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The effect of varying inositol supplementation on *Saccharomyces cerevisiae* grown in chemically defined media

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ABSTRACT

The present study investigates yeast membrane fluidity and ethanol tolerance, particularly in relation to inositol supplementation. Three *Saccharomyces cerevisiae* strains were selected, based on reported stress tolerance and ethanol productivity; an ethanol tolerant baker's yeast (A12), a wine yeast (PDM) and a sake yeast (K7), the latter two producing up to 17 and 17.5 %(v/v) ethanol, respectively. When cultured in yeast nitrogen base (YNB) medium with 2% (w/v) glucose, the three strains had similar growth rates and performance, although K7 maintained significantly higher viability. Comparison of generalized polarization (GP) of laurdan-labelled cells indicated that PDM had the highest membrane fluidity, followed in order by K7 and A12. Conversely, A12 had the highest ethanol tolerance, followed in order by K7 and PDM. Furthermore, in comparison to 6 h cultures, 24 h cultures of all strains had lower membrane fluidity and higher ethanol tolerance. The present study failed to confirm reports that inositol supplementation increases ethanol tolerance. No significant changes of either GP or viability reduction upon ethanol stress were found when the medium was supplemented with various levels of inositol. Further investigation, including more variations in concentration and zero level of inositol, is needed to elucidate this possibility.

Keywords:inositol, membrane fluidity, fluoresence spectroscopy, *Saccharomyces cerevisiae*

1. INTRODUCTION

Due to depletion of fossil fuels availability, many efforts have been conducted to find their replacement (Arnold *et al*., 2019). The alternative fuels that has been developed including biogas, biodiesel, and bioethanol (Arshad *et al*., 2018). Bioethanol is produced mainly from sugary, starchy, and lignocellulosic materials and converted to ethanol by the activity of microorganisms, especially the yeast *S. cerevisiae* (Cho &Strezov, 2018; Kumar *et al*., 2014). During fermentation, yeast cells are exposed to various environmental stress factors such as ethanol, weak acid, and hyperosmotic stresses (Nakamura &Shima, 2018).

Myo-Inositol (referred to simply as inositol) has been reported to have protective effects against ethanol stress (Chi *et al.*, 1999; Furukawa *et al.*, 2004; Ji *et al.*, 2008; Krause *et al.*, 2007). Supplementation of inositol was found to change phospholipid composition by increasing the phosphatidylinositol (PI) content of the plasma membrane, while phosphatidylcholine (PC) and phosphatidylethanolamine (PE) levels decreased (Chi *et al.*, 1999). Another study also revealed that inositol supplementation increases H^+-ATPase activity and protects yeast plasma membranes against leaking (Furukawa *et al.*, 2004).

Mutant yeast strains capable of accumulating higher levels of intracellular inositol were also found to have better tolerance against high ethanol concentrations (Krause *et al.*, 2007). Chi *et al.* (1999) found that inositol supplementation not only increased tolerance to high ethanol concentrations, but also increased ethanol productivity. However, another study showed that while inositol supplementation improved viability in the presence of high ethanol concentrations, it did not affect the final ethanol production and fermentation rate (Furukawa *et al.*, 2004).

Previous researchers used different concentrations of inositol in the growth media. Chi *et al.* (1999) used 0.1 g/L, Furukawa *et al.* (2004) used 10 µM (0.0018 m g/L) and 90 µM (0.0162 mg/L) and Krause *et al.* (2007) used 75 µM (0.0135 mg/L) inositol.

Ji *et al.* (2008) investigated effect of inositol supplementation on a different yeast cell species, *Pachysolen tannophilus*, and used several inositol concentrations in their growth media. They found that inositol provided optimal effects at 0.15 g/L for cell growth and ethanol tolerance, and 0.1 g/L for ethanol productivity, while excessive inositol tended to have negative effects by lowering growth rate and ethanol productivity.

To the best of our knowledge, only few published works have reported effects of more than two levels of inositol supplementation on *S. cerevisiae* (Ishmayana *et al.*, 2015; Wang *et al.*, 2015). Therefore, in the present study we investigated several levels of inositol supplementation to more closely investigate its effect on ethanol tolerance in *S. cerevisiae* strains.

