45
GrpE-*HB8*	7.6 ± 0.67	8.6 ± 0.64	12.4 ± 0.54

2.3. Cell concentration and cell viability analysis of lab *S. cerevisiae*

The culture of seed broth was the same to the method 2.3. The cultures were then diluted to OD_{660} of 0.1 in 250 mL-Erlenmeyer flasks containing 100 mL fresh YPD medium with 300 mg/L G418 and incubated at 30 °C for initial 12 h. For heat resistant experiments, two different sets of fermentation conditions were used: (1) the fermentation temperature was increased to 35 °C for 12 h, and then gradually increased by 2 °C every 12 h, increased up to 43 °C for 72 h. During this process, cell samples were taken at 12 h intervals to measure OD_{660}. Cell concentration of the sample was determined at OD_{660} by using a spectrophotometer, model U-2900 (HITACHI, Chiyoda, Tokyo). (2) The fermentation temperature was directly increased to 37 °C and 42 °C for 72 h and the cell samples were taken at 12 h intervals to measure OD_{660}. Meanwhile, samples which were taken at 60 h of 37 °C and 42 °C were serially diluted and spread onto YPD plates with 300 mg/L G418, and colonies were counted after 3 days of incubation at 30 °C. Viable cell counts (CFU/mL) was used as a unit to represent cell viability. A serial dilution assay was investigated through taking samples at 60 h of 37 °C and 42 °C to serially dilute 10-fold and 2.5 μL of the dilutions were then spotted onto YPD-G418 plates. Thermotolerant ability of the engineered lab *S. cerevisiae* was characterized by cell concentration, cell

viability and serial dilution assay. All data represent the mean standard deviation from three independent experiments.

2.4. Fluorimetric measurement of ROS production of the heat shocked lab S. cerevisiae

The culture of seed broth was the same to the method 2.3. The cultures were then diluted to OD_{660} of 0.1 in 100 mL-Erlenmeyer flasks containing 40 mL fresh YPD medium with 300 mg/L G418 and incubated at 30 °C. After cultured for 12 h, the cells were washed and diluted using pH 7.4 phosphate buffered saline (PBS) to the cell concentration of 10^7 cells/mL. Then samples were treated with 1 μL/mL of 2 mM stock solution of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) dissolved in DMSO for 15 min at 30 °C, in order to allow entry of the probe into the cells. Then cells were washed three times using PBS. After that, the positive control was treated with 2.5 μL/mL of 12 mM stock solution of H_2O_2, while other samples were heat shocked at 42 °C for 30 min. And cells were finally harvested and the fluorescence intensity was tested via flow cytometry with the excitation wavelength of 488 nm and the emission wavelength of 525 nm (CyAn ADP, BD, USA). As a control, background fluorescence of heat shocked cells not treated with DCFH-DA was also recorded. The relative fluorescence intensity of *S. cerecisiae* was represented as a ratio to the positive control [30]. All experiments were performed in triplicate, and differences between the means were considered significant at $p < 0.05$.

2.5. Survival against oxidative stress of lab S. cerevisiae

The culture of seed broth was the same to the method 2.3. The cultures were then diluted to OD_{660} of 0.2 in fresh 40 mL YPD medium containing 300 mg/L G418 and added with H_2O_2 to the final concentration of 2 mM. Cells were then incubated for 1 h at 30 °C, 170 rpm. Cells were taken every 15 min and serially diluted 10-fold and spread on the YPD plates to monitor the cell viability. After incubation of 48 h at 30 °C, the number of colonies were counted to measure the survival rate and presented as a ratio to that of untreated cells at 0 min. All experiments were performed in triplicate, and differences between the means were considered significant at $p < 0.05$.

2.6. High temperature ethanol fermentation of lab S. cerevisiae

The culture of seed broth was the same to the method 2.3. The cultures were then diluted to OD_{660} of 0.1 in 250 mL-Erlenmeyer flasks containing 100 mL fresh YPD medium with 300 mg/L G418 and 40 g/L glucose. After incubated at 30 °C for initial 12 h, the control was cultured at 30 °C and the engineered lab *S. cerevisiae* were fermented at 40 °C up to 60 h under anaerobic state and the cell samples were taken at 12 h intervals to measure OD_{660} and ethanol concentration. The method of determining OD_{660} was same with the method 2.4. Fermentation broth was then centrifuged for 1 min. Supernatant was diluted and the concentration of ethanol was measured using SBA-40E biosensor (Institute of Biology, Shandong Academy of Sciences, China). All experiments were performed in triplicate, and differences between the means were considered significant at $p < 0.05$.

