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Full Length Article

Effects of Zinc Combining with Specific Metal Ions on Ethanol Tolerance of Yeast *Saccharomyces cerevisiae*

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Abstract

The effects of zinc combining with other metal ions on ethanol tolerance of yeast *Saccharomyces cerevisiae* 1308 and *S. cerevisiae* BY4741 were studied for the first time. Addition of zinc increased ethanol tolerance of two yeast strains and reached the maximum viability, biomass and ethanol production at 0.1 m*M* Zn^{2+}. While ethanol tolerance weakened when zinc concentration was greater than 0.5 m*M*. When combined with 0.1 m*M* Ca^{2+}, 0.001 m*M* Cu^{2+} or 0.001 m*M* Mn^{2+}, the effect of zinc was further enhanced. Addition of 1 m*M*, 0.01 m*M* Cu^{2+} or 0.01 m*M* Mn^{2+} decreased ethanol tolerance. However, the negative effect could be alleviated by combining with Zn^{2+}. Effect of zinc on ethanol tolerance was also related to concentration and yeast strain. The optimum zinc level for growth and fermentation of yeast depended on kinds and concentration of other ions. © 2020 Friends Science Publishers

Keywords: Ethanol tolerance; Interaction; Metal ions; *Saccharomyces cerevisiae*; Zinc

Introduction

Saccharomyces cerevisiae is preferred yeast for most ethanol fermentation such as beer, wine and distilled spirits due to fast fermentation rate and high ethanol yield, which has important applications in both agricultural and industrial productions. However, ethanol accumulation can become a distinct stress for yeast cell growth and viability, fermentation productivity and ethanol yield (Ansanay-Galeote *et al.* 2001; Stanley *et al.* 2010). It is generally believed that the highest levels of ethanol are determined by the tolerance of different yeast strains used in ethanol industry. Although ethanol tolerance is a genetic determinant, fermentation conditions, which vary greatly from sub-industry to sub-industry, also profoundly affect ethanol tolerance of the strains, and each yeast strain responds differently to each factor (Casey and Ingledew 1986).

Ethanol mainly inhibits growth of yeast by affecting the fluidity and permeability of membrane. In the process of ethanol fermentation, yeast cells can continuously reconstruct the composition and structure of membrane to achieve higher alcohol tolerance (Chen *et al.* 2016). In previous studies, several compounds including unsaturated fatty acids, sterols, amino acids, vitamins and metal ions may increase yeast ethanol tolerance by affecting structure of membrane (You *et al.* 2003; Ma and Liu 2010; Yamaoka *et al.* 2014; Chen *et al.* 2016).

For optimal growth and fermentation, yeasts require different metal ions. These metal ions play roles as cofactors of the enzymes involved in metabolic activity and structure of yeast cell (Walker 2004). Among these, zinc is essential to growth and fermentstion of yeast. Addition of exogenous zinc may lead to reprogramming of the cellular metabolic network, including reducing glycerol biosynthesis, guiding carbon flux to ethanol production and enhancing biosynthesis of protective molecules such as ergosterol and trehalose which could stabilize the cell membrane structure of yeast in the presence of ethanol (Nicola *et al.* 2009; Zhao *et al.* 2009; Xue *et al.* 2010; Zhao and Bai 2012). It has been stated by many researches that Zn^{2+} in culture medium enhanced growth rate of yeast cells and production of ethanol by increasing ethanol tolerance of yeast (Nicola *et al.* 2009; Zhao *et al.* 2009). Other metal ions, such as magnesium, calcium, copper and manganese, were also proved to improve yeast ethanol tolerance to some extent (Nabais *et al.* 1988; Costa *et al.* 1997; Ma and Liu 2010).

