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Enhanced pathway efficiency of *Saccharomyces cerevisiae* by introducing thermo-tolerant devices

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HIGHLIGHTS

- Thermo-tolerant devices were constructed to refactor the thermo-tolerance of yeast.
- Improved thermo-tolerance of *S. cerevisiae* preserved by thermo-tolerant devices.
- Pathway efficiency of thermo-tolerant engineered *S. cerevisiae* was enhanced.
- The optimal growth temperature range of *S. cerevisiae* was broadened to 28–35 °C.

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ABSTRACT

In this study, thermo-tolerant devices consisting of heat shock genes from thermophiles were designed and introduced into *Saccharomyces cerevisiae* for improving its thermo-tolerance. Among ten engineered thermo-tolerant yeasts, *T.te*-TTE2469, *T.te*-GroS2 and *T.te*-IbpA displayed over 25% increased cell density and 1.5–4-fold cell viability compared with the control. Physiological characteristics of thermo-tolerant strains revealed that better cell wall integrity, higher trehalose content and enhanced metabolic energy were preserved by thermo-tolerant devices. Engineered thermo-tolerant strain was used to investigate the impact of thermo-tolerant device on pathway efficiency by introducing β-amyrin synthesis pathway, showed 28.1% increased β-amyrin titer, 28–35 °C broadened growth temperature range and 72 h shortened fermentation period. The results indicated that implanting heat shock proteins from thermophiles to *S. cerevisiae* would be an efficient approach to improve its thermo-tolerance.

1. Introduction

Microbial fermentations play an important role in various aspects of social and economic development including energy, chemicals, materials and medicines, but there still exists some disadvantages such as few chemicals types and low yield. In view of this, promising microbial cellular factories should be constructed to meet human demand for these chemicals to achieve sustainable development. The microbial cells face a variety of environmental stresses during fermentation processes, resulting in reduced production capacity. Improving cellular robustness of microbes is of great importance for efficient production of biofuels and biochemicals (Zhu et al., 2012). One of the most required traits for microorganisms to be used in industrial processes is their ability to survive at higher temperature. Higher temperature fermentations not only effectively accelerate the chemical reaction rate, facilitate downstream product recovery, shorten microbial fermentation period (Postmus et al., 2012), but also mitigate the cooling costs, avoid or minimize microbial contamination (Hasunuma and Kondo, 2012; Lin and Xu, 2013). Take million tons of chemicals enterprise for example, according to the calculations, the cooling water and cooling power consumption will be reduced 15% and 10% respectively every year if the fermentation temperature is increased 5 °C. However, higher temperature affects cell metabolism, reduce cell viability and fermentation capability, and consequently reduce the production stability which determines the amount of the end-product (Amillastre et al., 2012). Among industrial

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microorganisms, model strain *Saccharomyces cerevisiae* exhibits optimal growth between 28 and 30 °C. It is more sensitive to elevated temperature and unable to cope with the chronic exposure to higher temperature. Moreover, the optimal growth temperature of *S. cerevisiae* is also dismatch with the optimum enzymatic temperature (35–50 °C) leading to the poor enzymatic efficiency. Therefore, the possibility of performing fermentation at higher temperatures using thermo-tolerant yeasts capable of fermentation and growth at temperatures compatible with optimal enzyme activities would greatly improve the enzymatic efficiency and increase the chemicals production in microbial fermentation processes, and thereby make the production processes more economically feasible (Shahsavarai et al., 2013). Hence, improving the thermo-tolerance of microorganism cells is very important for improving biological catalytic efficiency, reducing energy consumption and production cost.

Several breeding methods such as mutagenesis, adaptation evolution, protoplast fusion, and genetic engineering strategies (Benjaphokee et al., 2012a; Auesukaree et al., 2012) had been previously used to improve the thermo-tolerance of *S. cerevisiae*. Although some thermo-tolerant *S. cerevisiae* have been applied in industrial fermentation processes (Hasunuma and Kondo, 2012; Shahsavarai et al., 2013), such strains still cannot meet the industrial requirement of direct application in consolidated bioprocessing (CBP) and simultaneous saccharification and fermentation (SSF).

In nature, thermophilic bacteria show superior robustness under harsh conditions, possibly owing to their well adapted stress-response genes during long-term natural evolution (Egorova and Antranikian, 2005). The thermophiles such as *Thermoanaerobacter tengcongensis* thrive under optimal temperature of $\geqslant$50 °C, they resist high temperature by various thermo-tolerance mechanisms including spore, the components of cell membranes, thermophilic enzyme, protein spatial structure, heat shock protein and genetic material (Saunders et al., 2003; Berezovsky and Shakhnovich, 2005; Lin and Xu, 2013). Reportedly, producing a large amount of heat shock proteins is a widely adapted mechanism. Heat shock proteins (HSPs) are a family of proteins that protect proteins from being irrevocably denatured, misfolded or aggregated under various exogenetic, abiotic stress conditions, and help organisms to modulate stress responses and protect organisms from cellular damages, thus considered to play an important role in conferring thermo-tolerance (Meng et al., 2009; Smith et al., 2012). Additionaly, some molecules such as trehalose and heat shock glycoprotein also can refactor thermo-tolerance to microorganisms but HSPs showed higher thermo-tolerance (Morano et al., 2012). Although wild thermophilic microorganisms have been employed in biochemicals production (Argyros et al., 2011), their industrial applications still hampered by many disadvantages such as sensitive to solvent and osmotic, low cellular biomass and difficult genetic manipulation (Lin and Xu, 2013). There are also very few engineered *S. cerevisiae* to grow at temperature higher than 41 °C (Shi et al., 2009). Therefore, if the thermo-tolerance characteristics of heat shock genes from thermophiles are implanted to *S. cerevisiae* by constructing thermo-tolerant devices, it would be a novel way to engineer a thermo-tolerant strain. Currently, there are a few literature reports describing the improvement of industrial fermentation process at higher temperature via implanting thermo-tolerant devices into microorganisms (Luan et al., 2014).