2.7. High temperature ethanol fermentation of industrial S. cerevisiae

The HSP genetic device, FBA1-*shsp-HB8*, the SOD genetic device, FBA1-*sod-MB4* and the HSP and SOD combination genetic device (FBA1p-*sod-MB4*-FBA1p-*shsp-HB8*) were transformed into the industrial *S. cerevisiae* (IS) which could transform xylose to ethanol. These engineered strains were named IS-sHSP-*HB8*, IS-SOD-*MB4* and IS-SOD-*MB4*-sHSP-*HB8*, respectively. The culture of seed broth was the same to the method 2.3, while the incubation time was reduced to 14 h. The cultures were then diluted to OD_{660} of 0.1 in 250 mL-Erlenmeyer flasks containing fresh 100 mL YPD medium with 300 mg/L G418, 75 g/L glucose and 35 g/L xylose. After incubated at 30 °C for initial 12 h, the control was cultured at 30 °C and the engineered strains were fermented at 35 °C up to 60 h and the cell samples were taken every 12 h to measure OD_{660}, ethanol concentration and xylose concentration. The measurement of OD_{660} and ethanol was same to the method 2.4 and method 2.7, respectively. Meanwhile, the xylose concentration was determined by HPLC system, model Shimadzu SCL-10A (Kyoto, Tokyo, Japan) with a RID-10A detector, a Bio-Rad Aminex HPX-87H (300 mm × 7.8 mm) column. A 5 mM H_2SO_4 solution was used as mobile phase at a flow rate of 0.6 mL/min and the analysis was carried out at 65 °C. All experiments were performed in triplicate, and differences between the means were considered significant at $p < 0.05$.

3. Results and discussion

Thermophiles exhibit excellent thermotolerance, offering us considerably rich gene resources which may help us improving stress resistance of industrial mesophilic microbes. In order to equip the host with the bio-compatibly heat-resistant devices, a number of heat-resistant genes and anti-oxidative genes, *hsp* and *sod*, were mined from different kinds of sequenced thermophiles' genomes. The genes are ubiquitous in various microorganisms and involved in different stress conditions. Especially, molecular chaperones that help proteins folding or refolding were mainly considered, like the GroeSL system (GroeS, GroeL), DnaKJE system (DnaK, DnaJ and GrpE) and small heat shock proteins family. In order to deal with the heat induced oxidative stress, the SOD proteins from all the thermophiles were also taken as candidates. Then the homologies of HSPs and SODs, between thermophiles and *S. cerevisiae* were compared (Supplementary Table S1). Because the SOD genes from *Thermoplasma acidophilum* and *Methanothermobacter thermautotrophicus* were difficult to obtain. Although they have a little higher homology than that from *Thermoanaerobacter tengcongensis* MB4, we had to remove the two genes from the candidates list. Finally, ten high homologous genes (eight *hsp* and two *sod*) from *T. tengcongensis* and *T. thermophilus* were chosen as candidates to improve high temperature survivability of yeast and they were listed in Supplementary Table S2.

3.1. Enhancing the thermotolerance of lab S. cerevisiae by HSP genetic devices

T. thermophiles HB8 as an extremely thermophilic microbe, with an optimal growth temperature of about 65 °C [31], maintains much more excellent thermotolerant system than yeast. The genome is sequenced and well characterized. Meanwhile, heat shock proteins from *T. thermophilus* have a higher homology with *S. cerevisiae* than other thermophiles. Therefore, *shsp*-HB8, *hsp20*-HB8, *hsp33*-HB8, *groes*-HB8, *groel*-HB8, *dnak*-HB8, *dnaj*-HB8 and *grpe*-HB8 were cloned from the genome of *T. thermophiles* HB8. Then they were assembled with a strong constitutive promoter FBA1p and terminator SLM5t (cloned from the genome of *S. cerevisiae* INVSc1) (Fig. 2a). Therefore, the genes controlled with the constitutive promoter can be continuous expressed at high temperature, which can be the back of the temporary endogenous thermotolerant system and help cell resist heat stress for long period. These devices were constructed into the pRS42K plasmid and then transformed into yeast.

