Bioresource Technology 152 (2014) 371–376

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Relationship of trehalose accumulation with ethanol fermentation in industrial *Saccharomyces cerevisiae* yeast strains

Pin-Mei Wang [a,b,1], Dao-Qiong Zheng [b,1], Xiao-Qin Chi [d], Ou Li [b], Chao-Dong Qian [b], Tian-Zhe Liu [b], Xiao-Yang Zhang [c], Feng-Guang Du [c], Pei-Yong Sun [c], Ai-Min Qu [c], Xue-Chang Wu [b,*]

[a] Ocean College, Zhejiang University, Hangzhou 310058, Zhejiang Province, China
[b] Institute of Microbiology, College of Life Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, China
[c] State Key Laboratory of Motor Vehicle Biofuel Technology, Nanyang 473000, Henan Province, China
[d] Fujian Provincial Key Laboratory of Chronic Liver Disease and Hepatocellular Carcinoma, Xiamen University Affiliated Zhongshan Hospital, Xiamen 361004, Fujian Province, China

HIGHLIGHTS

- Industrial yeast strains were engineered for more intercellular trehalose content.
- The protective effect of trehalose depends on its content and the level of stress.
- Trehalose accumulation is more important for VHG fermentation than regular one.
- It had great potential for improving VHG performances of industrial yeast strains.

ARTICLE INFO

Article history:
Received 7 September 2013
Received in revised form 11 November 2013
Accepted 13 November 2013
Available online 21 November 2013

Keywords:
Saccharomyces cerevisiae
Trehalose
Very high gravity fermentation
Ethanol
Osmotic stress

ABSTRACT

The protective effect and the mechanisms of trehalose accumulation in industrial *Saccharomyces cerevisiae* strains were investigated during ethanol fermentation. The engineered strains with more intercellular trehalose achieved significantly higher fermentation rates and ethanol yields than their wild strain ZS during very high gravity (VHG) fermentation, while their performances were not different during regular fermentation. The VHG fermentation performances of these strains were consistent with their growth capacity under osmotic stress and ethanol stress, the key stress factors during VHG fermentation. These results suggest that trehalose accumulation is more important for VHG fermentation of industrial yeast strains than regular one. The differences in membrane integrity and antioxidative capacity of these strains indicated the possible mechanisms of trehalose as a protectant under VHG condition. Therefore, trehalose metabolic engineering may be a useful strategy for improving the VHG fermentation performance of industrial yeast strains.

1. Introduction

Microbe-based ethanol production is involved in large and diverse industries from alcoholic beverages to biofuel production. *Saccharomyces cerevisiae* remains the most exploited and primary microbe known to the industry for potable and industrial ethanol production. From an economic perspective, increasing the substrate concentration in an existing process is a cost-effective approach to increasing productivity (Puligundla et al., 2011). A substrate for ethanol fermentation typically contains 180–220 g/L total sugar and achieves an ethanol concentration of 10–14% (v/v) (Puligundla et al., 2011; Zhao and Bai, 2009), whereas very high gravity (VHG) fermentation uses at least 270 g/L total sugar, which makes it very attractive and promising for optimizing ethanol production (Puligundla et al., 2011).

During VHG fermentation, yeast cells are subjected to high osmotic pressure from the substrate sugar during the initial stages and strong ethanol inhibition during the production stage. The high osmotic pressure of sugar and the toxic effects of high ethanol concentrations are the key factors that lower yeast cell viability, as well as prolong and slow down fermentation, along with other stress factors (Puligundla et al., 2011). Elucidating the underlying mechanisms of the yeast stress response and resistance, as well as breeding stress-tolerant industrial yeast strains, could improve ethanol productivity and facilitate the use of biofuels in a wide range of applications (Kim et al., 2013).

The stressors encountered by yeasts during fermentation are associated with the suppression of macromolecule biosynthesis, denaturation of cytoplasmic proteins, reduction of glycolytic enzyme activity, alterations in membrane composition (Gibson

* Corresponding author. Tel.: +86 571 88206636; fax: +86 571 88206627.
E-mail address: mblab@zju.edu.cn (X.-C. Wu).
[1] These two authors contributed equally.

