The viability and recovery of *Saccharomyces Cerevisiae* after freezing at -84°C with different concentrations of glycerol

Jeong, S., Le, A., Lee, K.

Abstract

We studied the survival of *Saccharomyces cerevisiae* cells after cryopreservation in different concentrations of glycerol. We wanted to determine which concentration of glycerol would be most protective against very cold temperatures. The concentration of glycerol varied from 0%, 5%, and 40%. Counts were done after cryopreservation for one week at -84 ºC to observe the percentage of dead yeast cells. Then we used hemocytometers to count the number of cells per mL. Upon data analysis, we found that immediately after thawing, the cell count per mL did not show any significant difference. However, after a 17-hour recovery period, the 0 and 40% glycerol treatments showed significantly different cell counts. We also looked at the percentage of dead cells after thawing, 1 hour, and 17 hours. Although not significant, the overall trend was that there were more dead cells when glycerol concentration was low. At 40% glycerol, the percentage of dead cells was below 3% for all treatments. The fewest cells died in 40% glycerol, however the yeast with no glycerol in its medium had more cells per mL after 17 hours.

Introduction

Cryopreservation is used for long-term storage of a variety of cells such as sperm, embryos and small tissue fragments. The process of cryopreservation requires freezing and thawing of cells. During the freezing process, damage to the cell may occur due to the water in the cell turning into ice. Ice forms at different rates during the gradual cooling process. The outer parts of the cell freezes before the intracellular ice begins to form (Wood 1980). As ice forms, the osmotic imbalance across the cell membrane causes water to be transferred out of the cell into the solution. This process can lead to the death of the cell if too much water remains in the cell. The excess water may cause damage to the contents of the cell due to ice crystal formation and it may also cause recrystallization during thawing (Mazar *et al.* 1972). Using cryoprotective

additives can minimize detrimental effects of increased solute concentration as well as ice crystal formation. Some of the cryoprotective additives that are used widely include dimethylsulfoxide (Me_2SO), glycerol, blood serum, methanol, polyvinylpyrrolidone (PVP), sorbitol and malt extract (Hubalek 2003).

We wanted to study the survival rate of *Saccharomyces cerevisiae,* using glycerol as our cryoprotective additive. Glycerol penetrates the cells and reduces the severity of dehydration and prevents ice crystal formation (Clark *et al.* 2009). By varying the concentration of glycerol, we wanted to determine the concentration at which cryopreservation was most protective. We observed the effects of cryopreservation at -84 ºC after one week. Three concentrations we experimented with were 0%, 5%, and 40%. We also had three sets of hypotheses. The null hypothesis was that the survival rate of *S. cerevisiae* will be less or the same with 40% glycerol at -84 ºC Our alternate hypothesis was that, the survival rate of *S. cerevisiae* will be greater with 40% glycerol at -84 ºC. The second null hypothesis is that the recovery rate of *S. cerevisiae* will be less or the same with 40% glycerol at -84 ºC. The alternate hypothesis was that the recovery rate of *S. cerevisiae* will be greater with 40% glycerol at -84 ºC. The third null hypothesis is that the 40% concentration of glycerol has little or no effect on the reproduction rate of *S. cerevisiae*. The alternate hypothesis is that *S. cerevisiae* in a glycerol concentration of 40% will be reproducing at a greater rate.

Knowing the effects of cryopreservation is important because it allows extrapolation into other cells such as sperm and embryos. In doing so, it broadens the knowledge of which concentrations of glycerol to use during cryopreservation

Methods

Measurement of Stock Culture Concentration

To determine the stock culture concentration, we put 10 µL of *S. cerevisiae* culture into the haemocytometer using a Zeiss Axiostar microscope. We made sure that each time the culture was well mixed to ensure an even concentration of cells. Next, the stock culture was placed on ice to prevent further reproduction. We then counted the cells in four randomly selected squares from the haemocytometer per sample and used the average of three samples to obtain the cell concentration of the *S. cerevisiae* stock culture.