On the other hand, analysis of variance shows that yeast fermentation performance depends on the complex interactions between metal ions studied (Chandrasena *et al.* 1997). Similarly, yeast ethanol tolerance is also the result of the interaction between factors. The effects of metal ions on yeast ethanol tolerance may depend not only on metal ion itself but also on interaction with other metal ions. Most of researches focused on the effect of certain metal ion on yeast ethanol tolerance or optimization of medium composition by statistical experimental design (Xue *et al.* 2008; Soyuduru *et al.* 2009). There is a lack of enough

information about the effect of certain ion combining with other specific ions on yeast ethanol tolerance. Based on this, we studied effect of zinc combining with calcium, copper and manganese on ethanol tolerance by measuring viability, growth and fermentation ability of two typical *S. cerevisiae* strains which were used in industrial field and laboratory. The effect of ion interactions on alcohol tolerance will help to control yeast availability in industrial fermentation to optimal levels.

Materials and Methods

Yeast Strain and Culture Condition

An industrial yeast strain *S. cerevisiae* 1308 (Institute of Microbiology Chinese Academy of Science, China), used for production of Chinese liquor, and a laboratory yeast strain *S. cerevisiae* BY4741 (Sichuan University of Science and Engineering, Zigong, China) were used in this study.

Yeast strain was activated at 30°C for 24 h in YPD medium which contained D-glucose 20 g/L (Amresco), peptone 20 g/L (Oxoid), yeast extract10 g/L (Oxoid) and agar 20 g/L (Oxoid). Then the 5% of activated liquid yeast culture were inoculated to 200 mL liquid YPD and cultured to exponential phase. After centrifugation, cell suspension was washed twice with deionized water. The cells were adjusted to 2×10^6 viable cells per mL and inoculated into culture solution containing yeast extract (10 g/L), D-glucose (20 g/L), $MgSO_4\cdot7H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 (3 g/L). Base medium was used for cell growth and alcoholic fermentation.

Effect of Zinc

To test the effect of zinc on ethanol tolerance of yeast, yeast strain was inoculated at 5% volume to base medium which was added various concentrations $ZnSO_4\cdot$(0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 m*M*). The cultures were incubated at 30°C and 150 rpm for 48 h. Ethanol tolerance of yeast was assessed by measuring the viability, growth and fermentation.

Effect of Zinc Combining Other Ions

Yeast strain was inoculated to base medium which was added various concentrations $ZnSO_4\cdot$and another metal ion. Metal ions of different concentration included $CaCl_2$ (0.01, 0.1, 1.0 m*M*), $CuSO_4\cdot$(0.001, 0.01 m*M*) and $MnSO_4$ (0.001, 0.01 m*M*). Cultures without addition of another metal ion were used as control. Ethanol tolerance of yeast was assessed as described above.

Ethanol Tolerance Assays

Viability was measured as follow: In addition to ions, lethal concentration of 15% ethanol was added to base medium. After incubating at 30°C and 150 rpm for 48 h, base medium was coated on YPD medium plates and incubated at 30°C for 3 days. Viability was determined by using methylene blue staining method to count viable cells.

Base medium containing 4% ethanol was used for measuring yeast growth. The growth rate was evaluated by measuring culture optical density at 640 nm. The OD_{640} values were converted to cell mass using a calibration curve established for this particular strain (Aleksander *et al.* 2009).

Fermentation was measured by determining ethanol production of yeast. Alcoholic fermentation was performed in 500 mL conical flasks at condition of 25℃, pH 4.0 and 80 rpm. The culture was sampled periodically for measuring ethanol content until the end of fermentation. The ethanol content (% v/v) was determined by gas-liquid chromatography.

Statistical Analysis

Statistical analyses were performed according to the SPSS user's guide. Analysis of variance was performed using ANOVA procedure. Significant differences ($P < 0.01$) between means were determined using Levene's multiple range tests.