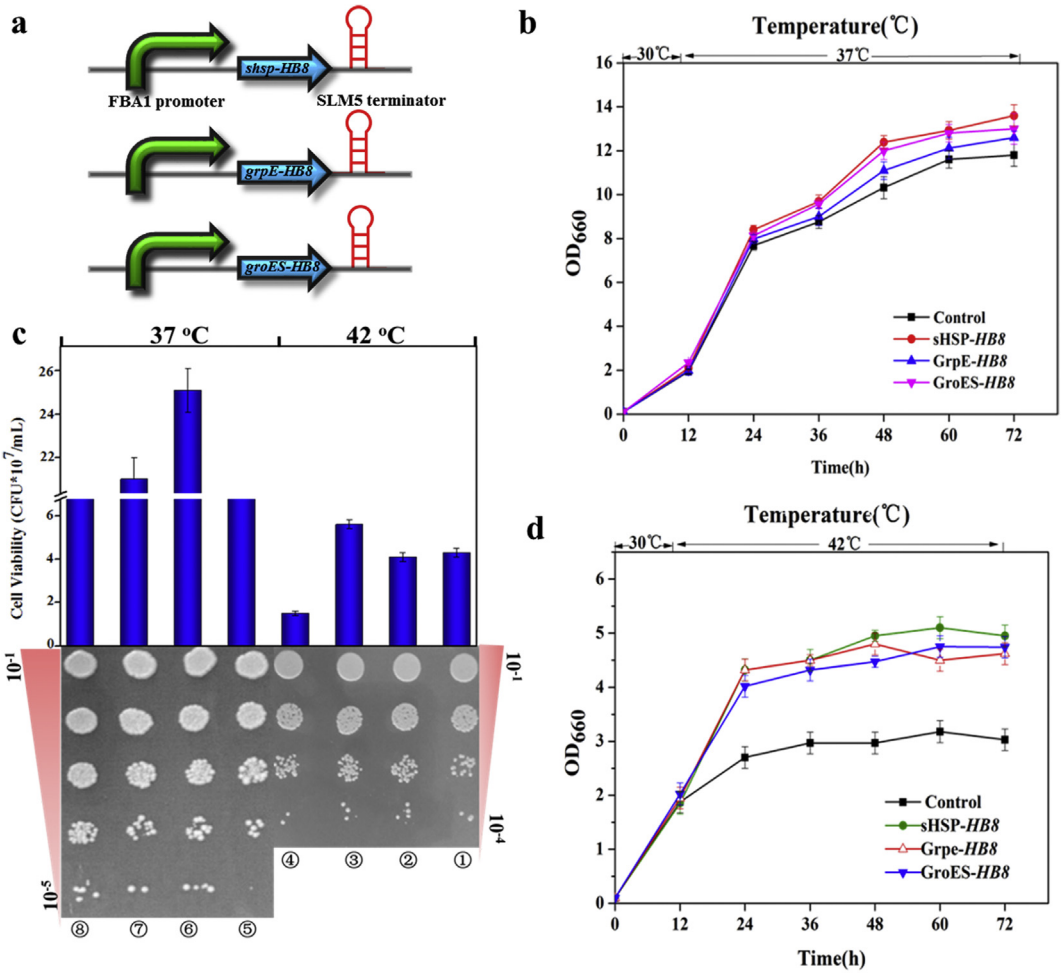

Fig. 2. Design and functional verification of HSP genetic devices. (a) HSP genetic devices; The cell growth of lab *S. cerevisiae* at 37 °C (b) and 42 °C (d); (c) The cell viability of the lab *S. cerevisiae* at 37 °C and 42 °C measured by spread plate and plot plate. (①GroES-*HB8*-42 °C ②GrpE-*HB8*-42 °C ③sHSP-*HB8*-42 °C ④Control-42 °C ⑤Control-37 °C ⑥sHSP-*HB8*-37 °C ⑦GrpE-*HB8*-37 °C ⑧GroES-*HB8*-37 °C).