In the present study, thermo-tolerant genes were cloned from *T. tengcongensis* to construct thermo-tolerant devices and expressed in *S. cerevisiae*. Effects of thermo-tolerant devices on growth and physiological characteristics of *S. cerevisiae* were investigated. Meanwhile, the function of thermo-tolerant device in value added compound producing strain was verified though enhanced pathway efficiency. This work significantly improved the thermo-tolerance of *S. cerevisiae*, which will greatly reduce pollution and energy consumption, ultimately lower the industrial production cost.

2. Methods

2.1. Strains, vectors, media, and reagents

The strains of *T. tengcongensis MB4* (provided by Dr. Ma, Institute of Microbiology Chinese Academy of Sciences), *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 thermo-tolerant devices and thermo-tolerant engineered strains

The genome of *T. tengcongensis MB4* and *S. cerevisiae INVSc1* were used as templates for amplification of full-length functional genes and constitutive promoter/terminator respectively. Functional genes, constitutive promoter and terminator were gel purified by DNA gel purification kit (BioTeKe, Beijing, China) and constructed expression cassettes (thermo-tolerant devices) via overlap extension PCR. Thermo-tolerant devices *were* double-digested with *EcoR* I and *BamH* I, subsequently ligated into linearized pRS42K that digested with the same restriction endonuclease, and then transformed into *E. coli* Top10. Positive transformants were screened on LB-kanamycin plates and confirmed by colony PCR. The recombinant plasmid pRS42K was extracted and electrotransformed into *S. cerevisiae INVSc1*, then positive transformants were selected on YPD medium containing G418 and confirmed via PCR.

2.3. Determination of cell concentration and cell viability

Single colony was inoculated into 100 mL-Erlenmeyer flask containing 40 mL YPD medium, and grew for 36 h at 30 °C and 170 rpm. An aliquot of the resulting culture was inoculated in 250 mL-Erlenmeyer flask containing 50 mL fresh medium, starting with an $OD_{660\ nm}$ of 0.1 for further cultivation at 30 °C and 170 rpm for initial 12 h. For heat shock response experiments, two different sets of fermentation conditions were used: (A) the fermentation temperature was increased to 35 °C in the first 12 h, then gradually increased 2 °C every 12 h, up to 45 °C for 72 h. During this process, cell samples were taken at 12 h-intervals to measure $OD_{660\ nm}$. Cell concentration of the sample was determined at $OD_{660\ nm}$ by using a spectrophotometer, model U-2900(HITACHI, Chiyoda, Tokyo). The control was the fermentation medium except cells. Meanwhile, samples were taken at 24, 36, 48, 60 and 72 h and 10-fold serially diluted to obtain a cell concentration of 2×10^3 cells/mL, for each of these cultures, approximately 300 of viable cells were spread onto YPD-G418 plates and colonies were counted after 3 days of incubation at 30 °C; (B) the fermentation temperature was directly increased to 42, 44 and 46 °C for 72 h and the cell samples were taken at 12 h-intervals to measure $OD_{660\ nm}$. In addition, yeast cells that cultured at 42 °C were also taken at 24, 36, 48, 60 and 72 h and

10-fold serially diluted to obtain a cell concentration of 1×10^3 cells/mL, for each of these cultures, approximately 150 of viable cells were spread on YPD-G418 plates and colonies were counted after 3 days of incubation at 30 °C, the cell viability was expressed as a relative value of viable-cell number. All data represent the mean standard deviation (SD) from three independent experiments.

2.4. Congo Red sensitivity of thermo-tolerant engineered strains

To further determine the physiological characteristic of thermo-tolerant strains, Congo Red sensitivity of three engineered strains (*T.te*-TTE2469, *T.te*-GroS2, *T.te*-IbpA) and control strain (WT/Vector) was investigated. The mid-logarithmic phase cells were taken and diluted in 10^{-1} steps so that aliquots (2.5 μL) of the 10-fold serially-diluted culture could be dropped onto YPD-G418 plates supplemented with Congo Red at a final concentration of 50 mg/L for incubation at 30 °C and 42 °C for 3 days. Besides, the cell viability of these strains to Congo Red was also determined via counting the colonies after 3 days of incubation at 42 °C.

2.5. Trehalose accumulation in thermo-tolerant engineered strains

Trehalose concentration was estimated via anthrone method (Zheng et al., 2011a). The yeast cells cultured at 42 °C were harvested at 84 h and quickly chilled, collected, washed and stored at −20 °C until use. Trehalose was extracted from cells and a 200 μL aliquot of each extraction was mixed with 1.8 mL of chilled distilled water and 8 mL cold anthrone reagent (0.2% anthrone-H_2SO_4). After incubating in boiling water for 2 min, each sample was cooled immediately in water, and its absorbance was measured at $OD_{620\ nm}$.

2.6. Quantitative PCR

Quantitative PCR was performed using method described by Li et al. (2013). Approximately 1×10^7 yeast cells were harvested and used for the total RNA extraction using Yeast RNA Kit (OMEGA, Doraville, GA). Genomic DNA contamination was eliminated by DNase I treatment. RNA was quantified by measuring the absorbance at 260 nm using NanoDrop 2000c (Thermo Scientific, Waltham, MA). Five hundred nanogram of RNA from each sample was used as template for Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR analysis was performed with the LightCycler SYBR Green I Master Kit (Roche) according to the manufacturer's instructions. The reactions were run with the LightCycler 96 real-time system using fast 96 well plates (Roche). The data were analyzed using LightCycler Software (v. 1.5). The housekeeping gene act1 was used as a reference gene and reaction without reverse transcriptase was used as a negative control. All experiments were performed in triplicate.

