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ORIGINAL ARTICLE

Changes in intracellular metabolism underlying the adaptation of *Saccharomyces cerevisiae* strains to ethanol stress

Juan Tian[1] · **Shuxian Zhang**[1] · **Hao Li**[1]

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Abstract *Saccharomyces cerevisiae* is often stressed by the ethanol which accumulates during the production of bioethanol by the fermentation process. The study of ethanol-adapted *S. cerevisiae* strains provide an opportunity to clarify the molecular mechanism underlying the adaptation or tolerance of *S. cerevisiae* to ethanol stress. The aim of this study was to clarify this molecular mechanism by investigating the ethanol adaptation-associated intracellular metabolic changes in *S. cerevisiae* using a gas chromatography–mass spectrometry-based metabolomics strategy. A partial least-squares-discriminant analysis between the parental strain and ethanol-adapted strains identified 12 differential metabolites of variable importance with a projection value of >1. The ethanol-adapted strains had a more activated glycolysis pathway and higher energy production than the parental strain, suggesting the possibility that an increased energy production and energy requirement might be partly responsible for an increased ethanol tolerance. An increased glycine content also partly contributed to the higher ethanol tolerance of the ethanol-adapted strains. The decreased oleic acid content may be a self-protection mechanism of ethanol-adapted strains to maintain membrane integrity through decreasing membrane fluidity. We suggest that while being exposed to ethanol stress, ethanol-adapted *S. cerevisiae* cells may remodel their metabolic phenotype and the composition of their cell membrane to adapt to ethanol stress and acquire higher ethanol tolerance.

Keywords *Saccharomyces cerevisiae* · Bioethanol · Ethanol tolerance · Ethanol-adapted strains · Metabolomics

Introduction

The energy crisis and environmental problems secondary to the overuse of traditional fossil energy sources have promoted the need for alternative renewable energy sources (Hill et al. 2006; Islam et al. 2014). Bioethanol, which is used in a clean and renewable way, can be a good alternative to traditional energy sources (Simas-Rodrigues et al. 2015).

Saccharomyces cerevisiae is the most commonly used fermentation yeast and is widely applied for the industrial-level production of bioethanol, alcoholic beverages and other industrial products (Bai et al. 2008; Westman et al. 2014). However, during the fermentation process, *S. cerevisiae* cells have to cope with a variety of stresses, such as the accumulation of ethanol (Ma and Liu 2010; Semkiv et al. 2014), high temperature (Wimalasena et al. 2014; Kanshin et al. 2015), high osmotic stress (Demeke et al. 2013), a large amount of sugar (Williams et al. 2015), inoculation amount (Favaro et al. 2013) and oxidation (Martín et al. 2008). Among these stresses, the accumulation of ethanol may be the main inhibitor of the bioethanol fermentation process, with accumulated ethanol possibly inhibiting *S. cerevisiae* cell growth and the complete bioethanol fermentation.

Consequently, ethanologenic strains with higher ethanol tolerance are needed to increase the final bioethanol yield (Swinnen et al. 2012; Yang et al. 2011). Attempts have been made to improve the ethanol tolerance of *S. cerevisiae* (Kajiwara et al. 2000; Nevoigt 2008; Parawira and Tekere

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✉ Hao Li
lihao@mail.buct.edu.cn; lihaoh@163.com

[1] Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China

2011), among which exposing *S. cerevisiae* cells to a step-wise increase in ethanol concentration has been found to be an effective way to obtain *S. cerevisiae* strains with a higher ethanol tolerance (Dinh et al. 2008; Wang et al. 2015). Microorganisms, including *S. cerevisiae*, have evolved a range of stress responses to environmental challenges and can tolerate or adapt to environmental perturbations through direct evolutionary engineering in response to a gradual inducing process (Dinh et al. 2008; Morano et al. 2012). In our previous study, we used directed evolutionary engineering to generate a series of ethanol-adapted *S. cerevisiae* strains that have a higher ethanol tolerance than the parental strain (Wang et al. 2015).