Then the HSP genetic devices were preliminarily screened through gradually enhanced temperature fermentation. As shown in Table 1, the engineered lab *S. cerevisiae* displayed better thermotolerance than the control except HSP20-*HB8* and DnaJ-*HB8*. Especially, the cell densities of sHSP-*HB8*, GroES-*HB8* and Grpe-*HB8* were increased by 36.9%, 24.5% and 31.9% compared with the control at 72 h, respectively. Moreover, sHSP-*HB8*, GroES-*HB8* and GrpE-*HB8* cultured at gradually enhanced temperature could grow even nearly as well as the control cultured at 30 °C after 48 h. The possible reason is that Grpe, GroES and sHSP are much smaller than the others and easier to fold at high temperature, which could assist other HSPs to refold denatured proteins and finally rescue heat shocked cells [32–34]. These results show that the HSP genetic devices, FBA1p-*shsp-HB8*, FBA1p-*groes-HB8* and FBA1p-*grpe-HB8*, could endow lab *S. cerevisiae* with excellent thermotolerance and they were chosen as the candidates for the further experiments.

The three excellent strains (sHSP-*HB8*, GroES-*HB8* and Grpe-*HB8*) were further investigated at constant high temperature 37 °C (the heat shock temperature) [35] and 42 °C (the heat lethal temperature) [36] to confirm whether the engineered strains had truly acquired thermotolerance through introducing HSP genetic devices. Observed from Fig. 2b and 2d, all the screened engineered lab *S. cerevisiae* showed better cell growth than the control under constant high temperature fermentation, especially sHSP-*HB8* displayed the excellent thermotolerance. The cell density of sHSP-*HB8* at 72 h was increased by 15.2% more than the control at 37 °C. Although the growth of all the strains performed a little worse at 42 °C than that at 37 °C, the cell density was enhanced by 63.3% compared with the control at 42 °C. Additionally, the OD_{660} of control at 42 °C was decreased by 74.4% compared to that at 37 °C, while sHSP-*HB8* was decreased by 63.6%. All the comparisons indicate that the HSP genetic device FBA1p-*shsp-HB8* could protect cells from heat damage.

The cell viability was also measured to authentically reflect the thermotolerance of the engineered lab *S. cerevisiae*. The cell viability of all engineered strains was higher than the control at both 37 °C and 42 °C. Moreover, the cell viability of sHSP-*HB8* exhibited 1.6-fold and 3-fold higher than the control at 37 °C and 42 °C, respectively (Fig. 2c). Meanwhile, there was no colony formation of the control on the YPD medium plate after serial diluting at 37 °C, while many colonies of sHSP-*HB8* were observed (Fig. 2c). The cell viability of sHSP-*HB8* is in good accordance with the above cell growth curve.

In short, the above gradually enhanced temperature fermentation and constant high temperature fermentation results all show that HSP genetic devices especially FBA1p-*shsp-HB8* could enhance the thermotolerance of yeasts and could be applied to the variously high temperature fermentation.

3.2. Intensifying the anti-oxidative ability of the lab S. cerevisiae by SOD genetic devices

As mentioned in the introduction, heat stress could lead to accumulate intracellular ROS, and yeasts need SOD at high temperature to defense the heat induced oxidative stress by clearing extra ROS [37]. Therefore, in this research SOD genetic devices were constructed to protect lab *S. cerevisiae* from ROS damage. The intracellular ROS levels of the control and the engineered lab *S. cerevisiae* with SOD genetic devices were verified at 42 °C. As shown in Fig. 3b, the ROS levels of SOD-*HB8* and SOD-*MB4* were significantly lower than the positive control and heat shocked control. especially, the ROS level of SOD-*MB4* was decreased by 64.1% compared with that of the control at 42 °C, representing the SOD genetic device, FBA1p-*sod-MB4* could efficiently scavenge intracellular ROS ignited by high temperature.