2.7. Functional verification of thermo-tolerant device in thermo-tolerant engineered strain

One thermo-tolerant device (FBA1p-*gros2*-SLM5t) was constructed into multiple plasmid pRS42k, subsequently transformed in β-amyrin producing *S. cerevisiae* (Sg-sb) to engender the strain named Sg-sb-*T.te*-GroS2. Then, a single colony of thermo-tolerant β-amyrin engineered and control strain was pre-cultured and an aliquot of the resulting culture was used to inoculate in 40 mL YPD medium, starting with an $OD_{660\ nm}$ of 0.1, then the culture was cultivated at 30 °C, 35 °C and 38 °C for 120 h respectively. During these processes, growth was examined by monitoring the $OD_{660\ nm}$. For the determination of β-amyrin concentration, the product was extracted and quantified using method described by Kirby et al. (2008) with some modifications. Yeast culture (40 mL) in a 50 mL centrifugal tube was centrifuged for 5 min at 10,000 rpm to pellet cells. The cells were resuspended in 10 mL of a fresh solution of 20% (w/v) KOH in 50% ethanol and boiled for 10 min in this solution. After cooling, the β-amyrin was extracted by vortexing with 10 mL hexane for 5 min at room temperature. Then, the supernatant containing β-amyrin was extracted and transferred into a new 50 mL centrifugal tube. The pellet was extracted again with same program and then supernatants from the two extractions were mixed together, swirled and evaporated at 45–50 °C condensed to 1 mL. The extractions were centrifuged and transferred to a glass vial and used for the β-amyrin assay using gas chromatography (GC-2010, shimadzu) installed with thermoelectric chromatography column TG-5MS (30 m × 0.25 mm × 0.25 μm). The oven temperature was held at 80 °C for 1 min after injection, and was then ramped to 280 °C at 20 °C min^{-1}, held at 280 °C for 20 min, ramped to 300 °C at 20 °C min^{-1} and held at 300 °C for 2 min. Standard curves for β-amyrin was run at the start and end of each batch of samples.

2.8. Statistical analysis

Results were analyzed via one-way ANOVA. Post hoc analysis was performed via a least significance difference using the SPSS 13.0 software.

3. Results and discussion

3.1. Mining of thermo-tolerant genes and designing of thermo-tolerant devices

Previous studies have proved that the appearance and disappearance of strain thermo-tolerance were consistent with the generation and degradation of HSPs (Auesukaree et al., 2012; Smith et al., 2012). Thermophilic archaea *T. tengcongensis* was chosen to clone thermo-tolerant genes because of its complete genomic information, clear thermo-tolerant mechanism and diverse heat shock proteins. As a result, ten heat shock protein genes such as *tte2469*, *thif*, *gros2*, *ibpa* and *hslo* were obtained by PCR. To construct thermo-tolerant devices, thermo-tolerant genes were expressed downstream of a strong constitutive promoter FBA1p because a certain amount HSPs expressed before heat shock treatment would be a better strategy for microorganisms to pre-acquire thermo-tolerance (Lindquist, 1986). Transforming thermo-tolerant

Table 1
Construction of thermo-tolerant devices and engineered *S. cerevisiae*.

Thermo-tolerant genes[a]	Thermo-tolerant devices[b]	Engineered strains[c]
		WT/Vector
tte2469	FBA1p-*tte2469*-SLM5t	*T.te*-TTE2469
thif	FBA1p-*thif*-SLM5t	*T.te*-ThiF
gros2	FBA1p-*gros2*-SLM5t	*T.te*-GroS2
ibpa	FBA1p-*ibpa*-SLM5t	*T.te*-IbpA
hslo	FBA1p-*hslo*-SLM5t	*T.te*-HslO
dnaj	FBA1p-*dnaj*-SLM5t	*T.te*-DnaJ
groel	FBA1p-*groel*-SLM5t	*T.te*-GroEL
groes	FBA1p-*groes*-SLM5t	*T.te*-GroES
grpe	FBA1p-*grpe*-SLM5t	*T.te*-GrpE
dnak	FBA1p-*dnak*-SLM5t	*T.te*-DnaK

[a] *tte2469 and thif* genes encoding ubiquitin protein; *gros2*, *ibpa*, *hslo* and *dnaj* genes encoding the heat shock proteins of HSP10, HSP20, HSP33 and HSP40 family respectively; *groel* and *groes* genes encoding HSP60; *grpe* and *dnak* genes encoding HSP70.
[b] Promoter-thermo-tolerant gene-terminator.
[c] Strains with plasmid of pRS42K containing thermo-tolerant devices; WT/Vector (wild type strain with plasmid of pRS42K); *T.te* (*Thermoanaerobacter tengcongensis* MB4).

devices linked to plasmid pRS42K into *S. cerevisiae* resulted in ten different thermo-tolerant engineered strains. All thermo-tolerant genes, devices and engineered strains are listed in Table 1. In order to determine whether these thermo-tolerant genes were transcribed in *S. cerevisiae*, transcription level of each gene was determined via qRT-PCR. The results showed that ten genes were expressed significantly different after heat shock at 42°Cfor 24 h. Gene *tte2469* showed highest expression level (1201 ± 56.7), followed by *groel* (1109.2 ± 87.6), *dnak* (786.4 ± 70.4), *dnaj* (687.6 ± 50.2), *grpe* (556.4 ± 66.3), *hslo* (432.6 ± 46.1), *groes* (332.8 ± 26.6), *thif* (220.5 ± 35.2), *gros2* (210.8 ± 26.1) and *ibpa* (180.2 ± 19.4).

