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ORIGINAL ARTICLE

Differences between flocculating yeast and regular industrial yeast in transcription and metabolite profiling during ethanol fermentation



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Abstract *Objectives:* To improve ethanolic fermentation performance of self-flocculating yeast, difference between a flocculating yeast strain and a regular industrial yeast strain was analyzed by transcriptional and metabolic approaches. *Results:* The number of down-regulated (industrial yeast YIC10 vs. flocculating yeast GIM2.71) and up-regulated genes were 4503 and 228, respectively. It is the economic regulation for YIC10 that non-essential genes were down-regulated, and cells put more “energy” into growth and ethanol production. Hexose transport and phosphorylation were not the limiting-steps in ethanol fermentation for GIM2.71 compared to YIC10, whereas the reaction of 1,3-disphosphoglycerate to 3-phosphoglycerate, the decarboxylation of pyruvate to acetaldehyde and its subsequent reduction to ethanol were the most limiting steps. GIM2.71 had stronger stress response than non-flocculating yeast and much more carbohydrate was distributed to other bypass, such as glycerol, acetate and trehalose synthesis. *Conclusions:* Differences between flocculating yeast and regular industrial yeast in transcription and metabolite profiling will provide clues for improving the fermentation performance of GIM2.71.

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1. Introduction

Bioethanol production by *Saccharomyces cerevisiae* is currently, by volume, the single largest fermentative process in

industrial biotechnology. The major portion of total expenditure in today's bioethanol industry is allotted to feedstock costs (Galbe et al., 2007). A global research effort is under way to expand the substrate range of *S. cerevisiae* to include nonfood feedstocks, such as Jerusalem artichoke. Jerusalem artichoke (*Helianthus tuberosus* L.) can grow well in non-fertile land and is resistant to frost, drought, salt-alkaline and plant diseases (Yu et al., 2011). It is superior to the other inulin-accumulating crops in terms of its output of biomass production, inulin content, and tolerance of a relatively wide range of environmental conditions. The tuber yield of Jerusalem artichokes can be up to 90 t/ha resulting in 5–14 t carbohydrates/ha (Stephe et al., 2006). Besides its economic value, it

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also has a function of soil remediation, such as salt adsorption. To date, Jerusalem artichoke has predominantly been cultivated in North America, Northern Europe, Korea, Australia, New Zealand and China (Li et al., 2013). The principle storage carbohydrate of Jerusalem artichoke is inulin, which consists of linear chains of β -2, 1-linked D-fructofuranose molecules terminated by a glucose residue. It preserves carbohydrate in a 9:1 average ratio of fructose to glucose. Improving of fermentation performance with Jerusalem artichoke would have significant impacts on profits in large scale ethanol production.

Flocculating yeast separated from fermentation broth by self-flocculating at the end of fermentation and was re-used in consecutive fermentation, and therefore high density cell was obtained without increasing operating costs. High density cells exponentially shortened the fermentation time and increased cells resistance to ethanol stress (Li et al., 2009a). This work provides the first demonstration of the differences in transcriptic and metabolic profiles between flocculating yeast and regular industrial yeast. The result will provide clues to improve fermentative performance of flocculating yeast.

2. Materials and methods

2.1. Strain and cell culture

Industrial *S. cerevisiae* YIC10 is presented by Bincheng alcohol company (Shandong Province, China), self-flocculating *S. cerevisiae* GIM2.71 is obtained from Guangdong Microbiology Culture Center. Yeasts were grown overnight before inoculated in fresh medium (1% yeast extract, 2% peptone, 0.4% glucose, 3.6% fructose, ratio of fructose/glucose is 9 in order to stimulate hydrolysates of Jerusalem artichoke) to an initial OD₆₀₀ of 0.1. Samples for microarray analysis were collected at exponential growth phase (7 h) and total RNA was then isolated. Samples for monitoring cell growth and fermentation were taken at 0, 2, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 21 and 23 h.

2.2. RNA extraction

After the sample was taken, it was immediately centrifuged at 4000 rpm for 3 min at 4 °C, the cells were then stored in liquid nitrogen until total RNA was extracted. Total RNA was extracted using Yeast RNAiso Kit (TaKaRa, Japan) after partially thawing the samples on ice, and RNA was purified using NucleoSpin Extract II kits (Machery-Nagel, Germany) according to the manufacturers' instructions. Then total RNA was assessed by formaldehyde agarose gel (1.2%, w/v) electrophoresis and was quantitated spectrophotometrically ($A_{260\text{ nm}}/A_{280\text{ nm}} \geq 1.80$).