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.11.033

et al., 2007), disruption of membrane integrity (Hohmann, 2002), and increased production of reactive oxygen species (ROS) (Fierro-Risco et al., 2013). Yeast cells have evolved defense strategies against several types of stress damage. Factors that stabilize or repair denatured proteins in yeast cells, such as trehalose and heat shock proteins, are increased in stressed yeast cells (Zhao and Bai, 2009).

Trehalose, a well-known storage carbohydrate in bacteria, fungi, insects, and plants, functions as a potential stress protectant by preserving the integrity of the plasma membrane and stabilizing proteins (Mahmud et al., 2009). In yeasts, trehalose is synthesized by a large enzyme complex comprising four subunits, which are encoded by *TPS1*, *TPS2*, *TPS3*, and *TSL1* (Bell et al., 1998). Trehalose is hydrolyzed into glucose by neutral trehalases encoded by *NTH1* and *NTH2* or by an vacuolar acid trehalase encoded by *ATH1* (Nwaka and Holzer, 1998). Many laboratory yeast strains are genetically engineered to explore the correlation between trehalose content and stress resistance, such as heat stress (Ding et al., 2009), saline stress (Mahmud et al., 2009), freeze–thaw stress (Momose et al., 2010), ethanol stress (Bandara et al., 2009; Jung and Park, 2005; Kim et al., 1996), and various environmental stresses (Mahmud et al., 2010; Soto et al., 1999). Industrial yeast strains may differ from laboratory strains in the stress response because they commonly encounter more complex stress conditions simultaneously or sequentially and often are selected to have better and faster stress responses to ensure their superior ethanol fermentation performance (Zhao and Bai, 2009). However, the biological effects and protective mechanisms of trehalose accumulation of industrial yeast strains during VHG fermentation remain unknown. In the present paper, industrial yeast strains were engineered with high intracellular trehalose content through the overexpression of the trehalose-6-phosphate synthase gene *TPS1* and deletion of the acid trehalase gene *ATH1* to determine the protective effect and the mechanisms of trehalose accumulation in industrial yeast strains during fermentation. This study is the first to report the protective effect of trehalose depends on its content and the level of stress that industrial yeast strains were exposed to.

2. Methods

2.1. Strains and media

S. cerevisiae haploid strain ZS (MAT α) were derived from industrial strains (from Henan Tianguan Fuel Ethanol Co. Ltd.). The engineered strains in this study were engineered from the wild strain ZS. All strains unless otherwise noted were grown on YPD medium, which contains (m/v) 2% peptone, 1% yeast extract, and 2% glucose.

2.2. Genetic manipulation

To overexpress *TPS1* gene, its ORF was amplified by PCR and then cloned into the *Kpn* I and *Apa* I sites behind the *PGK* promoter of the pUG6-derived plasmid pUG6E (contains resistant gene *kanMX*). The *TPS1*-overexpression strain was obtained through the homologous recombination of the *Stu* I-linearized plasmid pUG6E-TPS1 and the *TPS1* gene in genome (Fig. 1a). The transformants were selected on YPD plate containing 300 μg/mL G418. The engineered haploid was named ZS*ptps1* (MAT α, *PGK-TPS1-kanMX*).

The *ath1* deletion strain was obtained by a one-step disruption of *ATH1*. The *ATH1* disruption cassette contains, from left to right, fragment *ATH1A* (500 bp upstream of the ATG start codon of *ATH1*), the *ble* gene, and fragment *ATH1B* (500 bp downstream of *ATH1*) (Fig. 1b). The strains ZS and ZS*ptps1* were transformed by the *ATH1* deletion cassette, respectively. The *ath1Δ* mutant strains were screened on YPD medium containing 50 μg/mL zeocin. The *ath1Δ* mutant strain was named ZS*Δath1* (MAT α, *ath1*Δ::*ble*), and the mutant strain with *TPS1* overexpression and *ath1* deletion was named ZS*pTΔA* (MAT α, *PGK-TPS1-kanMX, ath1*Δ::*ble*).