3.2. Growth and cell viability of the engineered strains

The growth behavior of engineered strains and control strain was investigated in YPD liquid medium by gradual increasing temperature from 35 °C to 45 °C or constant temperature 42 °C. As shown in Table 2, among ten engineered strains, these engineered strains *T.te*-TTE2469 (15.2 ± 1.4, 9.6 ± 0.7), *T.te*-ThiF (15.9 ± 0.6, 9.9 ± 0.8), *T.te*-GroS2 (16.7 ± 1.2, 10.5 ± 0.7) and *T.te*-IbpA (14.7 ± 0.9, 9.4 ± 0.6) displayed over 25% increased cell density compared with the control strain (11.5 ± 0.8, 7.5 ± 0.8) ($p < 0.05$). The genes *tte2469* and *thif* encoding ubiquitin protein with similar function, hence only *T.te*-TTE2469 engineered strain with other two engineered strains *T.te*-GroS2 and *T.te*-IbpA were selected for further study. To evaluate the survival potential of aforementioned three engineered strains at higher temperature, cell samples were collected to investigate the cell viability. The results revealed that the cell viability decreased drastically with prolonged heat treatment time and three engineered strains showed significantly higher cell viability compared with the control. Especially, strain *T.te*-GroS2 had 3.2-fold and 4-fold cell viability at 72 h compared with the control under two different sets of fermentation conditions (Fig. 1a,b). Afterward, the precise upper limit growth temperature of these engineered strains was determined at 44 °C and 46 °C. It was evident that engineered strains grew better than the control ($p < 0.05$), although they displayed severe growth inhibition at higher temperatures (Table 2). These results revealed that implanting the thermo-tolerant devices in *S. cerevisiae* can improve its thermo-tolerance.

3.3. Protection of thermo-tolerant devices on cell wall integrity

Stress factors gradually reduce cell viability by affecting cell wall integrity (Ding et al., 2009). The cell wall, a dynamic structure and essential for the maintenance of cellular integrity and shape, need to be remodeled and repaired for a cell to attempt to respond to the heat stress (Hall and Gow, 2013). Heat stress can activate the cell wall integrity (CWI) pathway of *S. cerevisiae*, which in turn

Table 2
Comparsion of cell density of engineered and control strains after 84 h at various temperatures.

Strains	OD_{660}			
	35 °C–45 °C	42 °C	44 °C	46 °C
WT/Vector	11.5 ± 0.78	7.5 ± 0.76	4.8 ± 0.36	4.6 ± 0.19
T.te-TTE2469	15.2 ± 1.42	9.6 ± 0.72	5.9 ± 0.42	5.6 ± 0.25
T.te-GroS2	16.7 ± 1.22	10.5 ± 0.67	5.8 ± 0.21	5.4 ± 0.34
T.te-IbpA	14.7 ± 0.89	9.4 ± 0.56	5.5 ± 0.38	5.3 ± 0.17
T.te-THiF	15.9 ± 0.58	9.9 ± 0.78		
T.te-HslO	13.9 ± 0.86	8.9 ± 0.62		
T.te-DnaJ	13.1 ± 1.13	8.8 ± 0.35		
T.te-GroEL	10.6 ± 0.82	7.9 ± 0.66		
T.te-GroES	10.9 ± 0.89	7.2 ± 0.28		
T.te-GrpE	12.6 ± 0.58	9.1 ± 0.51		
T.te-DnaK	12.2 ± 0.64	8.3 ± 0.73		

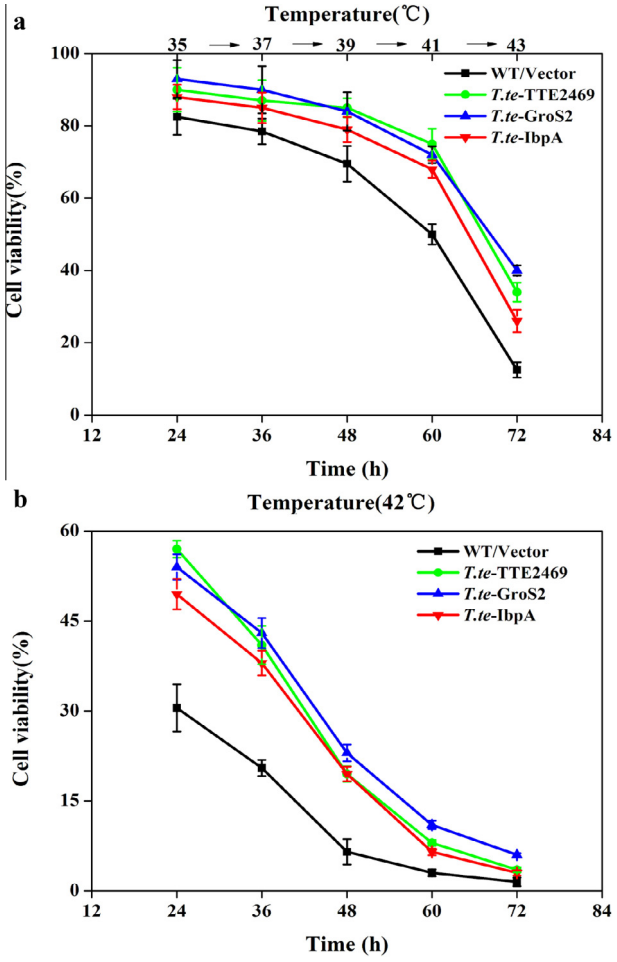

Fig. 1. Cell viability of strains cultured at temperatures from 35 °C to 43 °C (a) and 42 °C (b).