As described previously, inositol supplementation tends to increase PI and decrease PC and PE content of the yeast cell

plasma membrane (Chi *et al.*, 1999). Fatty acid compositions are generally different for each class of phospholipid. The main fatty acid components varied from one study to another, but in general, PI is mainly composed of C18:0 and C20:4, PE is mainly composed of C16:0, C18:0 and C20:4, PC is mainly composed of C16:0 and PS is mainly composed of C18:0 (Christie, 2010). Therefore, changes in phospholipid class composition might also change the membrane fluidity (Ishmayana *et al.*, 2015), since different degrees of fatty acid saturation will result. This led us to investigate the membrane fluidity in addition to the ethanol tolerance of yeasts grown in inositol supplemented media.

2. MATERIALS AND METHODS

Yeast strains

Yeast strains used in this experiment were A12, PDM and K7. A12 is an ethanol tolerant baker's yeast according to previous studies (Lewis, 1993), PDM is an industrial wine strain (Mauri Yeast) which can produce up to 17% (v/v) ethanol, and K7 is a sake strain (ATCC 26422) that can produce up to 17.5% ethanol.

Growth media and culture conditions

Cells were grown in the defined medium Yeast Nitrogen Base (YNB) broth containing 2% glucose and 0.67% YNB with ammonium sulfate and amino acid (Difco). Inositol was added to experimental cultures at various final concentrations, i.e. 0.002, 0.005, 0.05, 0.1, 0.15 and 0.2 g/L. As the YNB medium already included some inositol the media without inositol supplementation actually had about 0.002 g/L inositol.

YNB media were prepared by weighing out the required amount and dissolving it in MilliQ grade water, filter sterilizing using 0.22 µm pore size sterile syringe filters (Sarstedt) and storing at 4°C. Sterilization via autoclaving could not be performed, as this resulted in an increased autofluorescence which interfered with the interpretation of steady-state fluorescence results. Media were prepared on a monthly basis or as required. Supplements were freshly prepared and sterilized by filtering through 0.22 µm pore size sterile syringe filters.

Starter cultures were inoculated from YEP slopes and grown overnight (~16 h) at 30°C and 180 opm in an orbital shaker (Paton). For inositol addition experiments, inositol was added to the experimental culture at a final concentration as mentioned above at a time designated as 0 h.

Experimental batch culture conditions and sampling

Aerobic cultures were prepared by aseptically adding YNB media to sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and then inoculating to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was 4:1 to ensure adequate oxygen mixing.

Samples from the cultures were aseptically removed by drawing off with a micro pipette every 6 hours from 0 to 30 hours. Examination of the samples included measuring growth rate by measuring optical density, viable cell numbers, and glucose and ethanol concentrations. Detailed analysis including ethanol tolerance and membrane fluidity was performed at 6 and 24 h.

Growth Rate

Yeast growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}) using a Beckman DU 650 spectrophotometer, making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt).

Viable Cell Numbers

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) using a Neubauer-type haemocytometer. Methylene violet staining has been proposed as a better method for monitoring yeast cell viability compared to the traditional methylene blue staining method (Smart *et al.*, 1999).

Determination of membrane fluidity by spectrofluorometric analysis

Membrane fluidity was assessed using steady-state fluorescence spectroscopy, measuring generalized polarization of 6-dodecanoyl-2-dimethylamino-naphthalene (laurdan) following incorporation of the probe into yeast plasma membranes, as outlined by Learmonth (2012). Cell suspensions were standardized by diluting with centrifuged (10000 *g*) supernatant to an OD_{600nm} of 0.4 and a volume of 3 mL in a cuvette. Incorporation of the fluorescent probe into yeast cell membranes was accomplished by incubating the standardized washed cell sample with a final concentration of 5 µM laurdan for 60 min at 30°C in the dark with stirring. Unlabeled cell suspension at the same cell density was used to measure background fluorescence, which was subtracted from the experimental readings. The results were expressed as Generalized Polarization (GP) determined using Equation 1.

$$GP = \frac{I_{440nm} - I_{490nm}}{I_{440nm} + I_{490nm}} \quad \ldots \text{(Eq. 1)}$$

Where I_{440nm} : Emission intensity at 440 nm
I_{490nm} : Emission intensity at 490 nm

Ethanol tolerance

During growth in batch culture, the composition of the growth medium changes markedly and may affect the tolerance of cells to stress. In order to minimize these types of effects when comparing stress tolerance of cells from different growth phases, stress tolerance of all cells was tested in a standard medium, namely yeast nitrogen base without glucose (YNBNG) (Lewis *et al.*, 1997). Samples (1 mL) of culture were centrifuged at 1500 *g* for 2-3 minutes, the supernatant growth medium was decanted and the pellet resuspended in the original volume of YNBNG. Resuspended cells were then tested for stress tolerance.

