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Deletion of acetate transporter gene *ADY2* improved tolerance of *Saccharomyces cerevisiae* against multiple stresses and enhanced ethanol production in the presence of acetic acid

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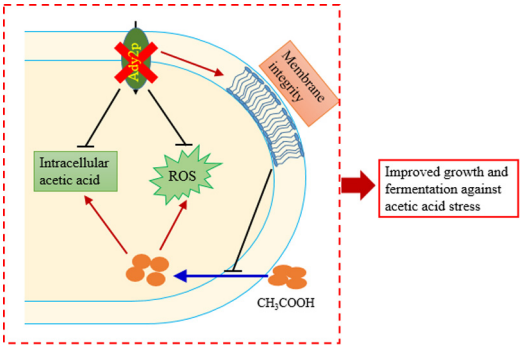
HIGHLIGHTS

- Zinc sulfate supplementation repressed transcription of acetate transporter gene *ADY2*.
- *ADY2* deletion improved yeast tolerance to acetic acid and hydrogen peroxide.
- *ADY2* deletion led to improved ethanol fermentation in the presence of acetic acid.
- Decreased intracellular acetic acid and ROS was revealed by *ADY2* deletion.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this work was to study the effects of deleting acetate transporter gene *ADY2* on growth and fermentation of *Saccharomyces cerevisiae* in the presence of inhibitors. Comparative transcriptome analysis revealed that three genes encoding plasma membrane carboxylic acid transporters, especially *ADY2*, were significantly downregulated under the zinc sulfate addition condition in the presence of acetic acid stress, and the deletion of *ADY2* improved growth of *S. cerevisiae* under acetic acid, ethanol and hydrogen peroxide stresses. Consistently, a concomitant increase in ethanol production by 14.7% in the presence of 3.6 g/L acetic acid was observed in the *ADY2* deletion mutant of *S. cerevisiae* BY4741. Decreased intracellular acetic acid, ROS accumulation, and plasma membrane permeability were observed in the *ADY2* deletion mutant. These findings would be useful for developing robust yeast strains for efficient ethanol production.

1. Introduction

Acetic acid is produced by budding yeast *Saccharomyces cerevisiae* during ethanol fermentation, which also widely exists in cellulosic hydrolysate as a potent fermentation inhibitor (dos Santos

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and Sá-Correia, 2015; Jönsson and Martín, 2016), since high concentrations of acetic acid induces accumulation of reactive oxygen species (ROS) (Wan et al., 2015), which then damages structures and functions of proteins and DNA (Sousa et al., 2012; Giannattasio et al., 2013). Toxicity of acetic acid at a pH lower than its pKa (4.75) is particularly severe, which disrupts the function of cellular membranes by altering the conformations of membrane proteins as well as damaging membrane lipid organization (Mira et al., 2010). Moreover, higher concentrations of acetic acid also causes energy deficiency and nutrient starvation of yeast cells, thereby hampers their growth and metabolism, even leads to programmed cell death (Giannattasio et al., 2013). Therefore, improved tolerance against acetic acid would render yeast cells better growth and metabolism for more efficient production of bioethanol from cellulosic biomass.

Both process optimization and strain development are of great concern for efficient cellulosic ethanol production (Zhao et al., 2016). Divalent metal ions (Zn^{2+}, Mg^{2+} and Ca^{2+}) in the culture medium exert positive effects on yeast stress tolerance (Zhao et al., 2009; Ismail et al., 2014). Previously, it was shown that zinc sulfate supplementation at a suitable concentration renders tolerance in yeast cells against high concentrations of acetic acid (Wan et al., 2015). Comparative metabolic profiling analysis revealed global changes in amino acid and carbohydrate metabolism (Wan et al., 2015), but related studies on global gene transcription have not been performed.

Multiple genes were shown to be involved in acetic acid stress response in *S. cerevisiae* (Chen et al., 2016; González-Ramos et al., 2016; Meijnen et al., 2016; Mira et al., 2010), indicating that acetic acid tolerance could be improved by manipulating the expression of key genes. For instance, our recent studies demonstrated that overexpression of *SET5* and *PPR1* enhanced ethanol production under inhibitory levels of acetic acid (Zhang et al., 2015). Alternatively, the deletion of *RTT109* and *QDR3* improved cell growth of *S. cerevisiae* under acetic acid stress (Cheng et al., 2016; Ma et al., 2015). It is hypothesized that still, more functional genes related to acetic acid stress tolerance might exist in *S. cerevisiae*, and it is of keen interest in elucidating the molecular mechanism underlying acetic acid stress tolerance in response to zinc sulfate addition to identify novel functional genes, which may be engineered to enhance the stress tolerance and ethanol production from inhibitor-containing feedstock.