To some extent, *S. cerevisiae* cells undergo domestication through their gradual adaption to a step-wise increasing ethanol concentration during the directed evolutionary process. During such domestication, *S. cerevisiae* cellular behavior, including metabolic phenotype or other cell physiological processes, may change or be remodeled to acquire higher ethanol tolerance. Our previous study focused primarily on the relationship between variation in cell membrane structure or composition and ethanol tolerance during the directed evolutionary process, with the results indicating that while being exposed to step-wise increased levels of ethanol, *S. cerevisiae* cells may remodel membrane components or structure to adapt to the ethanol stress (Wang et al. 2015). However, the molecular mechanism underlying the ethanol tolerance of *S. cerevisiae* is very complex, and many genes, proteins, metabolites and bio-processes might be involved (Teixeira et al. 2009; Lam et al. 2014). As a complex living system, the adaption and toleration of *S. cerevisiae* cells to ethanol stress would be not only related to the membrane, but also to the cell as a whole, which led us to study how *S. cerevisiae* cells acquired higher ethanol tolerance during the directed evolutionary process at the systemic level.

An analysis of metabolomics can reflect the last step among a series of changes that result from external stimuli or a pathological insult (Winder et al. 2011; Toya and Shimizu 2013). Metabolite contents or composition can directly reflect the phenotype changes which have occurred in living systems. Metabolomics analysis by gas chromatography–mass spectrometry (GC-MS) can reveal the stress-associated responses in *S. cerevisiae* with high sensitivity and resolution (Castro et al. 2012; Wei et al. 2013), as we demonstrated in our earlier studies in which we used a GC-MS-based metabolomics strategy to determine the effects of environmental stress on the metabolism of *S. cerevisiae* (Li et al. 2012; Cui et al. 2015). These previous results showed that GC-MS-based metabolomics strategy can provide a powerful platform for determining the directed evolutionary process-associated biochemical changes which occur in *S. cerevisiae*.

The aim of the study reported here was to clarify the molecular mechanism underlying the adaptation or tolerance of *S. cerevisiae* to ethanol stress. To this end, we compared the global metabolite profiles and metabolite contents or composition of the *S. cerevisiae* CEC B9S-15 parental strain and the ethanol-adapted M5 and M10 strains by a GC-MS-based metabolomics strategy. We also examined the metabolic relevance of these compounds in the response of *S. cerevisiae* to ethanol stress.

Materials and methods

Strains

The ethanol-adapted *S. cerevisiae* strains (i.e. M5 and M10) were obtained from the *S. cerevisiae* CEC B9S-15 parental strain through a directed evolutionary engineering process (Wang et al. 2015).

Grouping design

To study the metabolites of the *S. cerevisiae* CEC B9S-15 parental strain and the two ethanol-adapted strains (M5 and M10), we cultured all three strains under the same conditions, namely, in 250 ml cotton-plugged flasks containing YPD broth (2% glucose, 1% yeast extract, 2% peptone) at 30°C with shaking (150 rpm). Six biological replicates were performed for each group.

Preparation of metabolome samples

Metabolome samples of the *S. cerevisiae* strains were prepared according to the previously reported procedures (Villas-Bôas et al. 2005) with some modifications. After the primary cultures had been incubated for 8 h, we quickly harvested 2 ml of the parental strain and ethanol-adapted M5 and M10 strains and immediately transferred the samples to 15-ml tubes containing 8 ml of −40°C pre-chilled 60% methanol to quench the culture. After quenching, the yeast cells were collected by centrifugation (8 000 *g*, −4°C, 10 min) and washed with 1 ml of pre-chilled 60% methanol. The supernatant was discarded, and the pellet spiked with internal standards (50 μl of ribitol in water, 0.5 mg/ml) was prepared for the extraction of intracellular metabolites. The samples were suspended in 0.75 ml of −40°C pre-chilled pure methanol and then frozen in liquid nitrogen. The frozen suspension was thawed in an ice bath and this freeze–thaw process was repeated three times before centrifugation (8 000 *g*, −4°C, 10 min). The supernatant was collected and an additional 0.75 ml of pre-chilled pure methanol was added to the pellet. The mixture was vortexed for 30 s prior to centrifugation (8 000 *g*, −4°C, 10 min). Both supernatants were pooled together and stored at −20°C until use.

Sample derivatization

Samples were dried in a vacuum centrifuge dryer. Derivatization was performed according to our previously reported procedure (Li et al. 2012). For derivatization, 100 μl of methoxylamine hydrochloride in pyridine (20 mg/ml), the first agent, was added to the dried samples prior to incubation at 30°C for 2 h. Then, a 100-μl aliquot of the second derivatizing agent, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide, was then added to the samples and the mixtures incubated at 30°C for 3 h. The samples were diluted in 400 μl of acetonitrile.