The concentration of ethanol and time exposure to ethanol used in the ethanol tolerance test were based on the work of Chi & Arneborg (2000) and Lewis (1993) with slight modification. A 410 µL sample cells was added to a tube containing 90 µL of absolute ethanol and the sample was mixed immediately, exposing the cells to 18% v/v ethanol. The tube was incubated at 30°C for 60 minutes. The number of surviving cells was determined using two methods which were methylene violet staining and total plate count.

The method for measuring ethanol tolerance based on methylene violet staining and total plate count method was described by Ishmayana *et al*. (2017). Briefly for the methylene violet staining method, the stress was relieved by making a five- (for 6 h culture) or ten-fold (for 24 h culture) dilution in MilliQ water. The percentage of viable cells was then calculated. The result of this viability calculation was then expressed as "viability reduction" (referred as viability reduction by methylene violet staining (VR MVS)) as calculated using equation (2).

$$\text{VR MVS}\,(\%) = \frac{\text{viability control - viability test}}{\text{viability control}} \times 100\% \qquad \ldots \text{(Eq. 2)}$$

For the total plate count method, after the stress period the samples were diluted using YNBNG to give serial ten-fold dilutions. Then, 100 µL of each diluted sample was spread on YEP agar plates (containing 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% ammonium sulfate, 0.3% potassium dihydrogen phosphate, 1% glucose and 1.5% bacteriological agar), and incubated for about 72 hours at room temperature before counting the resultant colonies. The result was also expressed as "viability reduction" (referred to as viability reduction by total plate count (VR TPC) as calculated using equation (3).

$$\text{VR TPC}\,(\%) = \frac{\text{total viable cell control - total viable cell test}}{\text{total viable cell control}} \times 100\% \qquad \ldots \text{(Eq. 3)}$$

The extra 18% dilution of the cells resulting from the addition of ethanol was taken into account during the calculation of the viable count. The tubes with 90 µL of ethanol were prepared immediately before the experiment as the solvent may evaporate quickly and the small volume could allow substantial changes in the final concentration.

Measurement of glucose and ethanol using HPLC

The amount of glucose and ethanol was determined by measuring the compounds in the growth media compared to the initial concentration at the beginning of the fermentation (0 h) using HPLC.

The HPLC system (Shimadzu) consisted of SIL-20A auto sampler, DGU 20A5 in-line degasser, LC-20AD solvent delivery module, CTO 20A column oven, RID 10A refractive index detector, and Class-VP software. A Waters Sugar-Pak I HPLC column (part no. 85118) with dimensions of 6.5 × 300 mm was used for the separation of analytes. The stationary phase of the column is sulfonated styrene divinylbenzene resin in calcium form. The column was maintained at 80°C. A guard column was used to prevent column damage. The mobile phase was deionized MilliQ water (resistivity ~ 18 Mohm) containing 5 mg/L $CaNa_2$-EDTA (Sigma Aldrich) filtered through a 0.45 µm pore size filter. The mobile phase was passed through an in-line degasser to ensure that the mobile phase was gas free. The flow rate was maintained at 0.6 mL/min. Prior to the initial use, and after running about 150 samples, the column was reconditioned by passing through a 500 mg/L $CaNa_2$-EDTA solution at 80°C at a 0.5 mL/min flow rate for at least 2 hours in the reverse direction.