Preparation for Freezing

To obtain significant statistical values, each treatment of 0%, 5% and 40% concentration was replicated four times. To prepare 5% and 40% glycerol concentrations, we added 500 µL of 10% glycerol and 80% glycerol to 500 µL of *S. cerevisiae* culture. The 80% glycerol was made by combining 8 mL of glycerol with 2 mL of distilled water. To make the 10% glycerol, we did successive dilutions taking out 1 mL of 80% glycerol and combining it with 7 mL of distilled water. As a control, we added 500 µL of yeast media to the 0% concentration set and resuspended all of the samples to ensure that they were well mixed. We made sure to place all samples on ice immediately after every step to prevent reproduction and growth of the cells. We placed all of the samples in the freezer at -84ºC for a week.

Stain Control

To determine the viability of the stock culture, we placed 50 µL of well-mixed *S. cerevisiae* culture onto a microscope slide, focused using the 40X objective lens, and added 1 drop of 0.01% methylene blue stain. We noted the shape and color of live and dead cells to assist in the data collection for our samples.

Centrifuge Control

To determine whether the process of centrifuging damaged the cells, we did a centrifuge control by performing four replications of the procedure. In order to count the number of dead and live cells, we prepared a microscope slide with 50 µL of well-mixed *S. cerevisiae* culture and one drop of 0.01 methylene blue. We then looked under the microscope at 400X total magnification to record the number of dead and live cells. To centrifuge, we used four replicates of 500 µL of *S. cerevisiae* culture with 500 µL of media and centrifuged at 10000 rotations per minute (rpm) for 30 seconds. We then moved 50 µL of sample from the centrifuged tubes to a microscope slide along with one drop of 0.01% methylene blue dye and recorded the number of dead and live cells while viewing the sample under the microscope at 40X magnification. A comparison was made between the two sets of data.

Removing Glycerol

After taking the samples out of the freezer, we thawed them completely at 37ºC using our hands. In order to remove the glycerol, the thawed samples were centrifuged at 320000 rpm for 60 seconds and the supernatant was replaced with 1 mL of fresh yeast medium. Making sure to re-suspend the sample every time, we repeated the centrifuging process three times to remove all of the glycerol.

Data Collection

We removed 200 μL of each sample while keeping the stock at room temperature after the transfer to determine the condition of cells after a one hour and 17 hour recovery period. Next, we put the samples to be analyzed on ice immediately to prevent any growth of the cells. Data collection was done one sample at a time to ensure that results could be collected quickly at room temperature before the cells began to grow. To collect the data, the group first made a microscope slide with 50 μL of *S. cerevisiae* sample and 0.01% methylene blue and examined it under the microscope at 400X magnification. Then we recorded the number of dead and live cells. We then placed 10 μL of sample into the haemocytometer to count the number of cells, taking the average of 4 squares. In addition, the team averaged all of the results from each treatment. The procedure for data collection for the one-hour recovery period is identical to the procedure directly after thawing. After data collection from the one-hour recovery period, the samples were incubated on a shaker for 17 hours. The procedure for data collection was identical to the previous procedure but the 0% concentration was diluted to 1/50 and the 40% concentration was diluted to 1/100 due to the high number of cells. We calculated 95% confidence intervals for all of our data to determine their statistical significance.

Results

Statistical Analysis

We calculated the mean, standard deviation and 95% confidence interval to determine whether our data is statistically significant or not using Microsoft Excel 2007. For example, to calculate the mean for 0% glycerol concentration (Fig. 1), we used the values from the four

different replicates, converting them to concentration in cells/mL and divided by four to get the mean. To convert to concentration, we used the equation cell count x 1000000/4, where the 4 represents the 4 μL of sample that was present in the hemocytometer. After we got all the means, we took the 12 values (from 3 treatments and 4 replicates per treatment) and calculated an overall mean for the 0% treatment resulting in 6484375 cells/mL. To calculate the standard deviation, we used statistical functions in Excel, which gave a result of 1150606. For the 95% confidence interval, we used the equation 1.96 x (standard deviation/sqrt of n) to obtain 1127592.