Then, to directly investigate the role of SOD on the protection against the oxidative stress, the cell survivability of the engineered lab *S. cerevisiae* was studied under the artificial oxidative stress. The engineered lab *S. cerevisiae* and the control were exposed to H_2O_2 with the final concentration of 2 mM. The cell viability of both SOD-*HB8* and SOD-*MB4* was higher than the control. Moreover, SOD-*MB4* displayed 1.2-fold higher cell viability than SOD-*HB8* after exposure with H_2O_2 for 1 h, indicating that the yeast implanted with FBA1p-*sod-MB4* could be efficiently protected from oxidative stress (Fig. 3c). The engineered lab *S. cerevisiae* containing SOD

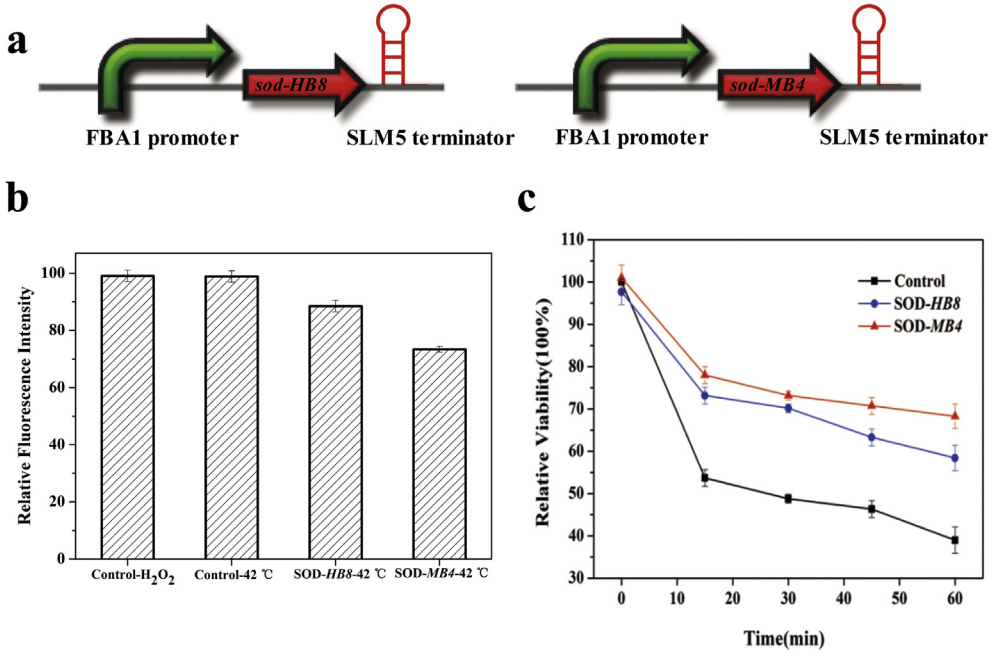

Fig. 3. SOD genetic devices design(a) and the anti-oxidative ability testing of the engineering strains. (b)The intracellular ROS levels of the engineered lab *S. cerevisiae* and the control after heat treating at 42 °C. (c)Cell viability of the engineered lab *S. cerevisiae* and the control with additional H_2O_2 (The colonies number were counted and presented as a ratio to that of untreated cells).