GC-MS analysis

Chromatography was performed on a GC-MS solution system (Shimadzu Corp., Kyoto, Japan) equipped with a DB-5 capillary column (30 m × 250 μm i.d., 0.25-μm film thickness; Agilent J&W columns, Aglilent Technologies, Santa Clara, CA). Samples (1 μl) were injected into the DB-5 capillary column by split injection mode with a split ratio of 30:1 using an AOC-20i autoinjector (Shimadzu Corp.). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The injection, ion source and ion source surface temperatures were set to 300, 200 and 280 °C, respectively. The GC oven temperature was heated to 80°C for 1 min, raised to 100°C at a rate of 2°C/min, then raised to 300°C at a rate of 15°C/min and finally maintained at 300°C for 6 min. Mass spectra were recorded by full scan mode (*m*/*z* 80–500) at a rate of 20 scans/s. Ribitol was served as an internal standard to monitor batch reproducibility and correct for minor variations that occurred during sample preparation and analysis.

Data analysis

GC-MS Real Time Analysis software (Shimadzu Corp.) was used to acquire mass spectrometric data. National Institute of Standards and Technology standard reference databases 27 and 147 (www.nist.gov/srd/nist27.cfm, www.nist.gov/srd/nist147.cfm) were used to search and identify metabolites restricted to peaks detected with a total ion current (TIC). The compounds were also identified by comparing their mass spectra and retention times with those of commercially available reference compounds. The generated normalized peak areas (variables) were imported into the SIMCA package (ver 10.0) (Umetrics, Umea, Sweden) for the multivariate statistical analysis. Partial least-squares-discriminant analysis (PLS-DA) was applied to the data after mean-centering and UV-scaling. These analyses employed a default three-fold internal cross validation from which the R^2 and Q^2 (goodness of prediction) values, representing the explained variance and the predictive capability, respectively, were extracted. A supervised PLS-DA model was initially performed to obtain an overview of the GC-MS data from the parental strain and ethanol-adapted strains.

To specify the metabolic variations, we performed a supervised PLS-DA pairwise comparison between *S. cerevisiae* parental strain and ethanol-adapted strains (M5 and M10). On the basis of the loading plots and variable importance in the projection (VIP) value threshold (VIP>1) from the three-fold cross-validated PLS-DA models, variables that were responsible for distinguishing between the parental and ethanol-adapted groups were selected. Accuracy and predictive ability of the models were evaluated by R^2 and Q^2 values.

Statistical analysis

Data were analyzed using the independent-samples *t* test with SPSS version 13.0 software for Windows (IBM Corp., Armonk, NY), and standard error of the mean was used as the error bar. Differences showing *P* values of <0.05 were considered to be statistically significant.

Results

Differences in the intracellular metabolite profiles among the *S. cerevisiae* parental and ethanol-adapted M5 and M10 strains

For the metabolomics analysis, *S. cerevisiae* cells were harvested after 8 h of incubation. Typical GC-MS TIC chromatograms from the *S. cerevisiae* parental and ethanol-adapted M5 and M10 strains are shown in Electronic Supplementary Material Fig. S1. The PLS-DA scatter plot with excellent fit and satisfactory predictive ability ($R^2X_{cum} = 0.949$, $Q^2_{cum} = 0.402$) (Table 1) was performed to represent the overview of the different distributions of the strain samples in the new multivariate space (Fig. 1). The plot showed that the biological replicates of different strains (i.e., the parental, M5 and M10 strains) were distinctly clustered in the plot along principal component 1 (Fig. 1).