Results

Effect of Zinc

The ethanol tolerance of yeast was evaluated by measuring the viability, growth and fermentation of yeast when different concentrations of zinc were added to medium. The results showed (Fig. 1) that addition of zinc enhanced ethanol tolerance of both yeast strains. In the range of 0-0.1 m*M* zinc concentration, viability, biomass and ethanol production all increased with the increase of zinc concentration. The maximum viability reached 46% for *S. cerevisiae* 1308 and 42% for *S. cerevisiae* BY4741 (Fig. 1A) when 0.1 m*M* Zn^{2+} was supplemented in the medium. The biomass of yeast reached to maximum 7.34 g/L (*S. cerevisiae* 1308) and 6.23 g/L (*S. cerevisiae* BY4741) (Fig 1B). And ethanol concentration raised to maximum 8.4% in *S. cerevisiae*1308 and to maximum 7.0% in *S. cerevisiae* BY4741 (Fig. 1C). However, a further increase in zinc concentration did not result in a corresponding increase in ethanol tolerance. In our studies, negative effect caused by Zn^{2+} above 0.1 m*M* was observed in *S. cerevisiae* BY4741. Compared with 0.1 m*M* Zn^{2+}, addition of 1.0 m*M* Zn^{2+} concentration led to a drop in viability to 33% (Fig. 1A), biomass to 4.88 g/L (Fig. 1B) and ethanol production to 6.4% (Fig. 1C). In contrast, negative effect caused by Zn^{2+} above 0.1 m*M* was not observed markedly in *S. cerevisiae* 1308. Viability, biomass and ethanol production were almost unchanged with addition of 0.5 m*M* and 1.0 m*M* zinc compared with 0.1 m*M* zinc.

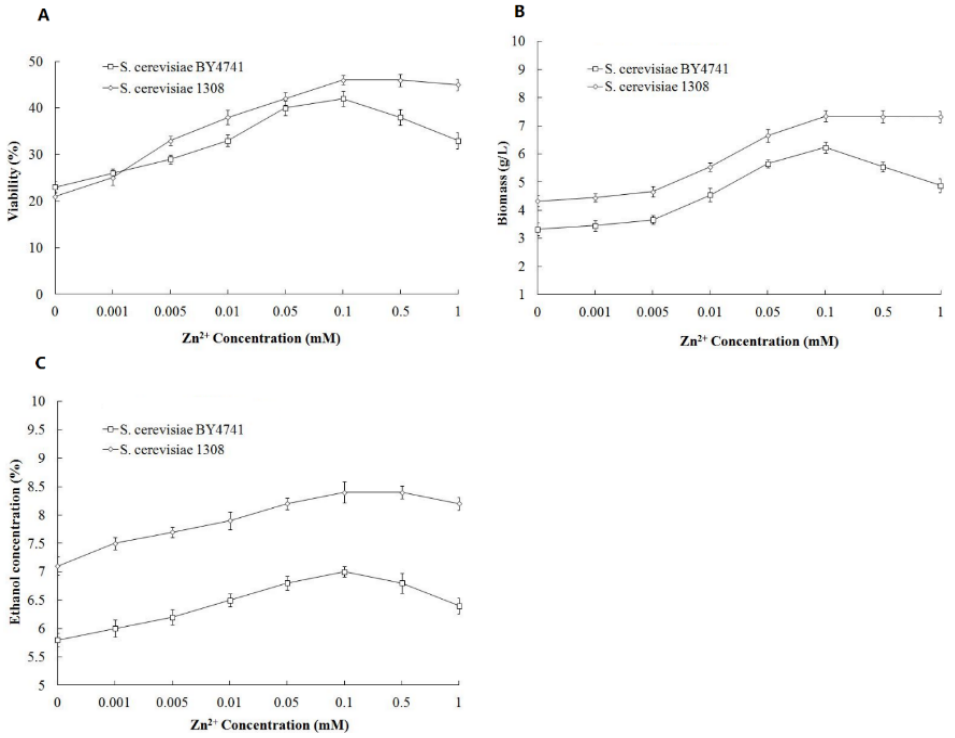

Fig. 1: Effect of zinc on ethanol tolerance of two yeast strains. The two yeast strains were *Saccharomyces cerevisiae* 1308 and *Saccharomyces cerevisiae* BY4741. Ethanol tolerance was assessed by measuring cell viability in lethal concentration of ethanol (**A**), biomass (**B**) and ethanol production (**C**) in the presence of 5% ethanol

