

THE IMPACT OF CALCIUM ION ON FERMENTATION PERFORMANCE AND STRESS TOLERANCE OF THE YEAST *Saccharomyces cerevisiae*

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Abstract

Metal ions in fermentation media is very crucial for yeast growth and fermentation performance. Other than that, they also have roles for protecting yeast cells against environmental stress factors. In the present study, we investigate the role of calcium ions on fermentation performance and stress tolerance. Most study focus only on one stress factor, while in the present study we tested the effect of calcium ion supplementation against four stress factors, namely ethanol, hyperosmotic, weak acid, and oxidative stresses. The results of the present study indicate that calcium ion has positive role in cell growth and fermentation performance as indicated by significantly higher OD_{600 nm} and higher ethanol produced when calcium ion present in the fermentation media. Calcium ion was also found improving the yeast cell tolerance to all stress factors tested. Therefore, we propose that calcium ion act as general stress protectant for the yeast strain used in this study.

Keywords: Calcium, ethanol fermentation, *Saccharomyces cerevisiae*, stress tolerance

Introduction

Currently, bioethanol is considered as one of favorable alternative energy sources because its renewable property and more environmentally friendly (Hahn-Hägerdal *et al.*, 2006). Bioethanol is ethanol produced from various biomass feedstocks such as sugary, starchy or cellulosic material, through fermentation process by microorganisms including yeasts and bacteria (Cazetta *et al.*, 2007; Jambo *et al.*, 2016). *Saccharomyces cerevisiae* is being the most utilized microorganism due to its ability to produce higher ethanol concentration and recognized as GRAS microorganism, and therefore waste from the fermentation process is easily

processed, including converted to animal feed (Natesuntorn *et al.*, 2019).

Some factors such as high sugar concentration, temperature, weak acid, and ethanol itself can act as stress factors during fermentation and therefore can inhibit the activity of yeast cells to ferment sugar (Stanley *et al.*, 2010; Morano *et al.*, 2012; Giannattasio *et al.*, 2013; Tilloy *et al.*, 2014; Rachman *et al.*, 2018). Other than that, by products from hydrolysis of feedstock can also become a stress factor that inhibit the fermentation process (Pornpukdeewattana *et al.*, 2014). When the yeast cells are exposed to the stress factors, it will be

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sensed by the yeast cells, and signal transduction process will occur which lead to expression of genes that change the metabolisms of the yeast cells. These changes will protect the cells against the stress factor and repair of the damages caused by the stress and eventually they can resume their growth (Siderius and Mager, 2003).

During the fermentation process, composition of the fermentation media is affecting physiological condition of the yeast cells and therefore can affect stress tolerance and the fermentation performance in general (Birch *et al.*, 2003; Ishmayana *et al.*, 2011). Some efforts have been proposed to increase yeast stress tolerance including supplementation of the fermentation media. One of the supplements that widely used in fermentation is metal ions (Xue *et al.*, 2008; Ismail *et al.*, 2014). Some metal ions, including zinc, magnesium, and calcium were reported to have protective effects against stress factors (Ismail *et al.*, 2014). Other than that, it can increase fermentation performance by increasing the rate of glycolysis (Nikolić *et al.*, 2009).

Calcium ion is known to have a role in protecting membrane of the cell (Li *et al.*, 2010) and also flocculating ability (Soares, 2011). Ismail *et al.* (2014) reported that the optimum concentration of calcium ions in the fermentation media is 2 mM which can increase ethanol production even though the cell were exposed to weak acid stress.

As a continuation of our study on the effect of metal ion on fermentation performance and stress tolerance (Rachman *et al.*, 2018), the present study was directed to investigate the effects of calcium ion supplementation on fermentation performance and stress tolerance of a baker's yeast strain. Most of stress tolerance investigation only examine the effect of supplementation on response of the stress tolerance against one stress factor (Ciesarová *et al.*, 1996; Stanley *et al.*, 2010; Giannattasio *et al.*, 2013; Ismail *et al.*, 2014). In the present study, we investigated the effects of calcium ion supplementation on fermentation performance and stress tolerance to four stress factors, namely ethanol, hyperosmotic stress, weak acid and oxidative stress.

