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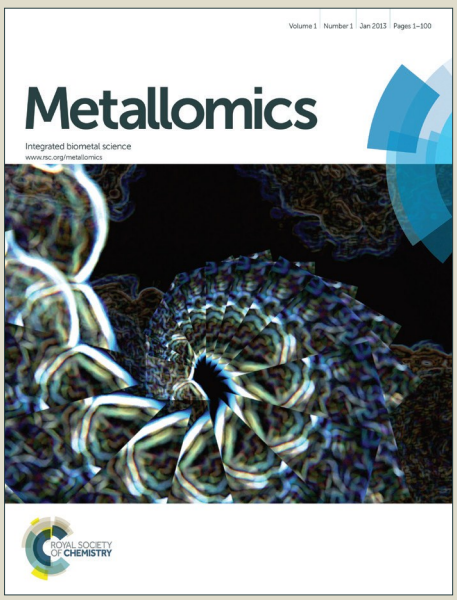

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Impact of zinc sulfate addition on dynamic metabolic profiling of *Saccharomyces cerevisiae* subjected to long term acetic acid stress treatment and identification of key metabolites involved in antioxidant effect of zinc

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Abstract

The mechanisms of zinc to protect the cells against acetic acid toxicity and how zinc acts as an antioxidant are still not clear. Here we present results of metabolic profiling of the eukaryotic model yeast species *Saccharomyces cerevisiae* subjected to long term high concentration acetic acid stress treatment in presence and absence of zinc supplementation. Zinc addition decreased the release of reactive oxygen species (ROS) in the presence of chronic acetic acid stress. The dynamic changes in the accumulation of intermediates in central carbon metabolism were observed, and higher contents of intracellular alanine, valine and serine were observed with zinc supplementation. The most significant change was observed in alanine content, which is 3.51-fold of that of the control culture in cells at stationary phase. Subsequently, it was found that 0.5 g L^{-1} alanine addition resulted in faster glucose consumption in presence of 5 g L^{-1} acetic acid, and apparently decreased ROS accumulation in zinc-supplemented cells. This indicates that alanine exerted its antioxidant effect at least partially through detoxification of acetic acid. In addition, intracellular glutathione (GSH) accumulation was enhanced by zinc addition, which is related to the protection of yeast cells from the oxidative injury caused by acetic acid. Our studies revealed for the first time that zinc modulates cellular amino acid metabolism and redox balance, especially biosynthesis of alanine and glutathione to exert its antioxidant effect.

Key words: *Saccharomyces cerevisiae*, zinc, acetic acid, stress tolerance, reactive oxygen species (ROS).

1. Introduction

Zinc is an important micronutrient for cell growth and metabolism of almost all living organisms, and is the cofactor of a myriad of enzymes. It also serves as a critical structural component of many essential proteins, including some ribosomal proteins and zinc-finger regulatory proteins[1-4]. Therefore, zinc homeostasis is important for cell growth and metabolism[3, 4]. Yeast strains of *Saccharomyces cerevisiae* are widely used as a eukaryotic model to study fundamental aspects of metal uptake and homeostasis[5-9], and molecular events related to zinc deficiency and zinc toxicity have been revealed[3, 6]. We recently reported the protective effect of zinc against ethanol toxicity and heat shock stress in brewing yeast *Saccharomyces cerevisiae*[10, 11], and suggested that zinc status contributed to the activation of defense systems against various harsh environmental conditions such as toxic level of ethanol and heat shock treatments[10-12].

Yeast strains of *S. cerevisiae* are widely used for production of beer, wine, as well as bioethanol. Fuel ethanol production by *S. cerevisiae* strains using lignocellulosic feedstock has been extensively studied in the recent years[13]. However, the economy of bioethanol production is still not satisfactory, and improvement of cell viability against various stressful factors is important for large scale production. The inhibitory compounds (e.g., week acids, aldehydes, and phenols) released from hydrolysis of lignocellulosic materials are well known to inhibit cell viability and ethanol production[14-16]. Acetic acid is one of the major weak acids in the lignocellulosic hydrolysate that exerts inhibitory effect on *S. cerevisiae*, which can

inhibit nutrient uptake, leading to energy depletion as well as decreasing activities of metabolic enzymes[17]. In addition, acetic acid also inhibits both cell growth and ethanol fermentation of *S. cerevisiae*[18, 19]. It can also induce reactive oxygen species (ROS) production[20] and thereby results in oxidative stress in yeast cells and programmed cell death[21]. Therefore, understanding the molecular events related to yeast acetic acid stress and improvement of acetic acid tolerance of *S. cerevisiae* is of great concern for both studies on the mechanism and biotechnological applications.

Various studies reported that acetic acid provokes oxidative stress of *S. cerevisiae*[19], and zinc is known as an antioxidant in mammalian cells[4, 22, 23], we therefore attempted to explore the function of zinc in acetic acid stress protection in *S. cerevisiae*. Studies of zinc in protection against oxidative stress of *S. cerevisiae* are still very limited. It was revealed that zinc deficiency induces ROS generation in *S. cerevisiae*[24-26], and the possible sources of ROS under zinc-limited conditions were deduced[4], which include the decreased activity of antioxidant enzyme(s) such as Cu/Zn superoxide dismutase (SOD1p) and reduced expression of metallothioneins (MTs). In addition, zinc can compete with redox active metal ions such as copper and iron ions, and bind to free sulfhydryl (-SH) groups of proteins to avoid their oxidation, thus may also contribute to ROS reduction. Consequently, disruption of the function or expression of key proteins in mitochondrial electron transport chain, formation of misfolding proteins with incorrect disulfide bonds in endoplasmic reticulum (ER), as well as the increased NADPH oxidase activity, is also proposed as the source of ROS in low zinc yeast cells[4]. However, so far, it is still not clear whether zinc can act as an

antioxidant under stressful conditions other than zinc deficiency. What's more, regulation of cell metabolism by zinc, which is related to the antioxidative effect of zinc, is largely unknown.

In this study, the effects of zinc addition on cell metabolism of *S. cerevisiae* during ethanol fermentation in presence of acetic acid were investigated, and the molecular mechanisms underlying the protective effect of zinc against acetic acid stress of *S. cerevisiae* were explored by comparison of metabolic profiling in presence and absence of zinc.

2. Materials and methods

2.1 Yeast strains and culture conditions

The yeast strain used in this study was the self-flocculating yeast *S. cerevisiae* SPSC01, which was deposited at China General Microorganisms Culture Collection center (CGMCC) with the accession number of CGMCC1602. The components of medium for seed preparation and activation was (g L^{-1}): glucose 30, yeast extract 4, peptone 3. The ethanol production medium in bioreactor consisted of (g L^{-1}): glucose 100, yeast extract 4, peptone 3, and the pH value was adjusted to 4.5. Acetic acid at final concentration of 10 g L^{-1} was used for evaluation of stress tolerance of *S. cerevisiae* SPSC01 in bioreactor.

For metabolites determination, *S. cerevisiae* SPSC01 growing on the slant for 16-20 h was inoculated into a 250 mL Erlenmeyer flask containing 100 mL medium and cultivated at 30 ºC, 150 rpm overnight. Next, the strains were deflocculated in 0.2

M sodium citrate, after which the cells were distributed in several flasks for final inoculation. Fermentation was performed in 3 L bioreactors with 1.5 L production medium containing 10 g L^{-1} acetic acid supplemented with or without 0.03 g L^{-1} zinc sulfate, and the initial optical density at 620 nm (OD_{620}) was adjusted to around 0.075 (~2×10^6 cells mL^{-1}). The fermentation was carried out at 30 ºC, 200 rpm, 0.04 vvm, and pH 4.5, and was stopped when the residual sugar was less than 1 g L^{-1}. Samples were taken at interval of 6-12 h, and yeast cells and fermentation broth collected at lag phase, log phase, and stationary phase were used for further analysis.

2.2 Analysis of biomass, ethanol, glucose, acetic acid, succinic acid, glycerol and zinc content.

To determine biomass of *S. cerevisiae* SPSC01, yeast cells were harvested by centrifugation at 13, 500 g, 4 ºC for 5 min, and washed three times with MilliQ water, followed by freeze-drying overnight, and then weighted. The supernatant from the fermentation broth of *S. cerevisiae* SPSC01 was applied to an ultrafast liquid chromatography (UFLC) system (Shimadzu, Japan) equipped with an Aminex HPX-87H column (300 mm× 7.8 mm, Bio-Rad, U.S.A.) and an RID-10A refractive index detector (Shimadzu) to measure the concentrations of ethanol, glucose, acetic acid, succinic acid and glycerol. The UFLC system was operated at 60 ºC, and 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL min^{-1}.