2.3. DNA microarray assays

An aliquot of 2 μ g of total RNA was used to synthesize double-stranded cDNA, and cDNA was used to produce biotin-tagged cRNA by MessageAmp™ II aRNA Amplification Kit (Ambion, USA). The resulting biotin-tagged cRNA were fragmented to strands of 35–200 bases in length according to the protocols from Affymetrix. The fragmented cRNA was hybridized to Affymetrix GeneChip Yeast Genome 2.0

Arrays. Hybridization was performed at 45 °C using Affymetrix GeneChip Hybridization Oven 640 for 16 h. The GeneChip arrays were washed and then stained by Affymetrix Fluidics Station 450 followed by scanning with Affymetrix GeneChip Scanner 3000.

2.4. Microarray data processing

Hybridization data were analyzed using Affymetrix GeneChip Command Console Software. An invariant set normalization procedure was performed to normalize different arrays using DNA-chip analyzer 2010 (<http://www.dchip.org>, Harvard University). A multiclass method for analysis of microarray software (Significant Analysis of Microarray method, developed by Stanford University) was used to identify significant differences. Genes with false discovery rate <0.05 and a fold-change >2 were identified as differentially expressed genes. Differentially expressed genes were clustered hierarchically using Gene Cluster 3.0 (Stanford University). Gene ontology (GO) analysis of differentially expressed genes was done with DAVID (<http://david.abcc.ncifcrf.gov/list.jsp>).

2.5. Real-Time quantitative PCR

Based on microarray results, seven genes (*HXT1-7*) were selected for quantitative transcription analysis. The primers used in RT-qPCR analyses are listed in Table 1. Real-Time quantitative PCR (RT-qPCR) was performed according to the method described by Ye et al. (2009). *ACT1* was used as an internal reference for normalizing gene expression (Liu et al., 2007).

2.6. Metabolites preparation and analysis

Intracellular and extracellular metabolites including glucose, fructose, ethanol, glycerol, acetate and trehalose were prepared by methods reported by our previous study (Li et al., 2009b). Samples were analyzed by a high-performance liquid chromatography (HPLC, Waters, USA) system with an Aminex HPX-87H column (Bio-Rad), 2414 refractive index detector

Table 1 Genes and primers used in RT-qPCR.

Gene	ID	Primer Sequence 5' → 3'	Amplicon (bp)
<i>ACT1</i>	F	AACATCGTTATGTCCGGTGGT	144
	R	ACCACCAATCCAGACGGAGTA	
<i>HXT1</i>	F	GTTGCTTTCGGTGGTTTCAT	101
	R	TCGTGGTGCTTCATACCAAA	
<i>HXT2</i>	F	ATTCGCTACTAGCCCGT	140
	R	TTGGCTTTGCTGGGAGTCA	
<i>HXT3</i>	F	GGCCGACCAAGTACTTACCA	85
	R	ACCGAAGGCAACCATAACAC	
<i>HXT4</i>	F	TACCGTTTTCACTGCTGTCC	145
	R	GGAAGCAGCACCCATAATA	
<i>HXT5</i>	F	TCTGAAGTGTCCCTAAGCA	139
	R	ATGGTACCCTCCATTGGACA	
<i>HXT6/7</i>	F	GGGCTGTTTGGTCTTCATGT	94
	R	TTCTTCCACATGGTGTGA	

and 515 HPLC pump. Column was kept at 50 °C and 5 mM H₂SO₄ was used as eluent at a flow rate of 0.5 ml/min.

3. Results and discussion

3.1. Fermentation behavior

YIC10 was superior to GIM2.71 in cell growth rate, sugar consumption and ethanol production performance (Fig. 1). YIC10 and GIM2.71 reached their highest ethanol yield at 12 h (16.2 g/L) and 21 h (16.0 g/L), respectively. Both strains showed indeed a similar behavior in terms of ethanol yield.