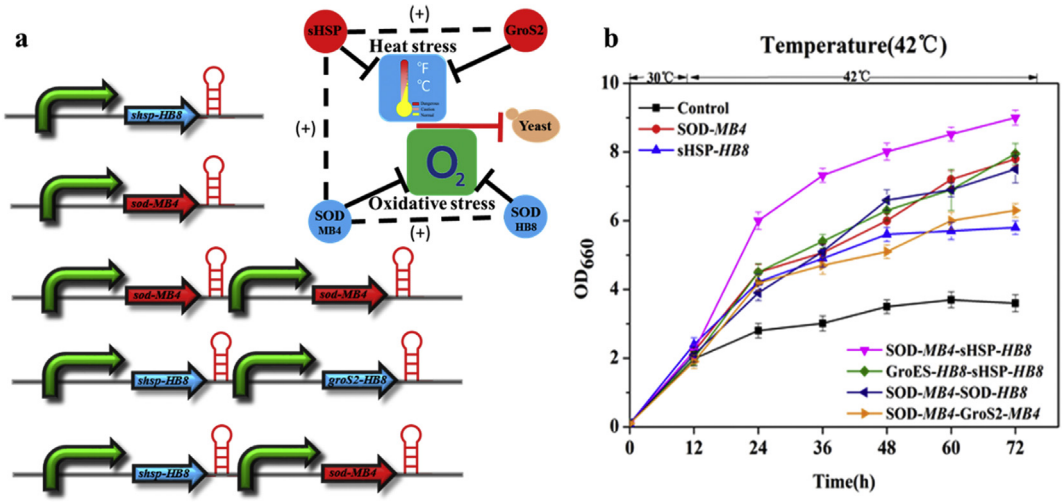

Fig. 4. (a) Rational combination of genetic device and the growth verification. (b)Cell growth curve of the control, the engineered lab *S. cerevisiae* with single genetic device and the engineered lab *S. cerevisiae* with the combination genetic devices at 42 °C.