Materials and Methods

Yeast Strain and Maintenance

A baker's yeast strain (*S. cerevisiae* strain A12) was used in the present study. It was selected due to its ability to produce high ethanol concentration. The yeast strain was maintained on slopes of yeast extract peptone (YEP) media containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% potassium dihydrogen phosphate, 1% glucose and 1.5% agar

(Rachman *et al.*, 2018). The slopes were stored at 4°C.

Growth Media and Culture Conditions

The cells were grown in a defined fermentation media, i.e. yeast nitrogen base (YNB), without calcium ions containing 10% w/v glucose as described previously (Rachman *et al.*, 2019). Starter cultures were inoculated from the slopes and grown overnight (~16 h) in a 180 orbital per min (opm) orbital shaker at room temperature.

Experimental Batch Culture Conditions and Sampling

Aerobic cultures were prepared by aseptically adding YNB media to a sterile Erlenmeyer flask and inoculating these with overnight cultures to give an initial viable cell number of $\sim 10^6$ cells/mL. The ratio of flask size to culture volume was maintained at a minimum of 4:1 to ensure adequate oxygen mixing. To investigate the effects of calcium ion supplementation, calcium chloride was added to the experimental cultures to give final concentrations of calcium ion as follows: 0 (control), 25, 50, and 100 ppm. Samples from each culture were aseptically drawn off with a sterile micro pipette every 6 h for the first 24 h and the rest was sampled every 12 h until 96 h were reached. Examination of the samples included measuring the growth rate by measuring optical density at 600 nm ($OD_{600\text{ nm}}$), total cell count, viable cell count, and glucose and ethanol concentrations.

Cell Count

Cell count was determined using light microscope (400 × magnification) using a Neubauer-type haemocytometer. Viable cell numbers were assessed using the methylene violet staining (Smart *et al.*, 1999). When counting, both live and dead cells were recorded to obtain the total number of cells per mL. The number of viable cells were then divided by the total cells and multiplied by 100 to give the percentage of viable cells.

Determination of Glucose Concentration

Glucose concentrations were determined using the alkaline potassium ferricyanide method (Walker and Harmon, 1996). This method was chosen due to its simplicity of the reagent used and procedure. As much as 0.2 mL of sample and 0.6 mL of potassium ferricyanide solutions were added to a tube and mixed thoroughly. The reaction mixture was heated at 100°C for 10 min and cooled to room temperature. Then, it was diluted with 4 mL of distilled water and mixed thoroughly. The absorbance was then recorded at 420 nm after setting the spectrophotometer to zero with distilled

water as reagent blank. The glucose standard solutions were made in concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL

Determination of Ethanol Concentration

Alcohol dehydrogenase assay was used to determine ethanol concentration (Ishmayana *et al.*, 2015). As much as 25 μ L of sample and 25 μ L of NAD^+ solution were added to a tube containing 1.25 mL semicarbazide buffer solution and mixed thoroughly. After being finely mixed, 5 μ L of alcohol dehydrogenase solution (4,000 unit/mL) was added and mixed thoroughly. The reaction mixture was then incubated at 30°C for 40 min. The absorbance was then read at 340 nm after setting the spectrophotometer to zero with the reagent blank. The ethanol standard solutions were made in concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05% v/v.

Yeast Cell Stress Tolerance Assay

The method for stress tolerance assay used was proposed by Garcia *et al.* (1997) with slight modification. Cells were drawn from 24 h cultures. YNB media containing 2% glucose were prepared and divided into four treatments. Each treatment had different stress factors including 7% v/v ethanol (ethanol stress), 27% w/v sorbitol (hyperosmotic stress), 67 mM acetic acid (weak acid stress), and 4 mM hydrogen peroxide (oxidative stress). To investigate the effects of calcium ion, one pair of the samples, with and without calcium ion supplementation, was set for each stress condition. The cell culture was diluted until ~ 0.1 OD_{600 nm} was reached and this was designated as 0 h. The cells were then grown at room temperature with 180 rpm of shaking speeds for 24 h at room temperature. The OD_{600 nm} was measured and relative growth were calculated as a percentage ratio of OD_{600 nm} of yeast

cell grown in the presence of stress factor to OD_{600 nm} of yeast cell grown without the corresponding stress factor.