3.2. Overview and GO analysis of microarray data

Microarray analysis showed that the number of down-regulated (YIC10 vs. GIM2.71) and up-regulated genes were 4503 and 228, respectively. It is the economic regulation for YIC10 that non-essential genes were down-regulated, and cells put more “energy” into growth and ethanol production. GO analysis was carried out with the up-regulated genes and the significant GO terms obtained were sorted according to their corresponding GO categories (Table 2). According to that analysis, most of genes focused on monosaccharide, hexose and glucose metabolic process, generation of precursor metabolites and energy and ion transport (Table 2), which

indicated that these pathways may have some contributions for fermentative performance.

3.3. Hexose transport

Gene expression analysis using RT-qPCR method was well corresponded with microarray means (Fig. 2a). Transport is suggested as the rate-limiting step of glycolysis in metabolic control analysis and transport exerts a high degree of control on glycolytic flux (Oehlen et al., 1994). The results showed that the detected transporter genes were all down-regulated in YIC10 vs. GIM2.71 comparisons, except *HXT5* (Fig. 2a). It was consistent with the report that *HXT5* was regulated by the growth rate of cells, where the growth rate of YIC10 was significantly higher than GIM2.71. However, different from *HXT5*, *HXT1-4* and *HXT6/7* were regulated by extracellular glucose (Diderich et al., 2001). Investigations using single transport mutants also showed that Hxt1-4, 6 and 7 are the major hexose transporters in yeast transporting glucose and fructose (Reifenberger et al., 1997, 1995). Furthermore, analysis of intracellular glucose and fructose showed that both sugars levels were always higher in GIM2.71 than in YIC10 (Fig. 2b and c), which was consistent with the higher expression of major genes involved in hexose transporter. It concluded that hexose transport was not the limiting-step in sugar consumption and ethanol production for GIM2.71, compared to YIC10.

