Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.1590/1678-457X.24217

Effect of high Cu^{2+} stress on fermentation performance and copper biosorption of *Saccharomyces cerevisiae* during wine fermentation

Xiangyu SUN[1,2,3], Lingling LIU[1], Tingting MA[1,4], Jing YU[1], Weidong HUANG[1], Yulin FANG[1,2], Jicheng ZHAN[1]*

Abstract

The effect of high Cu^{2+} stress on fermentation performance and copper biosorption of *Saccharomyces cerevisiae* BH8 during wine fermentation was investigated in this paper. Under high Cu^{2+} stress, the cell growth and survival rate of yeast BH8 were inhibited. With different copper concentration, the effect on cell growth of yeast BH8 was different. What's more, high Cu^{2+} stress could inhibit the fermentation property, alcohol production and reducing sugar utilization of yeast BH8 in Chardonnay grape must, but not significant. And the trend of the copper ion concentration falling down was consistent with the trend of yeast growth rising up, meanwhile the copper content was still much less than the initial concentration after fermentation. In addition, yeast BH8 showed a good fermenting property and copper removal ability under high Cu^{2+} stress. These results could provide certain reference and some new data for the wine industry to face the copper pollution risk.

Keywords: wine; copper; *Saccharomyces cerevisiae*; growth activity; biosorption.

Practical Application: It could give some help to wine making and lay a foundation.

1 Introduction

Copper is one of the richest heavy metal in wines. As there are so many copper sources during the whole wine production process, such as the copper absorbed from soil to grapes, the copper pesticide attached on the grape surface, the bronze wine brewing equipment and the copper sulfate or copper citrate added in wines for removal of reduction smell caused by hydrogen sulfide, mercaptan, etc (International Organisation of Vine and Wine, 2013; Tamasi et al., 2010; Volpe et al., 2009). Cupric pesticides, especially the Bordeaux mixture ($CuSO_4$ mixture and Ca $(OH)_2$) are the longest and most commonly applied vineyard fungicides. As they have been long term in large doses and unrestricted use, meanwhile copper rarely degrade or move in arable layer soils, which lead to copper accumulation in soil of the vineyard. Some vineyards have been far beyond the European Union regulations limit of 140 mg/kg, even 1500 mg/kg (Ash et al., 2012; García-Esparza et al., 2006; Machado et al., 2003; Mirlean et al., 2007; Nogueirol et al., 2010; Probst et al., 2008). When copper accumulation exceeds a certain limit, excess Cu^{2+} will be shipped to grapes through the transportation system from the root. The toxicity will not only affect the normal metabolism of grape cells, directly affect the quality of grapes, but also these copper will be carried into the grape must with soils, grapes, etc, ultimately affect the wine fermentation and wine quality (Mirlean et al., 2005; Pyrzynska, 2004).

In a narrow range of low concentration, copper is an essential trace element in almost all organisms and plays an important positive role for organisms (Azenha et al., 2000; Ferreira et al., 2006). However, it would have inhibitory effect on cell when out of the useful range, even toxicity (Robinson & Winge, 2010). In the process of fermentation, when Cu^{2+} concentration is more than 0.5 mg/L grape must, it might produce copper broken, wine oxidative browning and a series of reactions that reduced the quality of wines (Li et al., 2008). When Cu^{2+} concentration is more than 20 mg/L, it might inhibit the growth of yeast, resulted in slow fermentation, even stagnation (Ferreira et al., 2006). At the same time, with the increasing of copper content in wines, particularly existence with other heavy metals such as iron, manganese, zinc, nickel, lead, scandium etc, will cause harm to the health of consumers (Naughton & Petróczi, 2008). The European Union and South African stipulated that the copper content in grape must should not exceed 20 mg/L, not exceed 1 mg/L in wines (Ferreira et al., 2006; García-Esparza et al., 2006; Li et al., 2008). In fact, the copper content exceeding in the grape must happened from time to time, García-Esparza et al. (2006) found that about 13% of grapes and 18% of wines exceeded the maximum copper residue in Italy. In Nebbiolo, Australian and China, the phenomenon started to appear too (Marengo & Aceto, 2003; Sauvage et al., 2002; Sun et al, 2015b, 2017, 2018). And in a short period of time, Bordeaux mixture pesticides are still difficult to be replaced (Li et al., 2008); this also means that the copper content in the vineyard as well as in the grape must will still continue to rise. In future, we will probably need to face the wine fermentation

Received 26 July, 2017
Accepted 11 Oct., 2017
[1] *College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China*
[2] *College of Enology, Northwest Agricultural and Forestry University, Yangling Shaanxi, China*
[3] *Key Laboratory of Agro-products Quality and Safety Control in Storage and Transport Process, Beijing, China*
[4] *College of Food Science and Engineering, Northwest Agricultural and Forestry University, Yangling Shaanxi, China*
*Corresponding author: zhanjicheng@cau.edu.cn

under high copper concentration and how to reduce the copper concentration of wines.

In order to remove the excessive copper ions in wines, the current method is to add the adsorbent such as glue and then remove it by filtering. For example, when potassium ferrocyanide was added into wines, it will combine with copper as insoluble compounds, the rest of it will combine with iron ions existed in wines into insoluble compounds, and then the precipitation can be removed by filtering. But when the iron ions in wines were not enough, highly toxic cyanide may remain in wine, detrimental to drinkers' health (Benítez et al., 2002; Mira et al., 2007; Schubert & Glomb, 2010). Bentonite, gum Arabic, polyvinylimidazole polyvinylpyrrolidone copolymers, chitin, chitosan et al. were also allowed to add in wines to reduce the copper content by OIV, but these additives will affect wine sensory quality in different degrees (Benítez et al., 2002). And *Saccharomyces cerevisiae* has the capability of adsorption of copper ions (Veglio & Beolchini, 1997; Naja et al., 2010). Use *Saccharomyces cerevisiae* to complete the alcoholic fermentation and remove the redundant copper ions at the same time, can not only ensure the safety and the quality of the wine, but also retain the original color and flavor of wine furthest. Also it conforms to the requirements of the organic wine production, so it is a kind of environmental protection and effective method.