To measure the zinc content in yeast cells, 200 μL of 10 M nitric acid was added into the tube containing freeze-dried cells and mixed for 1 min. After centrifugation at

13, 500 g for 5 min, the supernatant was diluted by 100 times with 1 M nitric acid and tested using the Polarized Zeeman Atomic Absorption Spectrophotometer (AAS) (Z-2000, Hitachi High-Tech, Japan) equipped with a 7.5 mm (in height) burner. Zinc standard for AAS (TraceCERT®) was purchased from Sigma-Aldrich, U.S.A. The wavelength for detection was set at 213.9 nm, while both the delay and measurement time were 5 s, and the flow rates of air and C_2H_2 were 15 and 1.8 L min^{-1}, respectively. The detection limit (DL) for Zn is 3 μg L^{-1}, while the coefficient of variations (CV) was 99.9%. The distribution of zinc was calculated and indicated by the percentage of zinc in yeast cells and broth.

To determine the total metal content, yeast cells collected at exponential growth phase were treated with nitrate acid according to the above-mentioned method and the total metal content was analyzed by inductively coupled plasma spectrometer (ICP Optima 2000DV, PerkinElmer, U.S.A.) equipped with a concentric nebulizer. The radio frequency power of ICP was 1300 W, and the flow rates of the carrier gas (Ar) and auxiliary gas (air) were set at 0.8 and 0.2 L min^{-1}, respectively, and the measurement time was 30 s. The DLs for Zn, Fe, Ca, Mg, Na, K are 1, 2, 0.02, 0.1, 3 and 20 μg L^{-1}, respectively. Multi-element standard solution (TraceCERT®) was obtained from Sigma-Aldrich, U.S.A. Three biological replications were performed and the average values were used for analysis.

2.3 Evaluation of ethanol fermentation

The fermentation performance was evaluated through the following equations:

$Y_{E/CS}$ = [EtOH] $_{max}$/ [sugar] $_{consumed}$ (1)

$Y_{E/CS}$, ethanol yield; [EtOH] $_{max}$, the maximum concentration of ethanol during fermentation; [sugar] $_{consumed}$, sugar consumed at the end of fermentation.

$Y_{B/CS}$ = [Biomass] $_{max}$/ [sugar] $_{consumed}$ (2)

$Y_{B/CS}$, biomass yield; [Biomass] $_{max}$, the maximum biomass during fermentation.

Both of ethanol and biomass yield obtained from zinc addition experiment were determined as the percentage of control.

$Y_{E/T}$ = [EtOH] $_{max}$/t (3)

$Y_{E/T}$, ethanol productivity; t, the fermentation time that is corresponding to the maximum concentration of ethanol.

2.4 Metabolites extraction and analysis

The intracellular metabolites of *S. cerevisiae* SPSC01 were extracted with the protocols according to the previous study[27]. The freeze-dried metabolites were analyzed by GC-MS system (GC, Agilent 7890A equipped with a 30 m × 0.25 mm i.d. fused silica capillary column from Varian Inc., Palo Alto, CA; MS, Pegasus HT time of flight mass spectrometer from Leco Corp., St Joseph, MI) following the methods developed elsewhere[27], while the re-dissolved metabolites in 50 μL MilliQ water were analyzed with a LC-QqQ-MS system (LC, Agilent 1200 series; MS, Agilent 6460 with Jet Stream Technology; Agilent Technologies, Germany) following the protocols described previously[28]. The peak of a target metabolite was identified and quantified by comparing with the standard using the Pegasus ChromaTOF ver. 4.21 software

(Leco) and MassHunter Quantitative Analysis software package ver. 04.00 (Agilent Technologies). The Certified Reference Materials (CRM) for GC-MS and LC-QqQ-MS were adipic acid and D-camphor sulfonic acid, respectively, both of which were purchased from Nacalai Tesque, Inc., Kyoto, Japan.

2.5 Determination of ROS release in yeast cells grown in acetic acid

To detect ROS release of the yeast cells grown in the presence of acetic acid, yeast cells were collected and deflocculated by 0.2 M sodium citrate and washed with 1 mL of distilled water, then re-suspended in 50% 0.1 M sodium citrate and 0.1 mL phosphate buffered saline (PBS) pH 7.0. ROS release from different time points were stained with 2' 7'-dichlorofluorescein diacetate (DCFH-CA) following the methods described previously[29]. Cells were observed using the confocal laser scanning microscope (CLSM, Carl Zeiss, German) with fluorescein isothiocyanate (FITC) HYQ fluorescence filter (460-500 nm). For ROS determination, at least 100 cells from two time points were examined, and the results of both control group and the zinc-supplemented group of cells were obtained from two biological replications.

2.6 Investigation of the effect of alanine addition on ethanol fermentation of *S. cerevisiae* SPSC01 with acetic acid.

The concentrations of alanine used to test its effect on acetic acid tolerance were (g L^{-1}): 0, 0.2, 0.5 and 1.0, respectively. Acetic acid was added at the final concentrations of 5 g L^{-1} in the culture medium after autoclaving. Yeast cells of strain

SPSC01 were activated in seed medium by overnight cultivation for two times, which was then deflocculated by 0.2 M sodium citrate, and the initial inoculum was adjusted to OD_{620} of 2.0. Five milliliters seed culture was inoculated in 250 mL flasks containing 100 mL fermentation medium and the cultures were incubated at 150 rpm, 30 °C for 24 h. For ROS detection, yeast cells grown in the medium supplemented with 5 g L^{-1} acetic acid and 0.5 g L^{-1} alanine were collected at 12 h and 18 h, and were deflocculated using 0.1 M sodium citrate. Yeast cells grown in 5 g L^{-1} acetic acid without alanine addition were used as control. The cell density of the samples was adjusted to OD_{620} 1.0 with ddH_2O. DCFH-CA was added at a final concentration of 10 μM, after which the cells were further cultivated at 37 °C for 30~60 min, the cells were then collected at 10, 000 g and washed twice with PBS buffer solution. Subsequently, the cells were re-suspended in buffer solution (0.1 M PBS, pH 7.0, and 0.2 M sodium citrate with equal volume) before detecting the fluorescence value (excitation wavelength at 485 nm and emission wavelength at 525 nm).

2.7 Statistics

For metabolic profiling analysis and metal content determination, all values are expressed as mean ± standard deviation (SD) ($n \geq 3$). For ROS determination, we used at least 100 cells for counting. The differences between groups of discrete variables were evaluated by the Student's *t* test, while a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Effects of zinc addition on ROS release, cell growth and ethanol fermentation of *S. cerevisiae* SPSC01

Although it is known that acetic acid induces ROS release, so far there is no study on ROS accumulation in yeast cells during long term incubation in presence of high concentration of acetic acid under ethanol fermentation conditions. Therefore, ROS release was examined during various time points of ethanol fermentation in presence of 10 g L^{-1} acetic acid. The results of exponential phage cells are shown in Fig. 1. It can be clearly visualized that zinc supplementation decreased ROS release (Fig. 1A). Significant reduction of the percentage of cells showing clear ROS accumulation was observed (Fig. 1B).

Fig. 1 should be here.

Decreased ROS release is closely related to improved cell viability, which can be inferred from ethanol fermentation performance. As shown in Table 1 and Fig. 2, zinc addition resulted in a shorter lag phase of the yeast cells grown in higher concentration (10 g L^{-1}) acetic acid. Yeast cells with zinc addition started to grow about 23 h after inoculation, which is 12 h earlier than that of the control culture. Improved ethanol fermentation performance of *S. cerevisiae* SPSC01 with 10 g L^{-1} acetic acid was observed after zinc addition (Fig. 2). More precisely, zinc addition had significantly shortened the fermentation time to 45 h, and at the same time, the highest biomass obtained from zinc supplementation was around 3.5 g L^{-1}, which was 15%

higher than that from control culture (Table 1). Zinc addition did not affect ethanol titer very much. Nevertheless, due to the shortened fermentation time, ethanol productivity from zinc addition reached 1.096 g L^{-1} h^{-1}, which is 30.8% higher than that of control.

Fig. 2 and Table 1 should be here.

When extracellular metabolites in the culture broth were investigated, it was found that the concentration of acetic acid in control and zinc addition culture remained stable at around 6 g L^{-1} (data not shown). Zinc addition resulted in faster production of glycerol (~4.6 g L^{-1}) from 34 h to 45 h, whereas in the control culture, glycerol accumulated gradually during this time period. Similar glycerol level was retained at the end of fermentation in both the control culture and zinc-supplemented culture (Fig. 3). As for succinic acid production, it initiated earlier at 25 h in zinc supplemented culture, whereas in the control culture it started at 34 h (Fig. 3).

Zinc content in the cells of control culture was undetectable, and we therefore only detected zinc distribution in the zinc-supplemented culture. As illustrated in Fig. 3, most of the zinc existed in the culture broth at the beginning of cell growth, which accounts for 88% of total zinc. Yeast cells started to assimilate zinc vigorously during fast cell growth, accompanied with the linear decrease of zinc in fermentation broth. About 72% of zinc was located in yeast cells, while 28% of zinc distributed in the culture broth when glucose was completely consumed. After that, slight increase of zinc in cells was detected. We further analyzed the total metal contents of the yeast

cells grown at exponential phase using ICP-MS. It was found that potassium (K) content decreased to 50% of the control level by zinc addition, whereas magnesium (Mg) content increased (36%) in zinc-supplemented cells (p<0.05). No significant difference of content of other three metals (Ca, Fe and Na) was observed (Fig. 4).