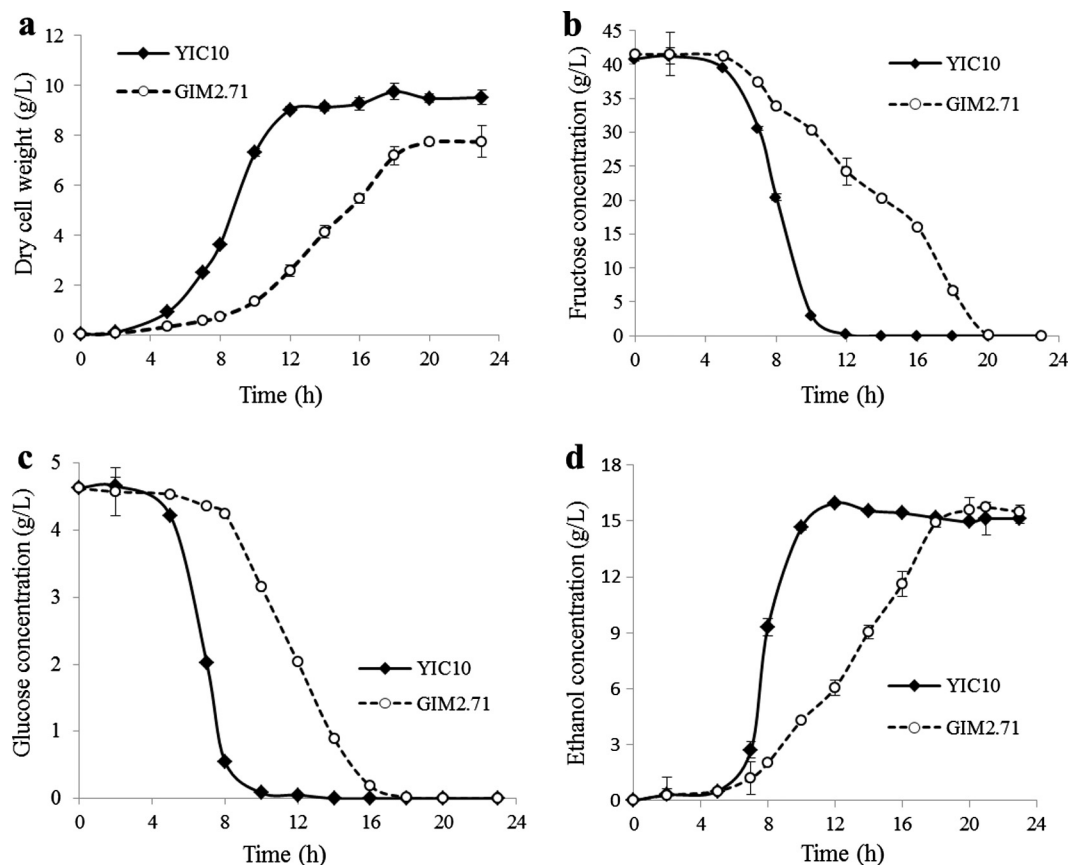


Figure 1 Fermentative performance of YIC10 and GIM2.71. Cell growth (a), fructose (b), glucose (c) and ethanol concentration (d) were determined.

Table 2 The GO analysis of up-regulated genes (Top 10).

Term	Count	%	P-value
<i>Metabolism</i>			
DNA metabolic process	26	13.3	2.90×10^{-2}
Monosaccharide metabolic process	15	7.7	4.40×10^{-4}
Hexose metabolic process	13	6.6	1.70×10^{-3}
Glucose metabolic process	11	5.6	3.60×10^{-3}
Cellular carbohydrate catabolic process	10	5.1	1.80×10^{-3}
Hexose catabolic process	8	4.1	4.20×10^{-3}
Monosaccharide catabolic process	8	4.1	6.20×10^{-3}
Alcohol catabolic process	8	4.1	9.60×10^{-3}
Glucose catabolic process	7	3.6	9.60×10^{-3}
Oxidoreduction coenzyme metabolic process	7	3.6	2.80×10^{-2}
<i>Energy</i>			
Generation of precursor metabolites and energy	16	8.2	6.20×10^{-2}
Energy reserve metabolic process	5	2.6	6.80×10^{-2}
<i>Transport</i>			
Ion transport	17	8.7	4.20×10^{-3}
Cation transport	15	7.7	5.10×10^{-4}
Metal ion transport	9	4.6	1.40×10^{-2}
Di-, tri-valent inorganic cation transport	7	3.6	7.90×10^{-3}
Transition metal ion transport	7	3.6	1.90×10^{-2}
Carboxylic acid transport	7	3.6	4.40×10^{-2}
Siderophore transport	5	2.6	2.00×10^{-4}
Anion transport	5	2.6	3.20×10^{-2}
Iron assimilation by chelation and transport	4	2	9.00×10^{-4}
Siderophore-iron transport	4	2	9.00×10^{-4}
<i>Protein</i>			
Protein modification by small protein conjugation or removal	11	5.6	2.70×10^{-2}
Protein modification by small protein conjugation	10	5.1	1.80×10^{-2}

3.4. Central carbon metabolism

Once sugars have been imported into cells, they are phosphorylated by one of three sugar kinases, Hxk1, Hxk2 and Glk1. Glucose and fructose are both phosphorylated by hexokinases Hxk1 and Hxk2 but with different efficiencies, and the glucokinase Glk1 phosphorylates glucose but not fructose (Rodriguez et al., 2001). The three genes were all down-regulated in YIC10 to GIM2.71 comparisons, which indicated that hexose phosphorylation was not the limiting steps in sugar consumption and ethanol production for GIM2.71.

Most genes in central carbon metabolism were down-regulated, only 3-phosphoglycerate kinase encoding genes *PGK1*, pyruvate decarboxylase encoding genes *PDC6*, alcohol dehydrogenase encoding genes *ADH5* were up-regulated (Fig. 3). During *S. cerevisiae* growth on fermentable carbon sources, six *PDC* genes were identified out of which three structural genes (*PDC1*, *PDC5* and *PDC6*) were encoded for active Pdc enzymes, independently (Milanovic et al., 2012). Pdc6p is the predominant isoenzyme form that catalyzes an