Therefore, research on the growth of *Saccharomyces cerevisiae* under high concentration copper stress condition, alcohol fermentation performance and adsorption of copper ions, can provide a new method of quality control for the wine industry, and also provide a new direction for microbial screening or modification. In this study, we used the yeast which was screened in the early stage of our laboratory and has a good ability to resist many different stresses, by studying the growth activity, the fermentation performance, copper adsorption situation of wine yeast under different Cu^{2+} concentration, analyzing the copper adsorption performance of yeast, thus to provide guidance for the choice of strains in the wine production, also provide a basis for the study of the microbial response to copper stress.

2 Materials and methods

2.1 Test strains and Medium

The *Saccharomyces cerevisiae strain* BH8 which have a certain ability to resist under many different stresses (Du et al., 2012; Li et al., 2010, 2011) was used in this study. It was isolated from BeiHong grape must and stored at the laboratory (China Agricultural University, Beijing), identified as *S. cerevisiae* by Institute of Microbiology, Chinese Academy of Sciences (Du et al., 2012; Li et al., 2010, 2011). Cells maintained on slants were pre-cultured aerobically to 6×10^7 cfu/mL in shaking flasks containing 60 mL YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 28 °C, 120 rpm (Du et al., 2012). Chardonnay grape must was devoted by Beijing Red leaf winery, the Brix was 22.7; the original copper concentration was 0.2625 mg/L.

2.2 Chemicals and standards

Standards of D-trehalose, D-glucose, D-fructose, sulfuric acid, and ethanol (chromatographically pure) were obtained from Sigma-Aldrich (St. Louis, MI, USA). HNO_3 (guarantee reagent) was purchased from Merck Co. (German). Deionized water was produced by Wahaha Co. (Hangzhou, China). All other reagents used were analytical grade unless specially noted.

2.3 High Cu^{2+} stress treatment and fermentation culture

The high Cu^{2+} stress was set with different Cu^{2+} concentration of 0.50 mM (32 mg/L, 1.00 mM (64 mg/L) and 1.50 mM (96 mg/L) by adding different volume of $CuSO_4{\cdot}5H_2O$ solution (0.1 mol/L) into 400 mL Chardonnay grape must. The control group was Chardonnay grape must without adding $CuSO_4{\cdot}5H_2O$ solution. 4 mL yeast precultures were inoculated in 500 mL flasks containing 400 mL grape must to obtain a density of 10^6 cells/mL (Li et al., 2011). Flasks were sealed with glass capillary stoppers filled with concentrated H_2SO_4 to prevent weight loss causing by water evaporation. Cultures were constantly shaken at 28 °C, 120 rpm in thermostatic shaker (SKY-2102C, Shsukun Co. Ltd., Shanghai) (Du et al., 2010). Fermentation experiments were separated into two groups: one group for weighing, the other group for sampling, and each group was carried out in triplicate.

2.4 Determination of cell growth, survival rate and fermenting property

Methylene blue staining method was used to distinguish the dead and live yeast (Marañon et al., 1999). Living ones were not-dyed, while dead ones were dyed blue. In the process of the fermentation process, take 1 mL fermented liquid from the sampling group every time appropriately (at early fermentation every 24 h, at later fermentation every 48 h). 5 times dilution, then take 100 μL dilution to trace centrifuge tube, add 100 μL 0.1% methylene blue dye solution, blending with pipetting gun. After that, take 100 μL dying cells suspension to XB-K-25 blood count sheet and observed under optical microscopes (XSZ-3G, COIC) under a 40× objective lens. Take four corners and center, a total of 5 medium lattices (80 small lattices) to count. The sum of number of living cells as N_L, the sum of number of living cells and dead cells as N, the total cells count of fermentation liquid = $5 \times 10^5 \times$ N/mL, the survival rate = $(N_L/N) \times 100\%$.

The determination of fermentation course by weighing the samples, due to the evolution of carbon dioxide (Brandolini et al., 2002). Mass loss caused by CO_2 evolution was monitored by weighing the fermentation flasks every 24 h. Fermentation was considered to have stopped when mass loss was less than 0.02 g for 3 days.

2.5 Determination of the alcohol produced by the fermentation and the reducing sugar residual after metabolism

The remaining reducing sugars and ethanol content in samples taken during alcoholic fermentation were determined by HPLC using Waters 2414 RI Detector and BIO-RAD Aminex HPX-87H resin-based column (300*7.8mm), which was eluted with 5 mM sulfuric acid (H_2SO_4) at 55 °C, 0.5 mL/min, sample quantity 10 μL, differential refraction detector was used (RID, Waters-414) (Ciani et al., 2006). Statistical differences for cell growth and fermentation performance of the strains were

analyzed using single variable general liner model with PASW Statistics 18.

2.6 Determination of copper content

Sample pretreatment

In order to separate the fermented liquid and yeast, 10 mL fermentation fluid were taken through 0.45 μm water membrane filter, collect filtrate in a 10 mL centrifuge tube. Then liquid nitrogen quick-freezing and stored at -40 °C for test together. Before ICP-OES determination, samples were thawed at 4 °C and shaked well, and then diluted 4 mL sample to 25 mL with 5% HNO_3 (diluted multiples 6.25).

ICP-OES determination

The copper content was detected by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometr) method (Yilmaz et al., 2014). The instrumental specifications are given in Table 1 (Optima 7000DV, Perkin Elmer). The standard curve drawing: Diluted copper standard stock solution to different concentration of 0.00, 5.00, 10.00, 20.00 mg/L series copper standard solution with 5% HNO_3. The instrument drawing the standard curve automatically, the correlation coefficient of standard curve is more than 0.9999.

Table 1. Operating conditions of ICP-OES.