The yeast strains were transformed using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Proper integration was verified through diagnostic PCR and sequence analysis.

2.3. Fermentation and metabolites

Anaerobic fermentation was performed in Erlenmeyer flasks containing 200 g of converted mash at 33 °C (pH 5.0). The yeast cells were aerobically precultured in YPD for 15 h at 30 °C, and 0.05 g of cells (dry weight) was transferred to the fermentation media. Two fermentation conditions were used in the study, namely, regular fermentation with 160 g/L glucose and high gravity fermentation with 270 g/L glucose. Samples for metabolite measurement were immediately centrifuged, filtered through 0.45 μm filters, and stored at −20 °C until analysis. Glucose and ethanol concentrations were measured on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA), followed by a refractive index detector (Wang et al., 2012).

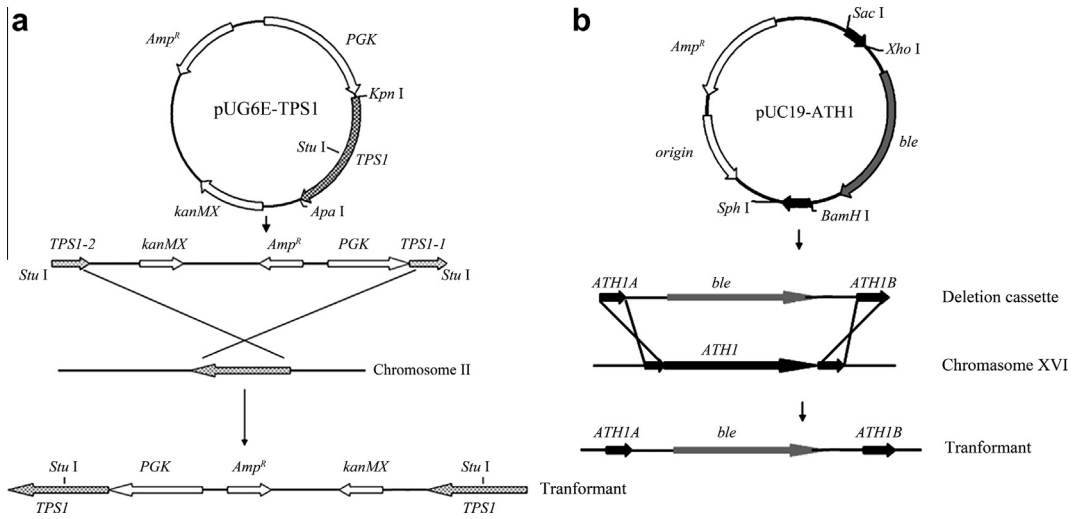

Fig. 1. The genetic manipulation in industrial *S.cerevisiae* stains (a) overexpression of *TPS1* and (b) deletion of *ATH1*.

2.4. Trehalose content

Trehalose was extracted from cells that were chilled and washed with cold 0.5 M trichloroacetic acid, and the trehalose concentration estimated using the anthrone method (Zheng et al., 2011a). Samples for dry weight analysis were washed with distilled water and dried at 80 °C for 12 h.

2.5. Protein extraction and enzyme assay

Proteins were released from yeast cells using Yeast Buster™ Protein Extraction Reagent according to the manufacturer's instructions (Novagen). BCA protein assay reagent (Pierce) was used to measure the protein concentration of the crude cell extract.

Yeast cells were cultivated in the YPD medium at 30 °C 200 rpm and harvested at stationary-phase for trehalose-6-phosphate synthase (TPS) and acid trehalase (ATH) enzyme assays. TPS and ATH activities were determined as reported previously (Tao et al., 2012). One unit of TPS activity was defined as the amount of enzyme that produces 1.0 μmol of NAD^+ per minute at 37 °C and pH 6.6. The activity of ATH was expressed as nmol of glucose liberated per min per mg total protein. The glucose concentration in the supernatant was measured using the glucose assay kit (Sigma–Aldrich).