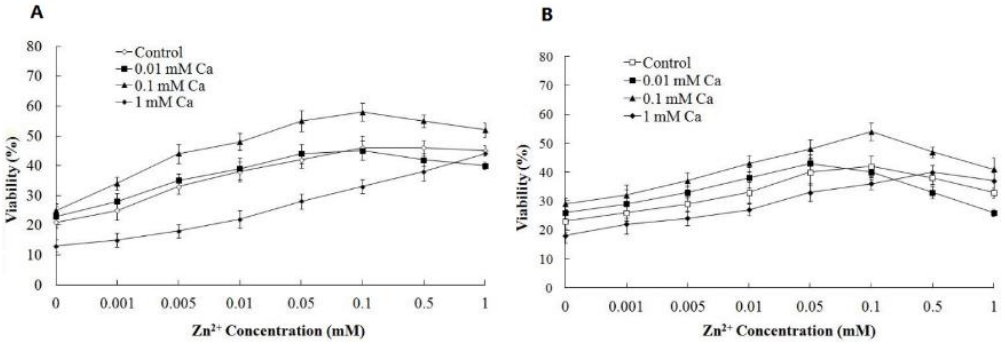

Fig. 2: Effect of zinc combining calcium on cell viability of both yeast strains. **A**) viability of *S. cerevisiae* 1308. **B**) viability of *S. cerevisiae* BY4741

Effect of zinc combining calcium

The results of effect of zinc combining calcium on yeast ethanol tolerance were showed in Fig. 2, 3 and 4. In our studies, addition of Ca^{2+} at low level (0.01 m*M* and 0.1 m*M*) enhanced ethanol tolerance of two yeast strains. Zinc combining with 0.1 m*M* Ca^{2+} led to more viability, biomass and ethanol production than with 0.01 m*M* Ca^{2+}. In the presence of 0.1 m*M* Ca^{2+}, viability increased to maximum 58% for *S. cerevisiae* 1308 (Fig. 2A) and 54% for *S. cerevisiae* BY4741 (Fig. 2B), while biomass increased to maximum 8.16g/L for *S. cerevisiae* 1308 (Fig. 3A) and 7.44g/L for *S. cerevisiae* BY4741 (Fig. 3B), ethanol production reached maximum 8.7% for *S. cerevisiae* 1308 (Fig. 4A) and 7.3% for *S. cerevisiae* BY4741 (Fig. 4B). Thus the optimum Ca^{2+} concentration mainly focused on 0.1 m*M*. Although low level calcium had a positive effect on yeast ethanol tolerance, addition of Zn^{2+} and Ca^{2+} did not synergistically enhance ethanol tolerance at all tested concentrations. Specifically, cell growth improved only when Zn^{2+} was added at less than 0.1 m*M* for *S. cerevisiae* BY4741 (Fig. 3B) or Ca/Zn ratio was in the range from 1:1 to 1:10 for *S. cerevisiae* 1308 (Fig. 3A). Furthermore, viability and ethanol production of both yeast were lower than that of control when 0.01 mM Ca^{2+} was added with 0.5 m*M* or 1.0 m*M* Zn^{2+} (Fig. 2A, 2B, 4A and 4B).

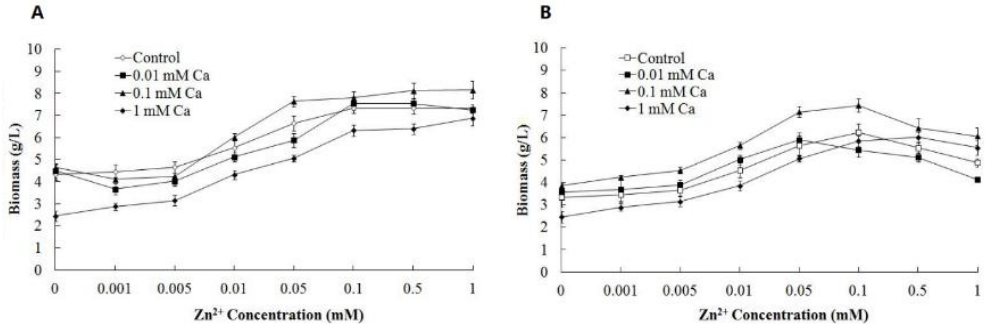

Fig. 3: Effect of zinc combining calcium on cell growth of both yeast strains. Cell growth was represented by biomass. **A**) Biomass of S. *cerevisiae* 1308. **B**) Biomass of *S. cerevisiae* BY4741