3. RESULTS AND DISCUSSION

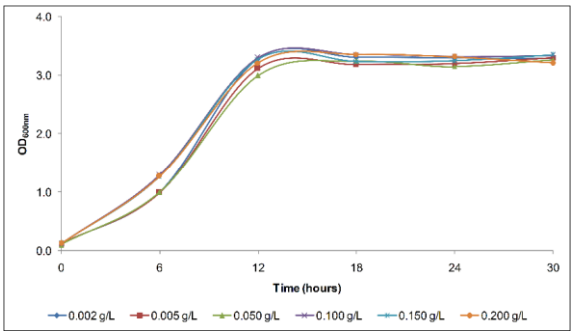
Effect of Inositol on Fermentation Performance

When compared to cells grown without inositol, cells grown in the presence of inositol are reported to have better growth and fermentation performance (Chi *et al.*, 1999; Ishmayana *et al.*, 2015; Krause *et al.*, 2007). In the present study we investigated cell growth and fermentation performance with different levels of inositol supplementation, from 0.002 g/L (the basal level in YNB) to 0.2 g. Cell growth was monitored by measuring OD at 600 nm and the result is presented in Figure 1.

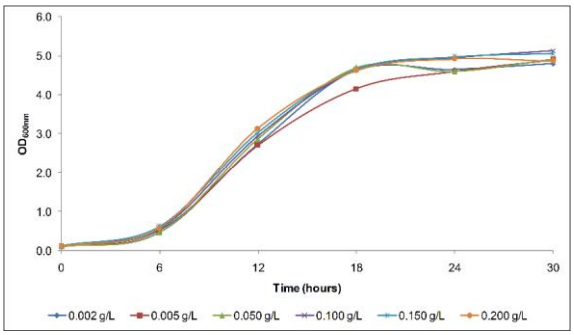
Generally, no differences were seen in cell growth of any of the strains used in the present study when grown in media with different inositol concentrations of up to 0.200 g/L. K7 reached the highest OD value compared to the other strains. A previously published study using a different yeast strain (A15, ATCC 38554), indicated that when cells were grown in the presence of inositol, they had relatively higher OD values (Ishmayana *et al.*, 2015). Together these findings indicate indicates that inositol is required in only a very small quantity to improve cell growth, and that higher concentrations do not stimulate further increases in growth response.

Cell viability of the yeast strains was also investigated and the result is presented in Figure 2. No significant difference in cell viability was detected between cells grown in the different inositol concentrations. For A12 and PDM, viability at 0 hours was about 80%, increasing to nearly 100% at 6 hours and decreasing to about 70% at 12 hours, thereafter maintaining relatively the same level until 30 hours. In contrast, K7 maintained high viability throughout the fermentation (at about 90%). This showed that K7 has better ability to maintain cell viability compared to the other strains, an intrinsic properties of that strain.There were no significant effects of increasing inositol in fermentation media on glucose consumption and ethanol production of the strains used in the present study (Figure 3). Since the concentration of glucose used in the present study was relatively low, it was exhausted within 18 hours of fermentation, leaving no glucose at the end of fermentation. For all strains and inositol supplementation levels, ethanol reached the highest concentration at 18 hours, in agreement with the glucose concentration data. Furthermore, at 24 hours almost all combinations of strain and inositol level showed decreasing ethanol concentration, which indicate diauxic shift (Piškur *et al.*, 2006).

There were no marked differences between the three strains for the growth parameters assessed in this study. Generally, glucose was exhausted after 18 hours of fermentation and the highest ethanol concentration was detected at 18-24 hours. The only significant difference in growth parameters was for cell viability, where K7 maintained a high cell viability throughout the fermentation, and after 12 hours of culture, the cell viability of K7 was significantly higher than for the other two strains ($p < 0.05$). Also, inositol supplementation did not lead to any significant change in the growth parameters assessed in this experiment.



(A)