Superoxide dismutase (SOD) activity was measured by inhibiting nitroblue tetrazolium reduction by O_2^- generated by the xanthine/xanthine oxidase system using a commercial assay kit (Nanjing Jiancheng Biological Products, Nanjing, China). One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition in 1 mL of reaction solution per mg of protein (Peng et al., 2009). The catalase (CAT) activity of the cell extract was assayed by measuring the decrease in A240 using H_2O_2 as a substrate in 0.05 M PBS (pH 7.0) at 25 °C. One unit of CAT activity was defined as the amount of enzyme that catalyzes the conversion of 1 mol of H_2O_2 at 25 °C within 1 min (Zheng et al., 2011a).

2.6. Cell viability, membrane integrity, and intracellular ROS accumulation

Stationary-phase yeast cells were collected by centrifugation, washed, and exposed to stress conditions in YPD medium at 30 °C for 3 h. Cells were collected by centrifugation, washed twice with 0.1 M PBS (pH 7.4), and resuspended to adjust the OD_{600} 0.1. Then, the cells were stained with both 20 μg propidium iodide (PI)/mL and a fluorogenic dye [10 μg of fluorescein diacetate (FDA)/mL or 10 μg of dichlorodihydro fluorescent diacetate (DCFH-DA)/mL] at 30 °C for 1.5 h (Zheng et al., 2011b). Cell viability and membrane integrity, labeled with FDA and PI, and ROS accumulation, labeled by DCFH-DA, were observed and imaged under a laser scanning confocal microscope. Three fields of view from each coverslip were randomly chosen, and relative membrane integrity was calculated using the method of Wei et al. (2008).

2.7. Statistical analysis

Results were analyzed via one-way ANOVA. Post hoc analysis was performed via a least significance difference and a Duncan test statistical analysis using the SPSS 13.0 software (Erten and Tanguler, 2010).

3. Results and discussion

3.1. More trehalose accumulation in engineered strains

Previous reports successfully engineered laboratory yeast strains via the overexpression and/or deletion of trehalose metabolic genes (*TPS1*, *TPS2*, *TPS3*, *TSL1*, *ATH1*, *NTH1*, and *NTH2*) to investigate the protective function of trehalose under stress conditions (Bandara et al., 2009; Garre et al., 2009; Jules et al., 2008; Jung and Park, 2005; Kim et al., 1996; Mahmud et al., 2009, 2010; Pereira et al., 2001; Soto et al., 1999). The previous study also found that several genes associated with trehalose metabolism in industrial yeast strains were highly expressed (*TPS1*, *TPS2*, *TPS3*, *TSL1*, *ATH1*, and *NTH1*) under ethanol stress (Hirasawa et al., 2001). Among these genes, *TPS1* and *ATH1* were the most highly expressed synthetic and degradation genes, respectively. Therefore, the industrial ZS strain was engineered by overexpressing *TPS1* and/or deleting *ATH1* to improve the intercellular trehalose accumulation. Unlike most methods for modifying trehalose metabolism in yeast strains (Jules et al., 2008; Mahmud et al., 2009, 2010), the method in this study relied on the use of G418- and Zeocin-resistance markers (*kanMX* and *ble*) (Fig. 1) to avoid the use of auxotrophic markers, which are difficult to obtain from industrial strains and will probably affect the ethanol yield (Guo et al., 2009).