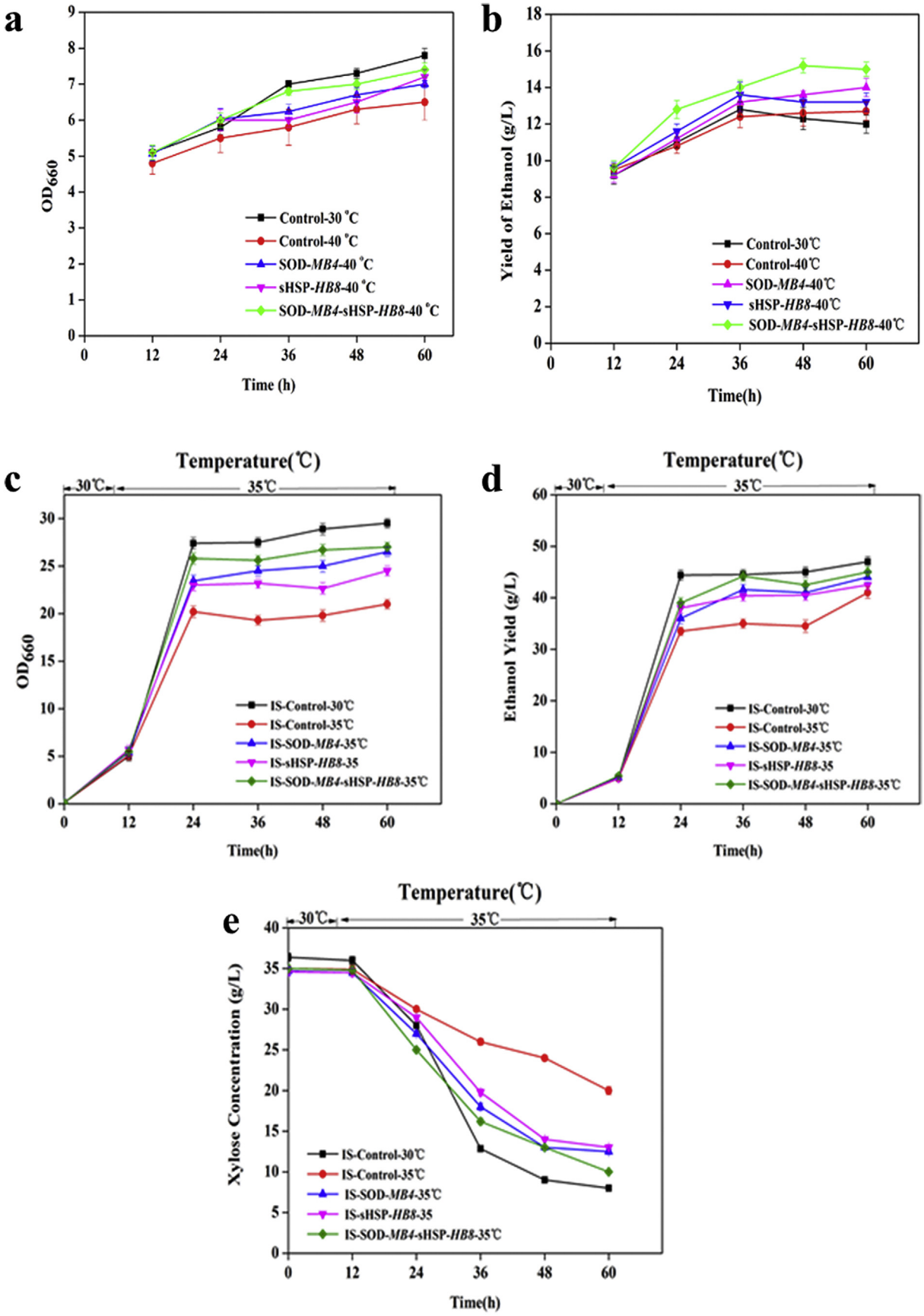

Fig. 5. The growth and ethanol fermentation verification of the lab and industrial *S. cerevisiae* with single genetic device and the combination genetic device. (a) the cell growth of the lab *S. cerevisiae*(INVSc1) at 30 °C and 40 °C; (b) the ethanol yield of the lab *S. cerevisiae*(INVSc1); (c) the cell growth of the industrial *S. cerevisiae* (30 °C and 35 °C); (d) the ethanol yield of the industrial *S. cerevisiae*; (e) the xylose concentration in medium.

genetic device FBA1p-*sod-MB4* could be protected from oxidative stress and kept low ROS level under the high temperature.

3.3. Rational combination genetic devices further strengthening the thermotolerance of S. cerevisiae

The above results displayed that HSP genetic devices and SOD genetic devices could endow lab *S. cerevisiae* with thermotolerance and anti-oxidation, respectively. To settle both heat stress and heat induced oxidative stress globally, the HSP and SOD combination genetic device (*sod-MB4-shsp-MB4*) was rationally constructed through combing the most excellent genetic devices, FBA1p-*shsp-HB8* and FBA1p-*sod-MB4*. Similarly, we also constructed *sod-MB4-gros$_2$-MB4* device. GroS$_2$ from *T. tengcongensis* was reported to greatly improve the thermotolerance of yeast [18]. Meanwhile, to test the cooperation between different kinds of devices, several other genetic devices with the same function were also combined, like *sod-MB4-sod-HB8*, *groes-HB8-shsp-HB8* (Fig. 4a). Then all the combination genetic devices were transformed into the lab *S. cerevisiae*. After grown at 42 °C, all the engineered lab *S. cerevisiae* grew better than the control, especially SOD-*MB4*-sHSP-*HB8* was 2.5 times higher cell density than the control (Fig. 4b). SOD-*MB4*-sHSP-*HB8* at 72 h exhibited the most excellent thermotolerance among the engineered strains and it displayed 15.3% and 55% increased cell growth than SOD-*MB4* and sHSP-*HB8* with single genetic device, respectively. Although SOD-*MB4*-GroS$_2$-*MB4* comprised the excellent HSP genetic device and the SOD genetic device, its cell density was 30% lower than SOD-*MB4*-sHSP-*HB8*. So the rational combination and compatibility of different devices is really important. SOD-*MB4* can also display thermotolerance, showing that alleviating oxidative stress ignited by heat shock could improve the high temperature growth ability. SOD-*MB4*-SOD-*HB8* and GroES-*HB8*-sHSP-*HB8* only contained either SOD genetic devices or HSP genetic devices, which could alleviate only one aspect of heat induced stress. Although the strains with *sod-MB4-sod-HB8* or groes-*HB8*-shsp-*HB8* grew better than the control, their thermo-ability are similar with the strains only with single genetic device. As these combination genetic device could only alleviate one aspect of heat induced stress, they couldn't customize thermotolerance to yeast thoroughly. That means the simply assemble of the same function genetic devices could not enhance the thermotolerance considerably.

These results indicate that when defensing both heat stress and heat induced oxidative stress, the HSP and SOD combination genetic device (*sod-MB4-shsp-HB8*) could efficiently customize better thermotolerance for the lab *S. cerevisiae*, which may owe to the synergistic effect of the two genetic devices (FBA1p-*sod-MB4* and FBA1p-*shsp-HB8*).