Among various cellular compartments exposed to acetic acid toxicity, plasma membranes are considered as the first assaulting target, and various membrane proteins of yeast have close interactions with acetic acid tolerance. Positive effects on acetic acid tolerance through *QDR3*, *YRO2* and *MRH1* deletion, as well as *PMA1* overexpression, has been revealed in previous studies (Ma et al., 2015; Takabatake et al., 2014; Lee et al., 2015; Lee et al., 2017). Among the genes encoding membrane transporters, *ADY2* (*ATO1*, *YCR010*) encodes a carboxylic acid transporter of *S. cerevisiae*. It was reported that acetate uptake was abolished by *ADY2* disruption when shifting the sole carbon source from glucose to acetic acid (Paiva et al., 2004). Furthermore, it was found that a mutant of Ady2p (Leu219Val and Ala252Gly) enabled a higher level of lactate uptake comparing to that observed in the wild-type (de Kok et al., 2012). Until now, studies on *ADY2* are still limited in the utilization of acetic acid as a non-fermentable carbon source and the role of this gene in response to environmental stimuli remains unclear.

In this study, comparative transcriptome analysis was performed to elucidate the underlying mechanism of improved acetic acid tolerance associated with zinc sulfate supplementation, which indicated that the transcription of *ADY2* was significantly repressed by zinc sulfate. To further reveal the molecular mechanism involved in this phenomenon, the impact of *ADY2* deletion on stress tolerance was evaluated. This work provides an evidence that plasma membrane transporter *ADY2* can be engineered to develop stress tolerant yeast strain for efficient ethanol production.

2. Materials and methods

2.1. Strains and culture media

Yeast strains (Table S1) were cultured and maintained in YNB medium (glucose 20 g/L and 6.7 g/L yeast nitrogen base without amino acids), YPD medium (yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L). For stress tolerance assay, YPD-II medium (yeast extract 4 g/L, peptone 3 g/L and glucose 20 g/L) was used which contained various inhibitory compounds without pH adjustment, and 3.6 g/L acetic acid, 5 mM H_2O_2, 1.2 g/L formic acid, 12 g/L lactic acid and 10% (v/v) ethanol, respectively, were added as stressors. To prepare solid agar plates, 20 g/L Bacto agar was added into the YPD medium. Ethanol fermentation medium was composed of yeast extract 4 g/L, peptone 3 g/L and glucose 100 g/L, and 10 g/L acetic acid was added into the medium, with the medium without acetic acid addition served as control. Fermentation medium II was composed of yeast extract 4 g/L, peptone 3 g/L, glucose 70 g/L, xylose 30 g/L, with the addition of major inhibitors detected during the pretreatment of lignocellulosic biomass (1.3 g/L furfural, 5.3 g/L acetic acid and 0.5 g/L phenol), and pH was adjusted to 4.5 (Wang et al., 2015).

2.2. Yeast culture and ethanol fermentation

For stress tolerance experiments, yeast cells were cultured overnight in 250 mL Erlenmeyer flasks containing 50 mL YPD medium at 30 °C and 150 rpm, which were harvested by centrifuging at 6000*g* for 5 min, and washed twice with sterilized distilled water.

Batch fermentation was performed in 250 mL flasks containing 100 mL fermentation medium, and the fermentation conditions were controlled at 30 °C and 150 rpm without pH adjustment. Samples were collected and analyzed as described previously (Wang et al., 2013).

Ethanol production experiments in the 2.5 L fermenter (KF-2.5 L, KoBio Tech, South Korea) filled with 1000 mL ethanol fermentation medium was performed at 30 °C, 150 rpm, 0.04 vvm, and pH 4.5. For acetic acid tolerance experiments, 7.5 or 10 g/L acetic acid was supplemented for each studied yeast strain, along with the control withoutacetic acid supplementation. Zinc sulfate was added into the fermentation medium at a final concentration of 104 μm, and the self-flocculating yeast SPSC01 was used for transcriptome analysis with culture without any zinc sulfate supplementation as the control.

2.3. Transcriptome analysis and qRT-PCR analysis

Total RNA was isolated from yeast cells through Spin Column Yeast total RNA Purification Kit (Sangon, Shanghai, China). Transcriptome analysis was performed by Beijing Genome Institute (BGI, Shenzhen, China) through RNA sequencing.

Relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method. All polymerase chain reaction (PCR) primers used in this study are listed in Supplementary Table S2. The mRNA transcript levels were normalized using *ACT1* as a reference. Relative expression levels of *ADY2*, *ATO2*, *JEN1*, *FPS1*, *ADE17*, *OGG1*, *CIN5* and *HSF1* were detected through quantitative real-time PCR (qRT-PCR) analysis.