Fig. 3 and 4 should be here.

3.2 Effects of zinc addition on the metabolic profiling of *S. cerevisiae* SPSC01 subjected to long term acetic acid stress treatment

Yeast cells in both the control culture and zinc addition culture were collected from the early log phase (Stage I), mid-log phase (Stage II), and late log phase (Stage III), respectively, and the time points were also selected according to different glucose consumption profile (indicated as red square in Fig. 2). More than 70 metabolites were quantified by GC-MS and LC-QqQ-MS, including intermediate metabolites derived from glycolysis, TCA cycle, phosphate pentose (PP) pathway, as well as amino acids and co-factors (e.g., NAD^+/NADH and $NADP^+$/NADPH). The major metabolites showing significant variation by zinc supplementation are summarized in Fig. 5 and Table S1. Intracellular glucose content in the zinc-supplemented cells was higher (12.7×10^3 nmol g^{-1} dry cell weight (DCW)) in Stage I, which was 1.48 times higher than that in the control culture, indicating faster glucose uptake by zinc addition. In contrast, the glucose content of the zinc-supplemented cells decreased dramatically in comparison with the control culture in Stage III, which was due to the active metabolism of glucose in zinc-supplemented cells. For intracellular

intermediates of glycolysis pathway and TCA cycle, as well as amino acids from glycolysis, significantly increased content was observed in the zinc-supplemented cells, which was consistent with the improved cell vitality by zinc addition. The most significant increase was found in the content of G6P and F6P in Stage II of the zinc-supplemented samples, which were 2.58 and 2.46-fold of that of the control culture, respectively, indicating that zinc could regulate the accumulation of these two metabolites to activate glycolysis when cells were subjected to chronic acetic acid stress.

Increased glycerol, succinic acid, alanine, valine and serine contents in cells resulted from zinc addition was also observed in various growth phases (Fig. 5 and Table S1). More specifically, glycerol in cells obtained from zinc addition was 1.24 times higher at Stage II than that from control. Succinic acid content reached to 7.2×10^3 nmol g^{-1} DCW at Stage I due to zinc addition, which was 1.5 times higher than that in the control. Among the amino acids detected, considerable accumulations of alanine in cells after zinc addition were revealed, which were 1.20, 1.25, and 3.51 times higher, respectively, in Stage I, Stage II, and Stage III than those in the control. Interestingly, the content of intracellular lactate displayed an opposite trend to that of alanine content. The contents of lactate in cells in the zinc-supplemented culture were dramatically lower than those in the control culture, especially in Stage I and Stage III. Since alanine and lactate are both derived from pyruvate node, it is clear that zinc regulates the carbon flux and directs more carbon to alanine biosynthesis.

Other amino acids that are significantly regulated by zinc addition include serine,

valine, and glycine. Differences in amino acid contents were observed in Stage III cells, and contents of glycine, serine and valine were significantly higher in zinc-addition cells than those in cells of the control culture (Table S1).

Contents of intermediates in non-oxidative PP pathways such as erythrose-4-phosphate (E4P), ribulose-5-phosphate (Ru5P), and xylulose-5-phosphate (Xu5P) showed a tendency to decrease in Stage I and increase in Stage II in zinc-rich cells, and were kept to similar level to those of the control cells in Stage III (Table S1). Specifically, Xu5P showed a significant decrease and increase in Stage I and II, respectively.

Higher accumulation of trehalose was observed in early growth Stage I, which was 1.58-fold of that of the control cells, consistent with our previous studies of increased trehalose biosynthesis by zinc addition in continuous high concentration ethanol fermentation[11]. Trehalose is known to confer acetic acid tolerance to *S. cerevisiae*[30]. The higher trehalose content in the early growth stage may benefit higher cell viability. It is worth noting that glutathione (GSH) in cells in zinc rich samples reached 3.6 and 9.1×10^3 nmol g^{-1} DCW in Stage II and III, which were 1.75 and 1.64 times higher than those of the control levels, respectively. An increase of oxidized glutathione (GSSG) in the cells from zinc addition was achieved in Stage II and III (Table S1). Nevertheless, the ratio between GSSG and GSH was much higher in control than that in zinc addition cells with respect to different stages, and the difference between control and zinc addition cells was the most evident at Stage I (Fig. 5).

Fig. 5 and Table 2 should be here.

The comparison of metabolites of cells grown with and without zinc addition has revealed some unique metabolites can either be detected in control or in the zinc-supplemented culture, and these metabolites were summarized in Table 2. The number of the unique metabolites was presented in Fig. S1. In all the three stages, more specific metabolites were found in zinc-supplemented culture, which was consistent with the higher cell viability and better fermentation performance of zinc-rich cells. When compared with control, most of the unique metabolites only detected in zinc-supplemented culture are amino acids, including tryptophan, γ-aminobutyric acid (GABA), lysine, tyrosine, and arginine.

3.3 Effects of alanine addition on the ethanol fermentation of *S. cerevisiae* SPSC01 treated with acetic acid

Among the amino acids that were increased by zinc addition in presence of long term acid stress, intracellular alanine content showed the most significant change. We proposed that intracellular alanine level was up-regulated by zinc addition during ethanol fermentation in presence of acetic acid, which could benefit cell viability against the toxicity of acetic acid. To test this hypothesis, ethanol fermentation was carried out to evaluate the fermentation performance of *S. cerevisiae* SPSC01 with the addition of alanine in presence of 5 g L^{-1} acetic acid. As shown in Fig. 6, it was obvious that alanine addition benefited glucose utilization and ethanol production, and

the fermentation time was shortened for almost 4 h when compared with control without alanine addition. More precisely, gradual consumption of glucose and production of ethanol were observed at the beginning of the fermentation, after which the glucose concentration in 16 h had reduced to 36.5, 22.0, and 25.0 g L^{-1} in the fermentation broth with 0.2, 0.5, and 1.0 g L^{-1} alanine, respectively, which was significantly lower than that from control (61.0 g L^{-1}). On the other hand, the ethanol concentration reached to 27.6, 33.9, and 32.6 g L^{-1} at this point with alanine added at dosages of 0.2, 0.5, and 1.0 g L^{-1}, respectively, while the ethanol in the control culture was only 17.0 g L^{-1}. When ethanol production was examined at 20 h, about 8.0 and 2.6 g L^{-1} of residual glucose were detected in the broth with 0.2 and 1.0 g L^{-1} alanine addition, whereas almost all glucose was consumed in the culture with 0.5 g L^{-1} alanine addition. Ethanol fermentation time of the control group is 4 h longer compared to the 0.5 g L^{-1} alanine addition group. No significant change in ethanol production level was observed with alanine addition, since the highest ethanol concentration from 0.5 g L^{-1} alanine addition was 49.0 g L^{-1} while that from control was 47.9 g L^{-1}. The effect of alanine was also tested using another industrial yeast *S. cerevisiae* ATCC4126, and similar promoting effect of alanine on ethanol fermentation in presence of acetic acid was observed (data not shown), indicating that alanine effect on acetic acid tolerance also applied to other yeast strains.

We also tested the effect of serine and valine on glucose consumption of *S. cerevisiae* SPSC01, and it was observed that addition of 1 g L^{-1} serine only slightly increased glucose consumption of the cells in the presence of acetic acid stress (Fig.

S2A). However, valine supplementation did not result in any significant change in glucose consumption (Fig. S2B). These results indicated that among the amino acids that were changed by zinc in the presence of acetic acid, alanine has the unique promoting effect.

To test whether alanine addition results in detoxification of acetic acid, release of ROS during chronic acetic acid stress in the presence and absence of alanine was examined. When ROS accumulation was examined using the cells collected from the control culture and 0.5 g L^{-1} alanine-supplemented cells grown for 12 h and 18 h, reduced ROS level was observed by alanine addition. The ROS fluorescence values of alanine-supplemented cells are 56.8 and 27.3 at 12 h and 18 h, respectively, whereas the values are 75.3 and 43.1 in the control group cells in the corresponding time points (Fig. 7). In other words, the ROS level in the cells with alanine addition was lower than that from control (p<0.05).

Fig. 6 and 7 should be here.

4. Discussion

Zinc is an important micronutrient for yeast cell growth and metabolism. The importance of zinc for ethanol fermentation has been widely recognized due to its role acting as cofactor for alcohol dehydrogenase ADH1p. However, the regulation of cell metabolisms by zinc, especially in relation to its antioxidant effect, is not well studied. ROS release was also observed in various growth stages during the fermentation (data not shown), which demonstrated that yeast cells indeed suffered from oxidative injury

when they were incubated long term with acetic acid. Addition of zinc decrease ROS accumulation inside the cells, and our results thus present the first evidence that zinc acts as antioxidant in yeast cells.

The metabolites profiling presented in this study showed that zinc exerts control of biosynthesis of various metabolites in glycolysis, TCA cycle, and pentose phosphate pathway, indicating that zinc serves a global regulation element and assists cells in altering carbon and energy flux to accumulate metabolites that render cells higher capability to get through adverse circumstances upon acetic acid stress.