Parameter	Value	Parameter	Value
Plasma gas flow rate (l/min)	15.0	Gas flow rate (ml/min)	1.5
Auxiliary gas flow rate (l/min)	0.2	Power (kW)	1.3
Pulverization gas flow rate (l/min)	0.75	Wavelengths (nm)	327.394
Nebulizer gas flow rate (l/min)	0.8	Observing mode	Axis
Refrigeration gas flow rate (l/min)	15		

2.7 Statistical analysis

The experimental results are expressed as the means ± standard deviations (SD) of three parallel measurements. Correlations were calculated using a linear regression. Statistical analyses were performed using Data Processing System software (DPS, version 7.05, Hangzhou, China) (Tang & Zhang, 2013).

3 Results

3.1 Influence of high Cu^{2+} stress on cell growth and survival rate

The results of cell growth and survival rate of strain BH8 in Chardonnay juice with high level Cu^{2+} were presented in Figure 1. On the whole, strain BH8 rapidly entered into logarithmic phase, after reaching the largest number of living yeast then entered decline phase in all treatment groups [0 (control), 0.50 mM (32 mg/L), 1.00 mM (64 mg/L), 1.50 mM (96 mg/L)]. The growth curve trend during the early stage of the logarithmic phase (0~24 h) and the late decline phase (240~288 h) was consistent among different groups (Figure 1a), as well as the survival rate (Figure 1b). Meanwhile the latency period and stable period were short. Jasna et al. (2007) also reported that Cu^{2+} stress could inhibit *Saccharomyces cerevisiae* cell growth.

In more details, under different high Cu^{2+} stress, the growth vitality of *Saccharomyces cerevisiae* was different. In the control group, after 48 h, the live strain count slowly rose along with the fermentation, and achieved the highest in 144 h which was closed to the end of fermentation (216 h, Figure 2), then the

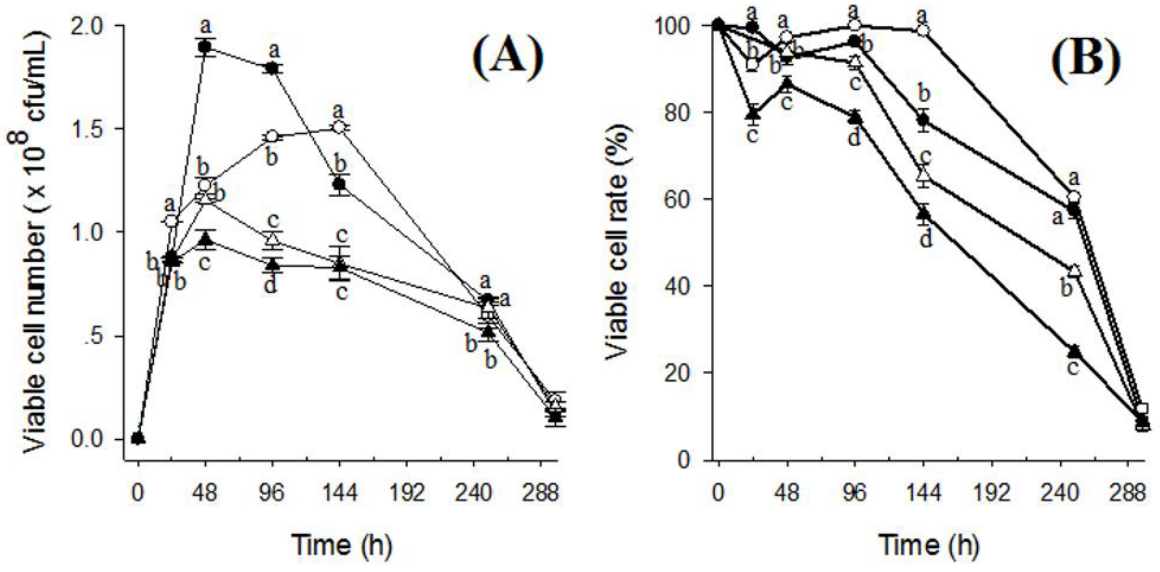

Figure 1. Cell growth (A) and survival rate (B) of strain BH8 in Chardonnay must with high level Cu^{2+} treatment. ○Control, ●32 mg/L, Δ64 mg/L, ▲96 mg/L. Different small letters indicate significant difference (Duncan's test: $P < 0.05$, performed by DPS software (version 7.55, China)).

live yeast count fell down along at the end of the fermentation. During 0~144h (before the end of fermentation), the survival rate of the control group was always around 90%, which was much higher than the high Cu^{2+} stress groups (Figure 1b).

Differently, in the high Cu^{2+} stress groups, the live yeast count stopped rising along with the fermentation after 48 h, then the live yeast count got down along with the fermentation (Figure 1a). What's more, the survival rate of the high Cu^{2+} stress groups descended at the first 24 h, then slowly rose, which indicated that the rapid proliferation of *Saccharomyces cerevisiae* was restrained under the high Cu^{2+} stress. Though gradually adaptation to the stress environment after 24 h (Du et al., 2012; Li et al., 2010, 2011), the rapid proliferation of *Saccharomyces cerevisiae* only happened in the first 48 h of fermentation prophase. After 96 h, the survival rate dropped sharply (Figure 1b).

With different copper concentration, the effect on cell growth of *Saccharomyces cerevisiae* was different. The yeast growth curve under 64 mg/L and 96 mg/L treatment was similar, the live yeast count and the survival rate were negatively related to the initial concentration of Cu^{2+}, and also significantly lower than control group. Differently from other groups, the survival rate of yeast did not fell down under 32 mg/L treatment (Figure 1b); on the contrary, the yeast continuous rapid proliferated during 24~48 h, and the live yeast count reached 1.89×10^8 cfu/mL, which was 55% higher than the control group (Figure 1a). But the survival rate at 48 h was still lower than the control group (Figure 1b). At 96 h, the live yeast count of 32 mg/L treatment group was about 1 times higher than 64 mg/L treatment group and 96 mg/L treatment group. Until 144 h, the live yeast count of 32 mg/L treatment group fell below the control group level (Figure 1a), the survival rate also had obvious difference with the control group, but was still higher than 64 mg/L treatment group and 96 mg/L treatment group (Figure 1b). From what we have mentioned above, we can see clearly that 32 mg/L (0.50 mM) treatment could promote the proliferation of *Saccharomyces cerevisiae* BH8 in Chardonnay grape must, which led to the live yeast count obviously increased; meanwhile the survival rate was still lower than the control group, which indicated that with the live yeast count increased, there were still a large number of yeast died at the same time.