Statistical Analysis

The raw data was compiled into Minitab 15® for Windows®. This software package was then used to perform statistical analysis. When significant ($p < 0.05$) differences were detected in the one-way ANOVA, the test was followed by the Fisher's post-hoc test to determine which data differed significantly.

Results and Discussion

Effect of Calcium Ion Supplementation on Yeast Growth

Nutritional composition of a fermentation media can affect the metabolism of the yeast cells and eventually their growth (Ishmayana *et al.*, 2011). Previous studies, indicate that supplementation of fermentation media with metal ion has positive effects on the cell growth (Birch *et al.*, 2003; Xue *et al.*, 2008; Ismail *et al.*, 2014; Rachman *et al.*, 2018). In the present study, 10% w/v initial glucose concentration was selected since a chemically defined fermentation media (YNB) was used and it was considered as a nutritionally poor media. The concentration was optimum to differentiate fermentation performance of the yeast cells in the media where glucose was not exhausted nor stuck fermentation was observed in this condition (Ishmayana *et al.*, 2018). The concentration of calcium ion used in this experiment was 0, 25, 50, and 100 ppm, and selected based on previous published result that concentration of 1 mM (~ 100 ppm) of calcium chloride already give positive effects on cell viability (Ismail *et al.*, 2014). Therefore, in the present study the range was lowered to see whether there are any effects of the calcium in the lower concentrations.

The result of optical density measurement during the fermentation is presented in Figure 1. From the figure it can be seen that for all treatments an adaptation phase was observed during the first 6 h of fermentation. An exponential phase was evidenced during the 6-18 h and followed by stationary phase. Calcium ion supplementation seems to promote cell growth as indicated by significantly higher OD_{600 nm} reading reached by cell grown in media with calcium ion supplementation during the stationary phase ($p < 0.001$). However, no significant difference was detected between the cells grown in different concentration of calcium ion supplementation.

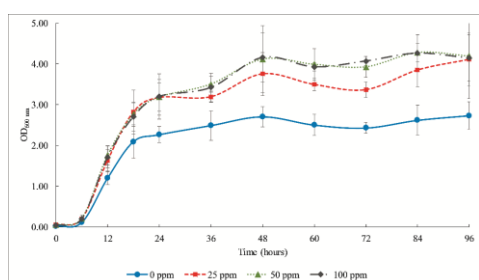


Figure 1. Growth of yeast cell as measured by optical density at 600 nm. The yeast cell grown in YNB media without and with calcium supplementation as indicated in the figure legend. The graphed data are means of three independent experiment and error bars represent standard deviation

Similar observation with optical density results was also observed for total cell count as presented in Figure 2. The adaptation phase was observed during the first 6 h of fermentation, followed by exponential phase at 6-18 h and finally from 18 to the end of fermentation (96 h) the total cell count was plateaued, indicating a stationary phase. The highest cell count was observed for yeast cell grown in media with 100 ppm calcium ion supplementation that reached about 80×10^6 cells/mL followed, in order, by yeast cell grown in 50 ppm ($\sim 75 \times 10^6$ cells/mL) and 25 ppm ($\sim 70 \times 10^6$ cells/mL). However, the difference observed are not statistically significant due to high variability of the data. Cells grown in media without calcium supplementation showed significantly lower total cell count ($\sim 50 \times 10^6$ cells/mL) during the stationary phase ($p < 0.05$). The result of total cell count is in a good agreement with the OD_{600 nm} result, which confirm that the two method are complimentary each other.

The fermentation performance in a fermentation process is actually only displayed by actively fermenting living cells that ferment glucose to ethanol. Therefore, we also examined viability during the fermentation process. Viability is a measure in percentage of living cells to total cell available in the fermentation media. The result of the viability measurement is presented in Figure 3. The viability of the yeast cells during the adaptation phase (0-6 h) are similar ($p > 0.05$). However, after entering the exponential phase (Figure 1) the viability of yeast cells grown in media with calcium ion supplementation are significantly higher than the yeast cells grown in media without calcium ion supplementation ($p < 0.05$). It is clear that calcium ion supplementation gives a positive impact on cell viability as indicated by significantly higher viability, even though no significant differences was observed between the cells grown in media without and with calcium supplementation. This might be due to increasing stress tolerance to ethanol as proposed by Nabais *et al.* (1988) and Li *et al.* (2010) who found better cell growth and viability when fermentation medium supplemented with calcium ions. Both authors suggest that the tolerance to ethanol is related to increasing the activity of plasma membrane ATPase and increasing plasma membrane stability when the media supplemented with calcium ions.