We have reported the impact of zinc on metabolic flux of *S. cerevisiae* SPSC01 during continuous high concentration ethanol fermentation[11]. The results in this study agree with the previous study that zinc exerts effect on trehalose biosynthesis[11]. Interestingly, in a recent study on nickel-resistant *S. cerevisiae* cells, it was found that the intracellular trehalose levels of the nickel-resistant strain in the absence of nickel stress were higher than those of the reference strain[8], suggesting that the higher trehalose levels even in the absence of any nickel stress may provide a general survival advantage to the nickel-resistant strain. Furthermore, new findings were reported in this study on the effect of zinc on acetic acid tolerance. The antioxidative effect of zinc in *S. cerevisiae* cells suffering from acetic acid stress was revealed by decreased ROS release after zinc addition. This is the first example of antioxidant effect of zinc in *S. cerevisiae* cells. Oxidative stress is a common experience during ethanol fermentation[31], which not only emerges in the toxicity of various inhibitory agents to yeast cell growth and metabolism including furfural and ethanol[29, 32], but

also in fungal pathogenesis[33] and cell aging[34]. The results presented in this study thus provide basis for further exploration of the impact of zinc status in cell metabolism and defense system in various biological and biotechnological studies.

Glycerol is a protective agent synthesized by yeast cells against environmental stress, which can function in maintaining redox balance[35]. The increased glycerol biosynthesis rate by zinc addition can thus benefit the stress defense of the cells against acetic acid toxicity. In our previous studies on continuous high concentration ethanol fermentation, zinc addition was found to decrease glycerol accumulation, and ethanol production was also improved by zinc addition[11]. In our current study, however, ethanol production level was not significantly changed by zinc addition. The different effect of zinc on glycerol biosynthesis may be due to the different experimental conditions as well as different stress response against ethanol and acetic acid.

Succinic acid is an important metabolite in TCA cycle for energy generation, and it also participates in GABA pathway involving ROS stress alleviation[36]. Indeed, we found that GABA content is increased in zinc-supplemented culture cells in Stage I when compared with the control culture cells (Table S1). On the other hand, increased flux to succinic acid may also direct more carbon to glyoxylate cycle. Glyoxylate was detected in early growth stage in zinc-rich cells (Table 2), whereas no glyoxylate was found in the control culture. Glyoxylate cycle is not well studied in yeast, and it is still not clear how zinc regulates glyoxylate cycle. We propose that activated glyoxylate cycle may act as an alternative carbon flux which introduces acetyl-CoA into TCA

cycle to generate energy for stress defense.

We also found an increase in glutathione (GSH) by zinc addition at Stage II and III. It was reported that GSH is a primary candidate to prevent yeast cells from oxidative stress[37, 38]. In addition, a declined GSSG:GSH ratio observed in zinc addition indicated low ROS level in yeast cells[4], causing less damage during ethanol fermentation. Moreover, the significant decrease of lactate (Fig. 5) in cells from the ethanol fermentation with zinc addition suggested zinc had accelerated carbon flux into glycolysis and TCA cycle, subsequently facilitated ATP synthesis which could enable the cells to combat stress from acetic acid. However, several metabolites, such as proline, which was reported as the biomarker for the status of yeast cells responding to thermal or osmotic environmental stresses[39], almost remained the same level at this fermentation point (Table S1), indicating that zinc did not influence metabolism of these metabolites under the conditions employed in this study to improve acetic acid tolerance.

Amino acid biosynthesis is important for cell stress response. It was reported that addition of tryptophan improved ethanol tolerance of *S. cerevisiae*[40], and importance of GABA metabolism in tolerance to inhibitor mixture (acetic acid, furfural, and phenol) was also reported recently[41], however, decreasing intracellular content of GABA in the both zinc-supplemented and the control cells was observed from Stage I to III, which might result from decreasing content of succinic acid (Fig. 5 and Table S1). GABA was only detected in the cells with zinc addition in Stage II and III cells, suggesting that GABA could also benefit the yeast cells in either tolerating acetic acid

or biosynthesizing other metabolites involved in acetic acid tolerance. Also, it will be interesting to study the involvement of zinc in the regulation of GABA metabolism and test the effect of GABA accumulation on acetic acid tolerance in various yeast host cells under different conditions. It has been reported that many genes associated with amino acids biosynthesis (e.g., arginine, histidine, and tryptophan) were up-regulated during the fermentation in presence of acetic acid[42], and up-regulated proteins involved in amino acids (e.g., methionine, asparagine, and glutamate) metabolism were also reported[43]. It is possible that zinc may promote protein degradation with more amino acids exacerbation to readjust metabolism and consequently defense oxidative stress resulted from acetic acid toxicity. However, we did not find significant beneficial effects of serine and valine addition against acetic acid toxicity and it seems that the beneficial effect of alanine is unique. Therefore, we assume that biosynthesis of key amino acids rather than protein degradation is one of the mechanism for cells to defense oxidative stress induced by acetic acid.

Alanine has been implicated as a biomarker for stress tolerant yeast which was subjected to combined inhibitors (acetic acid, furfural and phenol)[41], however, our current study is the first one that presents the connection of the stress tolerance of specific inhibitor, namely, acetic acid, with alanine accumulation. In addition, the involvement of biosynthesis of alanine in oxidative stress tolerance is also a first report. Alanine can be converted into pyruvate by alanine transaminase (Alt1p) which could provide ATP in the TCA cycle in energy-deficient environment through complete oxidation[44]. It was revealed in previous studies that *alt1*Δ mutants exhibited

the increased ROS generation, the accumulation of which could significantly affect the yeast cell growth[44]. Alt1p has functions in alanine utilization under fermentative conditions, therefore disruption of *ALT1* is supposed to affect alanine biosynthesis. This may explain why deletion of *ALT1* resulted in growth inhibition in presence of acetic acid stress. However, it is still not clear whether zinc exerts direct or indirect effect in alanine biosynthesis.

We found that non-oxidative PPP intermediates were decreased by acetic acid during early cell growth stage, which is consistent with the previous study[27]. However, we found that when cell entered into exponential growth phase and glucose was completely utilized, the contents of intermediates in PP pathway were increased, which may due to the adjustment of cell metabolism to provide enough NADPH for detoxification[45]. Recent study showed increased *TKL1* (encoding transketolase) transcription by zinc when yeast cells were grown in presence of acetic acid for xylose fermentation[46], which is in agreement with our current study that zinc exerts influence on non-oxidative PP pathway.

Zinc may also affect cell metabolism indirectly by changing the concentration of other important metals. In the previous studies, potassium supplementation of *S. cerevisiae* was found to improve acetic acid tolerance[47]. However, we found decreased total potassium content by zinc addition, which was accompanied with increased total zinc content. This observation could be attributed to the antioxidative effect of zinc on yeast cells to get through adverse circumstance, which results in less potassium requirement. It is worth noting that the distribution of metals in different

cellular compartments may also affect the physiological feature of the yeast cells, which deserves further investigations.

5. Conclusions

Our results provide the evidence that zinc exerts antioxidant effect on *S. cerevisiae* during long term incubation with acetic acid. Based on the data presented in this study, we conclude that zinc addition leads to reduced ROS accumulation of *S. cerevisiae* when exposed to long term acetic acid stress, and zinc exerts multiple effects on central carbon metabolism and redox balance of *S. cerevisiae* cells. The antioxidant mechanism of zinc was proposed to be related to the variation of biosynthesis of alanine and GSH. These results provide basis for further exploration of the impact of zinc status in cell metabolism and defense system in eukaryotic cells, and should be helpful for further exploration of the influence of zinc in cell metabolism during other circumstances of ROS induction, including fungal infection and cell aging.

Acknowledgements

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Table 1 The effect of zinc sulfate supplementation on the fermentation of self-flocculating yeast SPSC01 with 10 g L^{-1} acetic acid in bioreactor.

Zn^{2+} (g L^{-1})	Acetic acid (10 g L^{-1})						
	Lag time (h)	Fermentation time (h)	Biomass (g L^{-1})	$Y_{B/CS}$ (%) control	Ethanol (g L^{-1})	$Y_{E/CS}$ (%) control	$P_{E/T}$ g L^{-1} h^{-1}
0	35	58	3.03	100.0	48.6	100.0	0.838
0.03	23	45	3.47	115	49.3	101.4	1.096

Table 2 Unique metabolites detected in the control and zinc-supplemented cells at different cell growth stages.

Group	Stage I (0.1 g/L DCW)		Stage II (1.5 g/L DCW)		Stage III (3.0 g/L DCW)	
	CK	Zn	CK	Zn	CK	Zn
1	Maltose	Glyoxylate	Isoleucine	Cysteine		GABA
2	Ribitol	Galactose		Galactose		Arginine
3		Tryptophan		Lysine		Ornithine
4				Tyrosine		Citrulline
5				Uridine		Isoleucine
6				Phospholic acid		Homoserine
7				Xylose		Cytidine
8				GABA		Phospholic acid
9				Xylitol		Tryptophan
10				Cis-aconitate		

Figure legends

Fig. 1. Comparison of ROS accumulation in control and zinc addition. A, percentage of yeast cells in log phase that stained positively by DCFH-CA; B, selected images of yeast cells stained with DCFH-CA (*, $p<0.05$).