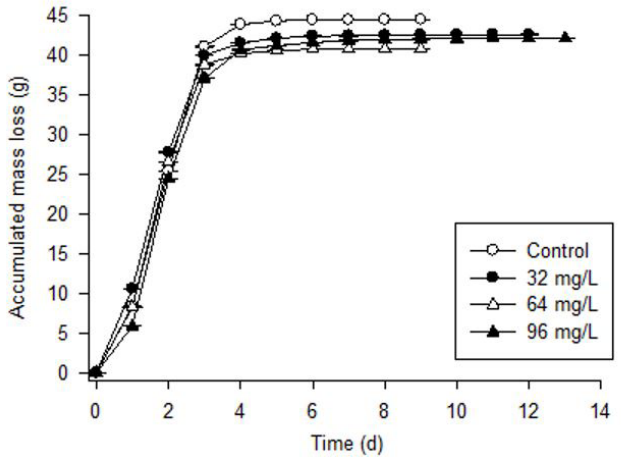

Figure 2. Fermenting property (accumulated mass loss) of BH8 fermenting Chardonnay must with high level Cu^{2+} treatment.

3.2 Influence of high Cu^{2+} stress on fermenting property

The results of CO_2 release percent of *Saccharomyces cerevisiae* BH8 in Chardonnay grape must under high Cu^{2+} treatment were presented in Figure 2. With different copper concentration, the trend of CO_2 release percent was basically the same, which means the fermentation trends were basically identical. In the first 3 days, the fermentation rate was the fastest. At 13 d, each treatment group and control group had completed alcohol fermentation. The total emission of CO_2 was around 43 g in all groups, which indicated that the *Saccharomyces cerevisiae* BH8 has a good performance on the chardonnay grape must fermentation.

Specifically, the fermenting property of high Cu^{2+} treatment groups were slower than the control group, meanwhile the total emission of CO_2 was also lower than the control group (44.38 g). The 64 mg/L treatment group generated the lowest CO_2 (40.71 g). The fermenting property at the first 3 days of 96 mg/L treatment group was lower, but after the 3 days rapid fermentation, its accumulated mass loss curve was basically identical with 32 mg/L treatment group; and the total emission of CO_2 (42.03 g) was no significant difference with the 32 mg/L treatment group (42.51 g). As can be seen from the above, results indicated that high Cu^{2+} treatment showed no significant inhibition on the fermentation of *Saccharomyces cerevisiae* BH8.

3.3 Influence of high Cu^{2+} stress on the alcohol production and reducing sugar utilization

The chromatography of fermentation products standards was shown as Figure 3. The label of peak was for material and the peak time. Sulfuric acid was mobile phase composition. Standard can be accurately distinguished, without delay or trailing. The correlation coefficients of standard curve were more than 0.9999 and showed good linear relationship.

The effect of high Cu^{2+} stress on the alcohol production and reducing sugar utilization were shown in Figure 4. Under different copper concentration groups, yeast were able to complete the alcoholic fermentation, the alcohol content was above 13% (Figure 4a) and reducing sugar residue was lower than 4 g/L (Figure 4b) after fermentation. Fermentation period did not exceed 12 days. The trend of alcohol production and reducing sugar utilization were consistent, and there was no significant delays or stagnation under different copper concentration groups, which indicated that reducing sugar was fully transform into alcohol, *Saccharomyces cerevisiae* performing well in the alcoholic fermentation. This result was consistent with Jasna et al.'s report (2007).

In more details, the control group produced the highest alcohol and utilized the highest reducing sugar, while the 96 mg/L treatment group produced the lowest alcohol and utilized the lowest reducing sugar. This result was consistent with the CO_2 release percent (Figure 2). For the 96 mg/L treatment group,

the amount of residual sugar (3.25 g/L) was significantly higher than other groups, but still accorded with standard of dry type wine residual sugar (4 g/L) (International Organisation of Vine and Wine, 2013). Results indicated that high Cu^{2+} stress could inhibit the process of transform reducing sugar into alcohol, but not significant, the main metabolites were not significantly suppressed ($P< 0.05$).

The fermenting property (Figure 2) under high Cu^{2+} stress in Chardonnay grape must was different with the result of our previous research in simulated grape juice (Zhao, 2012). In simulated grape juice, the alcoholic fermentation of *Saccharomyces cerevisiae* BH8 was inhibited significantly by high Cu^{2+} stress (Zhao, 2012). But in this research, in Chardonnay grape must, the fermentation rate and fermentation production did not significantly reduce. Du et al. (2010) also reported the similar results in Cabernet Sauvignon must.

With different yeast strains, the effect of Cu^{2+} stress on *Saccharomyces cerevisiae* was different (Azenha et al., 2000; Ferreira et al., 2006; Brandolini et al., 2002; Du et al., 2012; Li et al., 2010, 2011). In simulated grape juice, *Saccharomyces cerevisiae* BH8 had showed good copper resistance (Zhao, 2012). In Chardonnay grape must, *Saccharomyces cerevisiae* BH8 also showed good copper resistance. Though the real grape must could adsorb more copper than simulated grape juice (Figure 5, Zhao, 2012), it also proved that *Saccharomyces*