Effect of Calcium Ion Supplementation on Fermentation Performance

Glucose consumption and ethanol production is very important aspect in fermentation performance. The results of glucose and ethanol analysis during the fermentation is presented in Figure 4. No significant differences of glucose

consumption between cells grown in media without and with calcium ion supplementation. As for ethanol concentration, a significant difference was observed where ethanol produced at the end of fermentation was significantly lower in media without calcium ion supplementation ($p < 0.05$). The higher ethanol produced when calcium ion was supplemented to the fermentation media also reported in previous published study (Li *et al.*, 2010). The observed phenomena indicate that for the strain used in this study, calcium supplementation has effect on ethanol production but not glucose consumption. This might be due to calcium role in the metabolism of the yeast cells, especially the route of ethanol production. Calcium is known to affect fermentation through increasing

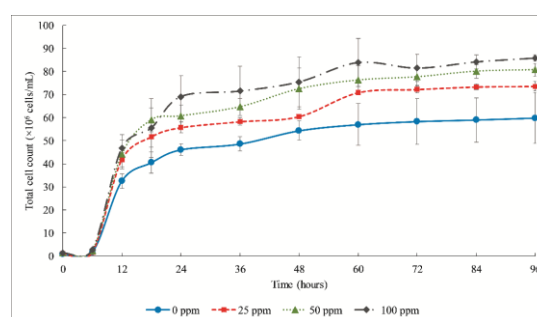


Figure 2. Growth of yeast cell as measured by total cell count using light microscopy with haemocytometer. The yeast cell grown in YNB media without and with calcium supplementation as indicated in the figure legend. The graphed data are means of three independent experiment and error bars represent standard deviation

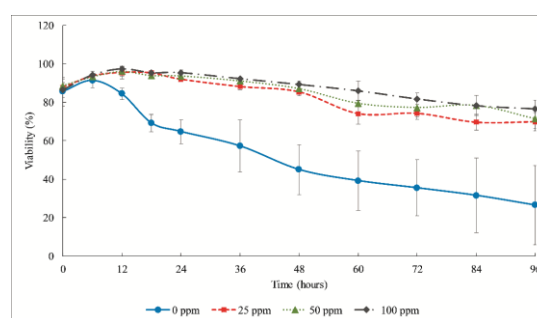


Figure 3. Viability of yeast cell as measured light microscopy with haemocytometer. The cell was stained using methylene violet and unstained cell considered as living cells. The yeast cell grown in YNB media without and with calcium supplementation as indicated in the figure legend. The graphed data are means of three independent experiment and error bars represent standard deviation

Table 1. Fermentation kinetic parameter of the yeast strain A12 grown in YNB media without and with calcium supplementation. The initial glucose concentration used was 10% w/v. The values presented in the table are means of data of three independent experiment $\pm$ standard deviation. The means value in the same column with different superscript letters are significantly different at $\alpha = 0.05$ as assessed using Fisher's post-hoc test

Ca ²⁺ concentration (ppm)	$\mu_{\max}$ ($\times 10^6$ cells.mL ⁻¹ .h ⁻¹)	Qs (mg.mL ⁻¹ .h ⁻¹)	Qp (mg.mL ⁻¹ .h ⁻¹)	Yp/s (mg.mg ⁻¹)
0	3.24 $\pm$ 0.38 ^b	0.526 $\pm$ 0.011 ^b	0.081 $\pm$ 0.017 ^b	0.135 $\pm$ 0.044 ^b
25	4.13 $\pm$ 0.38 ^{ab}	0.740 $\pm$ 0.050 ^a	0.156 $\pm$ 0.023 ^a	0.198 $\pm$ 0.038 ^{ab}
50	4.73 $\pm$ 0.43 ^a	0.724 $\pm$ 0.081 ^a	0.183 $\pm$ 0.011 ^a	0.232 $\pm$ 0.034 ^a
100	4.41 $\pm$ 1.02 ^a	0.731 $\pm$ 0.045 ^a	0.154 $\pm$ 0.018 ^a	0.203 $\pm$ 0.033 ^{ab}