affects the composition of cell wall and its proteins (Heilmann et al., 2013). Therefore, the cell wall integrity maintenance potential of engineered strains was estimated under heat stress by Congo Red dyeing because Congo Red interferes with cell wall integrity. The results showed that there was no obviously different between engineered strains and control strain when they cultured at 30 °C. However, three engineered strains grew better than the control when they cultured at 42 °C or supplemented with 50 mg/L Congo Red. Especially *T.te*-GroS2 strain showed the best growth. Meanwhile, the cells became smaller when strains cultured at 42 °C compared to 30 °C (see Fig. S1 in the supplementary material). Besides, the cell viability of engineered and control strains to Congo Red sensitivity was also compared. *T.te*-GroS2 strain (83.5 ± 4.2)% showed 1.5-fold more cell viability compared with the control (50 ± 6.5)% ($p < 0.05$). These results indicated that the thermo-tolerant devices effectively impeded the cell wall deformation by repairing certain cell wall associated proteins, resulting in stronger cell survival ability of thermo-tolerant strains at higher temperature.

3.4. Dual protection roles of thermo-tolerant devices on engineered strains

Previous studies have revealed that trehalose is an important compound involved in the survival of *S. cerevisiae* when exposed to various severely stressful environments, such as heat and hyperosmotic pressure. The protective role of this disaccharide lies in maintaining membrane stability, facilitating proper protein folding

by HSPs and preventing protein denaturation under stress conditions (Mahmud et al., 2010; Heilmann et al., 2013; Wang et al., 2014). To see whether *T.te*-TTE2469, *T.te*-GroS2 and *T.te*-IbpA strains accumulate a higher content of trehalose than WT/Vector, trehalose content of strains was compared at 42 °C. Trehalose accumulation in strains *T.te*-TTE2469, *T.te*-GroS2 and *T.te*-IbpA was higher by (14.5 ± 1.5), (21.7 ± 2.1) and (10.7 ± 1.3) mg/L respectively, compared with the control strain ($p < 0.05$). The results revealed that heat stress caused a significant increase in trehalose accumulation due to the function of thermo-tolerant devices. Trehalose and heat shock proteins may play a role in providing cross-protection against heat stress (Zhao and Bai, 2009). The previous study also demonstrated that several genes such as *tps1*, *tps2* and *tps3* associated with trehalose metabolism in yeast strains were highly expressed (Hirasawa et al., 2001). Among these genes, *tps1* (encoding trehalose-6-phosphate synthase) was highly expressed gene. Fig. 2a shows that the expression of *tps1* gene was up-regulated in three engineered strains after long-term heat exposure compared with the control ($p < 0.05$) and the trehalose content was strictly correlated with the gene expression level. Actually, higher expression of *tps1* in thermo-tolerant strain is a common phenomenon (Auesukaree et al., 2012). However, the increased trehalose content implied that glycolytic flux was altered and caused by a change in pyruvate kinase activity which associated with ATP metabolism. Interestingly, the *cdc19* gene encoding pyruvate kinase (involved in ATP production) was significantly up-regulated in three engineered strains compared with the control (Fig. 2b). Especially, strain *T.te*-GroS2 exhibited the highest expression level although the expression level decreased 48 h later ($p < 0.01$). The results suggested that the metabolic rate was enhanced due to the implanting of thermo-tolerant device, which may stimulate the energy generating pathway to maintain cellular homeostasis. Some studies also have shown that *cdc19* is important for high-temperature tolerance in *S. cerevisiae* (Benjaphokee et al., 2012b). Therefore, in this study, another engineered strain called *S.c*-CDC19 was also constructed by over-expressing *cdc19*. Fermentation experiment results showed that the cell density of *S.c*-CDC19 strain was increased (16.2 ± 1.3)% compared with the control. However, three thermo-tolerant engineered strains still grew significantly better than *S.c*-CDC19 strain (see Fig. S2 in the supplementary material). These results indicated that over-expression of *cdc19* can enhance the thermo-tolerance of strain, but the thermo-tolerance was significantly improved owing to the up-regulated expression of *cdc19* gene by implanting thermo-tolerant devices. Thermo-tolerant genes *tte2469*, *gros2* and *ibpa* encoding ubiquitin, HSP10 and HSP20 respectively. Ubiquitin plays a central role in controlling protein stability, activity, localization and protein–protein interactions. Over-expression of ubiquitin protects