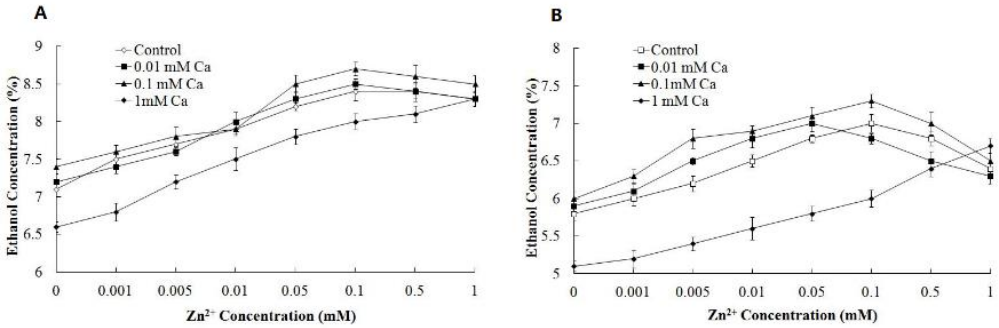

Fig. 4: Effect of zinc combining calcium on fermentation of both yeast strains. Fermentation was represented by ethanol production. **A**) ethanol production of *S. cerevisiae* 1308. **B**) ethanol production of *S. cerevisiae* BY4741

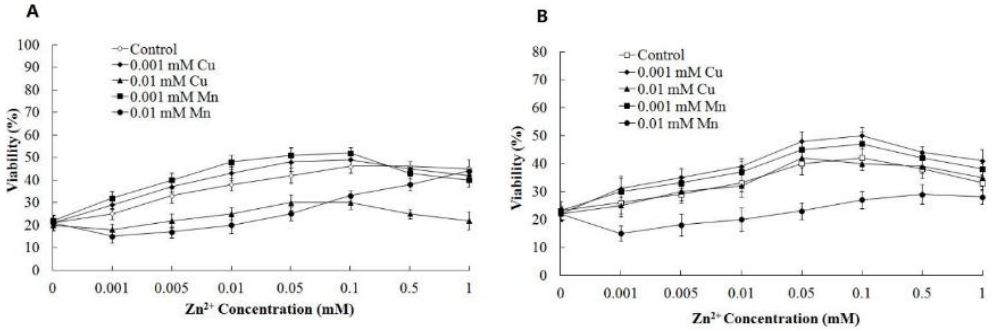

Fig. 5: Effect of zinc combining different concentration copper or manganese on cell viability of both yeast strains. **A**) viability of *S. cerevisiae* 1308. **B**) viability of *S. cerevisiae* BY4741

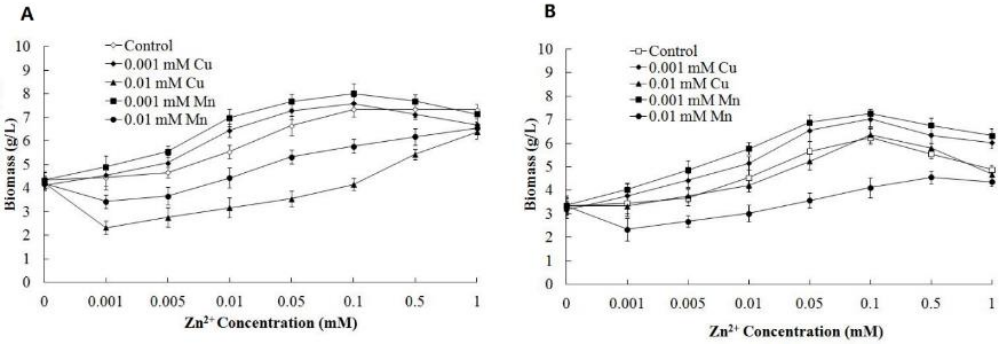

Fig. 6: Effect of zinc combining copper or manganese on cell growth of both yeast strains. Growth was represented by biomass. **A**) Biomass of S. *cerevisiae* 1308. **B**) Biomass of *S. cerevisiae* BY4741

