Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2018

www.elsevier.com/locate/jbiosc

Gene expression profiles of *Candida glycerinogenes* under combined heat and high-glucose stresses

Fei Yang,[1,2] Xinyao Lu,[1,2] Hong Zong,[1,2] Hao Ji,[1,2] and Bin Zhuge[1,2,*]

The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China[1] *and The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China*[2]

Received 10 January 2018; accepted 6 April 2018
Available online xxx

Low cell tolerance is a basic issue in high-glucose fermentation under high temperature to economically obtain high product titer. *Candida glycerinogenes*, an industrial yeast, has excellent tolerance to the combined heat and high-glucose stress than *Saccharomyces cerevisiae*. The potential mechanism responsible for the high tolerance was illustrated here. The transcription of the potential stress-responsive genes in two strains were varied under single stress (heat or high-glucose), especially the ribosome-related genes. Unlike *S. cerevisiae*, *C. glycerinogenes* up-regulated 17 genes, including most of the single stress responsive genes, and genes *Avt1* and *Pfk1* under the combined stress, indicating a more systematic stress-responsive system in *C. glycerinogenes*. Further down-regulating the 17 potential key responsive genes indicated that genes *Dip5*, *Gpd1*, *Pfk1*, *Hxt4*, *Hxt6*, and *Ino4* are important for cell tolerance to the combined stress. Furthermore, most of the ribosomal function related genes, such as *Mrt4*, *Nug1*, *Nop53*, *Rpa190*, *Rex4*, and *Nsr1*, play important role in cell tolerance. Therefore, the wider responsive gene spectrum and the activated expression of ribosomal function related genes might be key and prerequisite factors for the excellent tolerance to the combined stress of *C. glycerinogenes*.

[**Key words:** *Candida glycerinogenes*; Expression profiles; Heat; High-glucose; Stress response]

Fermentation under the conditions of high temperature and high concentration glucose is a promising technology, increasing product titer, minimizing or even avoiding microbial contamination, reducing the utilizations of cooling power and cooling water, and enabling simultaneous saccharification and fermentation (1). Cells have to encounter hyperosmotic and heat stresses simultaneously generated by high glucose concentration and high temperature. However, most microorganisms show poor tolerance to combined hyperosmotic and heat stress. Although many genetic engineering related studies have been done to improve cells tolerance to these stresses, the improvement of stress tolerance in microorganism was still unsatisfied.

Actually, most stress tolerance related studies were focused on the model yeast *Saccharomyces cerevisiae* which shows low tolerance to this combined stress. *Candida glycerinogenes* is an excellent industrial yeast used for the commercial glycerol production over 20 years in China (2–4). It can survive in 550 g/L glucose and also shows remarkable performance under high temperature. Notably, it also has salient ability to tolerate the combined stress of heat and high glucose concentration over *S. cerevisiae*. Understanding of cellular mechanisms required for protecting cells from the combined stress conditions is essential for the improvement of the stress tolerance in yeast. The main objective of this study was to investigate the response mechanisms responsible for the combined stress tolerance in *C. glycerinogenes*.

Previous studies have shown that the osmotic or thermal tolerance involve in regulation of metabolic enzymes, heat shock proteins (HSPs), compatible solutes accumulation, and most ribosomal protein (RP) genes (5–8). In this study, the expression levels of the genes involved in compatible solutes (trehalose, glycerol, lysine and dicarboxylic amino acid) syntheses and transportations, HSPs, glucose metabolism, membrane lipid metabolism, and ribosomal functions were investigated in *C. glycerinogenes* and *S. cerevisiae* exposed to various stresses. The expression levels of potential critical responsive genes were then down-regulated to further understand their detail roles in tolerance to the combined stress.

MATERIALS AND METHODS

Strains, media and plasmids The strains used here are listed in Supplementary Table S1. *S. cerevisiae* ZWA46 and *C. glycerinogenes* UA5 (9) were stored in our lab and activated prior to each experiment in YEPD medium (10 g/L yeast extract, 20 g/L tryptone and 20 g/L glucose). *E. coli* JM109 used for plasmid construction was grown in LB medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl). The plasmids used here are listed in Supplementary Table S2.

Cell culture and treatment Yeast cells were pre-cultivated in YEPD medium at 30 °C and 200 rpm for 14 h. The pre-incubated cells were inoculated into 100 mL YEPD medium (250 mL-Erlenmeyer flask) containing 250 g/L glucose at 42 °C under 100 rpm for ethanol production. Cells were also inoculated equally and cultivated in YEPD medium under standard conditions (20 g/L glucose, 30 °C) and stress conditions (250 g/L glucose, 42 °C), respectively, for tolerance analysis. All the

* Corresponding author at: The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China. Tel./fax: +86 0510 85918150.