Effects of Glycerol on Cell Counts

Directly after thawing from -84°C to 37°C, the cell count/mL did not show any significant differences (Fig. 1). However, after a one-hour recovery period, the treatments with 0% and 5% glycerol both increased in cell number, while the treatment with 40% decreased in cell number (Fig. 2). There are no significant differences in the means of the different treatments because the 95% confidence intervals overlap for all three treatments. After a 17-hour recovery period, all of the treatments increased dramatically in cell count with 0% having the most and 40% having the least (Fig. 3). There is a statistical significance between the 40% treatment with the 0% and 5% treatment. Overall, the 0% treatment has the greatest increase from 65 x 10^5 to 85 x 10^7 cells/mL while the 40% has the smallest increase from 68 x 10^5 to 9 x 10^7 cells/mL. The trend of the graphs indicates that the treatment with the least amount of glycerol results in the highest cell count.

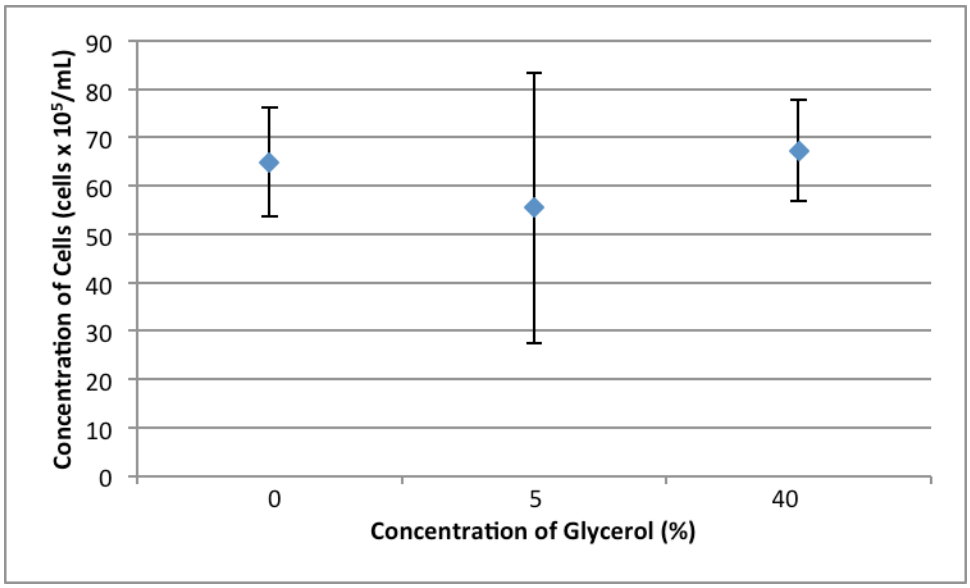

Figure 1: Cell count per mL of *S. cerevisiae* with 0%, 5% and 40% glycerol immediately after thawing to 37°C from -84°C. The points represent the mean taken from 4 replicates and the bars represent 95% confidence intervals.