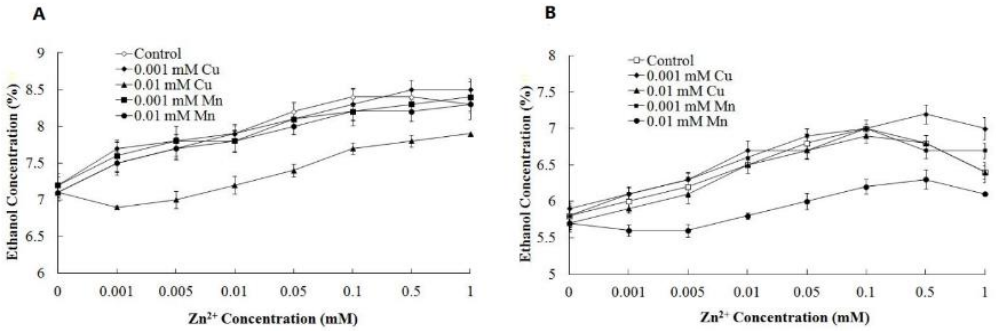

Fig. 7: Effect of zinc combining copper or manganese on fermentation of both yeast strains. Fermentation was represented by ethanol production. **A**) ethanol production of *S. cerevisiae* 1308. **B**) ethanol production of *S. cerevisiae* BY4741

On the other hand, negative effect of high concentration Ca^{2+} on yeast was displayed in this study. Addition of 1.0 m*M* Ca^{2+} caused negative effects on ethanol tolerance of both yeast strains, including lower viability (Fig. 2), less biomass (Fig. 3) and ethanol production than controls (Fig. 4), when zinc was added below 0.5 m*M*. However, the negative effect of 1.0 m*M* Ca^{2+} alleviated with increasing level of Zn^{2+}. When Zn^{2+} concentration reached above 0.5 m*M*, which should be an adverse concentration to both yeast strains, viability, biomass and ethanol production could exceed the controls.

Effect of zinc combining copper or manganese

Effects of zinc combining copper or manganese on yeast ethanol tolerance were showed in Fig. 5, 6 and 7. The results indicated that low level of Zn^{2+} combining with Cu^{2+} or Mn^{2+} improved ethanol tolerance of both yeast strains. Specifically, when 0.1 m*M* Zn^{2+} and 0.001 m*M* Cu^{2+} or 0.001 m*M* Mn^{2+} were added, viability reached maximum 52% (addition of Zn^{2+} and Mn^{2+}) and 49% (addition of Zn^{2+} and Cu^{2+}) for *S. cerevisiae* 1308 (Fig. 5A), 47% (addition of Zn^{2+} and Mn^{2+}) and 50% (addition of Zn^{2+} and Cu^{2+}) for *S. cerevisiae* BY4741 (Fig. 5B). Biomass reached 7.59g/L (addition of Zn^{2+} and Cu^{2+}) and 8.01 g/L (addition of Zn^{2+} and Mn^{2+}) for *S. cerevisiae* 1308 (Fig. 6A), 7.02g/L (addition of Zn^{2+} and Cu^{2+}) and 7.26 g/L (addition of Zn^{2+} and Mn^{2+}) for *S. cerevisiae* BY4741 (Fig. 6B).

Although 0.001 m*M* Mn^{2+} or Cu^{2+} enhanced ethanol tolerance of yeast by increasing viability and biomass, they did not promote fermentation in some cases. Addition of 0.001 m*M* Mn^{2+} produced no more ethanol for both strains than that of control, and 0.001 m*M* Cu^{2+} showed the same effect for *S. cerevisiae* 1308 (Fig. 7A and 7B). However, addition Zn^{2+} and 0.001 m*M* Cu^{2+} increased ethanol concentration to 7.2% for *S. cerevisiae* BY4741 (Fig. 7B).

For another, high level Cu^{2+} or Mn^{2+} reduced ethanol tolerance of two yeast strains to varying degrees. For *S. cerevisiae* 1308, 0.01 m*M* Cu^{2+} decreased viability, biomass and ethanol production, no matter how much zinc was added (Fig. 5A and 7A). Similar result was found only in the effect of 0.01 m*M* Mn^{2+} on biomass (Fig. 6A). For *S. cerevisiae* BY4741, 0.01 m*M* Mn^{2+} decreased viability, biomass and ethanol production when Zn^{2+} was below 0.5 m*M* (Fig. 5B, 6B and 7B).