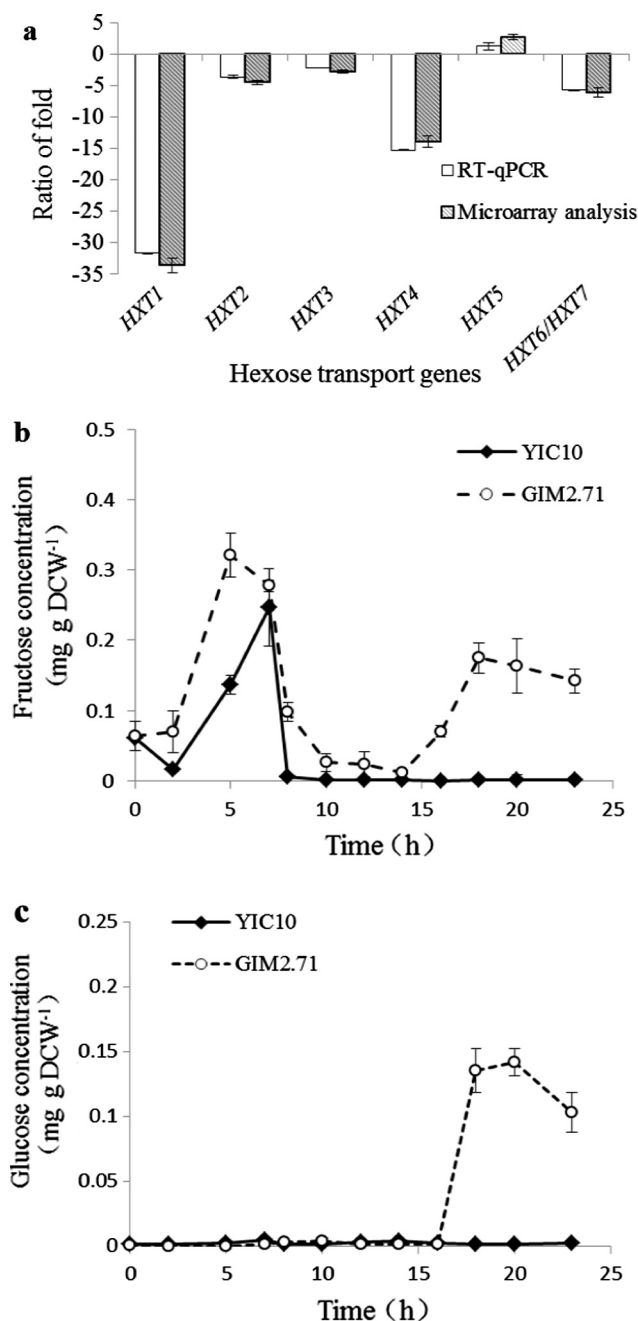


Figure 2 Expression of genes encoding hexose transport (A) and intracellular fructose (B) and glucose (C) levels.

irreversible reaction in which pyruvate is decarboxylated to acetaldehyde. Additionally, there are four genes (*ADH1*, *ADH3*, *ADH4* and *ADH5*) that encode alcohol dehydrogenases involved in ethanol synthesis. *ADH5* gene product is the major enzyme that is responsible for converting acetaldehyde to ethanol. It suggested that the most limiting steps of ethanol fermentation were the reaction of 1,3-disphosphoglycerate to 3-phosphoglycerate, the decarboxylation of pyruvate to acetaldehyde and its subsequent reduction to ethanol.