Fig. 2. Effects of zinc addition on ethanol fermentation of *S. cerevisiae* SPSC01 in presence of acetic acid. A, control, B, zinc addition. Red color indicates the time points of sampling for comparison.

Fig. 3. Concentrations of extracellular glycerol and succinic acid as well as intracellular and extracellular zinc distribution during the fermentation of *S. cerevisiae* SPSC01.

Fig. 4. Effects of zinc addition on the total cellular metal contents of *S. cerevisiae* SPSC01 (*, $p<0.05$; **, $p<0.01$).

Fig. 5. Effects of zinc addition on responsiveness changes in the metabolite levels of *S. cerevisiae* SPSC01 with acetic acid (*, $p<0.05$).

Abbreviations: 2PGA, 2-phosphoglycerate; Acetyl-CoA, acetyl-coenzyme A; AKG, α-ketoglutarate; Ala, alanine; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GABA, γ- aminobutyric acid; Glc, Glucose; Glu, glutamic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; Fum, fumarate; PP-pathway, pentose phosphate pathway; PYR, pyruvate; PEP, phosphopyruvate; Succ, succinate; TCA, tricarboxylic acid. Dot line means more than one steps are needed in the metabolic reaction, while solid line means one step in the reaction.

Fig. 6. Effects of alanine addition on the fermentation of *S. cerevisiae* SPSC01 with acetic acid. Red marked points indicate the samples used for ROS treatment.

Fig. 7. Reactive oxygen species (ROS) fluorescence in the cells from control and alanine addition (*, $p<0.05$; **, $p<0.01$).

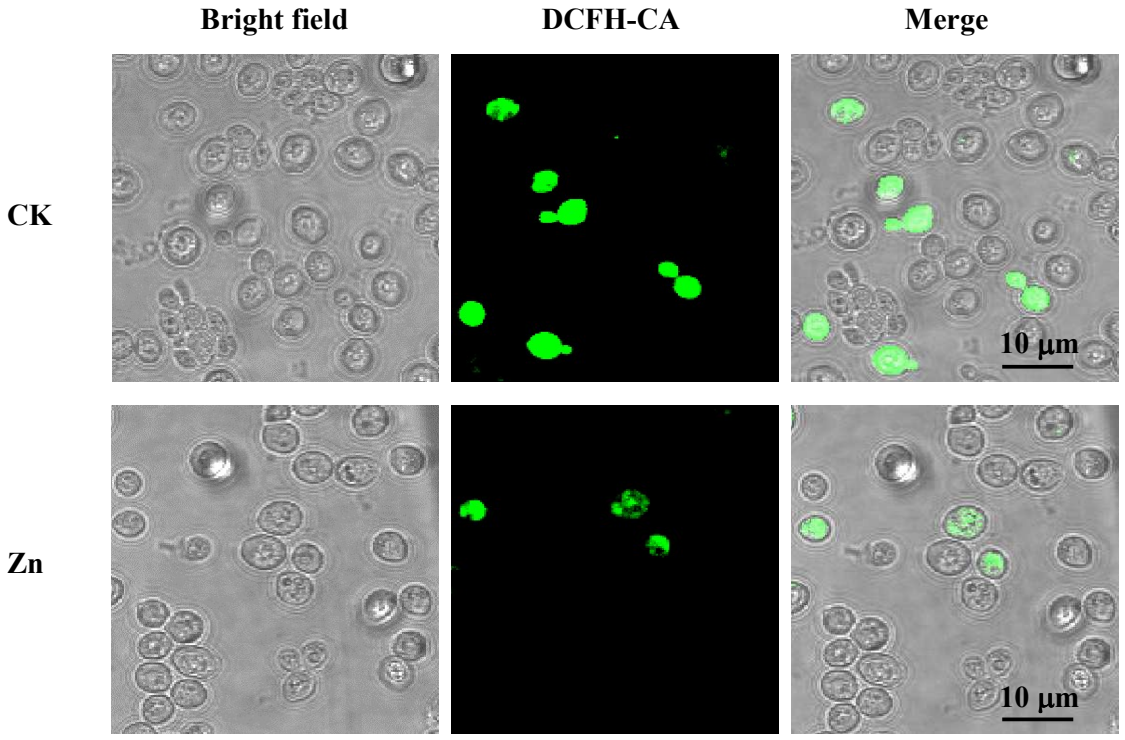


Fig. 1A.

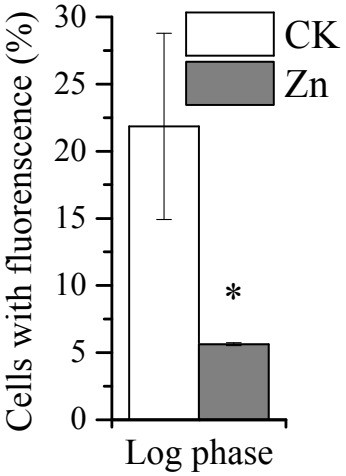


Fig. 1B.

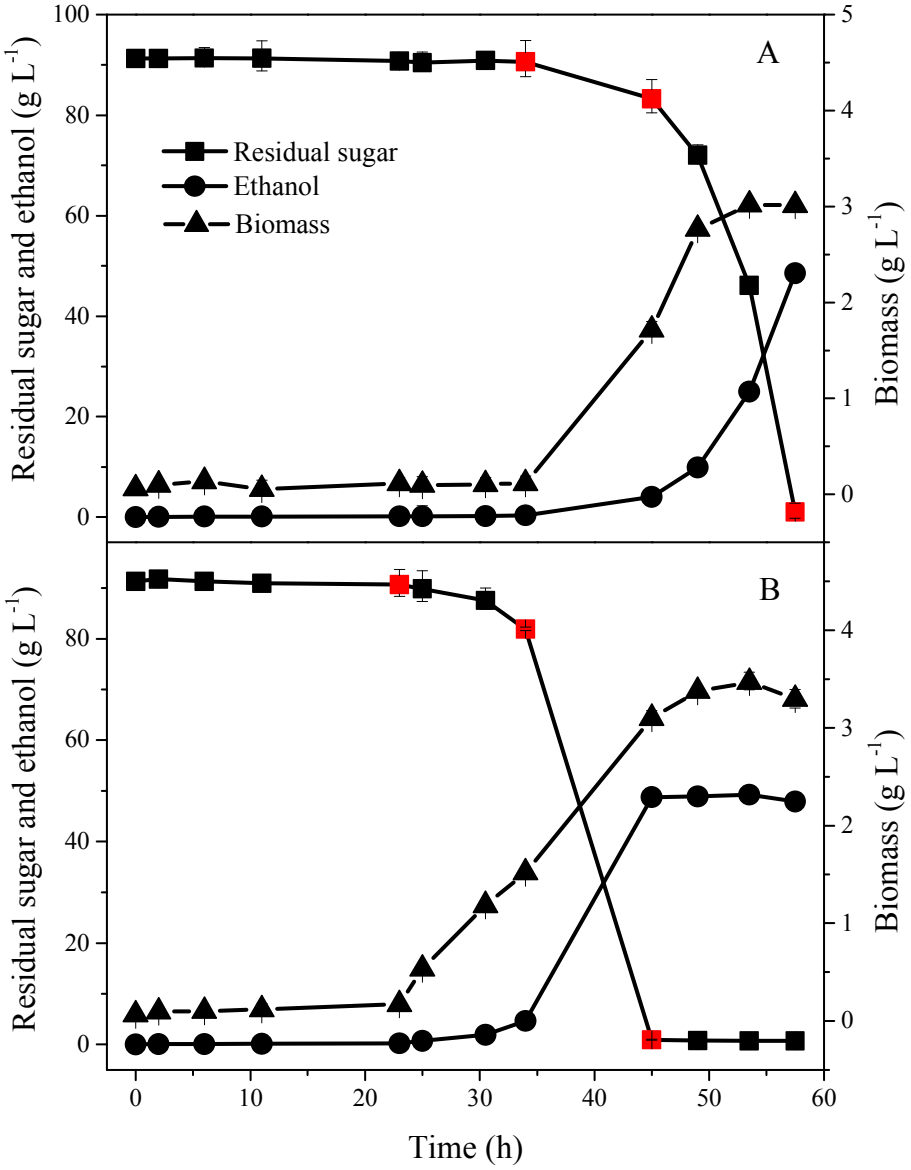


Fig. 2.