Unexpectedly, zinc could weaken the negative effect of 0.01 m*M* Cu^{2+} or Mn^{2+}. Viability, biomass and ethanol production increased with increasing zinc concentration in the presence of 0.01 m*M* Cu^{2+} for *S. cerevisiae* 1308 (Fig. 5A, 6A and 7A) and 0.01 m*M* Mn^{2+} for *S. cerevisiae* BY4741 (Fig. 5B, 6B and 7B).

Interestingly, negative effect of 0.01 m*M* Cu^{2+} and Mn^{2+} displayed strain dependent as well. Reduced ethanol content was not found with addition of 0.01 m*M* Mn^{2+} for *S. cerevisiae* 1308 (Fig. 7A). And 0.01 m*M* Cu^{2+} had no markedly effect not only on ethanol production but also on viability and biomass for *S. cerevisiae* BY4741 (Fig. 5B, 6B and 7B).

Discussion

Yeasts require a variety of metal ions in micro levels for optimum growth and fermentation. Among these ions, zinc is considered as one of the most critical factors (Nicola *et al.* 2009). Zinc is a part of Zn-finger DNA-binding proteins which involve in responding general stress. Zinc is cofactor of alcohol dehydrogenase which is essential to ethanol fermentation (Chandrasena *et al.* 1997). Addition of zinc increased cell survival in the presence of ethanol by inducing expression of genes that control cell membranes (Ismail *et al.* 2014) and improved ethanol production (Zhao *et al.* 2009; Zhao and Bai 2012).

The improvement of zinc on fermentation has been proved in many studies. Zinc could increase yeast growth fermentation rate for beer fermentation (Bromberg *et al.* 1997; Tosun and Ergun 2006; Nicola *et al.* 2009). Zinc supplementation further increased ethanol production and cell survival of yeast by enhancing ethanol tolerance (Zhao *et al.* 2009; Xue *et al.* 2010). Our study was consistent with the above studies, suggesting that zinc increased ethanol tolerance, and by improving viability, biomass, and ethanol production of tested yeast strains.

In our study, the optimum concentration of zinc was 0.1 m*M*, which was roughly in line with previous study (20 mg/L) (Xue *et al.* 2008). However, this number differed in other studies. The optimum concentration of zinc was 3.04 mg/L (Tosun and Ergun 2006) and 0.1–0.15 mg/L (Bromberg *et al.* 1997), respectively. While Rees and Stewart (1998) and Zhao *et al.* (2009) found that zinc supplement for maximum alcohol production was 50 mg/L.

High concentration of Zn^{2+} may be toxic and inhibited yeast growth and fermentation activity due to affecting the permeability of membranes to potassium (Tosun and Ergun 2006; Gibson 2011). The critical concentration of zinc is different in some studies. The activity of yeast strains did not decrease in the presence of 65.5 ppm of zinc (Rees and Stewart 1998). In some cases, zinc concentrations up to 1300 ppm still had a positive effect on fermentation (Gibson 2011). Our study showed that zinc concentration above 0.5 m*M* was harmful to *S. cerevisiae* BY4741 and decreased viability, biomass and ethanol production. However, the same concentration of zinc showed no negative effect on *S. cerevisiae* 1308. The differences in optimum zinc concentration and zinc tolerance may be due to the characteristics of the strain.

Like zinc, calcium is required by yeast as a macro-element. Calcium enhances ethanol tolerance of yeast by increasing the stability of cell membrane and protecting cell against the leaking of cellular materials caused by ethanol (Nabais *et al.* 1988). In our study, concentration range of calcium ions to increase ethanol tolerance was 0.01–0.1 m*M*, which was lower than other studies such as 3 m*M* Ca (Ciesarová *et al.* 1996) and 0.75–2.0 m*M* Ca (Nabais *et al.* 1988). However, the combined addition of calcium and zinc did not synergistically increase ethanol tolerance at all concentrations. Only when zinc was less than 0.1 m*M* did the addition of calcium further improve ethanol alcohol tolerance. Similar to Ca/Mg ratio, the appropriate Ca/Zn ratio was essential to the growth of yeast (Rees and Stewart 1997).