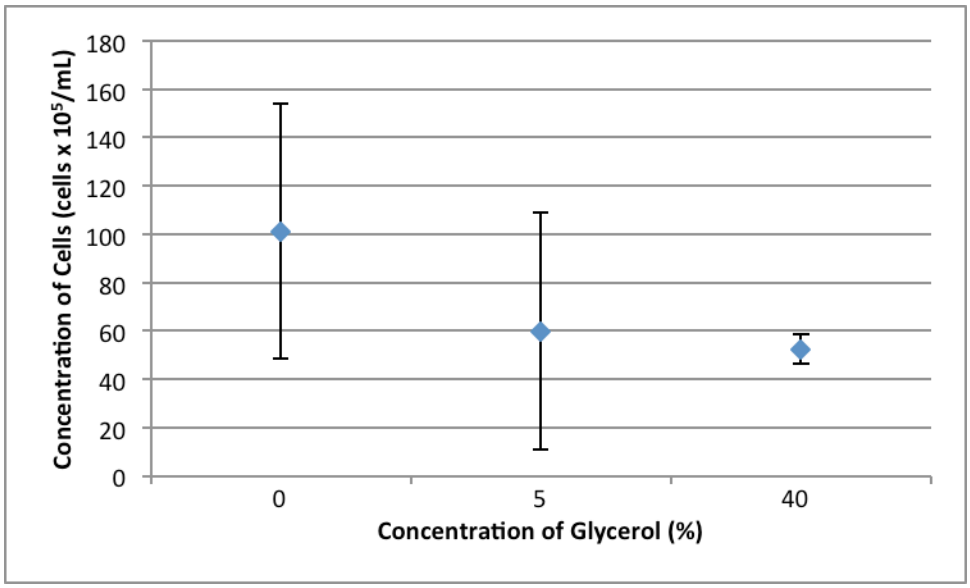

Figure 2: Cell count per mL of *S. cerevisiae* with 0%, 5% and 40% glycerol after a 1-hour recovery period at room temperature. The points represent the mean taken from 4 replicates and the bars represent a 95% confidence interval.

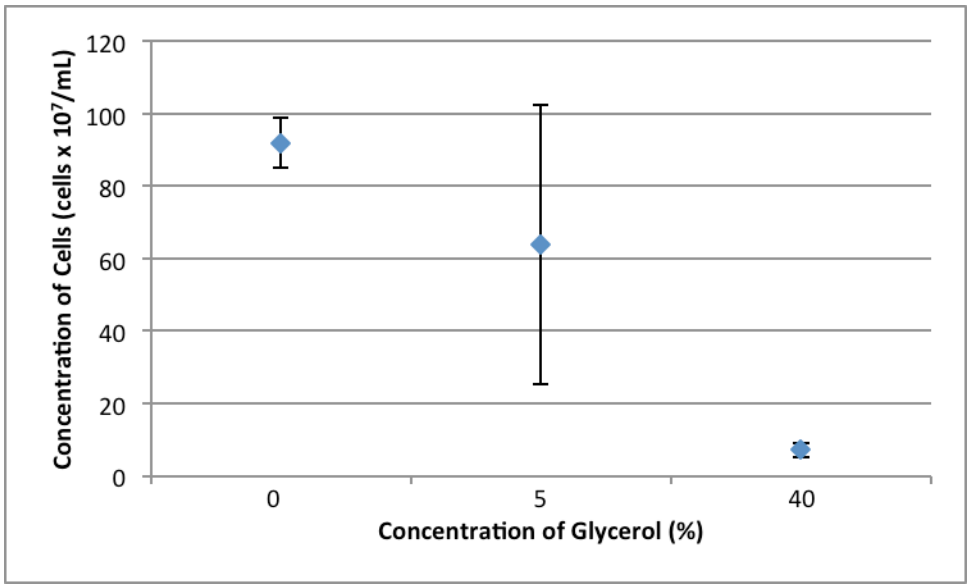

Figure 3: Cell count per mL of *S. cerevisiae* with 0%, 5% and 40% glycerol after a 17-hour recovery period on a shaker in the incubator at room temperature. The points represent the mean taken from 4 replicates and the bars represent a 95% confidence interval.