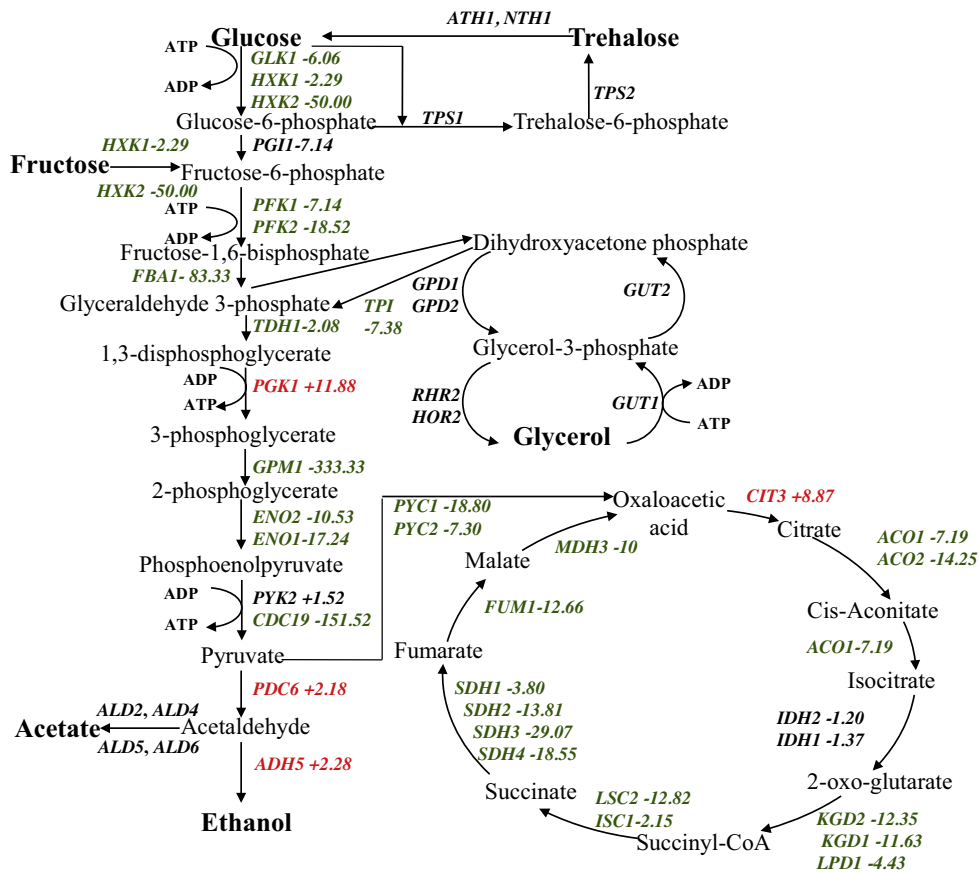


Figure 3 Expressions of genes involved in central carbon metabolism. “+” and “-” before regulation fold represented up-regulated and down-regulated genes, respectively.

3.5. Expression of genes involved in glycerol and its intracellular level

Glycerol was the major by-product in ethanol fermentation. The first step in glycerol synthesis is the most important as glycerol-3-phosphate dehydrogenase (encoded by *GPD1* and *GPD2*) activity controls the amount of glycerol produced. In this experiment, *GPD1* and *GPD2* were down-regulated significantly, and other genes involved in glycerol both synthesis (*RHR2* and *HOR2*) and degradation (*GUT1* and *GUT2*) were all down-regulated (Fig. 4a). Intracellular metabolic analysis showed that glycerol was at relatively low levels both for YIC10 and GIM2.71 at the onset of fermentation, whereas it was accumulated 83-fold compared to its initial level in GIM2.71 when ethanol was exponentially synthesized and carbon resource was exhausted (Fig. 4b). And this response was significantly stronger than YIC10.

3.6. Expression of genes involved in acetate and its intracellular level

Among genes encoding acetate synthesis, only *ALD4* was up-regulated and the other three genes (*ALD2*, *ALD5* and *ALD6*) were down-regulated (Fig. 4c). It was reported that the deletion of *ALD4* had no effect on the amount of acetate

formed (Remize et al., 2000). Intracellular metabolic analysis showed that acetate in YIC10 was always at a relatively low level, whereas acetate in GIM2.71 was accumulated quickly at late-logarithmic phase (Fig. 4d).

3.7. Expression of genes involved in trehalose and its intracellular level

Genes both were encoding trehalose synthesis (*TPS1* and *TPS2*) and hydrolysis (*ATH1* and *NTH1*) were all down-regulated (Fig. 4e). The intracellular trehalose in YIC10 was always at a relatively low level throughout the fermentation, whereas trehalose in GIM2.71 was accumulated rapidly at 10 h and 16 h (Fig. 4f).

Glycerol, acetate and trehalose were significantly accumulated in response to environmental stress in GIM2.71. Glycerol formation is the results of redox balance and stress response (Nevoigt and Stahl, 1997) and the observed differences suggest that the two strains could have a different stress response. This hypothesis is also supported by the formation of acetate, another significant redox-driven product, and the accumulation of trehalose, other potential stress protectants like glycerol.