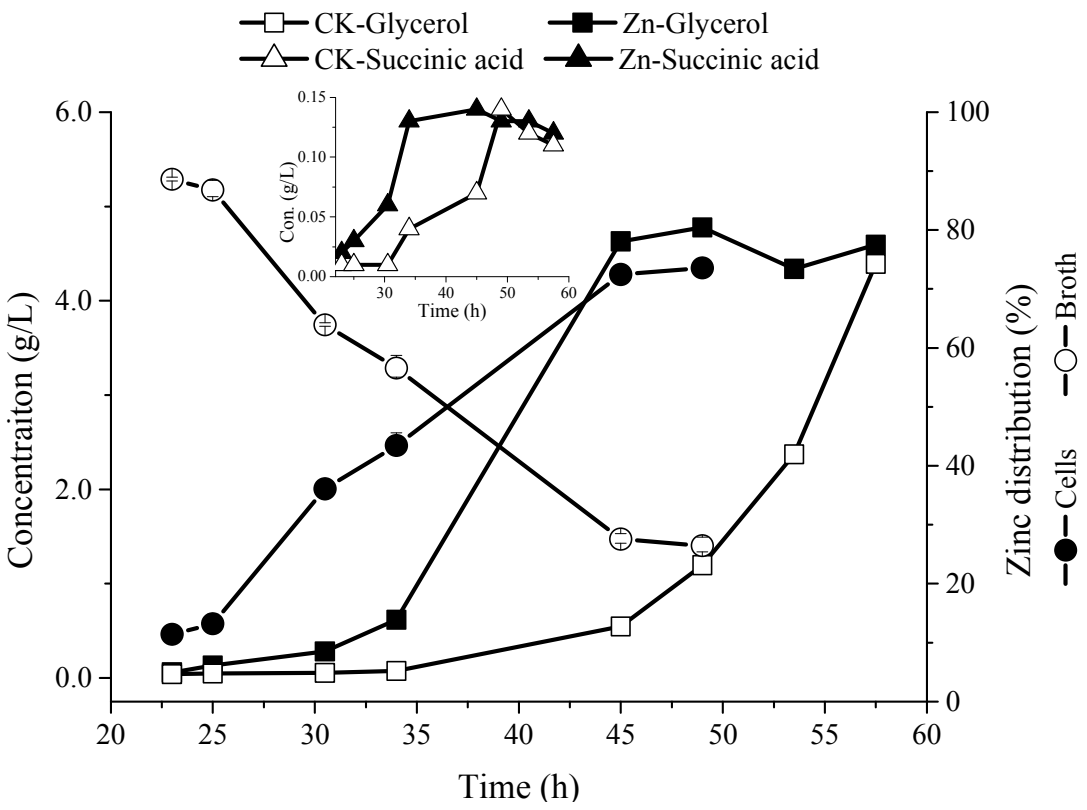


Fig. 3.

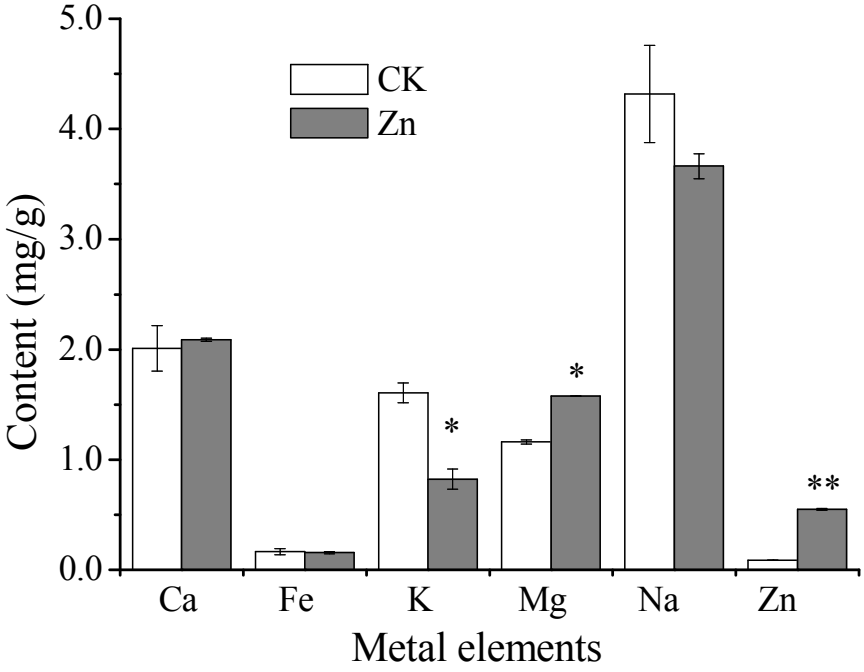


Fig. 4.

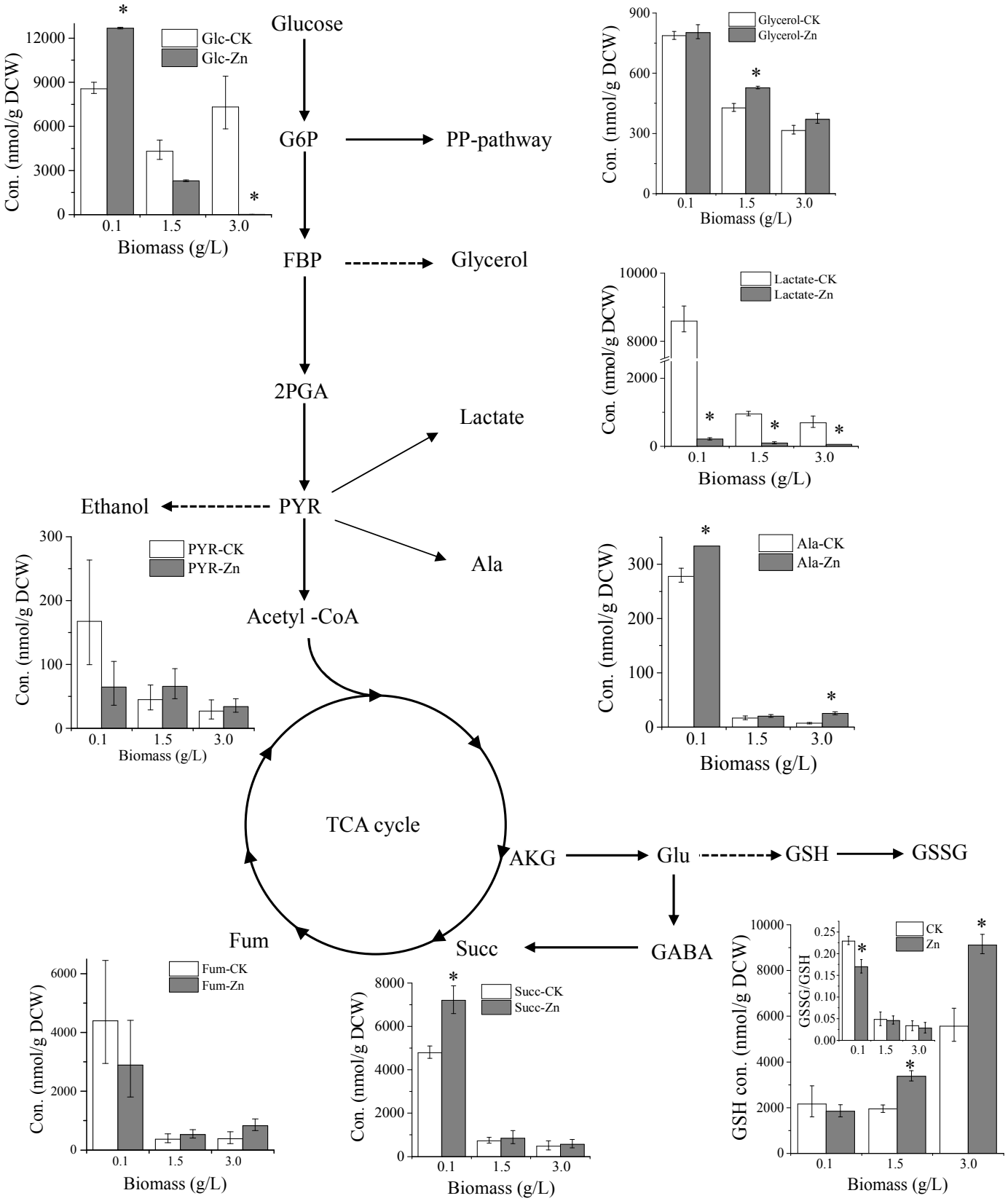


Fig. 5.

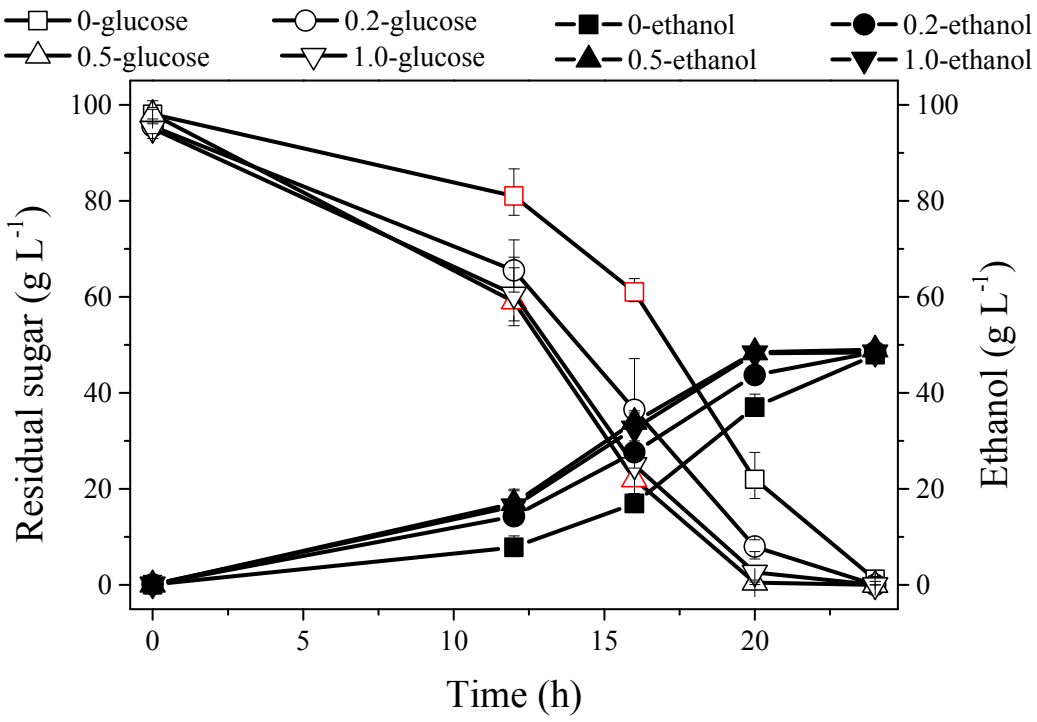


Fig. 6.