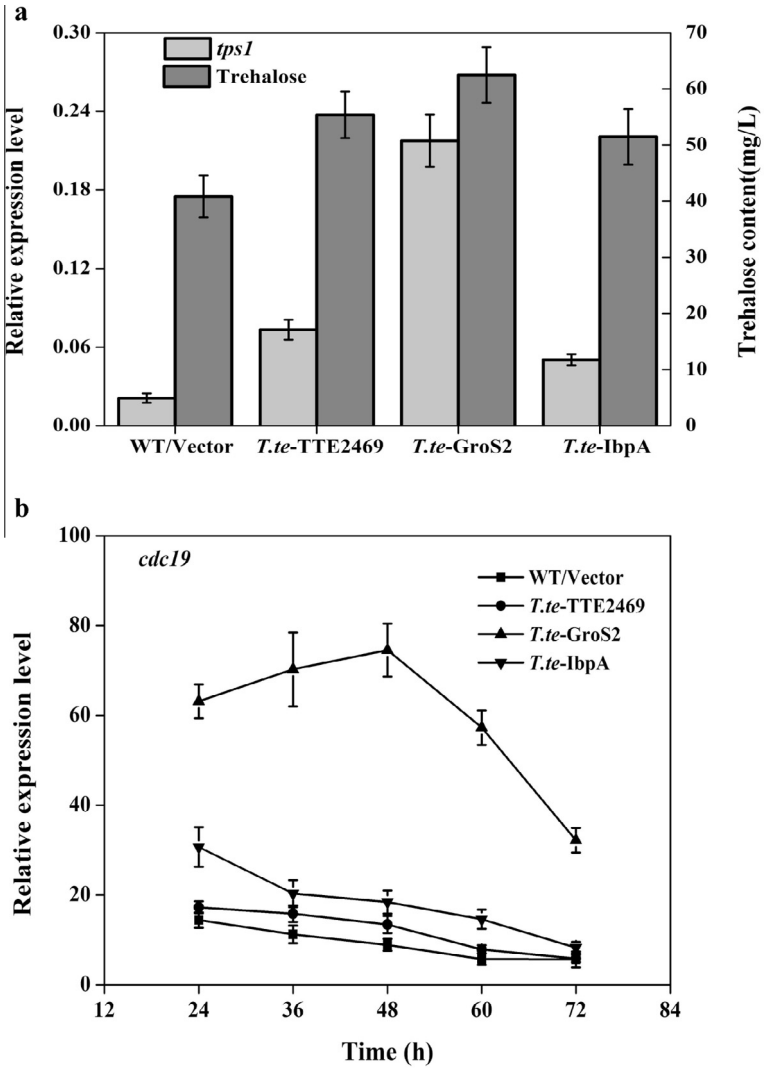

Fig. 2. Comparsion of trehalose accumulation and expression of *tps1* gene in three engineered strains *T.te*-TTE2469, *T.te*-GroS2 and *T.te*-IbpA and control stain WT/Vector (a), and expression level of *cdc19* gene in three engineered strains and control stain under heat stress condition (b).

yeast against multiple stresses (Hiraishi et al., 2006). HSP10 and HSP20 proteins belong to the small heat shock proteins (sHSPs), sHSPs are a superfamily found in all organisms such as Archaea, Bacteria, and Eukarya, and as molecular chaperones, they aid in binding partially denatured proteins and also prevent irreversible protein aggregation induced by various environmental stressors. Over-expression of small heat shock proteins protects microorganisms against heat stress (Ahn et al., 2012; Mayer et al., 2012). This property makes sHSPs critically important in cellular defenses against a wide variety of stresses. Therefore, these exogenous heat shock proteins may possess dual protective roles on cells, on one hand, they protected proteins from being irrevocably denatured, misfolded or aggregated, on the other hand, they indirectly affected stress-response genes such as *cdc19* and *tps1*, engaging the HSPs, trehalose and energy generating pathway synergically to withstand adverse heat stress.

3.5. Enhanced pathway efficiency in thermo-tolerant engineered strains

β-Amyrin is a triterpenes compound and possesses antibacterial, antiviral and antioxidant properties. In order to evaluate the protective role of thermo-tolerant device on value added compound producing strain, thermo-tolerant β-amyrin engineered strain Sg-sb-*T.te*-GroS2 was constructed. Reportedly, the actual fermentation temperature for *S. cerevisiae* applied in industrial processes was between 34 °C and 38 °C (Gu et al., 2014), the mandated temperature for *S. cerevisiae* fermentation in SSF was typically no more than 35 °C (Cannella and Jørgensen, 2014). Therefore, the fermentation profiles of thermo-tolerant engineered strain Sg-sb-*T.te*-GroS2 cultured at 35 °C and 38 °C and control strain Sg-sb cultured at 30 °C, 35 °C and 38 °C were compared. As shown in Fig. 3a,b, when Sg-sb-*T.te*-GroS2 strain was cultured at 35 °C, the cell density was increased (10.1 ± 0.6)% and the β-amyrin titer was apparently increased (28.1 ± 2.6)% compared with the control strain that cultured at 30 °C. Meanwhile, the cell density and β-amyrin titer were also increased (20.6 ± 1.8)% and (49.2 ± 1.4)% respectively compared with the control strain that cultured at 35 °C. In addition, the β-amyrin titer of Sg-sb-*T.te*-GroS2 strain (35 °C) was 16.3 mg/L at 48 h, which was slightly higher than the control strain (30 °C) at 120 h, namely, the fermentation period of Sg-sb-*T.te*-GroS2 strain was shortened to 48 h. The saved fermentation period demonstrated that the metabolic rate of Sg-sb-*T.te*-GroS2 strain was enhanced. When Sg-sb-*T.te*-GroS2 strain was cultivated at 38 °C, although the cell density was decreased (18.3 ± 1.3)%, the β-amyrin titer was merely decreased (2.9 ± 0.2)% compared with the control strain that cultured at 30 °C. Moreover, the cell density and β-amyrin titer were increased (11.3 ± 0.8)% and (25.7 ± 2.2)% respectively compared with the control strain that cultured at 38 °C. According to above results and calculation, the β-amyrin synthesis efficiency of Sg-sb-*T.te*-GroS2 strain cultured at 38 °C was increased (21.2 ± 1.5)% and (46.7 ± 3.3)% at 48 h compared with the control strain that cultured at 30 °C and 38 °C respectively. Therefore, the increased β-amyrin titer indicated that the pathway efficiency of β-amyrin synthesis was enhanced. Studies have shown that higher temperature increases glycolytic flux and enzyme activity to improve the efficiency of cell synthesis (Postmus et al., 2012; Amillastre et al., 2012; Lin and Xu, 2013). Therefore, these results indicated that the thermo-tolerance of *S. cerevisiae* was improved, pathway efficiency and metabolic rate were further enhanced due to the protective role of thermo-tolerant device, which reduced the energy consumption, increased the β-amyrin titer and shortened the fermentation period.