Table 1 Statistical data from partial least squares-discriminant analysis models

Sample	PLS PC'S	R^2X (cum)	R^2Y (cum)	Q^2 (cum)
All	3	0.949	0.893	0.402
PS vs. M5	3	0.979	0.995	0.811
PS vs. M10	5	0.973	0.997	0.587

PLS-DA Partial least squares-discriminant analysis, *PS* Parental strain, *M5* ethanol-adapted M5 strain, *M10* ethanol-adapted M10 strain

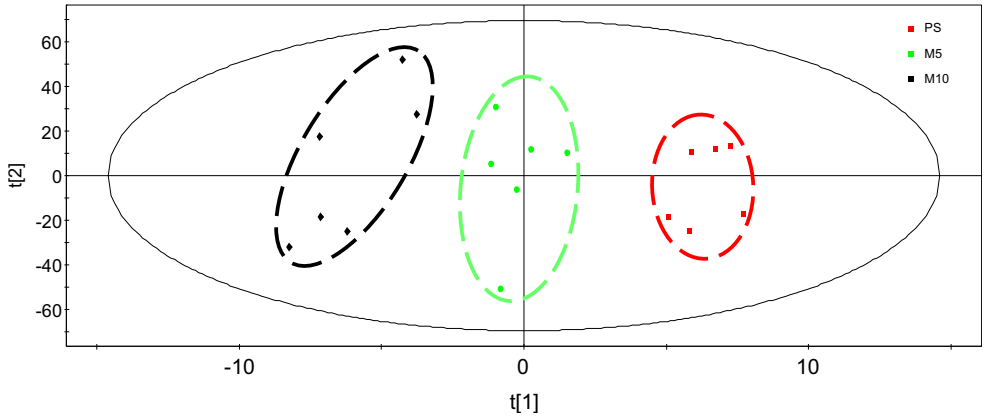

Fig. 1 Partial least squares-discriminant analysis (PLS-DA)-derived metabolite profiles for the parental strain *S. cerevisiae* CEC B9S-15 (*PS*) and the ethanol-adapted strains (*M5* and *M10*). In this scores plot, the confidence interval is defined by the Hotelling's T2 ellipse (95% confidence interval), and observations outside the confidence ellipse are considered to be outliers

Differences in intracellular metabolites among the *S. cerevisiae* parental and ethanol-adapted M5 and M10 strains

The PLS-DA pairwise comparisons between the parental strain and each ethanol-adapted strain suggested an obvious metabolic difference between the classes in each pairwise comparison on the first component (Fig. 2a, c). The PLS-DA models were well constructed with excellent fit and satisfactory predictive power (Table 1). The major metabolic perturbations that caused these discriminations were identified from the line plots of the *X*-loadings of the first component of the PLS-DA models (Fig. 2b, d).

Metabolites with a VIP value of >1 make a significant contribution to the separation of groups within the PLS-DA models (Szeto et al. 2010). The VIP plots demonstrated that some of identified metabolites contributed to the class separation. On the basis of the PLS-DA results with good pairwise discriminations between the parental strain and ethanol-adapted M5 and M10 strains, we selected a total of 12 paired retention time-mass to charge ratio (RT-*m/z*) variables according to the cutoff VIP value (VIP > 1) (Table 2).

Discussion

In the context of the industrial production of bioethanol in fermentation plants, ethanologenic strains with a higher ethanol tolerance are highly desirable as this would increase the final bioethanol yield. Directed evolutionary engineering can effectively facilitate the development of *S. cerevisiae* ethanol-adapted strains with a higher ethanol tolerance. In our previous study, a series of ethanol-adapted strains, including the M5 and M10 strains evaluated in the present study, were generated using a directed evolutionary strategy, with the M5 and M10 strains showing a higher survival rate than the parental strain while being exposed to 10%(v/v) ethanol stress for 2 h (Wang et al. 2015). How these the ethanol-adapted strains acquired their higher ethanol tolerance was question which we felt was worth exploring. Although our previous study demonstrated that *S. cerevisiae* cells might remodel their membrane components or structure to adapt to the increased ethanol stress (Wang et al. 2015), the mechanism underlying the higher ethanol tolerance of mutant strains could not be only limited to the variation in cell membrane composition or structure. In addition to changes in the cell membrane, many other changes, including those in cellular metabolisms, would also occur during the directed evolution process and might contribute to the acquired higher ethanol tolerance of the ethanol-adapted strains. In fact, the results of the present study do show that directed evolution did induce metabolic changes in the ethanol-adapted M5 and M10 strains, especially in the metabolism of carbohydrates, amino acids and lipids (Table 2).