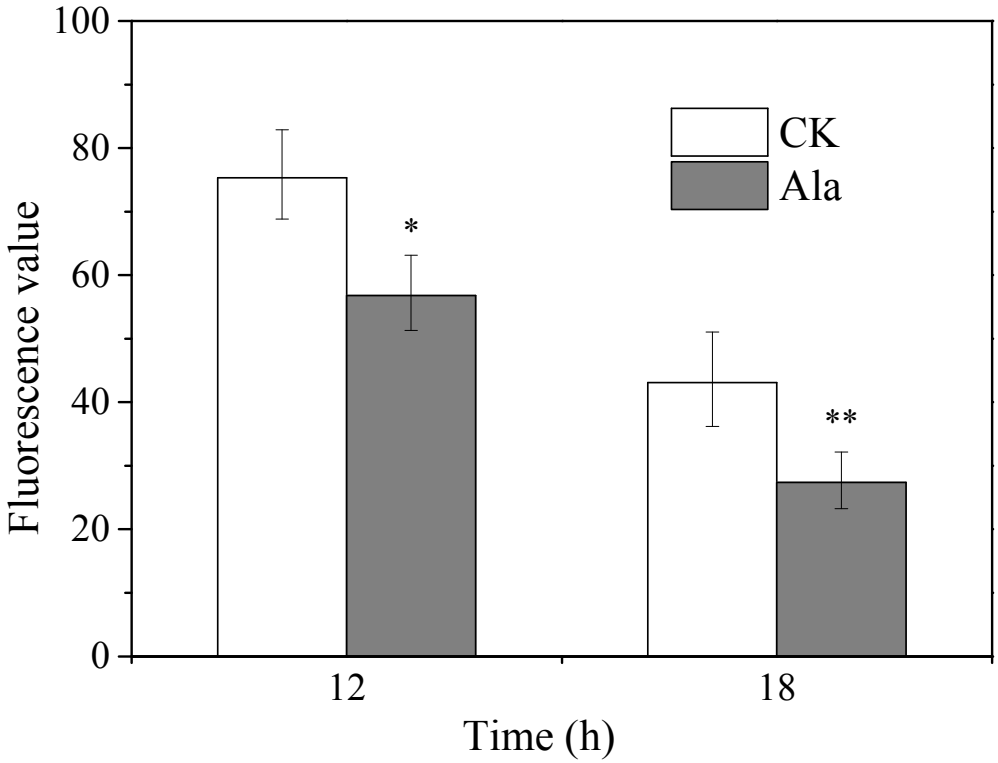


Fig. 7.

Table S1 Comparison of major metabolites quantified through GC-MS and LC-QqQ-MS between control and zinc addition based on various cell growth stages[§] ([*] $p<0.05$).

Metabolites		Stage I (0.1 g/L biomass)			Stage II (1.5 g/L biomass)			Stage III (3.0 g/L biomass)		
(nmol/g DCW)		CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)
Glycolysis	G6P	7.44±2	5.19±2	0.70	0.69±0.1	1.78±0.1	**2.58**[*]	1.17±0.5	1.27±0.6	1.09
	F6P	0.87±0.2	0.59±0.1	0.68	0.26±0.1	0.64±0.0	**2.46**[*]	0.86±0.3	0.41±0.1	0.48
	FBP	1.04±0.4	0.71±0.1	0.68	0.11±0.1	0.18±0.01	1.64	0.49±0.4	0.06±0.04	0.12
	2PGA	0.59±0.4	0.30±0.3	0.51	0.073±0.1	0.2±0.3	2.74	0.11±0.1	0.062±0.1	0.56
	PEP	0.65±0.4	0.22±0.1	0.34	0.17±0.1	0.25±0.1	1.47	0.23±0.1	0.16±0.04	0.70
PP-pathway	Ru5P	0.44±0.2	0.081±0.03	0.18	0.11±0.1	0.20±0.1	1.82	0.29±0.2	0.26±0.1	0.90
	R5P	0.53±0.3	0.18±0.1	0.34	0.06±0.03	0.08±0.01	1.33	0.08±0.03	0.05±0.01	0.63

Table S1 continued

Metabolites (nmol g^{-1} DCW)		Stage I (0.1 g L^{-1} biomass)			Stage II (1.5 g L^{-1} biomass)			Stage III (3.0 g L^{-1} biomass)		
		CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)
PP-pathway	Xu5P	0.73±0.1	0.27±0.03	**0.37***	0.15±0.03	0.27±0.01	**1.80***	0.40±0.05	0.34±0.01	0.85
	S7P	0.50±0.2	0.23±0.1	0.46	0.073±0.05	0.14±0.1	1.92	0.16±0.1	0.35±0.1	2.19
	E4P	0.64±0.3	0.16±0.1	0.25	0.060±0.1	0.082±0.01	1.37	0.03±0.01	0.03±0.01	1.00
Amino acids	Val	264.0±29	392.65±0.0	**1.49***	14.41±3	15.13±0.4	1.05	4.31±0.3	8.25±0.2	**1.91***
	Gly	654.87±95	806.18±14	1.23	55.96±7	42.01±1.5	0.75	20.02±3	33.78±3	**1.69***
	Ser	362.22±16	446.23±7	**1.23***	22.17±2	22.73±0.6	1.03	11.69±0.4	21.00±1.1	**1.80***
	Proline	310.04±14	270.88±0.0	0.87	23.04±2.6	23.69±0.5	1.03	12.56±0.9	13.71±1.1	1.09
Others	Trehalose	202.43±20	320.12±14	**1.58***	33.43±2.7	26.93±1.1	0.81	292.88±65	211.98±46	0.72

Table S1 continued

Metabolites		Stage I (0.1 g L^{-1} biomass)			Stage II (1.5 g L^{-1} biomass)			Stage III (3.0 g L^{-1} biomass)		
(nmol g^{-1} DCW)		CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)	CK	Zn	Ratio (Zn/CK)
Others	Mannose	370.70±156	308.76±7	0.83	41.50±6.4	32.18±9.7	0.78	12.88±2.1	9.52±0.98	0.74
	Fructose	3913.74±32	5444.41±21	**1.39***	287.94±7.5	220.17±15	**0.76***	82.54±18	23.13±5.9	**0.28***
	GSSG	490.71±174	303.31±103	0.62	98.14±50	160.90±54	1.64	163.94±10	242.80±7.6	**1.48***
	GABA	238.35±29	337.36±43	1.42	ND	31.44±5.2		ND	16.85±0.3	
	NAD^+	26.13±20	12.05±9.4	0.46	4.16±2.9	6.39±4.7	1.54	3.56±2.6	3.26±2.2	0.92
	NADH	0.38±0.1	0.29±0.1	0.76	0.06±0.03	0.16±0.1	2.67	0.33±0.1	0.25±0.1	0.76
	$NADP^+$	0.61±0.1	0.12±0.03	**0.20***	0.17±0.02	0.14±0.1	0.82	0.069±0.01	0.015±0.001	**0.21***
	NADPH	0.11±0.1	0.01±0.01	0.09	0.01±0.002	0.01±0.002	0.94	0.01±0.001	0.001±0.001	0.26

[§]ND, not detectable.

Supplementary figures

Fig. S1. Overview of the number of unique metabolites and common metabolites at different growth stages in control and zinc-supplemented cells.

Numbers in grey and orange background means numbers of the unique metabolites detected in the control and zinc-supplemented cells at different cell growth stages, respectively, whereas numbers in green background indicate numbers of common metabolites with or without zinc addition.

Fig. S2. Effects of serine (A) and valine (B) addition on the glucose consumption of *S. cerevisiae* SPSC01 in presence of 7.5 g L^{-1} acetic acid.