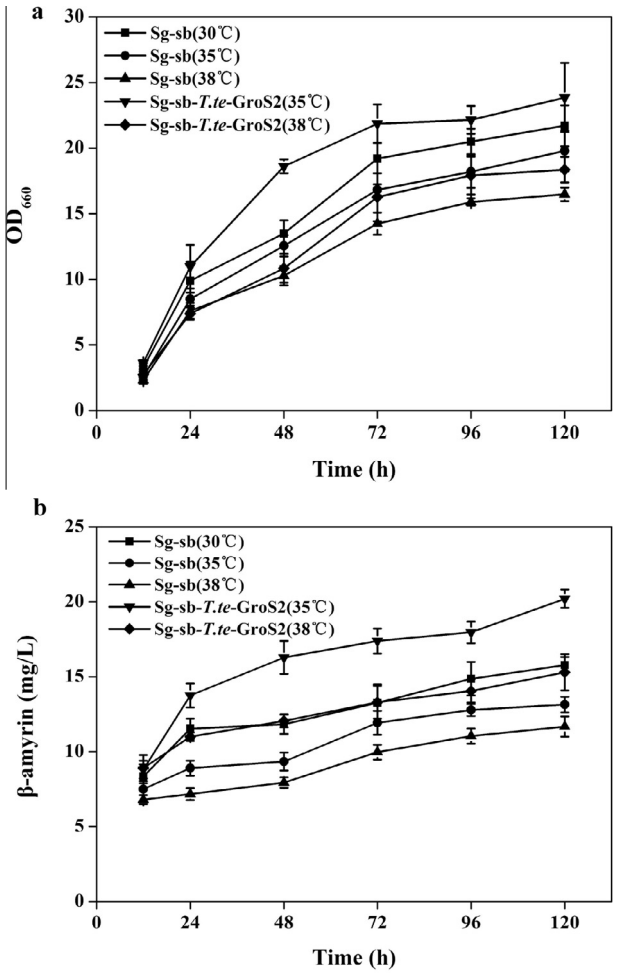

Fig. 3. Pathway efficiency verification of Sg-sb strain cultured at 30 °C, 35 °C and 38 °C and Sg-sb-*T.te*-GroS2 strain cultured at 35 °C and 38 °C for 120 h by the growth potential (a) and β-amyrin titer (b).

4. Conclusions

The main achievement of this work was that the optimal growth temperature range of *S. cerevisiae* was broadened to 28–35 °C via introducing thermo-tolerant devices. Three refactored engineered strains *T.te*-TTE2469, *T.te*-GroS2 and *T.te*-IbpA showed better growth at higher temperature. The better growth ability was the result of thermo-tolerant devices may possess dual protective roles on cells. Thermo-tolerant engineered strain showed 28.1% increased β-amyrin titer and 72 h shortened fermentation period. This study provides a platform to substantially reduce energy consumption and production cost and offers a valued scientific reference to construct other resistant strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.07.063.

References

Ahn, J., Won, M., Choi, J.H., Kyun, M.L., Cho, H.S., Park, H.M., Kang, C.M., Chung, K.S., 2012. Small heat-shock protein Hsp9 has dual functions in stress adaptation and stress-induced G2-M checkpoint regulation via Cdc25 inactivation in *Schizosaccharomyces pombe*. Biochem. Biophys. Res. Commun. 417 (1), 613–618.

Amillastre, E., Aceves-Lara, C.A., Uribelarrea, J.L., Alfenore, S., Guillouet, S.E., 2012. Dynamic model of temperature impact on cell viability and major product formation during fed-batch and continuous ethanolic fermentation in *Saccharomyces cerevisiae*. Bioresour. Technol. 117, 242–250.

Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., Foden, J.M., Miller, B.B., Lynd, L.R., Hogsett, D.A., 2011. High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl. Environ. Microbiol. 77 (23), 8288–8294.

Auesukaree, C., Koedrith, P., Saenpayavai, P., Asvarak, T., Benjaphokee, S., Sugiyama, M., Kaneko, Y., Harashima, S., Boonchird, C., 2012. Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits. J. Biosci. Bioeng. 114 (2), 144–149.

Benjaphokee, S., Hasegawa, D., Yokota, D., Asvarak, T., Auesukaree, C., Sugiyama, M., Kaneko, Y., Boonchird, C., Harashima, S., 2012a. Highly efficient bioethanol production by a *Saccharomyces cerevisiae* strain with multiple stress tolerance to high temperature, acid and ethanol. New Biotechnol. 29 (3), 379–386.

Benjaphokee, S., Koedrith, P., Auesukaree, C., Asvarak, T., Sugiyama, M., Kaneko, Y., Boonchird, C., Harashima, S., 2012b. CDC19 encoding pyruvate kinase is important for high-temperature tolerance in *Saccharomyces cerevisiae*. New Biotechnol. 29 (2), 166–176.

Berezovsky, I.N., Shakhnovich, E.I., 2005. Physics and evolution of thermophilic adaptation. PNAS 102 (36), 12742–12747.

Cannella, D., Jørgensen, H., 2014. Do new cellulolytic enzyme preparations affect the industrial strategies for high solids lignocellulosic ethanol production? Biotechnol. Bioeng. 111 (1), 59–68.

Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D., Zhang, K., 2009. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 85 (2), 253–263.

Egorova, K., Antranikian, G., 2005. Industrial relevance of thermophilic Archaea. Curr. Opin. Microbiol. 8 (6), 649–655.

Gu, H., Zhang, J., Bao, J., 2014. Inhibitor analysis and adaptive evolution of *Saccharomyces cerevisiae* for simultaneous saccharification and ethanol fermentation from industrial waste corncob residues. Bioresour. Technol. 157 (1), 6–13.