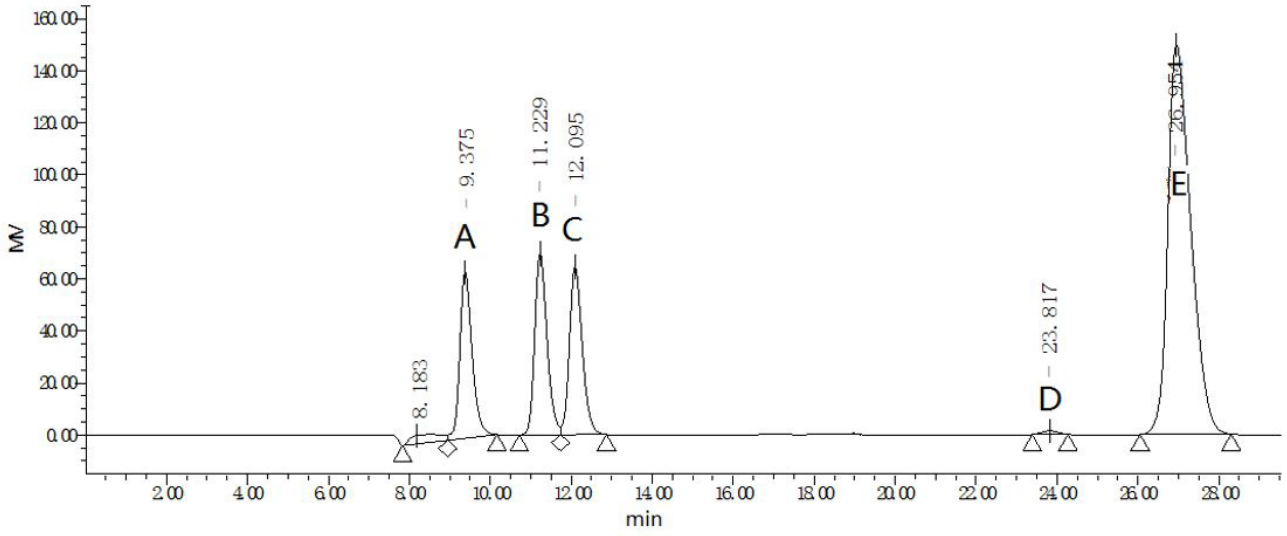

Figure 3. Chromatography of fermentation products standards. (A) D-trehalose (B) d-Glucose (C, D) fructose (D) sulfuric acid (E) ethanol.

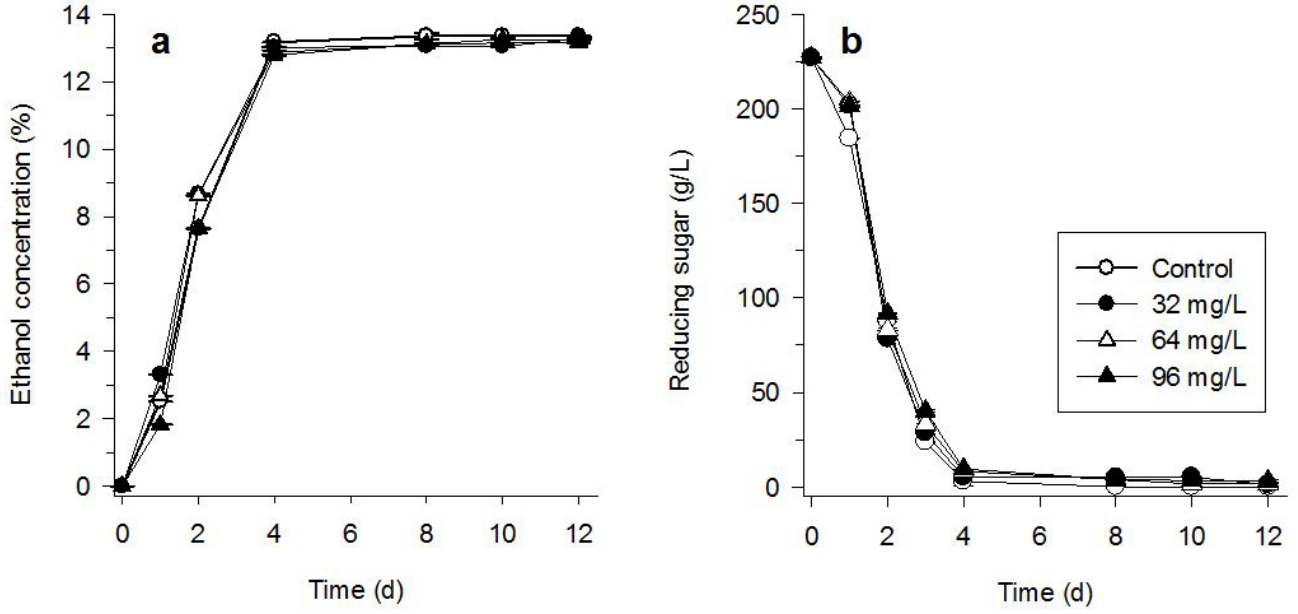

Figure 4. Ethanol (a) and reducing sugar (b) levels during fermentation in Chardonnay must at different Cu^{2+} concentrations.

cerevisiae BH8 was capable of finishing the wine fermentation under high Cu^{2+} stress.

32 mg/L copper treatment could improve the growth activity of *Saccharomyces cerevisiae* BH8 (Figure 1a), but had no effect on the alcoholic fermentation, the survival rate did not improve either (Figure 1b). One possible reason is that the influence mechanism of copper treatment on *Saccharomyces cerevisiae* fermentation system was complex; under low concentrations, copper is an essential trace element in almost all organisms and plays an important positive role for organisms (Azenha et al., 2000; Ferreira et al., 2006), but it would have inhibitory effect on cells when out of the useful range, even toxicity (Robinson & Winge, 2010). Under 32 mg/L copper concentration, on the one hand, copper stimulate the breeding of *Saccharomyces cerevisiae* BH8; on the other hand, it also accelerates yeast death. More research is needed, such as the facilitation and inhibition specific thresholds are needed.

3.4 Influence of high Cu^{2+} stress on copper biosorption of Saccharomyces cerevisiae during wine fermentation

The results of copper biosorption of *Saccharomyces cerevisiae* during wine fermentation were shown in Figure 5. The copper ion concentration in Chardonnay must fell sharply at first, then slowly risen up with the alcohol fermentation advancing. The falling speed and rallied speed were all positively related to the initial concentration of Cu^{2+}. What's more, the trend of the copper ion concentration falling down at the earlier stage (Figure 5) was consistent with the trend of yeast growth rising up at the earlier stage (Figure 1a), the maximum speed were all happened at the second day. Meanwhile, at the exuberant fermentation period (2~4 d), the copper ion concentration remained stable at the lowest level, which was also same with the fermentation efficiency remained stable period (Figure 2).