Effects of Viability and Recovery using Different Concentrations of Glycerol

Directly after thawing from -84°C to 37°C, the treatment with 0% concentration has about 15 times more dead cells than the 40% treatment; 44% and 3%, respectively (Fig. 4). The 5% treatment lies in the middle with 27% (Fig. 4). There is statistical significance between the 40% treatment with the 0% and 5% treatment. After an hour of recovery at room temperature, the percentage of dead cells in the 0% decreased dramatically from 44% to 5% while the 5% and 40% treatment decreased only slightly (Fig. 5). There is statistical significance between all three of the treatments. After a 17-hour recovery period in a shaker, all of the treatments decreased in the percentage of dead cells with the exception of the treatment with 0%, which increased by 4%. The 5% treatment has the most dramatic decrease dropping from 23% to 6% (Fig. 6). There is no statistical significance between the treatments due to the overlap of the 95% confidence intervals.

The trend in the graphs shows that the treatment with the highest concentration of glycerol gives the lowest percentage of dead cells.

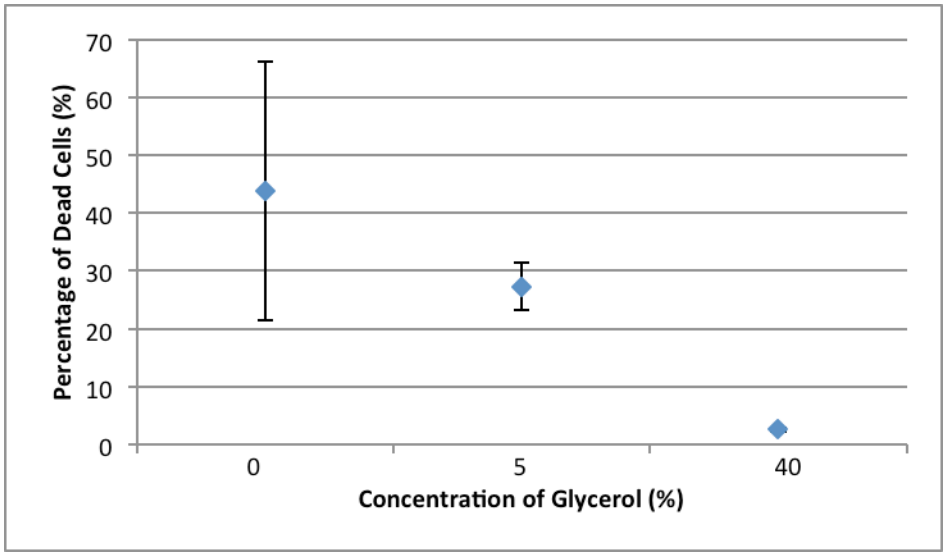

Figure 4: The percentage of dead *S. cerevisiae* with 0%, 5%, and 40% glycerol immediately after thawing to 37°C from -84°C. The points represent the mean taken from 4 replicates and the bars represent 95% confidence intervals.