Note: $\mu_{\max}$ = maximum growth rate, Qs = glucose consumption rate, Qp = ethanol production rate and Yp/s = ethanol yield. $\mu_{\max}$ was calculated as slope value of the total cell number curve from 0 to 18 h. Qs was calculated as the amount of glucose consumed during the fermentation divided by the time of fermentation. Qp was calculated as the amount of ethanol produced during the fermentation divided by the time of fermentation. Yp/s was calculated as ratio of the amount of ethanol produced to the amount of glucose consumed during the fermentation for 96 h.

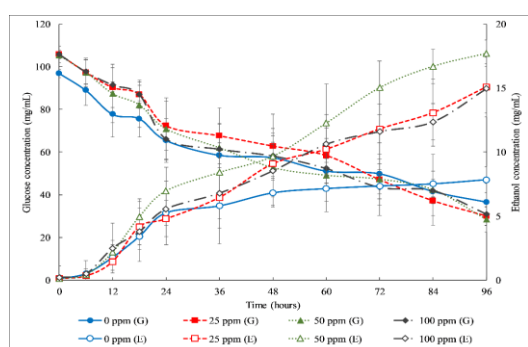


Figure 4. Glucose consumption (G) and ethanol production (E) of yeast strain A12 grown in YNB media without and with Ca²⁺ supplementation as indicated in the figure legend. The graphed data are means of three independent experiment and error bars represent standard deviation

tolerance of the yeast cells to ethanol, leading to higher ethanol production (Li *et al.*, 2010). The ethanol obtained in the present study is lower than the expected value (~20 mg/mL vs ~50 mg/mL). This gap might be due to the media used in this study. YNB media is considered as nutritionally poor media, and therefore repress the ability of yeast cell to ferment all sugar available in the fermentation media (Ishmayana *et al.*, 2011).

The fermentation kinetic data is presented in Table 1. The maximum growth rate was determined based on total cell count, and there is a statistically significant difference between maximum growth rate of yeast cell grown in media without calcium ion supplementation and with 50 and 100 ppm calcium ion supplementation. Only yeast cells grown with 25 ppm calcium ion showed no statistically significant difference with yeast cells grown in media without calcium ion

supplementation. This indicate that the effect of calcium ion supplementation appears only at concentration higher than 25 ppm.

The rate of glucose consumption (Qs) and ethanol production (Qp) of yeast cell grown in media without calcium ion supplementation is statistically lower compared to cell grown in media with calcium ion supplementation. No statistically significant difference of Qs and Qp between yeast cell grown in different concentration of calcium ion supplementation. As for ethanol productivity (Yp/s) the only statistical significance difference was observed between yeast cell grown without calcium supplementation and yeast cell grown with 50 ppm calcium ion supplementation. This result suggest that calcium ion generally enhances fermentation performance.

The results of the present study indicate that calcium ion supplementation have positive impact on promoting the cell growth and fermentation performance. Even though previously published result by Ciesarová *et al.* (1996) reported that calcium ion has negative impact on yeast cell growth and ethanol production, it might be that the strain that they used is very sensitive to calcium. According to Rees and Stewart (1997), the response of yeast cells to calcium ion is strain dependent which also might explain the difference result observed in this study. Calcium ion effect was also proposed related with the presence of magnesium ion in the fermentation media, higher magnesium to calcium ion ratio tend to promote yeast cell growth and fermentation performance (Birch *et al.*, 2003). The concentration of magnesium ion in the fermentation media used in the present study is about 85 ppm, and it seems that for the yeast strain studied in the present experiment the concentration of calcium ion used is still in acceptable ranges for the yeast cells to maintain good cell growth and fermentation performance.