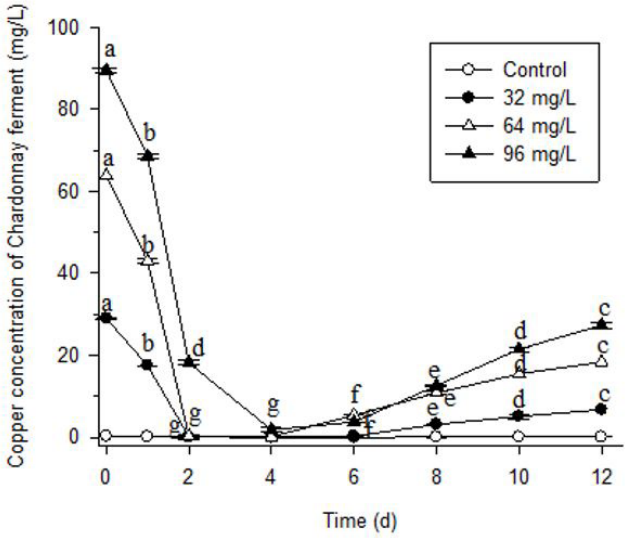

Figure 5. Copper concentration in Chardonnay must during fermentation at different Cu^{2+} levels. Different small letters indicate significant difference (Duncan's test: $P < 0.05$, performed by DPS software (version 7.55, China)).

After the fourth day, copper ion concentration began to recover slowly, but it was still much less than the initial concentration.

After alcoholic fermentation, the order of the removal rate of copper was as follows: the control group (80.95%), then 32 mg/L treatment group (64.55%), 96 mg/L treatment group (60.67%), 64 mg/L treatment group (58.14%). This order was same with the order of the efficiency of fermentation producing CO_2 (Figure 2), but inconsistent with the live yeast count (Figure 1a), which means that except biological adsorption (by living yeast), non-biological adsorption (including died yeast) might play a role.

For the reason that the real grape must was complicated with many solids compositions (Ferreira et al., 2006; Li et al., 2010), it was difficult to extract yeast, which means that it was difficult to detect the copper content of the yeast body directly. So we detected the change of copper content in grape must to reflect the copper ions absorption of *Saccharomyces cerevisiae* BH8. The rapidly falling of the copper content in grape must embodied that *Saccharomyces cerevisiae* BH8 had a good adsorption performance on copper. This is consistent with the results in simulated grape juice (Sun et al., 2015a, 2016). In addition, the copper ion removal rate was more than 58% in real grape must, far more than the result under the same initial concentration of Cu^{2+} in simulation grape juice (around 20%) (Zhao, 2012). The reason of this might be that simulation grape juice composition is relatively simple (Du et al., 2012; Ferreira et al., 2006), there was no effect of natural microbe, solids, etc, so reflect a good correlation between the copper ions removal rate and the initial concentration of Cu^{2+} (Jasna et al., 2007; Zhao, 2012). And in real grape must, as there are many other biological components (plant cell, microbial) and non-biological components (protein, free sulphur) that can be combined with copper existed, though the copper ions removal rate were still negative correlation with the increasing of initial concentration of Cu^{2+}, the removal rate was much higher than in simulation grape juice (Zhao, 2012).

At the late alcoholic fermentation stage, the copper ion concentration began to slowly rising up. As *Saccharomyces cerevisiae* began to autolysis in late fermentation (Ishii et al., 2014; Moon et al., 2013), and high Cu^{2+} stress accelerated the death of *Saccharomyces cerevisiae* cells (Figure 1b) (Robinson & Winge, 2010; Azenha et al., 2000; Ferreira et al., 2006), meanwhile the form of *Saccharomyces cerevisiae* adsorption copper ion including absorption copper to intracellular accumulation (Kapoor & Viraraghavan, 1995), so the slow rise of copper concentration may come from the intracellular accumulation of copper ion release again. There are many factors affecting the copper adsorption of *Saccharomyces cerevisiae*, therefore, the adsorption mechanism, including biological adsorption non-biological adsorption should be further studied.

4 Conclusions

Under high Cu^{2+} stress, the cell growth and survival rate of *Saccharomyces cerevisiae* BH8 were inhibited. With different copper concentration, the effect on cell growth of *Saccharomyces cerevisiae* BH8 was different. 64 mg/L and 96 mg/L treatment inhibited the yeast growth curve in the whole fermentation stage, while 32 mg/L treatment promoted the yeast continuous at early

fermentation stage, which led to the live strain count obviously increased, but the survival rate was still lower than the control group, which indicated that with the live yeast count increased, there were still a large number of yeast died at the same time.

What's more, high Cu^{2+} stress could inhibit the fermenting property, the alcohol production and reducing sugar utilization of *Saccharomyces cerevisiae* BH8 in Chardonnay grape must, but not significant. In all treatment groups, reducing sugar can be fully converted to alcohol. With the alcohol fermentation advancing, the copper ion concentration in Chardonnay must fell sharply at first, then slowly risen up. The falling speed and rallied speed were all positively related to the initial concentration of Cu^{2+}. Moreover, the trend of the copper ion concentration falling down at the earlier stage was consistent with the trend of yeast growth rising up at the earlier stage, and the copper content was still much less than the initial concentration in the end of fermentation. After alcoholic fermentation, the order of the removal rate of copper was same with the order of the efficiency of fermentation producing CO_2, but inconsistent with the live yeast count, which means that except biological adsorption (by living yeast), non-biological adsorption (including died yeast) might play a role. In addition, the *Saccharomyces cerevisiae* BH8 showed a good fermenting property and copper removal ability under high Cu^{2+} stress. These results could provide certain references and some new data for the wine industry to face the copper pollution risk.

Acknowledgements

This study was supported by the National Nature Science Foundation Project (31471835), the China Agriculture Research System for Grape Industry (CARS-30-zp-9), the Fundamental Research Funds for the Central Universities (2452017224, 2452017227) and the National Key Research and Development Program (2016YFD0400504, 2016YFD0400501).