Achieving high fermentative performance is a major challenge, particularly when it comes to modifications of the cen-

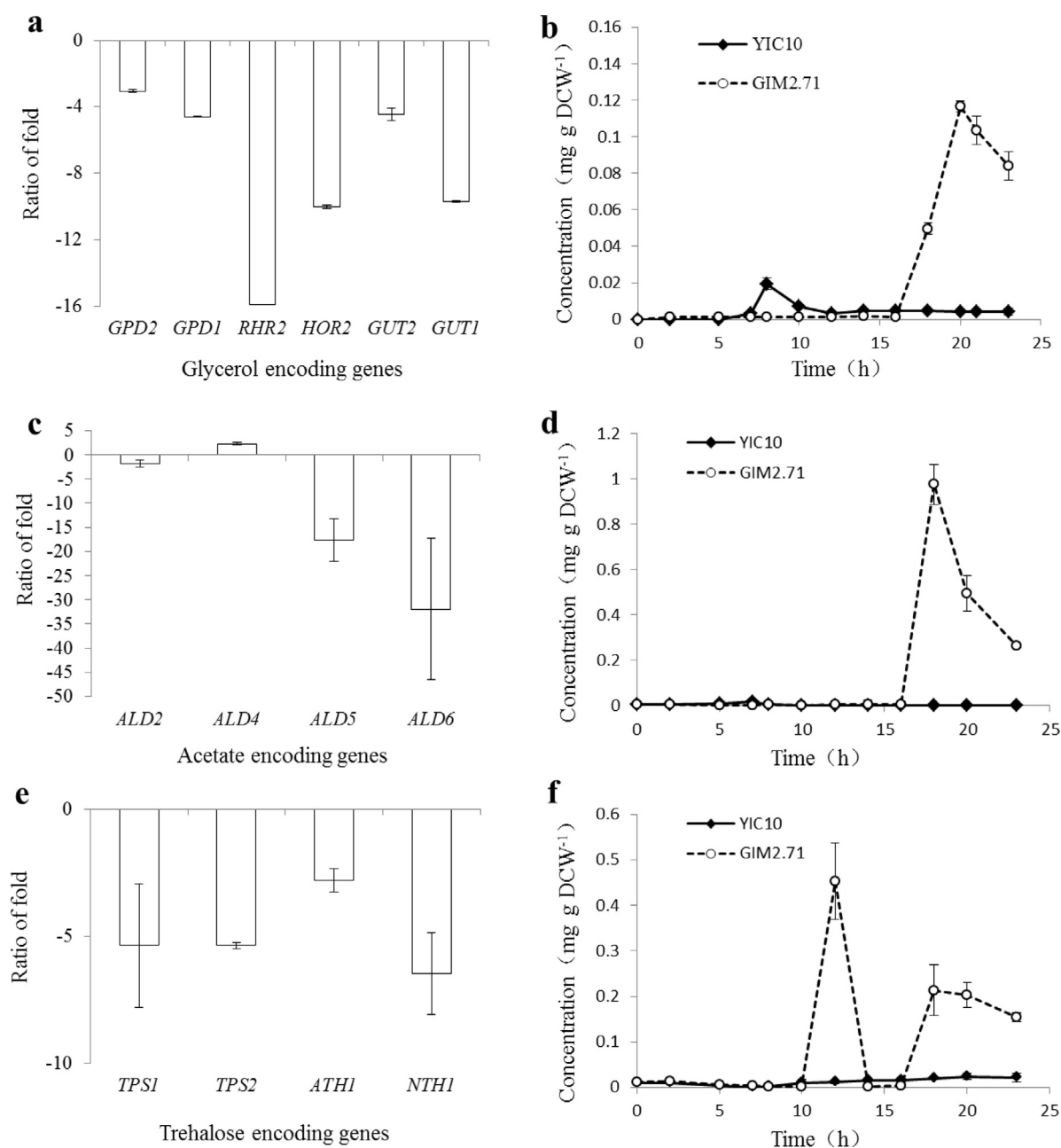


Figure 4 Expression of genes involved in glycerol (a), acetate (c) and trehalose (e), and intracellular levels of glycerol (b), acetate (d) and trehalose (f).

tral carbon metabolism which is inherently coupled to energy and redox issues. Glycerol is the major by-product accounting for up to 5% of the carbon in *S. cerevisiae* ethanolic fermentation. Decreasing glycerol formation may redirect part of the carbon toward ethanol production (Nissen et al., 2000). Pagliardini et al. (2010) reported that fine-tuning the glycerol synthesis pathway allowed the strains to keep their initial ethanol tolerance.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Diderich, J.A., Schuurmans, J.M., Van Gaalen, M.C., Kruckeberg, A. L., Van Dam, K., 2001. Functional analysis of the hexose transporter homologue *HXT5* in *Saccharomyces cerevisiae*. *Yeast* 18, 1515–1524.