To demonstrate the effects of genetic manipulation, the enzyme activities of TPS and ATH and the intercellular trehalose content between wild strain ZS and its engineered strains were compared. As expected, the engineered strain ZS*ptps1* had a higher TPS activity, strain ZS*Δath1* had a lower ATH activity, and strain ZS*pTΔA* had a higher TPS and a lower ATH activity, compared with its wild strain ZS (Fig. 2). Accordingly, the three engineered strains accumulated more trehalose than the wild strain ZS (Table 1). Moreover, before stress induction, the three engineered strains had higher trehalose content than the wild strain ZS (Table 1). Among these engineered strains, the strain ZS*pTΔA* exhibited the highest increase in trehalose content, which indicates the significant effect of *TPS1* overexpression combined with *ATH1* deletion in improving trehalose accumulation of industrial strains.

3.2. Fermentation performance and trehalose accumulation of the engineered strains

Numerous reports have focused on intracellular accumulation of trehalose in laboratory yeast strains under various stress conditions (Ding et al., 2009; Mahmud et al., 2009, 2010). Compared with laboratory strains, industrial strains are generally more adaptable to specific environments (Amillastre et al., 2012; Zhao and Bai, 2009; Zheng et al., 2012). However, the biological effects and protective mechanism of the trehalose accumulation of industrial yeast strains during VHG fermentation are still unreported. Therefore, the fermentation performances and intercellular treha-

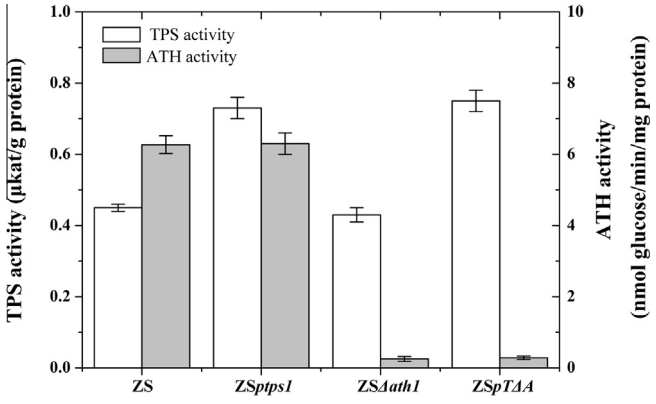

Fig. 2. TPS and ATH activity in the wild strain ZS and its engineered strains.

Table 1
Intracellular trehalose content of *S. cerevisiae* strain ZS and its engineered strains under different conditions.

Strains	Trehalose[a] (mg/g dry weight)		
	YPD	30% Glucose	20% Ethanol
ZS	11.3 ± 0.35	39.3 ± 0.38	29.5 ± 0.33
ZS*ptps1*	15.6 ± 0.27	47.9 ± 0.42	34.6 ± 0.30
ZS*Δath1*	14.9 ± 0.31	48.6 ± 0.24	35.3 ± 0.29
ZS*pTΔA*	18.9 ± 0.32	55.7 ± 0.26	40.2 ± 0.25
P[b]	0.00	0.00	0.00

[a] Strains were precultured in YPD medium to stationary-phase, and cells were collected and cultured in YPD with or without the stress factors at 30 °C with 200 rpm for 1 h.
[b] $P < 0.05$ indicates there are statistically significant differences between the four strains, while $P > 0.05$ indicates there is no significant difference between the four strains.

lose accumulation of the wild strain ZS and its engineered strains were investigated.

As shown in Fig. 3a, no significant difference was observed between the wild ZS strain and the engineered strains under regular fermentation condition with 160 g/L glucose. The residual glucose concentrations of all the strains were less than 2 g/L after the fermentation finished at 45 h (Fig. 3a). Under the VHG condition with 270 g/L glucose, the fermentation rate of all the strains decreased. By contrast, the fermentation rates of the three engineered strains ZS*ptps1*, ZS*Δath1*, and ZS*pTΔA* were significantly higher than that of the wild ZS strain. The ethanol yields of the three engineered strains ZS*ptps1*, ZS*Δath1*, and ZS*pTΔA* were higher by (8.7 ± 0.8)%, (7.4 ± 0.6)%, and (11.1 ± 0.9)%, respectively, compared with that of the wild strain ZS (Fig. 3b).