References

Ash, C., Vacek, O., Jaksik, O., Tejnecky, V., & Drabek, O. (2012). Elevated soil copper content in a bohemian vineyard as a result of fungicide application. *Soil and Water Research*, 7, 151-158.

Azenha, M., Vasconcelos, M., & Moradas-Ferreira, P. (2000). The influence of Cu concentration on ethanolic fermentation by *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering*, 90(2), 163-167. PMid:16232836. http://dx.doi.org/10.1016/S1389-1723(00)80104-8.

Benítez, P., Castro, R., & Barroso, C. (2002). Removal of iron, copper and manganese from white wines through ion exchange techniques: effects on their organoleptic characteristics and susceptibility to browning. *Analytica Chimica Acta*, 458(1), 197-202. http://dx.doi.org/10.1016/S0003-2670(01)01499-4.

Brandolini, V., Tedeschi, P., Capece, A., Maietti, A., Mazzotta, D., Salzano, G., Paparella, A., & Romano, P. (2002). *Saccharomyces cerevisiae* wine strains differing in copper resistance exhibit different capability to reduce copper content in wine. *World Journal of Microbiology & Biotechnology*, 18(6), 499-503. http://dx.doi.org/10.1023/A:1016306813502.

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology*, 108(2), 239-245. PMid:16487611. http://dx.doi.org/10.1016/j.ijfoodmicro.2005.11.012.

Du, G., Zhan, J. C., Li, J. Y., You, Y. L., Zhao, Y., & Huang, W. D. (2012). Effect of Fermentation Temperature and Culture Medium on Glycerol and Ethanol during Wine Fermentation. *American Journal of Enology and Viticulture*, 63(1), 132-138. http://dx.doi.org/10.5344/ajev.2011.11067.

Du, J., Li, H. L., Li, H., Zhan, J. C., & Huang, W. D. (2010). Influence of copper stress on *Saccharomyces cerevisiae* grape must. *Zhongguo Nong Ye Ke Xue*, 43, 3259-3265.

Ferreira, J., Toit, M., & Toit, W. (2006). The effects of copper and high sugar concentrations on growth, fermentation efficiency and volatile acidity production of different commercial wine yeast strains. *Australian Journal of Grape and Wine Research*, 12(1), 50-56. http://dx.doi.org/10.1111/j.1755-0238.2006.tb00043.x.

García-Esparza, M. A., Capri, E., Pirzadeh, P., & Trevisan, M. (2006). Copper content of grape and wine from Italian farms. *Food Additives and Contaminants*, 23(3), 274-280. PMid:16517529. http://dx.doi.org/10.1080/02652030500429117.

International Organisation of Vine and Wine – OIV. (2013). *International code of oenological practices*. Paris: OIV.

Ishii, J., Kondo, T., Makino, H., Ogura, A., Matsuda, F., & Kondo, A. (2014). Three gene expression vector sets for concurrently expressing multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, 14(3), 399-411. PMid:24447461. http://dx.doi.org/10.1111/1567-1364.12138.

Jasna, M., Damir, S., Vesna, S., Dubravka, Š., & Slobodan, G. (2007). Optimization of Bioprocess for Production of Copper-Enriched Biomass of Industrially Important Microorganism *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering*, 103(4), 331-337. PMid:17502274. http://dx.doi.org/10.1263/jbb.103.331.

Kapoor, A., & Viraraghavan, T. (1995). Fungal biosorption-an alternative treatment option for heavymetal bearing wastewaters: a review. *Bioresource Technology*, 53, 195-206.

Li, H., Du, G., Li, H., Wang, H., Yan, G., Zhan, J., & Huang, W. (2010). Physiological Response of Different Wine Yeasts to Hyperosmotic Stress. *American Journal of Enology and Viticulture*, 61(4), 529-535. http://dx.doi.org/10.5344/ajev.2010.09136.

Li, H., Guo, A. Q., & Wang, H. (2008). Mechanisms of oxidative browning of wine. *Food Chemistry*, 108(1), 1-13. http://dx.doi.org/10.1016/j.foodchem.2007.10.065.

Li, J., Du, G., Xiao, Y., & Huang, W. (2011). Effect of Proanthocyanidins on Yeast Metabolism, H+-ATPase Activity, and Wine Fermentation. *American Journal of Enology and Viticulture*, 62(4), 512-518. http://dx.doi.org/10.5344/ajev.2011.11021.

Machado, R., Santos, C., Correia, M., & Carvalho, J. (2003). Biosorption of copper by grape stalks and pine bark biomasses. *The European Journal of Mineral Processing and Environmental Protection*, 3, 108-118.

Marañon, I., Chaudansson, N., Joly, N., & Gervais, P. (1999). Slow heat rate increases yeast thermotolerance by maintaining the plasma membrane integrity. *Biotechnology and Bioengineering*, 65(2), 176-181. PMid:10458738. http://dx.doi.org/10.1002/(SICI)1097-0290(19991020)65:2<176::AID-BIT7>3.0.CO;2-5.

Marengo, E., & Aceto, M. (2003). Statistical investigation of the differences in the distribution of metals in Nebbiolo-based wines. *Food Chemistry*, 81(4), 621-630. http://dx.doi.org/10.1016/S0308-8146(02)00564-2.

Mira, H., Leite, P., Catarino, S., Ricardo, S., & Curvelo, G. (2007). Metal reduction in wine using PVI-PVP copolymer and its effects on chemical and sensory characters. *Vitis*, 46, 138-147.

Mirlean, N., Roisenberg, A., & Chies, J. (2005). Copper-based fungicide contamination and metal distribution in Brazilian grape products. *Environmental Contamination and Toxicology*, 75(5), 968-974. PMid:16400586. http://dx.doi.org/10.1007/s00128-005-0844-3.

Mirlean, N., Roisenberg, A., & Chies, J. (2007). Metal contamination of vineyard soils in wet subtropics (southern Brazil). *Environmental Pollution*, 149(1), 10-17. PMid:17321651. http://dx.doi.org/10.1016/j.envpol.2006.12.024.