The concentrations of serine and valine used in this study were (g L^{-1}): 0, 0.2, 0.5 and 1.0, respectively. Acetic acid was added at the final concentration of 7.5 g L^{-1} in the culture medium after autoclaving and cooling the medium to room temperature. Yeast strain of SPSC01 was activated in seed medium by overnight cultivation for two times, which was then deflocculated by 0.2 M sodium citrate, and the initial inoculum was adjusted to OD_{620nm} of 2.0. Five milliliter of the seed culture was inoculated to 250 ml flasks containing 100 ml fermentation medium and the culture was incubated at 150 rpm, 30 °C for 66 h. Samples were collected at a 12 h interval, and residual glucose concentrations were determined.

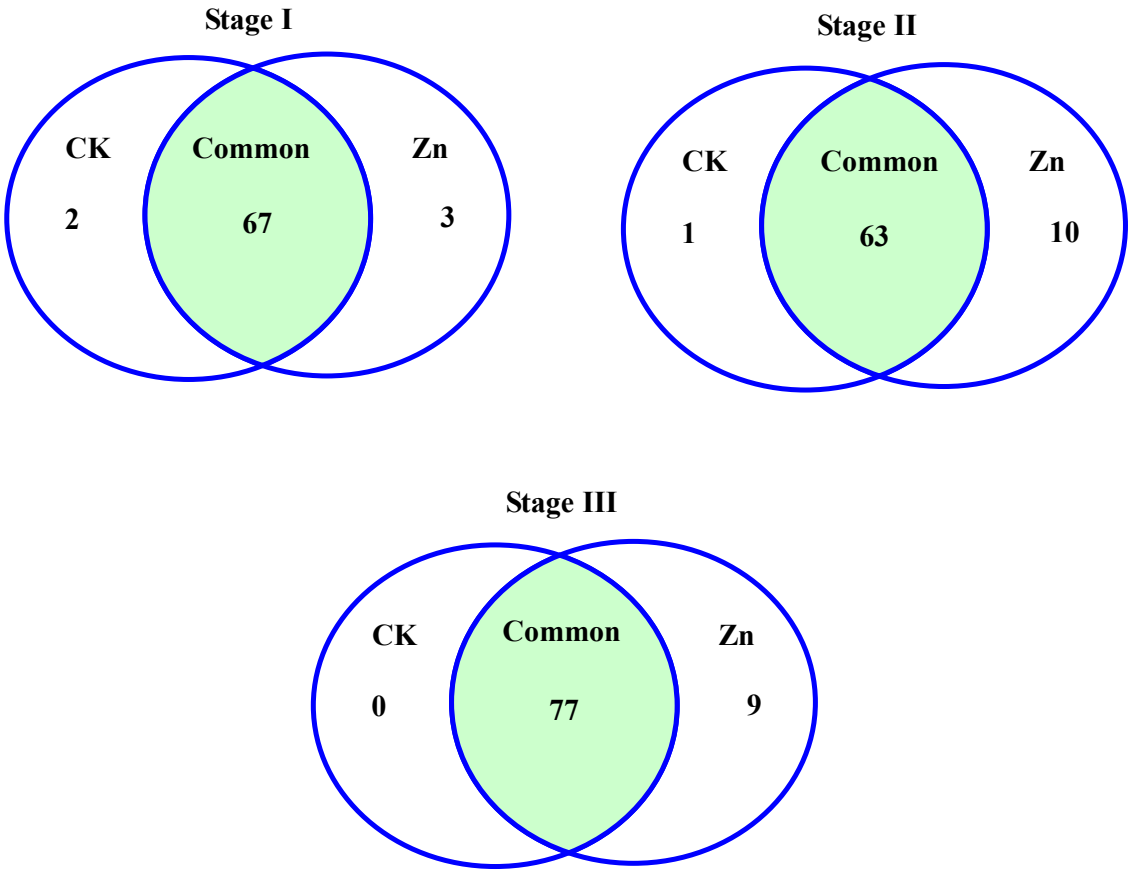


Fig. S1.

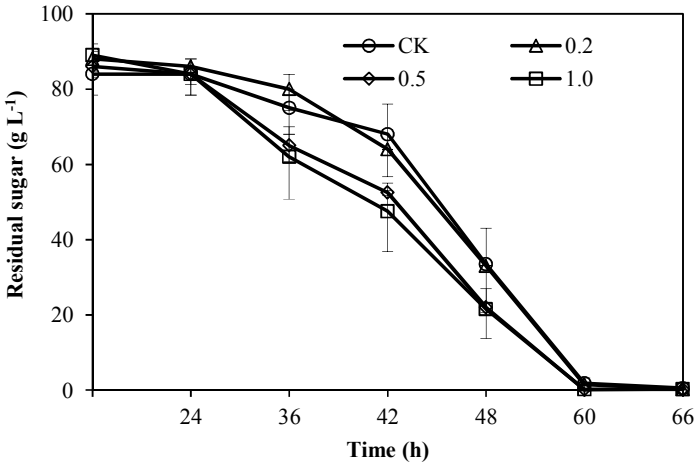


Fig. S2A

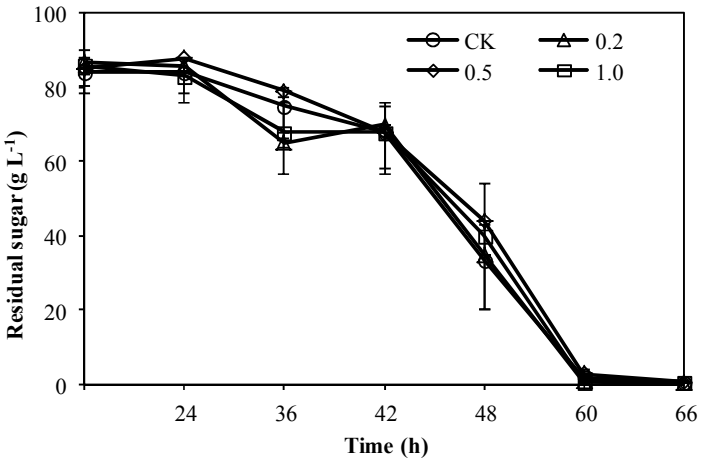
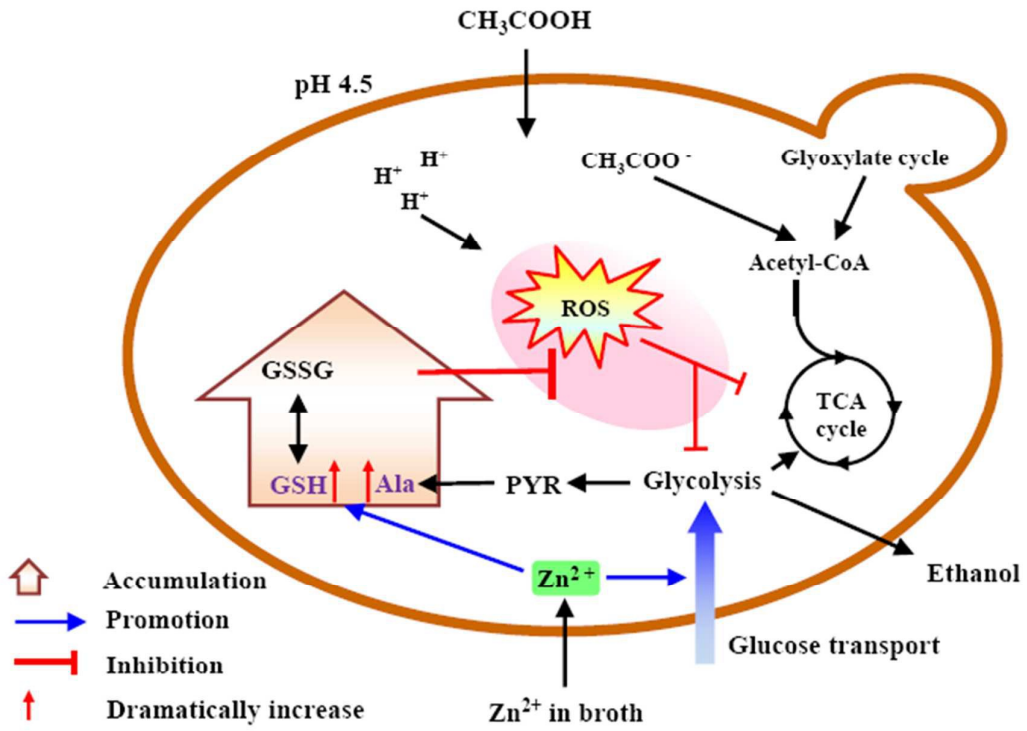


Fig. S2B

Table of contents entry

By Wan, et al., Impact of zinc sulfate addition on dynamic metabolic profiling of *Saccharomyces cerevisiae* subjected to long term acetic acid stress treatment and identification of key metabolites involved in antioxidant effect of zinc, submitted to Metallomics.

Zinc modulates cellular amino acid metabolism and redox balance, especially biosynthesis of alanine and glutathione to exert its antioxidant effect.



Ala, Alanine; GSH, glutathione; GSSG, oxidized glutathione; PYR, pyruvate; ROS, reactive oxygen species; TCA, tricarboxylic acid.

Figure for cover: Effect of zinc as an antioxidant in *Saccharomyces cerevisiae* upon acetic acid stress