2.4. Deletion of ADY2, ATO2, and JEN1

The *ADY2* deletion cassette was amplified by PCR using the pHO plasmid as a template (He et al., 2012) with primers ady2F and ady2R (Table S2), which was transformed into *S. cerevisiae* strains BY4741 and ATCC4126, separately, using the LiAc method (Gietz and Schiestl, 2007). Transformants were selected from the YPD agar plates containing 300 μg/mL Geneticin (Sigma–Aldrich, USA). The *ADY2* deletion was confirmed using PCR amplification with the primers YCR101CF/YCR101CR and subsequent sequencing. *JEN1* and *ATO2* deletion cassettes were amplified from the plasmid UG27 with primers T1-jen1F/LRS-jen1R and T1-ato2F/LRS-ato2R, respectively, which were transformed into BY4741 and BYady2, respectively. Transformants were selected from the YNB agar plates without histidine addition. The *ATO2* and *JEN1* deletion mutants were verified using diagnostic PCR with the primers T1 and LRS (Table S2), which were further confirmed by DNA sequencing.

2.5. Evaluation of stress tolerance

Stress tolerance of yeast cells was evaluated using both plate spot assay and liquid culture assay. For plate spot assay, the ten-fold diluted suspensions were spotted onto the YPD solid agar plates containing 3.6 g/L acetic acid or 5 mM H_2O_2, then cultured at 30 °C for 48 h. For liquid culture assay, the YPD-II medium containing various inhibitors without pH adjustment was inoculated with an initial OD_{600} of 0.1 in a microtiter plate and the culture was performed at 30 °C using medium speed for 2–3 days, which was monitored by a Bioscreen C machine (Bioscreen, Finland) with OD_{600} measured in an interval of 2 h. The culture was performed at 30 °C shaking at medium speed for 2–3 days.

2.6. Determination of intracellular acetic acid

The intracellular acetic acid of *S. cerevisiae* cells was determined as described previously with some modifications (Tanaka et al., 2012). In brief, overnight pre-cultured cells were transferred from the YPD medium without acid supplementation to the YPD medium containing 10 g/L acetic acid, and incubated at 30 C, 150 rpm for 60 min. Cells were harvested by centrifuging at 6000*g* for 5 min, and washed twice with chilled distilled water and resuspended in 0.5% (m/v) arabinose solution. Then cell suspension was boiled at 100 C for 10 min to extract intracellular acetic acid. Finally, the supernatant obtained by centrifuging at 12000*g* for 5 min was analyzed by HPLC analysis to detect intracellular acetic acid.

2.7. ROS accumulation and membrane integrity evaluation

The ROS accumulation of yeast cells was detected with the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method. In general, yeast cells were collected at the log phase by centrifugation and washed twice with 10 mM PBS (pH 7.0), followed by re-suspending in the PBS containing 10 μM DCFH-DA, and then cultured at 37 C for 30 min. ROS examination and quantification were followed the methods described previously (Zhang et al., 2015). Membrane integrity was evaluated using propidium iodide (PI) following the method: samples were re-suspended in PBS containing 15 μM PI, and then cultured at room temperature for 20 min. Membrane permeabilization was measured using Multiskan spectrum microplate spectrophotometer as described (Garcia-Gonzalez et al., 2010).

2.8. Statistical analysis

All experiments were performed in triplicates. The results of RT-qPCR, intracellular acetic acid detection, ROS accumulation and membrane integrity evaluation were showed as means with standard deviations (SD). Statistical analysis was performed using the student *t*-test at the significance of $P < 0.05$ and $P < 0.01$, respectively.

3. Results and discussion

3.1. Comparative transcriptome analysis revealed significant repression of transporter genes by zinc sulfate addition under acetic acid stress

Strain SPSC01 is a self-flocculating industrial strain developed in our group with good fermentation performance, which has been used in a commercial fuel ethanol plant (Xu et al., 2005). In the previous studies, we found improved acetic acid tolerance of SPSC01 by zinc sulfate addition (Wan et al., 2015), therefore this strain was used for transcriptome analysis. Ethanol fermentation was performed under acetic acid stress with or without zinc sulfate supplementation (Fig. S1A), and global gene transcription was investigated by RNA-seq analysis using cells that harvested at 72 h. As observed in the previous studies (Wan et al., 2015), ethanol production under acetic acid stress was enhanced by zinc sulfate addition. Consistent qRT-PCT data using primers to detect the transcription levels of *ADY2*, *ATO2*, *JEN1*, *ADE17*, *OGG1*, *CIN5* and *HSF1* were obtained with the transcriptome analysis, suggesting that the transcriptomic results are reliable. The RNA-seq data were provided as Supplementary file S1. The results showed that in total, 2219 genes changed their expression levels ($P < 0.05$; Log_2 Ratio > 1 or < -1), among which 715 genes were upregulated and 1504 were downregulated when compared to the control. Then KEGG and GO analysis was carried out to explore genes which may be critical for acetic acid resistance.