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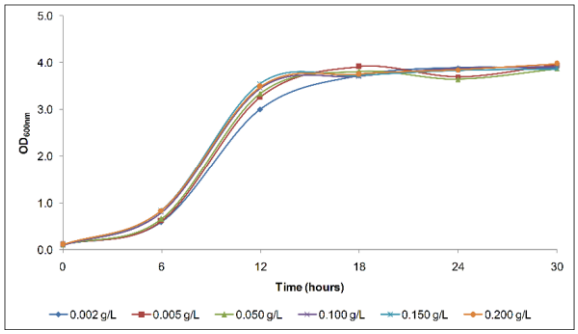
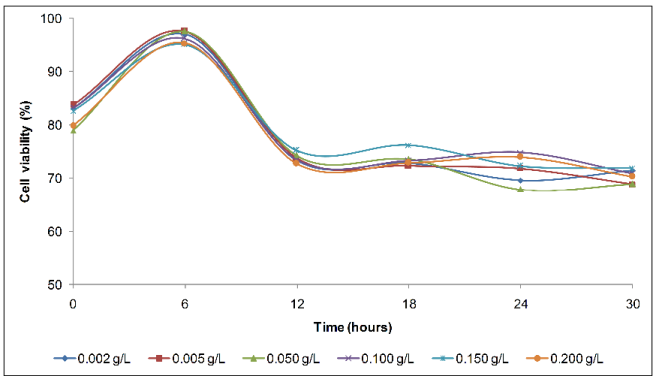
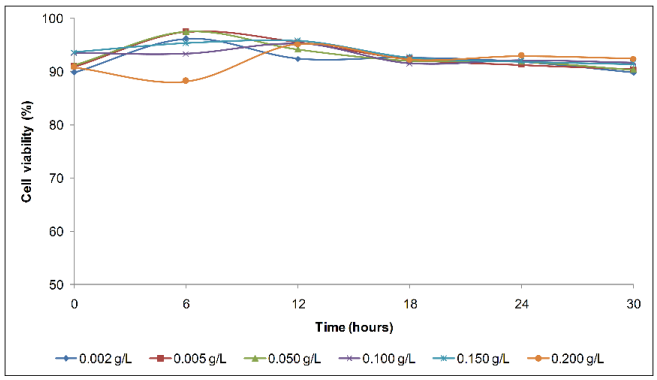


(C)

Figure 1. Cell growth of yeast cell strains A12 (A), K7 (B) and (C) PDM (C) in YNB media with varying inositol concentrations as indicated on the figure legend. Yeast cells were grown in YNB media with 2% w/v initial glucose. Data presented are means of two independent experiments.



(A)



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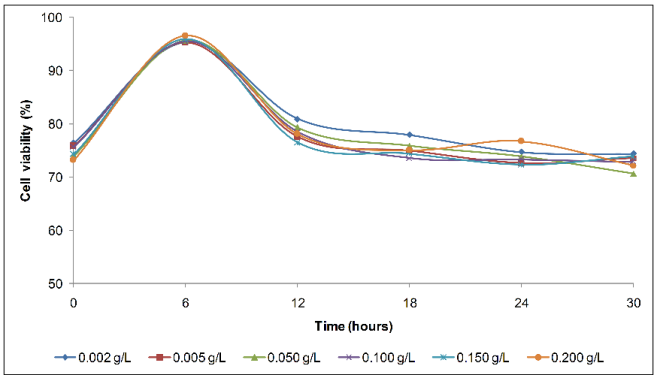


(C)

Figure 2. Cell viability of yeast cell strain A12(A), K7 (B) and PDM (C) in YNB media with varying inositol concentration as indicated on the figure legend. Yeast cells were grown in YNB media with 2% w/v initial glucose. Data presented are means of two independent experiments.