On the other hand, the adverse effect of high calcium concentrations on fermentation is well recognized. This is due to calcium antagonism to magnesium absorption and function, which caused damage to yeast. In this study, 1 m*M* of calcium inhibited ethanol tolerance of two yeast strains. Nevertheless, critical calcium concentration was not reported in detail and may depend on magnesium concentration (Rees and Stewart 1999; Gibson 2011).

Unexpectedly, the inhibition of 1 m*M* of calcium was reduced by increasing zinc levels. For *S. cerevisiae* BY4741, combined addition of 1 m*M* calcium and certain concentration of zinc can even made viability, biomass and ethanol production exceeding that of the control, even though this zinc concentration produced a negative effect when added alone. It was the first time to observe the inhibition of calcium was weakened by zinc. This may be due to zinc alleviating calcium antagonism to magnesium, resulting in increased ethanol tolerance. Previous researches showed that a divalent ion in yeast could impede uptake of another divalent ion (Brady *et al.* 2008; Reddy *et al.* 2011). There may be antagonism between zinc and calcium, which also indicated that the interaction between ions was complex.

Copper and manganese, as cofactor of some enzymes such as reductase, oxidase and peroxidase (Walker 2004; Ferreira *et al.* 2006), are required in micro level for yeast. In our study, addition of zinc combining plus 0.001 m*M* copper or 0.001 m*M* manganese increased ethanol tolerance. This may be attributed to response of MnSOD and Cu/ZnSOD to ethanol stress (Zhao and Bai 2012). Although Xue *et al.* (2008) indicated that manganese did not promote ethanol tolerance, adding manganese or copper increased yeast biomass and ethanol yield in other studies (Soares *et al.* 2003; Ferreira *et al.* 2006; Zhao and Bai 2012).

Many works have revealed toxicity of high concentration copper and manganese to yeast, even if the toxic level was disagreement (Akrida-Demertzi *et al.* 1990). In this study, 0.01 m*M* manganese and copper showed inhibition of ethanol tolerance on two yeast strains, leading to decreased viability, biomass and ethanol production. There is antagonism between manganese, copper and zinc, which could trigger competition for binding site. The replacement of zinc ion by these two ions may weaken even lost the activity of enzymes which were activated by zinc ion (Soares *et al.* 2003). It was noteworthy that zinc could reduce toxicity of high concentration copper and manganese. This could be because zinc reduced absorption rate of intracellular copper. Similar phenomena were observed in other ions such as calcium detoxified copper, manganese detoxified zinc and so on (Mirminachi *et al.* 2002; Soares *et al.* 2003; Reddi *et al.* 2009). These results implied that the effect caused by competitive ions may be varied. Though the details were not well understood, it provided a possibility to detoxify excessive amount of ions in yeast.

On the other hand, 0.01 m*M* copper had no toxicity on *S. cerevisiae* BY4741. The strain dependent of copper effect on growth and fermentation of several kinds of wine yeasts was mentioned before (Ferreira *et al.* 2006). Our results indicated that ethanol tolerance also was related yeast strain.

Conclusion

It is of great practical significance to study the optimal combination of metal ions to improve ethanol yield and cell viability. This study was the first to report the effect of zinc combining other metal ions on yeast ethanol tolerance. The results showed that zinc reinforced ethanol tolerance of yeast by increasing viability, growth and promoting fermentation in the presence of ethanol. Addition of zinc combining with calcium or copper or manganese ion could enhance or weaken ethanol tolerance of yeast, and these effects were, to some extent, concentration and yeast strain dependent. Our results differed from some previous studies and could be attributed to differences in yeast strain, medium or metal concentration. Therefore, a general

understanding of the effect of metal ions on ethanol tolerance of yeasts may require optimization of types and concentrations of metal ions and more detailed physiological and biochemical basis from more yeast strains, which can help to optimize yeast growth and fermentation processes.

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