KEGG pathway analysis showed that amino acid metabolism, purine and pyrimidine metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway and steroid biosynthesis changed significantly (Fig. S1B). Glycolysis and pentose phosphate pathway provide enough energy for cell growth and are important for anti-toxicity of acetic acid because yeast cells combat acetic acid stress by pumping out acid using energy from ATP hydrolysis (reviewed by Caspeta et al. (2015)). Positive effects of amino acids and peroxisome on acetic acid tolerance have been well studied previously (Wan et al., 2015; Wang et al., 2015; Guaragnella et al., 2008), and interestingly these pathways were also shown to be clustered in this study. Ergosterol is critical for maintaining yeast membranes stabilization and has a close relationship with membrane proteins (Grossmann et al., 2008). Meanwhile, ergosterol biosynthesis shows important function in improving growth in the presence of acids or ethanol stress (Fletcher et al., 2016; Caspeta et al., 2014). Under acetic acid stress, genes involved in ergosterol biosynthesis were highly changed in response to zinc sulfate addition. As shown in Fig. 1, *ERG1*, *ERG3*, *ERG6*, *ERG11* and *ERG25* were significantly upregulated in the zinc sulfate addition cells, and of these five genes, deletion of *ERG3* and *ERG6* were reported to lead to acetic acid sensitivity (Mira et al., 2010). Consistently, the content of ergosterol was 46.9 mg/g (on DCW basis) in the zinc sulfate addition yeast cells was detected, which was 14.6% higher than that of the control (Fig. S2).

GO analysis of changed genes was performed with DAVID and SGD database (Huang et al., 2008). In total 79 categories of GOTERM_BP (Biological Process), 39 categories of GOTERM_CC (Cellular Compound) and 43 categories of GOTERM_MF (Molecular

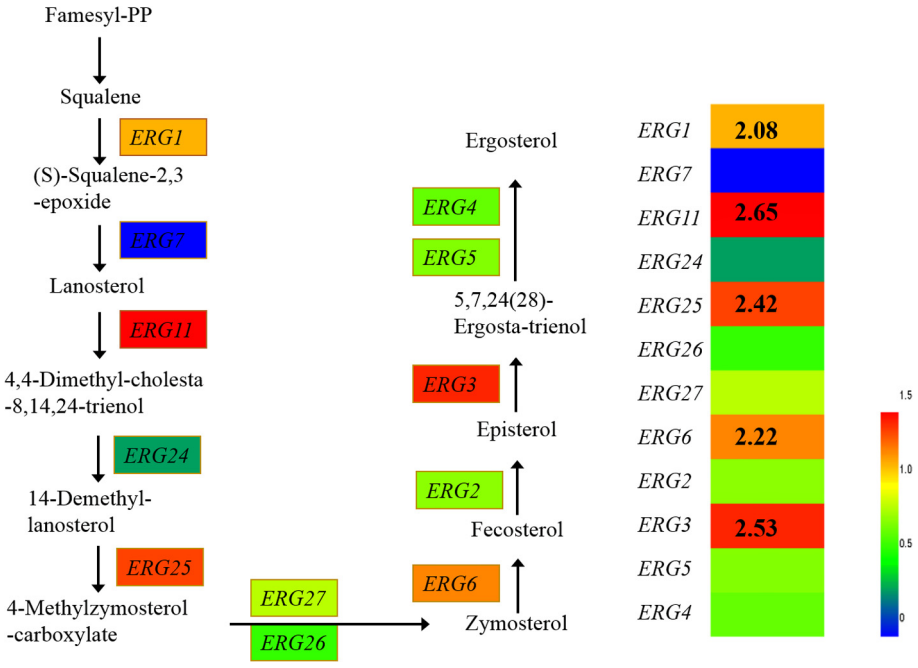

Fig. 1. Variation of gene transcription related to ergosterol biosynthesis pathway in *S. cerevisiae* by zinc sulfate addition under acetic acid stress.