E-mail addresses: 1242666736@qq.com (F. Yang), 370801962@qq.com (X. Lu), 275527376@qq.com (H. Zong), 417720438@qq.com (H. Ji), bzhuge@126.com (B. Zhuge).

1389-1723/$ – see front matter © 2018, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2018.04.006

cultivations starting with an OD_{600} of 0.25. Cells were collected by centrifugation at 5000 ×*g* for 1 min from the seed culture and then incubated in YEPD medium with different glucose concentrations and temperatures for qRT-PCR analysis, and intracellular chemicals quantification.

RNA preparation and qRT-PCR assays The cell samples were harvested after treatment and used for the total RNA extraction by Trizol reagent (BBI, Toronto, Canada). One microgram RNA from each sample was used to synthesize the cDNA by using Hiscript II Q RT SuperMix (Vazyme, Nanjing, China). Quantitative PCR were performed by using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). Primers used for Quantitative RT-PCR are shown in Supplementary Tables S3 and S4. Operational procedures were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s and a final heating cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. In *C. glycerinogenes* and *S. cerevisiae*, all the operations were performed in triplicate and the housekeeper gene 18S rDNA and *ACT1* were used as the internal reference, respectively.

Genetic manipulation The plasmids construction here is shown in Fig. S1. Briefly, the *GFP* coding region was cloned from commercial plasmid pCAMBIA1302 and then inserted into plasmid pURGAP, generating pURGAP-*GFP*. The transcription terminator amplified from pURGAP was inserted into the plasmid pURGAP-*GFP*, generating pURGAP-*GFP*-tT. The promoter P_{GAP} inserted into the plasmid pMD19-T (Takara, Dalian, China) to generate the vector pMD19-T-P_{GAP}. The antisense DNA which is reverse complementary DNA sequence of the −350 bp to the 350 bp of P_{GAP}-*GFP* were amplified from the plasmid pURGAP-*GFP* and then cloned into pMD19-T-P_{GAP}, obtaining the plasmid pMD19-T-P_{GAP} -antisense *GFP*. The DNA fragment P_{GAP} -antisense *GFP* were digested and inserted into the plasmid pURGAP-*GFP*-tT, resulting in the pURGAP-*GFP*-P_{GAP} -antisense *GFP* (Fig. S1A) for analyzing the effectiveness of antisense RNA technique. Similarly, the antisense DNAs of endogenous target genes were amplified from the genomic DNA of *C. glycerinogenes* and then inserted into pURGAP to generate different recombinant plasmids (Table S2, Fig. S1B).

The expression vector pURGAP, using *ura5* as a selection marker, was described in our previous study (2). Primers used for PCR shown in Supplementary Table S5. The obtained recombinant plasmids were linearized and integrated into the chromosome of *C. glycerinogenes* as described previously (9) to obtain the different recombinant yeasts (Table S1).

Analytical methods The biomass was represented by the cell dry weight. The contents of ethanol and glucose were determined by high performance liquid chromatography (HPLC) equipped with a refractive index detector (RI-2031Plus, JASCO, Tokyo, Japan) using an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The HPLC apparatus was operated at 60 °C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and 0.02 mL injection volume.

For the quantitation of intracellular amino acids, trehalose and glycerol, the samples were collected by centrifugation, washed and suspended in 1 mL of normal saline. The obtained samples were incubated in boiling water for 10 min to extract intracellular compounds (10). After centrifugation (10 min at 16,000 ×*g*), according to the instruction of the manufacturers, intracellular contents quantified with a trehalose assay kit (Megazyme International Ireland, Wicklow, Ireland) for trehalose, and a glycerol assay kit (Applygen, Beijing, China) for glycerol, an amino acid analyzer (L-8900; Hitachi, Tokyo, Japan) for amino acids.

Fluorescence microscopy UA5/*GFP* and UA5/*GFP*-anti *GFP* strains were grown in liquid YEPD medium containing 20 g/L glucose at 30 °C and 200 rpm. Cells were taken at set intervals and viewed under the microscope after washed with PBS (Excitation wavelength 488 nm, emission wavelength 520 nm). All slides were viewed with a Leica SP8 Confocal System (TCS SP8, Leica, Wetzlar, Germany).

RESULTS AND DISCUSSION

Cell growth and ethanol production under combined heat and high-glucose stress conditions Under aerobic fermentation conditions, the final biomass of *C. glycerinogenes* were about 1.5- and 1.7-fold of that of *S. cerevisiae* in the medium containing 20 g/L glucose at 30 °C and 250 g/L glucose at 42 °C, respectively (Fig. 1). The obvious growth lag phase and decreasing of biomass (24%) of *S. cerevisiae* were observed. In contrast, *C. glycerinogenes* exhibited shorter lag phase and lower biomass reduction (12%) under combined stress (Fig. 1). Meanwhile, the combined stress also significantly suppressed logarithmic growth of *S. cerevisiae* but showed slightly impact on that of *C. glycerinogenes* (Fig. 1). These results indicated that *C. glycerinogenes* has prominent tolerance to the combined stress.