The intracellular trehalose content of the engineered strains was detected during regular (Fig. 3c) and VHG (Fig. 3d) fermentation. The intracellular trehalose content of all the strains rapidly increased with time, peaking at 20 h under regular fermentation and at 30 h under VHG fermentation. Interestingly, the intracellular trehalose content of strains was increased again at the end of VHG fermentation (Fig. 3d), which may be related to the higher ethanol concentrations in the VHG fermentation medium. During the whole fermentation process, the intracellular trehalose content of the engineered strains was higher than that of the ZS strain. Trehalose accumulated in the ZS*pTΔA* up to (13.2 ± 0.3)% of the dry cell weight under regular fermentation and (23.2 ± 0.2)% under VHG fermentation conditions, which were higher by (25.7 ± 1.5)% and (30.5 ± 1.3)%, respectively, compared with those in the strain ZS.

The cycle of trehalose biosynthesis and degradation is active under stress conditions (Blomberg, 2000; Kaino and Takagi, 2008). Compared with regular YPD and regular fermentation, the intracellular trehalose content of the original strain ZS and its engineered strains all increased under high osmotic stress, ethanol stress, and VHG fermentation conditions (Table 1, Fig. 3c and d). This result suggests that this futile cycle is highly activated in industrial strains under VHG fermentation conditions, which is similar to previous reports under stress conditions (Blomberg, 2000; Kaino and Takagi, 2008).

3.3. Stress tolerance of engineered strains

During the ethanol fermentation process, yeast cells are exposed to high osmotic stress at the initial stage and then to high ethanol concentration (Wang et al., 2012; Zheng et al., 2011a). Previous studies reported that intercellular trehalose has a protective function in the survival of *S. cerevisiae* when exposed to stressful environments, such as heat and hyperosmotic pressure (Bandara et al., 2009; Zheng et al., 2011a). Consistent with previous studies (Bandara et al., 2009; Jung and Park, 2005; Kim et al., 1996; Pereira et al., 2001; Soto et al., 1999), the engineered industrial strains *ZSptps1*, *ZSΔath1*, and *ZSpTΔA* had significantly better growth performance under osmotic stress and ethanol stress compared with the wild strain ZS (Fig. S1 in Supplementary file).