3.4. HSP and SOD combination genetic device boosting high temperature ethanol fermentation

Reportedly, the actual fermentation temperature of *S. cerevisiae* in SSF for ethanol production is usually higher than 30 °C [6]. Therefore, we embedded the combination genetic device into *S. cerevisiae* and investigated the ethanol production of the engineered lab strain and the industrial strain at high temperature to verify their industrial application prospects. Firstly, the high temperature ethanol fermentation of the engineered lab strain (*S. cerevisiae* INVSc1) was performed in the YPD medium containing 40 g/L glucose. As observed from Fig. 5a, the cell growth of all the engineered lab strains was better than the control cultured at 40 °C, especially SOD-*MB4*-sHSP-*HB8* displayed 13.8% increased cell density compared with the control at 40 °C. In the batch fermentation process, when the original concentration of glucose was constant, the ethanol yield of the control (wild type *S. cerevisiae* INVSc1) at 30 °C initiated to decrease after 36 h; as a result, it was lower than the control cultured at 40 °C (Fig. 5b). This phenomenon is similar to the previous report [37]. 30 °C is the optimal temperature for yeast to assimilate nutrition for cell growth and ethanol can also be used as carbon resource when lack of glucose [38,39]. That is why the cell density of the control cultured at 30 °C is higher than the control cultured at 40 °C, while the ethanol yield is lower. All the engineered strains maintained ethanol production at higher level than the control at 40 °C. Especially, the ethanol yield of SOD-*MB4*-sHSP-*HB8* was 20.6% higher than the control at 40 °C and even increased by 11.7% and 15.1% compared with other engineered strains containing FBA1p-*sod-MB4* and FBA1p-*shsp-HB8* at 48 h, respectively (Fig. 5b). These above results indicate that the production of ethanol in the engineered lab *S. cerevisiae* with *sod-MB4-shsp-HB8* is increased during high temperature fermentation.

Then the HSP and SOD combination genetic device (*sod-MB4-shsp-HB8*) was further applied to the high temperature ethanol fermentation in the industrial *S. cerevisiae* (IS) which could transform xylose to ethanol in the YPD medium containing 75 g/L glucose and 35 g/L xylose. As observed from Fig. 5c, the cell density of all the engineered industrial strains(IS) exhibited higher than the control cultured at 35 °C. Meanwhile, the ethanol yield tendency of all the industrial strains was in accordance with cell growth, the higher cell density the higher ethanol production. Furthermore, the ethanol yield of the control (wild type industrial *S. cerevisiae*) at 30 °C reached steadily after 24 h while the highest ethanol yield of the control at 35 °C appeared at 60 h, representing fermentation period was prolonged under heat stress. However, the fermentation period of the engineered strains incorporated with genetic devices was shortened by 24 h compared with the heat shocked control under high temperature. Additionally, the ethanol yield of IS-SOD-*MB4*-sHSP-*HB8* was the highest among the other engineered strains contained single genetic device and increased by 26.3% compared with the control at 35 °C at 36 h (Fig. 5d). Moreover, the xylose utilization efficiency of control at 35 °C was lower than the control at 30 °C. After incorporated with genetic devices especially *sod-MB4-shsp-HB8*, the xylose utilization efficiency of the engineered strains was similar as the control at 30 °C (Fig. 5e). All these results show *sod-MB4-shsp-HB8* could efficiently protect cells from heat and oxidative stress, improve the ethanol yield and shorten the fermentation period of industrial *S. cerevisiae.* Even though the ethanol yield of IS-SOD-*MB4*-sHSP-*HB8* was slightly lower than the control cultured at 30 °C, high temperature fermentation has so many advantages [6]. Hence, IS-SOD-*MB4*-sHSP-*HB8* exhibited better industrial application value than yeast cultured at 30 °C.

In summary, the cell growth and ethanol production are different in the two kinds of *S. cerevisiae* owing to the divergence of the strains' properties and fermentation conditions. However, both the engineered lab *S. cerevisiae* and industrial *S. cerevisiae* containing HSP and SOD combination genetic device display enhanced thermotolerance and ethanol yield.

4. Conclusion

The dying cells were pulled back from the verge of death through rationally combing genetic devices to globally enhance thermotolerance of *S. cerevisiae* by defensing both heat stress and heat induced oxidative stress. Furthermore, FBA1p-*sod-MB4*-FBA1p-*shsp-HB8* can efficiently improve the thermotolerance and ethanol yield of the lab *S. cerevisiae* and the industrial *S. cerevisiae*. This method provides a platform to increase production efficiency and reduce energy consumption substantially and it can be expanded to other strains.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.synbio.2017.04.003.

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