In micro-aerobic fermentation with combined stress conditions, the cell growth and ethanol titer of *C. glycerinogenes* were 1.2- and 1.6-fold of that of *S. cerevisiae*, respectively (Fig. S2). Besides, the

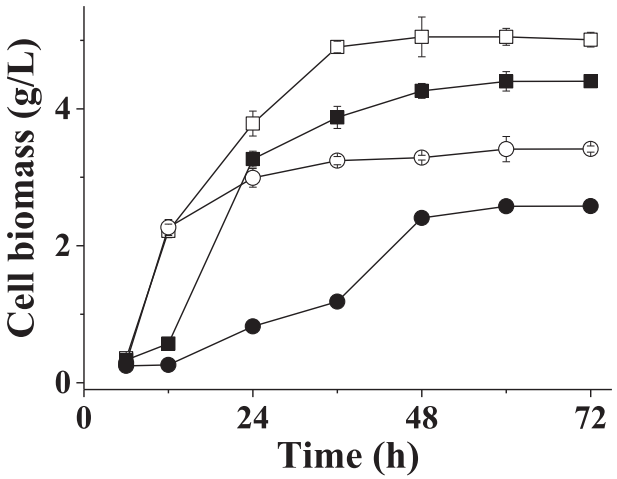

FIG. 1. Cell growth of *C. glycerinogenes* (squares) and *S. cerevisiae* (circles). Yeast strains were inoculated into a 250 mL-Erlenmeyer flask containing 50 mL YEPD with total 20 g/L glucose at 30 °C (open symbols) and with total glucose at 250 g/L at 42 °C (closed symbols), starting with an OD_{600} of 0.25 at 200 rpm. The results are expressed as the mean ± SD of three independent experiments.

maximum specific growth rate and maximum ethanol productivity rate of *C. glycerinogenes* were 1.27 h^{-1} and 3.15 g^{-1} L h^{-1}, respectively, while that of *S. cerevisiae* just reached 0.33 h^{-1} and 2.6 g^{-1} L h^{-1}, respectively. Moreover, the maximum glucose consumption rate of *C. glycerinogenes* was higher than that of *S. cerevisiae*, reached 7.62 g^{-1} L h^{-1}, suggesting that *C. glycerinogenes* consumed glucose more efficiently. These results demonstrated that *C. glycerinogenes* showed better performance on ethanol production under combined stress conditions, thus has extensive application prospect. Therefore, the understanding of the tolerance mechanism is necessary.

Gene expression levels of *C. glycerinogenes* in response to heat or high-glucose stress To elucidate the potential mechanism of the higher stress tolerance of *C. glycerinogenes*, we compared the expression levels of genes involved in the biosyntheses and transportations of compatible solutes (trehalose, amino acids, glycerol), HSPs, membrane lipid metabolism, glucose metabolism, ribosome biogenesis of *C. glycerinogenes* and *S. cerevisiae* under heat or high-glucose stresses.

Trehalose, an important compatible solute, was proved to be accumulated intracellularly to protect the structures of proteins and lipids under heat stress (7,11). The expression levels of the trehalose synthesis genes *Tps1* and *Tps2* was up-regulated in *S. cerevisiae* in response to heat stress (Fig. 2A), agreeing with the previous studies (7,12). However, the transcription levels of these trehalose synthesis genes in *C. glycerinogenes* were not up-regulated (Fig. 2A), implying the different roles of trehalose in heat stress tolerance. *C. glycerinogenes* might up-regulate the lysine synthesis genes *Lys21* (Fig. 2A) and the dicarboxylic amino acids permease gene *Dip5* (Fig. 2B) to accumulate lysine and dicarboxylic amino acids against heat stress and high-glucose stress, respectively, which is different to *S. cerevisiae*.

The high-glucose stress showed obviously positive regulations on the transcription levels of the glycerol synthesis gene *Gpd1* in both *S. cerevisiae* and *C. glycerinogenes* (Fig. 2B). Glycerol is the most important intracellular compatible solute to maintain the osmotic balance when cells were exposed to high external osmolarity (5). However, glycerol accumulation is not required for *C. glycerinogenes* under moderate osmolarity (<200 g/L glucose). In contrast, the intercellular glycerol gradually enhanced with the increase of osmotic stress (>200 g/L glucose) (Fig. S3). These results suggested the existence of different osmotic response mechanisms in

Please cite this article in press as: Yang, F., et al., Gene expression profiles of *Candida glycerinogenes* under combined heat and high-glucose stresses, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.04.006