Function) were revealed, suggesting the global function of zinc sulfate in cell resistance to acetic acid. The significant categories (Fig. S1B) include plasma membrane, oxidoreductase activity, and oxidantation-reduction process etc. Among all the categories, transmembrane transporter activity which belongs to GOTERM_MF was worth noticing (Table S3). Among all categories, changes in transmembrane transporter activities were apparent (Table. S3). Remarkably down-regulated carboxylic acid transporter encoding genes were revealed, which mainly include *ADY2*, *ATO2,* and *JEN1* (Table 1). *ADY2* has an important function in acetic acid uptake (Paiva et al., 2004). On the other hand, *ATO2* is the paralog of *ADY2*, and it was reported that mutations in *ATO2* were also known to trigger acetic acid hypersensitivity in *S. cerevisiae* (Gentsch et al., 2007). In addition, Jen1p was also known as one of the carboxylic acid transporters that are responsible for acetic acid uptake (Casal et al., 2016). It is therefore of interest to study whether deletion of these transporter encoding genes can improve acetic acid tolerance in *S. cerevisiae*.

3.2. Improved growth ability of yeast cells by ADY2 deletion

Putative transcription factor binding sites in the *ADY2* promoter region (599 bp upstream of the start codon) were analyzed based on the YEASTRACT database (Fig. S4), and it was found that *ADY2* is the potential target of Msn2p/Msn4p, which are key transcription factors responsible for general stress response (Martinez-Pastor et al., 1996), suggesting that *ADY2* might be a stress-responsive gene. Haa1p is the main player in the transcriptional regulation of multiple genes involved in acetic acid stress response and tolerance (Mira et al., 2011), while the putative Haa1p binding site is also present in the promoter region of *ADY2*. In addition, the putative Stb5p binding sites are also present in the *ADY2* promoter region, and the function of this regulator is related to NADPH production (Re et al., 2010). It is thus of interest whether *ADY2* is related to yeast tolerance of multiple stresses.

The effect on of *AYD2* deletion on cell growth and ethanol fermentation under acetic acid stress was investigated. As illustrated in Figs. 2 and S5, *ADY2* deletion did not influence the growth of *S. cerevisiae* BY4741 under no exposure to external stress. Contrastingly, the deletion clearly improved cell growth when cells were exposed to 3.6 g/L acetic acid (pH 3.7) and 5 mM H_2O_2 on the agar plates (Fig. S5). Similar results were observed in liquid culture when these two inhibitors were supplemented in the liquid medium (Fig. 2B and C). Furthermore, improvement of cell growth was also revealed when formic acid or ethanol was added in the medium (Fig. 2D and F). In contrast, no apparent difference of growth with supplementation of 12 g/L lactic acid between the two strains was observed (Fig. 2E). These results demonstrated for the first time that deletion of *ADY2* enhances tolerance of yeast cells to multiple stresses.

Table 1
Relative expression levels of genes involved in acid transport in zinc sulfate supplemented *S. cerevise* SPSC01 under acetic acid stress.

Systematic Name	Standard Name	Log2 ratio (Zn vs CK)	Function
YCR010C	*ADY2*	−3.76	Acetate transporter required for normal sporulation
YNR002C	*ATO2*	−3.28	Putative transmembrane protein involved in export of ammonia
YKL217W	*JEN1*	−3.47	Monocarboxylate/proton symporter of the plasma membrane

3.3. Improved fermentation efficiency by ADY2 deletion

The time-courses of ethanol fermentation after the deletion *ADY2* gene were measured in the laboratory strain *S. cerevisiae* BY4741. When 3.6 g/L acetic acid (pH 3.7) was added to the fermentation media I, the distinctly improved growth rate of BYady2 was observed when compared to BY4741, which was 5.7% higher in the maximum biomass (Fig. 3A). Resultantly, almost all glucose was consumed by the strain BYady2 after 48 h, whereas there was still 10.4 g/L residual glucose with the strain BY4741 after 48 h (Fig. 3B). The final concentrations of ethanol production were 42.4 g/L and 37.0 g/L by strains BYady2 and BY, respectively. To investigate the application of *ADY2* deletion in other *S. cerevisiae*