Compared to the parental strain, the M5 strain had lower levels of fructose and glucopyranoside, and the M10 strain had lower glucopyranoside content (Table 2), indicating that the glycolytic pathway might be enhanced in ethanol-adapted *S. cerevisiae* strains. It has been shown that cell growth and glycolysis in the parental *S. cerevisiae* strain can be markedly inhibited by ethanol stress (Li et al. 2012). Wang et al. (2015) observed that cell growth in the ethanol-adapted M5 and M10 strains was inhibited by 10% v/v ethanol, but interestingly these also authors noted that the growth superiority of M5 and M10 cells in comparison to those of the parental strain was not evident in the case of no ethanol stress, but only observed under ethanol stress. The detection of an enhanced glycolytic pathway demonstrated that a higher consumption rate of carbon sources was probably realized in the M5 and M10 strains and that the metabolic phenotype of ethanol-adapted strains might be also remodeled. As we did not detect any growth difference among the parental, M5 and M10 strains in the absence of an exogenous ethanol stress, it is possible that more carbon flux might reroute from biomass

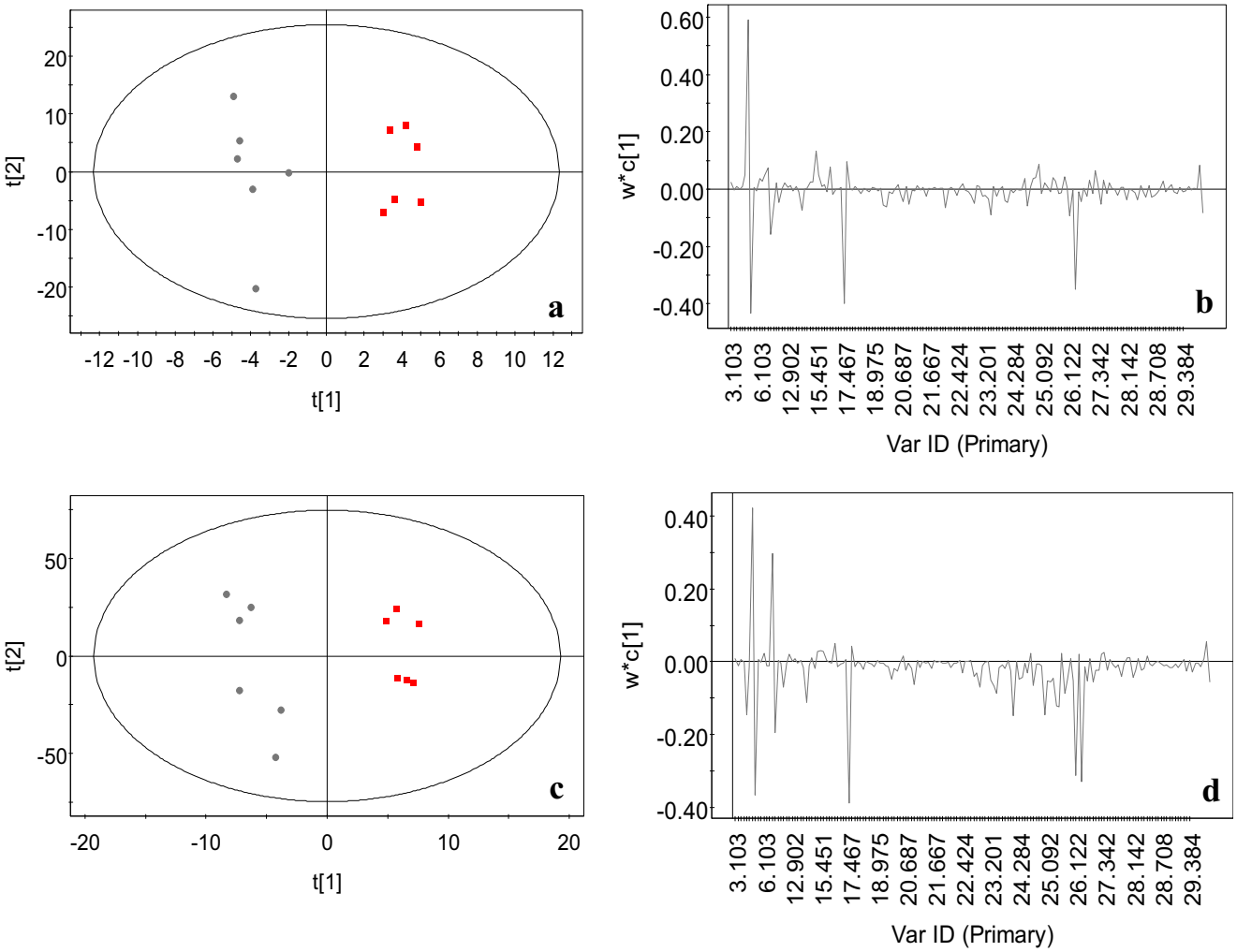

Fig. 2 PLS-DA model plots for the parental group (PS; *red symbols*) vs. ethanol-adapted groups (M5, M10; (*black symbols*). **a**, **c** Cross-validated scores plots of the pairwise comparison between PS and M5 (**a**) and between PS and M10 (**c**). Two groups in each scores plot were separated along the first component. In the scores plots, the confidence interval is defined by the Hotelling's T2 ellipse (95% confidence interval), and observations outside the confidence ellipse are considered to be outliers. **b**, **d** Loading plots of pairwise comparison between PS and M5 (**b**) and between PS and M10 (**d**). The metabolites with the largest intensities contributed to the clustering

towards other metabolic processes, such as the production of protectants (i.e. trehalose and ergosterol) against ethanol stress in *S. cerevisiae* (Fig. 3). In other words, in addition to a guaranteeing basic need for biomass, more carbon sources might be consumed by other metabolic processes. In fact, the M5 and M10 strains showed an increased content of trehalose and ergosterol in comparison to the parental strain (Wang et al. 2015).