- Galbe, M., Sassner, P., Wingren, A., Zacchi, G., 2007. Process engineering economics of bioethanol production. *Adv. Biochem. Eng. Biotechnol.* 108, 303–327.
- Li, F., Zhao, X.Q., Ge, X.M., Bai, F.W., 2009a. An innovative consecutive batch fermentation process for very high gravity ethanol fermentation with self-flocculating yeast. *Appl. Microbiol. Biotechnol.* 84, 1079–1086.
- Li, L.L., Ye, Y.R., Pan, L., Zhu, Y., Zheng, S.P., Lin, Y., 2009b. The induction of trehalose and glycerol in *Saccharomyces cerevisiae* in response to various stresses. *Biochem. Biophys. Res. Commun.* 387, 778–783.
- Li, L.L., Li, L., Wang, Y.P., Du, Y.G., Qin, S., 2013. Biorefinery products from the inulin-containing crop *Jerusalem artichoke*. *Biotechnol. Lett.* 35, 471–477.
- Liu, X.Y., Zhang, X.H., Wang, C., Liu, L.Y., Lei, M., Bao, X.M., 2007. Genetic and comparative transcriptome analysis of bromodomain factor 1 in the salt stress response of *Saccharomyces cerevisiae*. *Curr. Microbiol.* 54, 325–330.
- Milanovic, V., Ciani, M., Oro, L., Comitini, F., 2012. *Starmerella bombicola* influences the metabolism of *Saccharomyces cerevisiae* at pyruvate decarboxylase and alcohol dehydrogenase level during mixed wine fermentation. *Microb. Cell Fact.* 11.
- Nevoigt, E., Stahl, U., 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 21, 231–241.
- Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J., 2000. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. *Yeast* 16, 463–474.
- Oehlen, L.J.W.M., Scholte, M.E., Dekoning, W., Vandam, K., 1994. Decrease in glycolytic flux in *Saccharomyces cerevisiae* Cdc35-1 cells at restrictive temperature correlates with a decrease in glucose-transport. *Microbiology* 140, 1891–1898.
- Pagliardini, J., Hubmann, G., Bideaux, C., Alfenore, S., Nevoigt, E., Guillouet, S.E., 2010. Quantitative evaluation of yeast's requirement for glycerol formation in very high ethanol performance fed-batch process. *Microb. Cell Fact.* 9.
- Reifenberger, E., Freidel, K., Ciriacy, M., 1995. Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. *Mol. Microbiol.* 16, 157–167.
- Reifenberger, E., Boles, E., Ciriacy, M., 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *Eur. J. Biochem.* 245, 324–333.
- Remize, F., Andrieu, E., Dequin, S., 2000. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: role of the cytosolic Mg²⁺ and mitochondrial K⁺ acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Appl. Environ. Microbiol.* 66, 3151–3159.
- Rodriguez, A., de la Cera, T., Herrero, P., Moreno, F., 2001. The hexokinase 2 protein regulates the expression of the *GLK1*, *HXK1* and *HXK2* genes of *Saccharomyces cerevisiae*. *Biochem. J.* 355, 625–631.
- Stephe, A.M., Phillips, G.O., Willians, P.A., 2006. *Food Polysaccharides and their Applications*. CRC Press, Florida.
- Ye, Y.R., Zhu, Y., Pan, L., Li, L.L., Wang, X.I., Lin, Y., 2009. Gaining insight into the response logic of *Saccharomyces cerevisiae* to heat shock by combining expression profiles with metabolic pathways. *Biochem. Biophys. Res. Commun.* 385, 357–362.
- Yu, J., Jiang, J.X., Ji, W.M., Li, Y.Y., Liu, J.P., 2011. Glucose-free fructose production from Jerusalem artichoke using a recombinant inulinase-secreting *Saccharomyces cerevisiae* strain. *Biotechnol. Lett.* 33, 147–152.