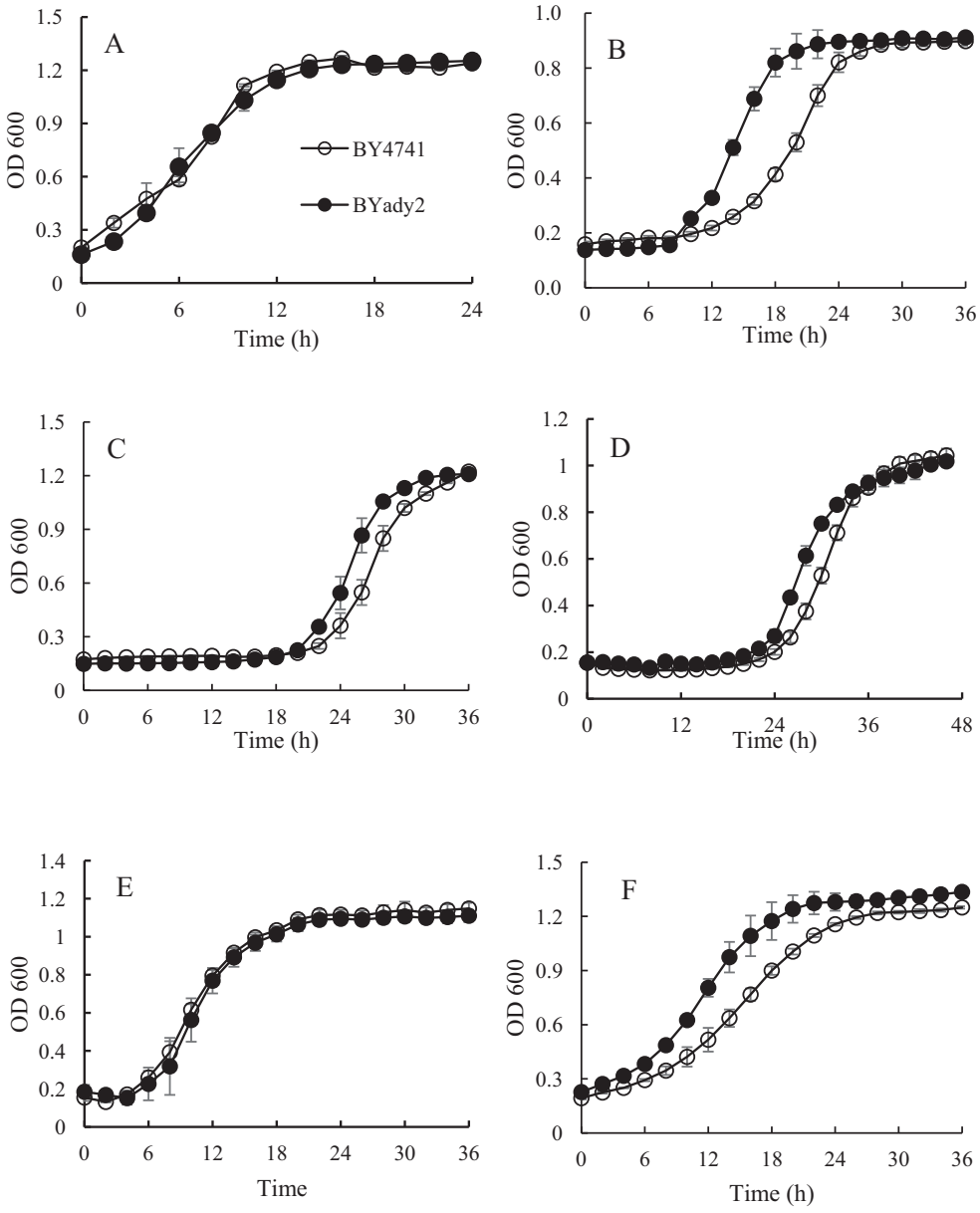

Fig. 2. Evaluation of growth performance with strains BYady2 and BY4741.The strains were cultured without external stress (A), supplemented with 3.6 g/L acetic acid (B), 5 mM H_2O_2 (C), 1.2 g/L formic acid (D), 12 g/L lactic acid (E) and 10% ethanol (F). The solid circle and square represent BY and BYady2, respectively. Results are the average of three independent experiments.

strains, *ADY2* deletion mutant of the industrial yeast strain *S. cerevisiae* 4126 was also constructed (named 4ady2). Fermentation data (Fig. 3C and D) showed a shorter lag phase, and higher fermentation efficiency in 4ady2 when compared to that of 4126, in the presence of 7.5 g/L acetic acid (pH 4.5) in the fermenter. The maximum biomass of 4ady2 reached 3.3 g/L at 18 h, in contrast to 3.2 g/L of control strain 4126 at 36 h. Although the similar final concentration of ethanol production was obtained by both strains, ethanol productivity of 4ady2 reached 1.8 g/L/h, in contrast to 1.1 g/L/h for control strain 4126. These results suggested that *ADY2* deletion enhanced production of ethanol under acetic acid stress. During pretreatment of lignocellulosic biomass, acetic acid, furfural and phenol could be released as inhibitors (Jönsson and Martín, 2016). Hence, simulated hydrolysis medium (pH 4.5) was used to evaluate fermentation performance of the *ADY2* deletion strain. It was observed that after 40 h, all glucose was consumed by BYady2, whereas 7.0 g/L residual glucose was detected for *S. cerevisiae* BY4741 (Fig. S6). These results indicated that *ADY2* deletion could be an effective method to develop robust yeast strain for cellulosic ethanol production.

