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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.