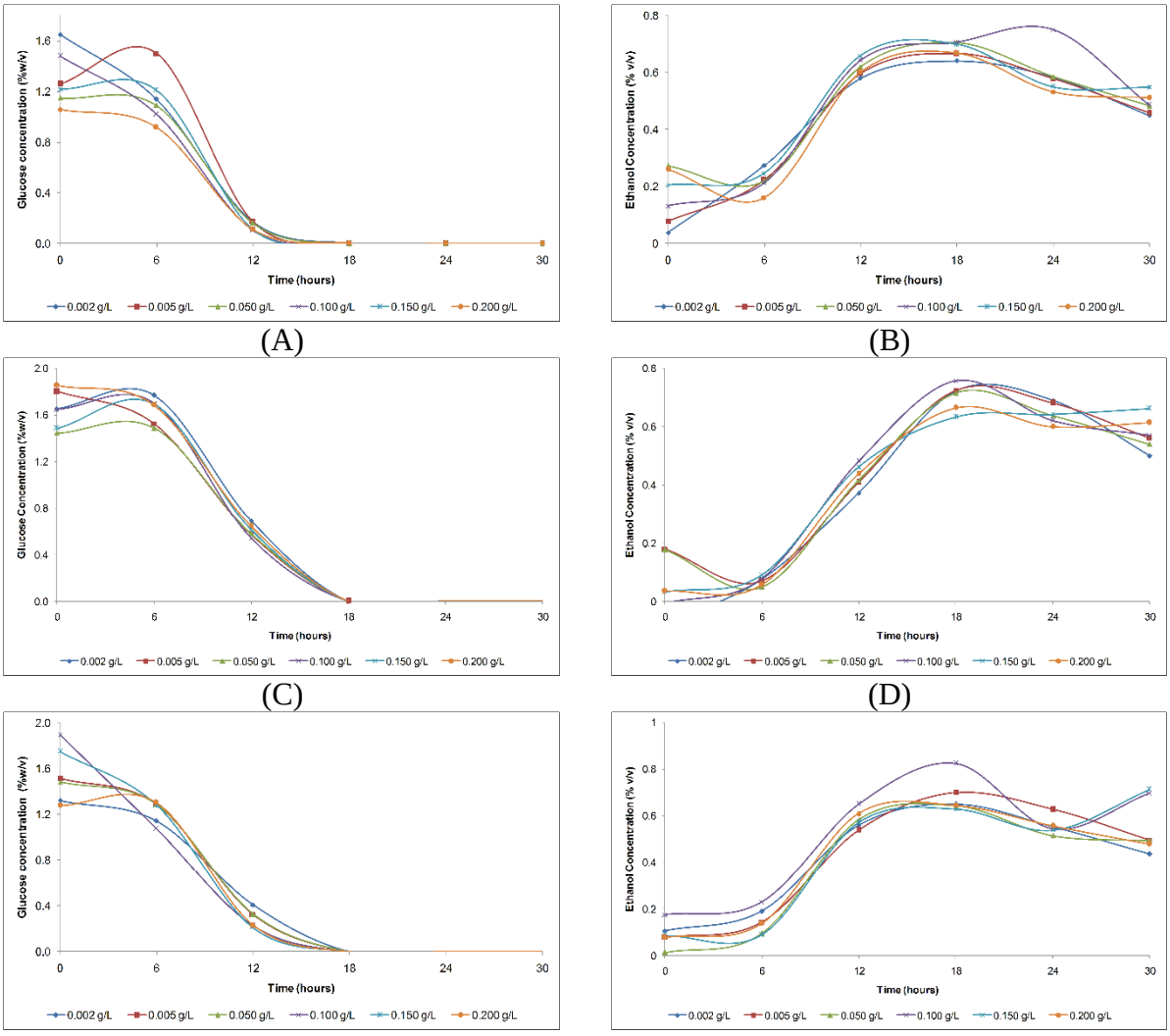


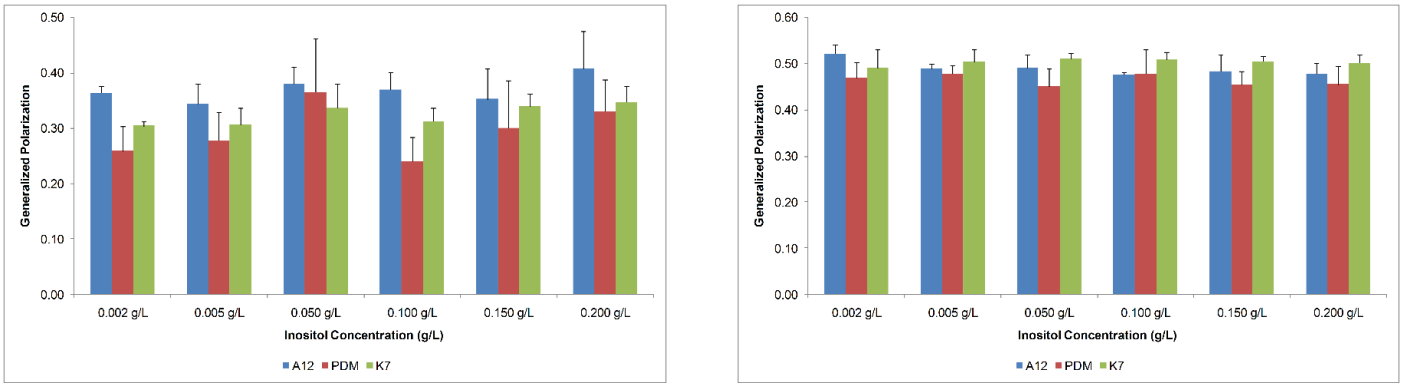
Figure 3. Glucose consumption of (A) A12, (C) K7, and (E) PDM and ethanol production of (B) A12, (D) K7 and (F) PDM yeasts strains in YNB media with varying inositol concentration as indicated on the figure legend. Yeast cells were grown in YNB media with 2% w/v initial glucose under aerobic conditions at 30°C. Data presented are means of two independent experiments.