One important mechanism of yeast cell tolerance to acetic acid is the export of acetic acid. Previous results revealed that the intracellular acetic acid level of was significantly lower in the *HAA1* overexpression mutant or *JJJ1* deletion mutant, which exhibited improved acetic acid tolerance (Tanaka et al., 2012; Wu et al., 2016). Hence, in this study, the acetic acid tolerance rendered by *ADY2* deletion was further studied by evaluating the intracellular level of acetic acid. Interestingly, a decrease of the intracellular acetic acid level in the deletion mutant BYady2 was observed, which was 12.9% lower than that of the control strain *S. cerevisiae* BY4741. This result was consistent with the previous finding that Ady2p serves as the acetic acid transporter (Paiva et al., 2004). However, these results are different from the previous report in the way that the function of *ADY2* was explored during adaptation

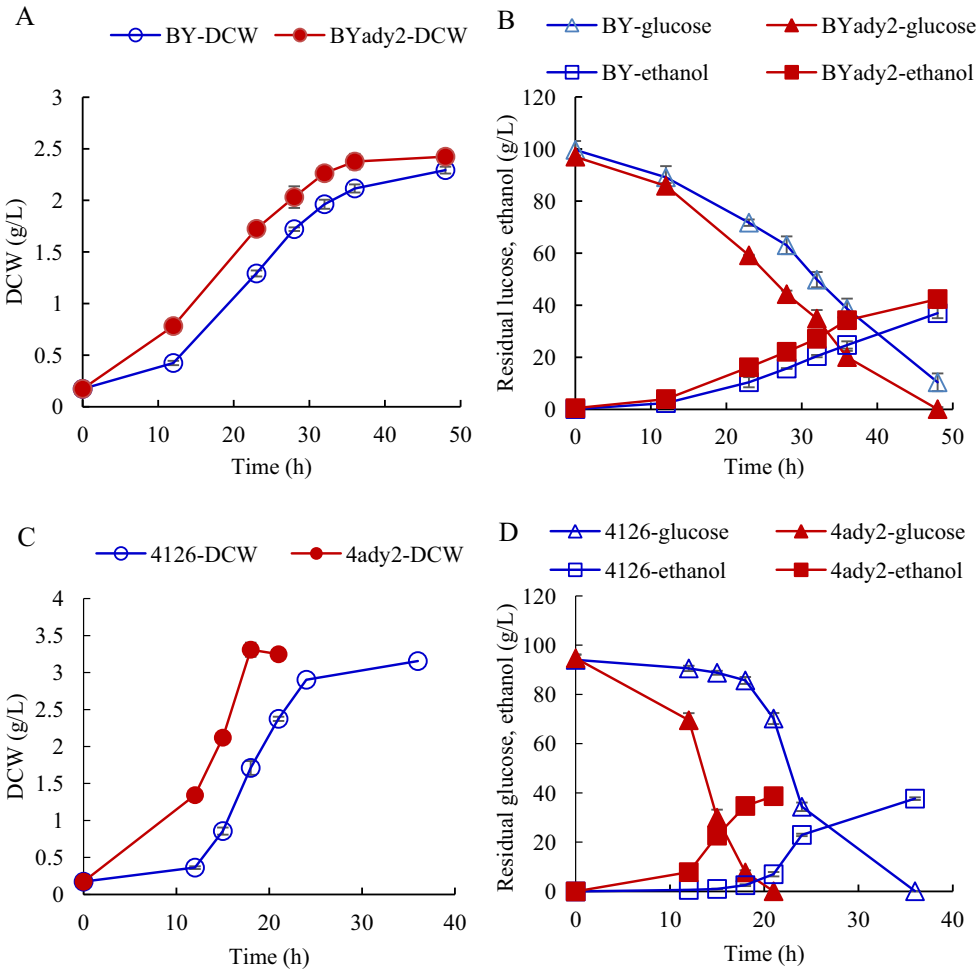

Fig. 3. The impact of *ADY2* deletion on ethanol fermentation of *S. cerevisiae*. Ethanol fermentation was performed from fermentation medium supplemented with 3.6 g/L acetic acid in the host strain *S. cerevisiae* BY4741 (A and B) and 7.5 g/L acetic acid in the host strain 4126 (C and D). Results are the average of three runs ± standard deviation.

of acetic acid stress, where the previous studies focused on the shift of glucose to acetic acid as an alternative carbon source (Paiva et al., 2004).