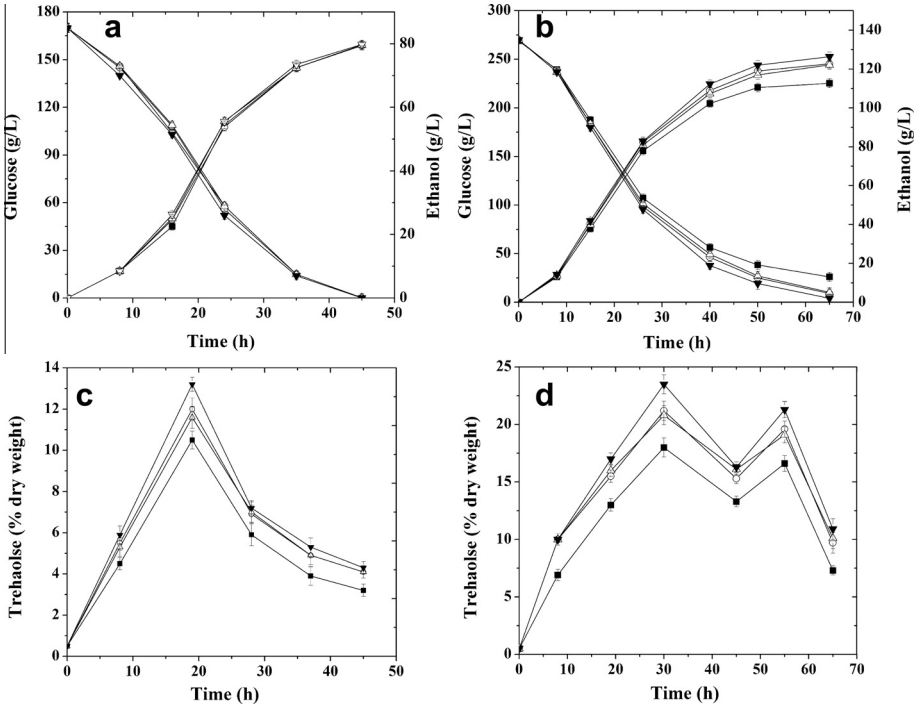

Fig. 3. Fermentation performance and intracellular trehalose content of the parent strain ZS (■) and the engineered strains ZS*ptps1* (○), ZS*Δath1* (△), and ZS*pTΔA* (▼) under regular (a, c) and very high gravity (VHG) conditions (b, d).

Moreover, the engineered strain *ZSpTΔA* showed the best growth performance under both stress conditions, which is consistent with it accumulating the highest intracellular trehalose content (Table 1). Under VHG fermentation conditions, the improvement in fermentation performance (including fermentation rate, ethanol yield, and residual glucose) of these strains was consistent with their intracellular trehalose content, similar to their performances under stress conditions (Table 1 and Fig. S1 in Supplementary file). No significant difference was observed among these strains under non-stress conditions (Fig. S1 in Supplementary file) or regular fermentation with lower level stress (compared with the stress from VHG fermentation; Fig. 3a) although their intercellular trehalose content was different (Table 1 and Fig. 3c). The results of tolerance and fermentation test in these industrial strains showed the effectiveness of trehalose as a protectant depends on its content and the level of stress that industrial yeast strains were exposed to. These findings are consistent with those of Bandara et al. (2009), which involved laboratory yeast strains under ethanol stress and the stress level-dependent nature of the effects of trehalose.

3.4. Cell viability and membrane integrity of the engineered ZSpTΔA strain under stress conditions

Previous studies indicated that stress factors gradually reduce cell viability by affecting cell membrane integrity and function (Ding et al., 2009). To determine the mechanism underlying the protective function of trehalose during VHG fermentation, the cell viability and membrane integrity between the wild strain ZS and its engineered strain *ZSpTΔA* were compared under stress conditions.

The ZS and *ZSpTΔA* strains were stained with FDA/PI after exposure to the high osmotic stress (3 M NaCl) and ethanol stress (20% v/v) treatments for 2 h. The viable cells were stained green with FDA and the cells that lost cell viability and membrane integrity were stained red with PI (Fig. S2a in Supplementary file). The cell viability and membrane integrity of the ZS cells were inferior to those of the *ZSpTΔA* strain after stress treatment. The viable cells of ZS and *ZSpTΔA* were (37.9 ± 1.2)% and (68.9 ± 2.4)%, respectively, after ethanol stress treatment. The viable cells of *ZSpTΔA* were 1.8 times higher than those of ZS, which indicate that the ethanol tolerance of the *ZSpTΔA* strain was significantly higher than that of the ZS strain. These results demonstrate that the engineered *ZSpTΔA* strain maintains cell viability and membrane integrity under stress conditions better than the wild ZS strain.

3.5. Antioxidative capacity of the engineered ZSpTΔA strain under stress conditions

ROS derived from exposure to ethanol, heat, and osmotic stress, appear to have a common function in stress-induced damage (Kim et al., 2013). Yeast cells generate antioxidant defenses, including CAT and SOD, under different types of stresses (Zheng et al., 2011a). ROS accumulations as well as CAT and SOD enzyme activities of the wild strain ZS and its engineered strain *ZSpTΔA* were compared after high osmotic (3 M NaCl) and ethanol (20% v/v) stress treatments for 2 h. The intercellular ROS of yeast cells was stained green with DCFH-DA (Fig. S2b in Supplementary file). Both ZS and *ZSpTΔA* accumulated more intercellular ROS after stress treatment than strains under YPD conditions without stress treatment. Under high osmotic (3 M NaCl) and ethanol (20% v/v) stress treatments, (24.4 ± 0.8)% and (86.6 ± 2.2)% of ZS yeast cells accumulated ROS and (19.0 ± 0.6)% and (52.1 ± 1.4)% of *ZSpTΔA* cells accumulated ROS, respectively ($P < 0.05$).