Moreover, a higher ethanol level could increase the energy requirement for *S. cerevisiae* cell growth but at the same time it might also decrease the capacity of cells to produce energy through inhibiting glycolytic enzymes (Pagliardini et al. 2013). However, the promoted glycolytic pathway in the ethanol-adapted M5 and M10 strains might indicate that energy production was not inhibited—and even increased in the M5 or M10 strains. Moreover, the M5 and M10 strains had a lower phosphoric acid content than the parental strain (Table 2). The decreased level of phosphoric acid, which participates in the phosphorylation of ADP to ATP, might indicate an increased level of ATP was production (Ding et al. 2009b). In the present study, the decreased phosphoric acid level probably also indicates that a higher energy production might be realized in M5 and M10 strains. An increased energy production and energy requirement might also be partly responsible for the increased ethanol tolerance of the M5 or M10 strain (Fig. 3).

The ethanol-adapted strains also had lower contents of alanine, isoleucine, serine and proline than the parental strain (Table 2). These amino acids could be converted from 3-P-glycerate and pyruvate, which are metabolic intermediates of the Embden–Meyerhof–Parnas (EMP) pathway. Viewed from another perspective, to some extent, the levels of these two metabolic intermediates of the EMP pathway increased in the ethanol-adapted strains in comparison to that in the parental

Table 2 Intracellular metabolites of *Saccharomyces cerevisiae* identified by gas chromatography-mass spectrometry that differ between the parental *S. cerevisiae* strain and the two ethanol-adapted *S. cerevisiae* strains (M5 and M10 strains)

Retention time	Metabolite	*Saccharomyces cerevisiae* strains		
		Parental strain	Ethanol-adapted M5 strain	Ethanol-adapted M10 strain
3.486	Propanoic acid	0.230 ± 0.060	0.000**	0.290 ± 0.030
4.235	L-Alanine	0.450 ± 0.040	0.310 ± 0.040**	0.300 ± 0.030**
6.103	Phosphoric acid	0.040 ± 0.003	0.000**	0.010 ± 0.001*
10.037	L-Isoleucine	0.050 ± 0.009	0.040 ± 0.030*	0.000**
10.367	Glycine	0.050 ± 0.006	0.070 ± 0.003	0.090 ± 0.006*
12.600	L-Serine	0.030 ± 0.009	0.020 ± 0.005	0.010 ± 0.002*
15.107	L-Proline	1.000 ± 0.020	0.000**	0.070 ± 0.009*
17.751	α-Glycerophosphoric acid	0.160 ± 0.010	0.070 ± 0.010**	0.070 ± 0.010**
18.564	Pentanedioic acid	0.880 ± 0.040	0.000**	0.040 ± 0.006*
20.765	Oleic acid	0.040 ± 0.006	0.060 ± 0.010	0.000**
23.316	D-Fructose	0.040 ± 0.006	0.000**	0.080 ± 0.012
24.027	α-D-Glucopyranoside	8.330 ± 0.260	6.880 ± 1.010**	5.560 ± 0.840**

*$P < 0.05$ compared with the parental strain; **$P < 0.01$ compared with the parental strain

The variable importance in the projection (VIP) scores of all listed metabolites are >1

The data represents the relative peak intensity and are presented as the mean ± standard error of the mean

strain. Such results also indicate the probable activation of glycolysis in the ethanol-adapted strains.