Effect of Inositol Supplementation on Membrane Fluidity

It is important to note that YNB medium used in the present experiment contains 0.002 g/L inositol. Therefore, the basal (unsupplemented) medium already contains 0.002 g/L inositol. Figure 4(A) shows the GP value of yeast strains grown in inositol-supplemented or -unsupplemented media at 6 hours of culture. No significant differences were detected in this data, but a possible trend can be observed at 0.05 g/L inositol-supplementation for PDM which resulted in a higher GP value compared to other levels of inositol supplementation. This difference was not statistically significant due to the relatively high variability in these readings.

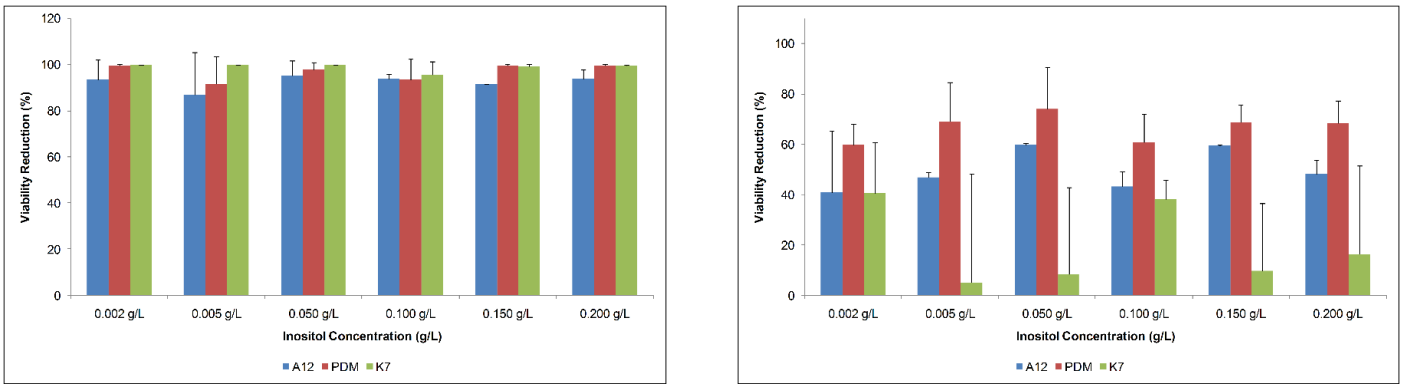
Generalized polarization values for 24 hours of culture are presented in Figure 4(B). From this figure, it can be seen that there were also no significant differences between the generalized polarization values for yeast strains or inositol levels at this time point. However, as expected, the GP values for 24 hours of culture were as expected markedly higher than at 6 hours of culture, indicating lower membrane fluidity in respiratory phase cells. The GP was increased significantly ($p < 0.05$) compared to 6 hours of culture, indicating significantly lower membrane fluidity. As in previous experiments, decreasing membrane fluidity was accompanied by increasing ethanol tolerance for all strains, independent of the level of inositol supplementation. This result supports previous study which indicated that ethanol tolerance is related to lower plasma membrane fluidity (Swan & Watson, 1999). It is expected that in respiro-fermentative phase, the cells were more sensitive to ethanol.

Generalized polarization data did not show any significant effects of inositol supplementation on membrane fluidity. Only slightly higher GP was detected and this was only for the PDM strain at 6 hours when supplemented with 0.05 g/L inositol, possibly indicating lower membrane fluidity compared to the other levels of supplementation. This result requires further study since a previous study (Chi *et al.*, 1999), indicated that phospholipid composition was altered following inositol addition, which could lead to a changed plasma membrane fluidity. However, in the present study, in which fluidity was determined by a biophysical technique, any factors counterbalancing compositional changes would be taken into account as part of the measurement. Therefore, further study is required to investigate changes of phospholipid composition due to inositol supplementation and its relationship to membrane fluidity.