Meanwhile, the intracellular ROS accumulation and membrane integrity of strains were also detected. Lower intracellular ROS accumulation (24.8%) and higher cell membrane integrity (17.8%) were shown in BYady2 when compared to control strain BY4741. ROS accumulation is usually induced by various stress conditions, including formic acid and ethanol stress (Du et al., 2008; Charoenbhakdi et al., 2016). It was reported that overexpression of *SET5* and *PPR1* or deletion of *RTT109* and *JJJ1* endowed yeast increased acetic acid tolerance which was accompanied with decreased ROS accumulation (Zhang et al., 2015; Cheng et al., 2016; Wu et al., 2016). Previous studies revealed that membrane disruption could be caused by acetic acid toxicity, and high membrane integrity was commonly consistent with high acetic acid tolerance (Zheng et al., 2011). It was also reported that many genes associated with acetic acid stress tolerance are also related to formic acid tolerance (Henriques et al., 2017). The common toxic effects of formic acid, ethanol, acetic acid and H_2O_2 are membrane injury and oxidative stress, therefore we proposed that improved stress tolerance to multiple conditions by *ADY2* deletion are related to the influence on ROS accumulation and membrane properties. The increased membrane integrity by deletion of *ADY2* suggests that the absence of Ady2p is associated with variation of plasma membrane properties, leading to improved cell viability. Interestingly, it was found that *ADY2* transcription was decreased by ergosterol addition (Fig. S3), which may be correlated with improved membrane integrity. It will be interesting to examine whether transcription of *ADY2* is affected by membrane properties. We propose that the reduction of membrane permeability and lower level of ROS accumulation are the possible mechanisms underlying *ADY2*-mediated improved acetic acid tolerance.

Among the detected plasma membrane proteins responded to zinc sulfate addition, there were other two membrane protein encoding genes, *ATO2* and *JEN1*. These two genes were also deleted separately in *S. cerevisiae* BY4741 and the obtained mutants were named as BYato2 and BYjen1, respectively. As observed after *ADY2* deletion, improved acetic acid tolerance was also revealed by deletion of *ATO2*, whereas double deletion of *ATO2* and *ADY2* did not further improve acetic acid tolerance (Table 2). Although

Table 2
The maximum specific growth rate of the wild type strain *S. cerevisiae* BY4741 and the mutant strains.*

Yeast strains	μ_{max}	
	CK	60 mM acetic acid
BY4741	0.389 ± 0.0094	0.141 ± 0.0018
BYady2	0.386 ± 0.0019	0.166 ± 0.0042
BYato2	0.382 ± 0.00060	0.157 ± 0.0019
BYaa	0.375 ± 0.029	0.172 ± 0.0057

* BYady2, the *ADY2* deletion mutant; BYato2, the *ATO2* deletion mutant; BYaa, the *ADY2* and *ATO2* double deletion mutant.

JEN1 deletion also improved acetic acid tolerance, but it could not be explained why *JEN1* deletion also improved cell growth when no acetic acid is present (data not shown). Hence, the function of gene *ADY2* was mainly detected in this study. Multi-drug resistance transporters *AQR1*, *TPO2*, *TPO3*, and *PDR12* were demonstrated to influence acetic acid tolerance (Mira et al., 2010). The current findings in this study have added new evidence of important functions of plasma membrane transporters Ady2p, Jen1p, and Ato2p on yeast acetic acid tolerance.

Interestingly, *ADY2*, *ATO2*, and *JEN1* all contain putative binding sites of Haa1p and Msn2p/Msn4p (Fig. S4), further studies on the transcriptional regulation of these genes by Haa1p will enrich the knowledge of the regulatory network of Haa1p under acetic acid stress. In addition, studies on the dynamics of the Ady2p-Ato2 complex in the acetic acid stress and their interaction with membrane ergosterol will be interesting. It was reported that overexpression of *PRS3*, *RPB4* and *ZWF1* improved ethanol production using cellulosic hydrolysates (Cunha et al., 2015). Our current work provide novel insights into the function of *ADY2* in yeast stress response and offer an alternative strategy for the development of stress tolerant yeast strains for production of bioethanol under various stress conditions.

4. Conclusions

Zinc sulfate addition down-regulated *ADY2* expression of *S. cerevisiae* SPSC01 under acetic acid stress. Improved growth of the *ADY2* deletion mutant was observed in the presence of multiple inhibitors, including acetic acid, ethanol, and H_2O_2. Meanwhile, positive effect of *ADY2* deletion on ethanol fermentation was revealed in the presence of acetic acid, which was associated with the decrease of intracellular acetic acid content, ROS accumulation and plasma membrane permeability. These results indicate that deletion of *ADY2* may be a useful strategy for stimulating ethanol production under stress conditions.

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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.2017.05.191.

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