Moreover, the CAT and SOD enzyme activity of both strains were determined after the high osmotic stress (3 M NaCl) and ethanol stress (20% v/v) treatments for 2 h. After stress treatment, the CAT and SOD activities of the ZS and *ZSpTΔA* strains were significantly higher than those without stress treatment. Without stress treatment, the CAT and SOD activity of the ZS strain did not significantly differ from those of the *ZSpTΔA* strain. However, after stress treatment, the CAT and SOD activity of the *ZSpTΔA* strain were significantly higher than those of the ZS strain (Fig. 4). The CAT activity of the *ZSpTΔA* strain under high osmotic stress was (20.3 ± 2.3)% higher than that of the ZS strain and (17.9 ± 2.2)% higher than that of the ZS strain under ethanol stress ($P < 0.05$). Similarly, the SOD activity of the *ZSpTΔA* strain under high osmotic stress was (12.1 ± 1.2)% higher than that of the ZS strain under high osmotic stress and (19.2 ± 2.2)% higher than that of the ZS strain under ethanol stress ($P < 0.05$). The higher CAT and SOD activities of *ZSpTΔA* were consistent with their higher trehalose accumulation.

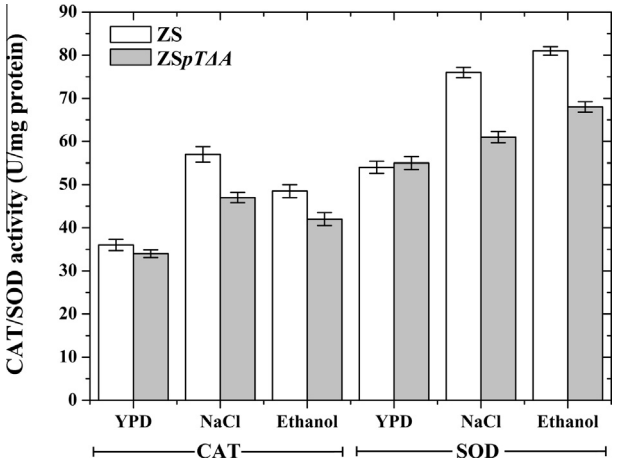

Fig. 4. CAT and SOD activity in the wild strain ZS and its engineered strain *ZSpTΔA* in response to 3 M NaCl and to 20% ethanol (v/v).

These results indicate that the protective function of intracellular trehalose for yeast cells during VHG fermentation may depend on maintaining membrane stability and improving the activity of key enzymes. Trehalose likely stabilizes proteins and the plasma membrane by replacing water and by hydrogen bonding with the polar residues of the protein (Hottiger et al., 1987) or by forming a glass-like structure (vitrification) to surround proteins (Ding et al., 2009) during industrial yeasts VHG fermentation. However, further studies are needed to determine whether trehalose functions as an antioxidant to decrease ROS under stress.

4. Conclusion

This study investigates the protective effect and mechanisms of trehalose in industrial yeast strains during ethanol fermentation. The results show that inducing trehalose accumulation is more important for VHG than regular fermentation in industrial yeast strains. The VHG fermentation performances of the strains with different trehalose contents were consistent with their growth capacity under osmotic and ethanol stresses, the key stressors during VHG fermentation. The differences in membrane integrity and antioxidative capacity of these strains indicated the possible mechanisms of trehalose as a protectant during VHG fermentation. Trehalose metabolic engineering may be a potential strategy for improving industrial VHG fermentation.

Acknowledgements

This work was financially supported by the Scientific Research Fund of Zhejiang Provincial Education Department (Y201327852),

State Key Laboratory of Motor Vehicle Biofuels Technology of China (2013013), Postdoctoral Science Foundation of China (2013M531450), and Postdoctoral Science Foundation of Zhejiang province (BSH1301026).

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.2013.11.033.

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