Hall, R.A., Gow, N.A., 2013. Mannosylation in Candida albicans: role in cell wall function and immune recognition. Mol. Microbiol. 90 (6), 1147–1161.

Hasunuma, T., Kondo, A., 2012. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. Process Biochem. 47 (9), 1287–1294.

Heilmann, C.J., Sorgo, A.G., Mohammadi, S., Sosinska, G.J., de Koster, C.G., Brul, S., de Koning, L.J., Klis, F.M., 2013. Surface stress induces a conserved cell wall stress response in the pathogenic fungus *Candida albicans*. Eukaryot. Cell 12 (2), 254–264.

Hiraishi, H., Mochizuki, M., Takagi, H., 2006. Enhancement of stress tolerance in *Saccharomyces cerevisiae* by overexpression of ubiquitin ligase rsp5 and ubiquitin-conjugating enzymes. Biosci. Biotechnol. Biochem. 70 (11), 2762–2765.

Hirasawa, R., Yokoigawa, K., Isobe, Y., Kawai, H., 2001. Improving the freeze tolerance of bakers' yeast by loading with trehalose. Biosci. Biotechnol. Biochem. 65 (3), 522–526.

Kirby, J., Romanini, D.W., Paradise, E.M., Keasling, J.D., 2008. Engineering triterpene production in *Saccharomyces cerevisiae* – β-amyrin synthase from *Artemisia annua*. J. FEBS 275 (8), 1852–1859.

Li, Z., Qu, H., Li, C., Zhou, X., 2013. Direct and efficient xylitol production from xylan by *Saccharomyces cerevisiae* through transcriptional level and fermentation processing optimizations. Bioresour. Technol. 149, 413–419.

Lin, L., Xu, J., 2013. Dissecting and engineering metabolic and regulatory networks of thermophilic bacteria for biofuel production. Biotechnol. Adv. 31 (6), 827–837.

Lindquist, S., 1986. The heat-shock response. Annu. Rev. Biochem. 55 (1), 1151–1191.

Luan, G., Dong, H., Zhang, T., Lin, Z., Zhang, Y., Li, Y., Cai, Z., 2014. Engineering cellular robustness of microbes by introducing the GroESL chaperonins from extremophilic bacteria. J. Biotechnol. 178, 38–40.

Mahmud, S.A., Hirasawa, T., Shimizu, H., 2010. Differential importance of trehalose accumulation in *Saccharomyces cerevisiae* in response to various environmental stresses. J. Biosci. Bioeng. 109 (3), 262–266.

Mayer, F.L., Wilson, D., Jacobsen, I.D., Miramon, P., Slesiona, S., Bohovych, I.M., Brown, A.J., Hube, B., 2012. Small but crucial: the novel small heat shock protein Hsp21 mediates stress adaptation and virulence in *Candida albicans*. PLoS. One 7 (6), e38584.

Meng, B., Qian, Z., Wei, F., Wang, W., Zhou, C., Wang, Z., Wang, Q., Tong, W., Wang, Q., Ma, Y., 2009. Proteomic analysis on the temperature-dependent complexes in *Thermoanaerobacter tengcongensis*. Proteomics 9 (11), 3189–3200.

Morano, K.A., Grant, C.M., Moye-Rowley, W.S., 2012. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190 (4), 1157–1195.

Postmus, J., Aardema, R., Koning, L.J., Koster, C.G., Brul, S., Smits, G.J., 2012. Isoenzyme expression changes in response to high temperature determine the metabolic regulation of increased glycolytic flux in yeast. FEMS Yeast Res. 12 (5), 571–581.

Saunders, N.F., Thomas, T., Curmi, P.M., Mattick, J.S., Kuczek, E., Slade, R., Davis, J., Franzmann, P.D., Boone, D., Rusterholtz, K., 2003. Mechanisms of thermal adaptation revealed from the genomes of the antarctic *Archaea Methanogenium frigidum* and *Methanococcoides burtonii*. Genome Res. 13 (7), 1580–1588.

Shahsavarai, H., Hasegawa, D., Yokota, D., Sugiyama, M., Kaneko, Y., Boonchird, C., Harashima, S., 2013. Enhanced bio-ethanol production from cellulosic materials by semi-simultaneous saccharification and fermentation using high temperature resistant *Saccharomyces cerevisiae* TJ14. J. Biosci. Bioeng. 115 (1), 20–23.

Shi, D.J., Wang, C.I., Wang, K.M., 2009. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. Ind. Microbiol. Biotechnol. 36 (1), 139–147.

Smith, H.A., Burns, A.R., Shearer, T.L., Snell, T.W., 2012. Three heat shock proteins are essential for rotifer thermotolerance. Exp. Mar. Biol. Ecol. 413, 1–6.

Wang, P.M., Zheng, D.Q., Chi, X.Q., Li, O., Qian, C.D., Liu, T.Z., Zhang, X.Y., Du, F.G., Sun, P.Y., Qu, A.M., 2014. Relationship of trehalose accumulation with ethanol fermentation in industrial *Saccharomyces cerevisiae* yeast strains. Bioresour. Technol. 152, 371–376.

Zhao, X., Bai, F., 2009. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J. Biotechnol. 144 (1), 23–30.

Zheng, D.Q., Wu, X.C., Tao, X.L., Wang, P.M., Li, P., Chi, X.Q., Li, Y.D., Yan, Q.F., Zhao, Y.H., 2011. Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance. Bioresour. Technol. 102 (3), 3020–3027.

Zhu, L., Zhu, Y., Zhang, Y., Li, Y., 2012. Engineering the robustness of industrial microbes through synthetic biology. Trends Microbiol. 20 (2), 94–101.