Moon, S. K., Lee, J., Song, H., Cho, J. H., Choi, G. W., & Seung, D. (2013). Characterization of ethanol fermentation waste and its application to lactic acid production by *Lactobacillus paracasei*. *Bioprocess and Biosystems Engineering*, 36(5), 547-554. PMid:22907566. http://dx.doi.org/10.1007/s00449-012-0810-5.

Naja, G. M., Murphy, V., & Volesky, B. (2010). Biosorption, metals. In M. C. Flickinger (Ed.), *Encyclopedia of industrial biotechnology: bioprocess, bioseparation, and cell technology* (pp. 1-29). Hoboken: John Wiley and Sons, Inc. http://dx.doi.org/10.1002/9780470054581.

Naughton, D., & Petróczi, A. (2008). Heavy metal ions in wines: meta-analysis of target hazard quotients reveal health risks. *Chemistry Central Journal*, 3, 1-7. PMid:18973648.

Nogueirol, R., Alleoni, L., Nachtigall, G., & de Melo, G. W. (2010). Sequential extraction and availability of copper in Cu fungicide-amended vineyard soil from Southern Brazil. *Journal of Hazardous Materials*, 181(1-3), 931-937. PMid:20579811. http://dx.doi.org/10.1016/j.jhazmat.2010.05.102.

Probst, B., Schüler, C., & Joergensen, R. (2008). Vineyard soils under organic and conventional management—microbial biomass and activity indices and their relation to soil chemical properties. *Biology and Fertility of Soils*, 44(3), 443-450. http://dx.doi.org/10.1007/s00374-007-0225-7.

Pyrzynska, K. (2004). Analytical methods for the determination of trace metals in wine. *Critical Reviews in Analytical Chemistry*, 34(2), 69-83. http://dx.doi.org/10.1080/10408340490475858.

Robinson, N., & Winge, D. (2010). Copper metallochaperones. *Annual Review of Biochemistry*, 79(1), 537-562. PMid:20205585. http://dx.doi.org/10.1146/annurev-biochem-030409-143539.

Sauvage, L., Frank, D., Stearne, J., & Millikan, M. B. (2002). Trace metal studies of selected white wines: an alternative approach. *Analytica Chimica Acta*, 458(1), 223-230. http://dx.doi.org/10.1016/S0003-2670(01)01607-5.

Schubert, M., & Glomb, M. (2010). Analysis and chemistry of migrants from wine fining polymers. *Journal of Agricultural and Food Chemistry*, 58(14), 8300-8304. PMid:20568775. http://dx.doi.org/10.1021/jf101127t.

Sun, X. Y., Liu, L. L., Zhao, Y., Ma, T. T., Zhao, F., Huang, W. D., & Zhan, J. C. (2016). Effect of copper stress on growth characteristics and fermentation properties of *Saccharomyces cerevisiae* and the pathway of copper adsorption during wine fermentation. *Food Chemistry*, 192, 43-52. PMid:26304318. http://dx.doi.org/10.1016/j.foodchem.2015.06.107.

Sun, X. Y., Ma, T. T., Han, L. Y., Huang, W. D., & Zhan, J. C. (2017). Effects of copper pollution on the phenolic compound contents, color and antioxidant activity of wine. *Molecules*, 22(5), 726. http://dx.doi.org/10.3390/molecules22050726.

Sun, X. Y., Ma, T. T., Yu, J., Huang, W. D., Fang, Y. L., & Zhan, J. C. (2018). Investigation of the copper contents in vineyard soil, grape must and wine and the relationship among them in the Huaizhuo Basin Region, China: A Preliminary Study. *Food Chemistry*, 241, 40-50. PMid:28958546. http://dx.doi.org/10.1016/j.foodchem.2017.08.074.

Sun, X. Y., Zhao, F., Ma, T. T., Wang, L. H., Wang, C., Sun, Y. Q., & Zhan, J. C. (2015b). Analysis of the Copper Content of Part of China's Main Wine Regions. *Modern Food Science and Technology*, 31(5), 278-284.

Sun, X. Y., Zhao, Y., Liu, L. L., Jia, B., Zhao, F., Huang, W. D., & Zhan, J. C. (2015a). Copper tolerance and biosorption of Saccharomyces cerevisiae during alcoholic fermentation. *PLoS One*, 10(6), e0128611. PMid:26030864. http://dx.doi.org/10.1371/journal.pone.0128611.

Tamasi, G., Pagni, D., Carapelli, C., Justice, N., & Cini, R. (2010). Investigation on possible relationships between the content of sulfate and selected metals in Chianti wines. *Journal of Food Composition and Analysis*, 23(4), 333-339. http://dx.doi.org/10.1016/j.jfca.2009.12.011.

Tang, Q., & Zhang, C. (2013). Data Processing System (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. *Insect Science*, 20(2), 254-260. PMid:23955865. http://dx.doi.org/10.1111/j.1744-7917.2012.01519.x.

Veglio, F., & Beolchini, F. (1997). Removal of metals by biosorption. *Hydrometallurgy*, 44(3), 301-316. http://dx.doi.org/10.1016/S0304-386X(96)00059-X.

Volpe, M. G., Cara, F., Volpe, F., Mattia, A., Serino, V., Petitto, F., Zavalloni, C., Limone, F., Pellecchia, R., Prisco, P., & Stasio, M. (2009). Heavy metal uptake in the enological food chain. *Food Chemistry*, 117(3), 553-560. http://dx.doi.org/10.1016/j.foodchem.2009.04.033.

Yilmaz, V., Arslan, Z., Hazer, O., & Yilmaz, H. (2014). Selective solid phase extraction of copper using a new Cu(II)-imprinted polymer and determination by inductively coupled plasma optical emission spectroscopy (ICP-OES). *Microchemical Journal*, 114, 65-72. PMid:24511158. http://dx.doi.org/10.1016/j.microc.2013.12.002.

Zhao, Y. (2012). Characteristics of Cu2+ biosorption by *Saccharomyces cerevisiae*. Beijing: China Agricultural University.