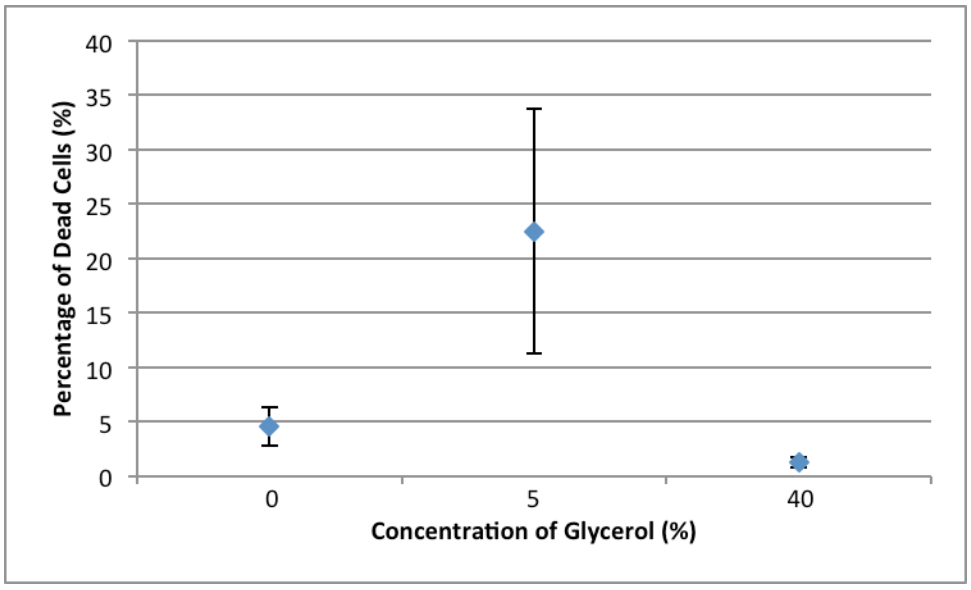

Figure 5: The percentage of dead *S. cerevisiae* with 0%, 5%, and 40% glycerol after a 1 hour recovery period at room temperature. The points represent the mean taken from 4 replicates and the bars represent 95% confidence intervals.

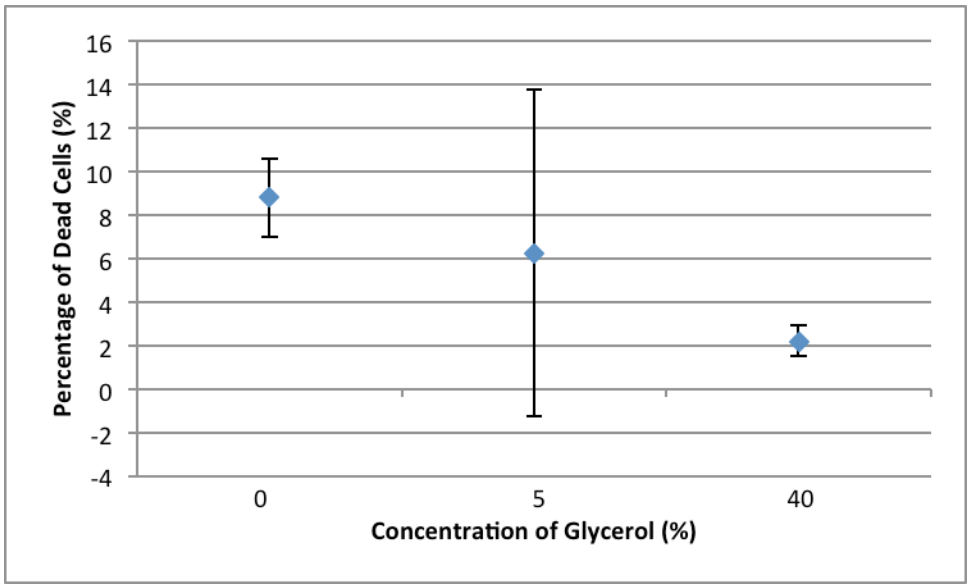

Figure 6: The percentage of dead *S. cerevisiae* with 0%, 5%, and 40% glycerol after a 17 hour recovery period on a shaker in the incubator at room temperature. The points represent the mean taken from 4 replicates and the bars represent 95% confidence intervals.

Growth and Reproduction in Different Concentrations of Glycerol

When compared with the 17 hour recovery period, a one-hour period resulted in a greater change in percentage of dead cells when treated with 0% and 40% glycerol relative to the percentage immediately after thawing while the 5% treatment has a lower change in percentage (Fig. 7). However, the 5% treatment resulted in the most dramatic change from 5% up to 22%.