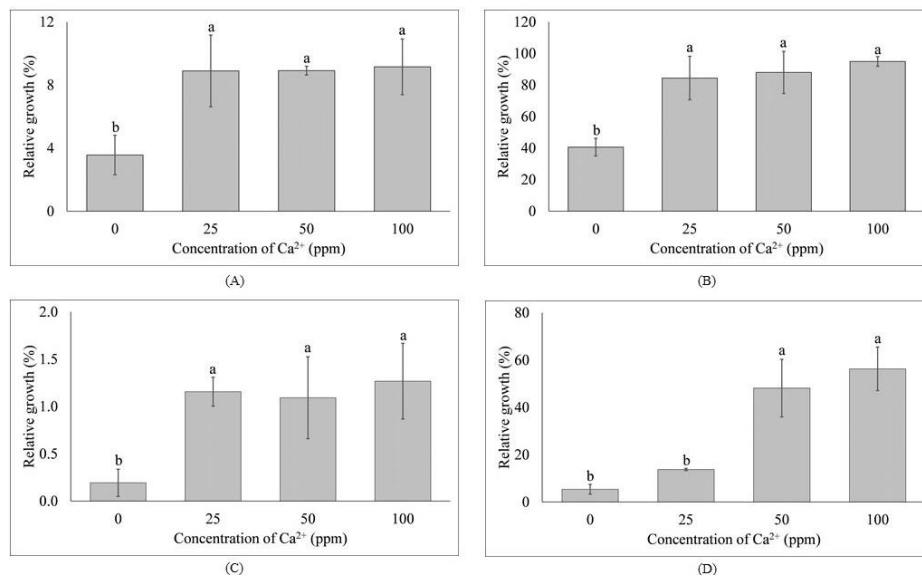


Figure 5. The effects of Ca^{2+} supplementation on yeast strain A12 cell growth grown in media with stress conditions: (A) ethanol stress (7% v/v ethanol), (B) hyperosmotic stress (27% w/v sorbitol), (C) weak acids stress (67 mM acetic acid), and (D) oxidative stress (4 mM hydrogen peroxide). The graphed data are means of three independent experiment of relative cell growths of cells grown in the presence of the stress factors compared to cells grown without stress factors. Error bars represent standard deviation. Bars with different letters exhibit differences that are statistically significant at $\alpha = 0.05$ as assessed using Fisher's post-hoc test

The Effect of Calcium Ion Supplementation on Stress Tolerance

Most stress tolerance studies focus only on one of stress factors that may encounter the yeast strain (Ciesarová *et al.*, 1996; Xue *et al.*, 2008; Ismail *et al.*, 2014). In the present study, we examine the stress tolerance of yeast cell grown in media without and with calcium ion supplementation to four stress factors, including ethanol, hyperosmotic, weak acid and oxidative stresses. The result of stress tolerance assessment is presented in Figure 5.

The yeast strain used in the present study has the highest sensitivity to acetic acid followed, in order, to ethanol, hydrogen peroxide and hyperosmotic stresses based on relative growth of the yeast cells in the presence of the stress factors. Calcium ion supplementation was proved to alleviate stress effects of all the stress factors assayed in the present study. Only for oxidative stress against hydrogen peroxide at 25 ppm the effect was not statistically significant. Therefore, it can be proposed that calcium act as a general stress protector. One of possibility of the mechanisms is proposed by Li *et al.* (2010) who stated that calcium ion can maintain plasma yeast plasma membrane structure leading to increasing H^+ -ATPase activity which has a role in maintaining proton homeostasis that is very crucial for yeast to maintain their vitality. Other than that, the presence of calcium

ions can increase the activity of oxidative stress protecting enzyme, such as glutathione peroxidase (Tanaka *et al.*, 2005), leading to more tolerant yeast cells when the cells grown in the presence of calcium ions.

Conclusions

The present study confirms previous published studies which stated that calcium ion has an impact on cell growth. Even though most of the studies propose that calcium ions tend to reduce cell growth and fermentation performance, this study find that for the strain used in the present study calcium ion tend to promote cell growth and fermentation performance. This might be another proof that the response to calcium ion is strain specific. Furthermore, stress tolerance results of yeast cells grown in the presence of calcium ion proved that calcium ion can act as general stress protector. This also may contribute to better cell growth and fermentation performance.

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