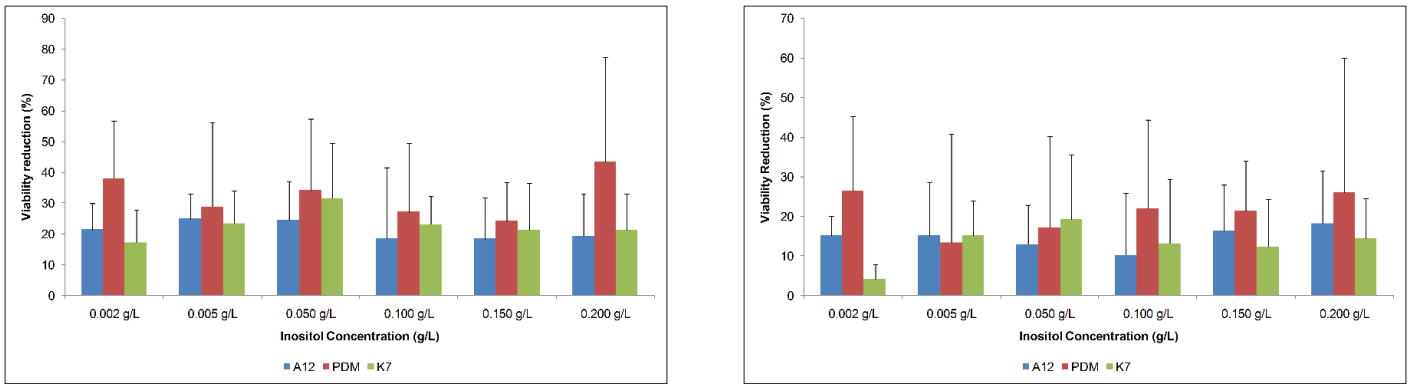
(A) (B)

Figure 4. Generalized polarization of yeast strains grown in inositol-supplemented media at (A) 6 and (B) 24 hours of culture. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations



(A) (B)

Figure 5. Viability reduction of yeast cells grown with various concentrations of inositol at (A) 6 and (B) 24 hours of culture. Viability reduction was determined by the TPC method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of two independent experiments. Error bars represent standard deviations.



(A) (B)

Figure 6. Viability reduction of yeast cells grown with various concentrations of inositol at (A) 6 and (B) 24 hours of culture. Viability reduction was determined by the methylene violet staining method after exposing yeast cells to 18% v/v ethanol. Cultures were grown in YNB medium with 2% (w/v) glucose under aerobic conditions at 30°C. Data are the means of four independent experiments. Error bars represent standard deviations.

Effect of Inositol Supplementation on Ethanol Tolerance

Figure 5 presents viability reduction induced by ethanol stress as determined by the TPC method. Viability reduction was very high at 6 hours (Figure 5(A)) of culture indicating that respiro-fermentative cells are very sensitive to high concentrations of ethanol. No significant differences in viability reduction were observed for the yeast strains as related to inositol concentration at this time point.

Viability reduction of 24 hour cultures as determined by the TPC method was generally lower than at 6 hours culture, as expected (Figure 5(B)). Again, no significant differences were detected between the cultures with or without inositol supplementation at this time point.

Furthermore, methylene violet staining did not show any significant differences in viability reduction between the cultures as related to inositol concentration, either at 6 or 24 hours of culture (Figure 6). However, like the TPC method, methylene violet staining also showed decreased viability reduction (i.e. higher viability) at 24 hours of culture, which indicates that ethanol tolerance is increased in respiratory phase cells. It should be noted that very high variability between replicate samples was observed for this data.

No significant difference in viability reduction, either as measured by TPC or methylene violet staining, were detected between yeast grown with the various concentrations of inositol. High variability between replicates of viability measurements were observed for TPC and methylene violet staining. Therefore, exploration of other methods for ethanol tolerance determination is required to achieve better results.

4. CONCLUSIONS

PDM was found to be the yeast strain most susceptible to ethanol compared to A12 and K7. The present study could not confirm the results of previous studies which indicated that inositol supplementation and accumulation improved ethanol tolerance of yeast cells. Even though not significant, 0.05 g/L inositol might slightly change the membrane fluidity of the PDM strain. Further study is required to test this result.

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