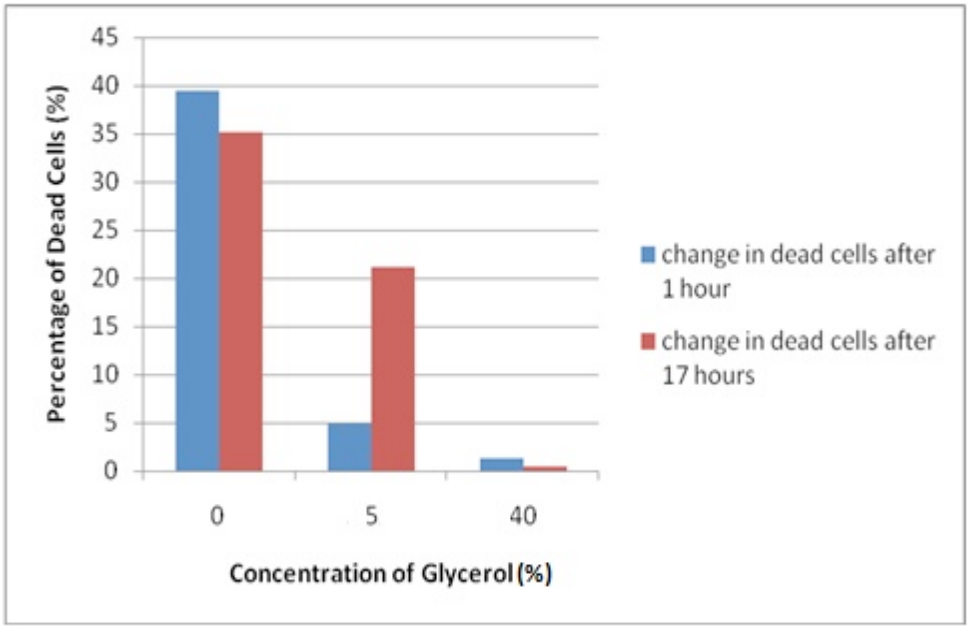

Figure 7: Percent change in *S. cerevisiae* after 1 and 17 hours of recovery in comparison to the percentage of cells that were thawed immediately to 37°C from -84°C. The bars represent a mean taken from 4 replicates for both recovery periods.

Discussion

The total cell concentration (cells/mL) after freezing is not significant at the different treatments (Fig. 1). Our graphs show both dead and live cell concentrations, which did not change before and after freezing regardless of glycerol presence. The cell concentration reduced after freezing due to severe dehydration, removing cellular water below a critical level from which the cell could not recover (Williams *et al*. 2011). Our data show no change in cell concentration at 0% glycerol because we did not remove cellular water below a critical level to kill the cells completely. The movement or transport of glycerol to the intracellular fluid could have a profound impact on maintaining cell water volume during dehydration or freezing. Therefore, our 5% and 40% samples were fairly close to each other, in terms of the total cell concentration (cells/mL). The results from the first hour after thawing showed little significance.

S. cerevisae in no glycerol reproduced more cells (~6.5 x 10^6cells/mL to ~1.0 x 10^7 cells/mL), even though the cells in the 0% glycerol sample died more compared to 40% glycerol (Fig. 2, Fig. 4). The live cells in the 0% were determined to be healthier and as a result, they budded and reproduced more at room temperature.

Cryoprotective agents, such as glycerol, should be selected to minimize freezing-induced deformation as well as to protect cells from cryoinjury (Teo *et al*. 2011). However, the cells in the 40% glycerol seem less healthy. The data show that they had trouble reproducing and their concentration decreased due to the toxicity of glycerol. Even after glycerol had been removed by successive washes after centrifugation, a small amount of glycerol remaining might affect the result. Although glycerol protects many cells in small amounts, there are consequences when too much glycerol is present. The high concentration of glycerol would have forced water to leave the cell, leading to further dehydration, greater membrane damage and osmotic shock at low temperatures (Clark *et al*. 2009). We believe that 40% glycerol may have been exposed to too much glycerol for *S. cerevisae* and led to a decreased numbers in cell concentration.