Glycine has been reported to be an osmoprotectant which alleviates the side effect of osmotic stress (Thomas et al. 1994) and can also improve the ethanol tolerance of *S. cerevisiae*. Moreover, the protection effect of glycine against stress has been found to be superior to that of proline (Xue and Jiang 2006). In our study, the increased glycine content in the M5 and M10 strains might also contribute to the higher ethanol tolerance of these ethanol-adapted strains.

Fatty acids (FAs), especially unsaturated FAs, are essential to the cell membrane (Tao et al. 2012) and play vital roles in the ethanol resistance of yeast cells (Ding et al. 2009a; Kim et al. 2011). Ethanol stress can affect the fluidity of the plasma membrane (Aguilera et al. 2006) and can also make cell membrane structure more loose. The plasma membrane should retain its structural integrity as much as possible to antagonize the inhibitory effect of ethanol stress. Repeated exposure to step-wise increased ethanol stress may result in remodeling the composition and structure of the cell membrane of the ethanol-adapted strains. In the present study, the levels of octadecanoic acid ($C_{18:0}$), hexadecanoic acid ($C_{16:0}$) and palmitoleic acid ($C_{16:1}$) did not change, and oleic acid ($C_{18:1}$) content decreased in the M10 strain. Oleic acid is an important monounsaturated fatty acid of *S. cerevisiae* involved in ethanol tolerance (You et al. 2003). Decreased oleic

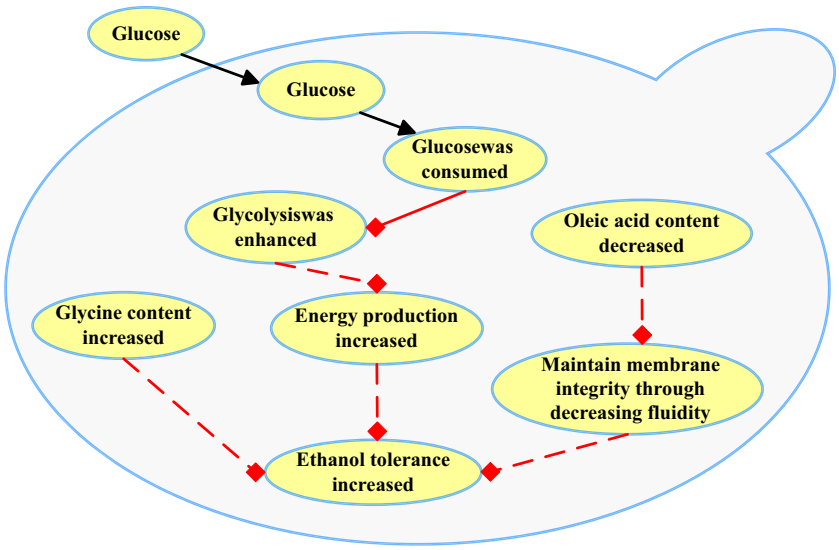

Fig. 3 Hypothesis model of metabolic changes in the adaptation of *Saccharomyces cerevisiae* to ethanol stress. *Black arrows* Mass flow, *red diamonds* positive regulation

acid content might be a self-protection process of ethanol-adapted strains to maintain their membrane integrity through decreasing membrane fluidity (Fig. 3).

In summary, this study demonstrates the utility of a GC-MS-based metabolomics strategy to evaluate the adaptation of *S. cerevisiae* to ethanol stress. Ethanol-adapted *S. cerevisiae* strains (M5 or M10 strain) acquired a higher ethanol tolerance through changing their cell membrane composition or structure and remodeling of their metabolism. The results described here highlight our current understanding of the mechanism underlying the adaptation of *S. cerevisiae* to ethanol stress. A full understanding of this mechanism would facilitate the engineering of metabolic regulation to improve the ethanol tolerance of S. cerevisiae or contribute to the construction of feasible ethanologenic strains with better fermentative capacities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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