After 17 hours of recovery, our results show a significant difference between 0% and 40% (Fig. 6). The 5% sample had some errors from the beginning so did the sample 2. This might have produced a big confidence interval. The difference of the cell concentration between the *S. cerevisae* sample without glycerol and with 40% glycerol is significantly greater (Fig. 3). Our results cause us to reject our third alternate hypothesis. The growth of the number of *S. cerevisae* cells at 40 % glycerol sample showed low reproduction rates after cryopreservation compared to 0% glycerol. The membrane water permeability responsible for thawing injuries is also an important factor in cryopreservation of competent cell (Suga *et al*. 2000).

The percentage of the dead cells after thawing seems significant without the 5% sample (Fig. 4). More cells in the absence of glycerol, after freezing and thawing, turned blue with methylene blue dye. At high concentrations of glycerol, greater morphologic changes in the cell membrane would occur (Oh *et al*. 2007), making the cell more susceptible of being non-viable. As a result, the survival rate of *S.cerevisiae* with 40% of glycerol at -84°C is determined to cryopreserve cells better than the other treatment. Therefore, we reject our first null hypothesis; the survival rate was greater for cells in 40% glycerol sample.

A high percentage of dead cells are present after an hour and 17 hours for 0% and 40% are significant (Fig. 5, Fig. 6). The difference shows the percentage of dead cells that recovered for the different levels of glycerol (Fig. 7). The recovery rate of *S. cerevisae* without glycerol seemed to be faster than the recovery rate for 5% and 40% glycerol.

Cryoprotectants can and sometimes do have toxic effects (Fahy 2009). However, many live cells were able to recover as in reproduction in the 0% sample in an hour and after 17 hours. The 5% sample cells did not recover well in the short period. The recovery may have started slowly since its cell had been affected by the toxic glycerol from the beginning. For 40% sample, almost no cells recovered because most of the cells were already alive and well protected from the beginning. Therefore, the experimental results cause us to fail to reject the second null hypothesis.

Throughout our experiment, 5% glycerol was determined to be a better cryoprotectant agent because *S. cerevisiae* cells survived and reproduced more compared to 0% and 40% samples. Glycerol level of 40% wasnot a good cryoprotectant because the reproductive rate was relatively small.

Even though some of the experimental results had good correlation with the expected results, small discrepancies may have been caused by experimental errors. The use of methylene blue for the viability assay in the presence of oxygen may have been reversed the reaction that converts blue coloured methylene blue to a colourless component. The dye could have killed the number of cells when counting. Another source of error might be from the centrifuge control, which was measured at 10000 rpm for 30seconds. However, the experiment was carried out with 13200 rpm for 60 seconds to obtain better pellets. According to the research, cell survival rate is lower at longer time in the centrifuge (Kim *et al*. 2009). This does not affect the experimental results but it is considered as a small control discrepancy.

Future studies should be concentrated in testing cryoprotective agents, such as glycerol, DMSO and sucrose combined. It should be determined whether DMSO combined with glycerol or glycerol with sucrose would result in an additive or reductive effect on the cryopreservation. Combination of the cryoprotectant was not significant but it is better than using one cryprotectant (Oskouei *et al*. 2010). In addition, all the different possible combinations of cryoprotective agents should be tested to find the most effective mixture to acquire the most optimal percentage of cell viability. Moreover, it would be better to use 20% of glycerol concentration instead of using 40% glycerol.

Conclusion

The survival rate of yeast in 40% glycerol was higher than ones with less glycerol. As the percentage of dead cells was lower in 40% concentration, we support our first alternate

hypothesis. The recovery rate of *S. cerevisiae* was higher in media with less glycerol. We have support for our second null hypothesis. Although cryoprotective agents, such as glycerol help minimize the effects of freezing-induced deformation, having the cryoprotective agent in excess harmed the population of *S. cerevisae*. We fail to reject our null hypothesis , as *S. cerevisiae* in 40% glycerol did not reproduce as much as the other concentrations of glycerol. Overall, the best concentration of glucose to use as a cyroprotectant was 5 % because *S. cerevisiae* cells survived and reproduced more compared to the other concentrations, 0% and 40%.

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