The effect of stress due to sodium chloride exposure on the growth of *Saccharomyces cerevisiae*

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Abstract

Throughout its many industrial applications, *Saccharomyces cerevisiae* is exposed to a variety of sodium chloride (NaCl) concentrations that limit its functionality. The objective of this experiment was to study the effect of increasing stress posed by sodium chloride on the growth rate of wild-type *S. cerevisiae*. We created three different media containing 0g/L, 30g/L, and 60g/L NaCl for *S. cerevisiae* growth while incubated at 30ºC. Samples were taken every hour for six hours and cell counts were determined using a haemocytometer. Our results revealed overall increasing growth. However, increasing salt concentrations resulted in slower growth rates with mean final concentrations of 3.14×10^6 cells/mL, 1.05×10^6 cells/mL, and 4.21×10^5 cells/mL in our 0g/L NaCl, 30g/L NaCl and 60g/L NaCl, respectively. A two-way ANOVA test provided three p-values<0.05 that allowed us to support our alternate hypotheses. NaCl dehydrates and disrupts *S. cerevisiae* cells which in turn rely on coping mechanisms involving ion uptake regulation and carbohydrate production for cell protection. We concluded that increasing NaCl concentration and increasing time, as well as the interaction between these two factors, had an effect on the growth rate of *S. cerevisiae*. All cultures had increasing cell concentrations as time progressed, but cultures exposed to higher NaCl concentrations experienced reduced growth rates, possibly due to the fact that energy was redirected from reproduction processes to the osmotic stress response.

Introduction

Saccharomyces cerevisiae, more commonly known as brewer's or baker's yeast, is a unicellular eukaryote that is found in a variety of environments ranging from plants, the human digestive tract and soil (Landry *et al*. 2006). *S. cerevisiae* tolerates a wide range of temperatures and pH environments while undergoing asexual reproduction via budding in both aerobic and anaerobic conditions (Altmann, et *al.* 2007). The generation time can be under 120 minutes in optimal conditions, making it a good candidate for studies involving growth and in optimizing characteristics such as stress tolerance for industrial purposes (Altmann *et al.* 2007).

Several abiotic factors such as temperature, pH and the presence of caffeine have received attention in previous studies in this course with respect to their effects on *S. cerevisiae*. Sodium chloride (NaCl), however, has not been commonly studied. This, added to the many

instances in which this organism is used in the presence of NaCl in the industry, led us to choose it as our factor. The objective of our experiment was to determine the effect of stress through increasing NaCl exposure on the growth rate of *S. cerevisiae*. Our study will increase knowledge on how certain yeast strains behave in the presence of NaCl. *S. cerevisiae* is commonly exposed to salts, particularly NaCl, during storage, production of breads and other products, and ocean water applications (Barnett 2003), which is why increased salt resistance is highly valued. We formulated three sets of hypotheses:

H_{01}: Time in NaCl has no effect on the growth rate of *Saccharomyces cerevisiae*.

H_{A1}: Time in NaCl has an effect on the growth rate of *Saccharomyces cerevisiae*.

H_{02}: NaCl concentration has no effect on the growth rate of *Saccharomyces cerevisiae*.

H_{A2}: NaCl concentration has an effect on the growth rate of *Saccharomyces cerevisiae*.

H_{03}: There is no interaction between time in NaCl and NaCl concentration with respect to the growth rate of *Saccharomyces cerevisiae*.

H_{A3}: There is an interaction between time and NaCl concentration in regards to the growth rate of *Saccharomyces cerevisiae*.

Fermentation by *S. cerevisiae* initially converts sugars into pyruvate via glycolysis, and then subsequently converts the pyruvate into ethanol and carbon dioxide (Landry *et al*. 2006). As Marshall and Odame-Darkwah (1994) found, the presence of NaCl decreases the overall fermentation rate and rise of the finished product. Additionally, the same study revealed that higher concentrations of NaCl inhibited the survival of several yeast strains present in dough (Marshall and Odame-Darkwah 1994). Nevitt *et al*. (1990) found that increasing concentrations of NaCl may have caused hyperosmotic stress which could have modified the transcription and expression of genes due to decreased or entirely arrested cell growth.
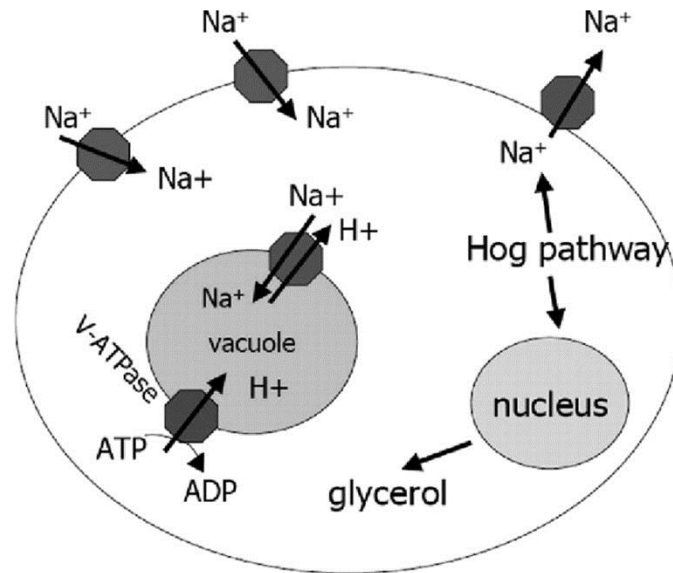
Figure 1. Activation of V-ATPase and Hog pathways upon osmotic stress in *Saccharomyces cerevisiae*. Model from Li *et al.* (2012).

A series of events, depicted in Figure 1, occur when a yeast cell is exposed to NaCl-containing medium. According to this model, a concentration gradient promotes movement of sodium ions into the cell that activate the high-osmolarity glycerol (Hog) pathway and promote V-ATPase activity (Li *et al.* 2012). During the osmotic stress reaction, yeast cells consume energy to power V-ATPase and collect Na+ ions into vacuoles, removing them from the cytosol and hence reducing their toxic effect on the cell (Hamilton *et al.* 2002). The Hog pathway upregulates genes for glycerol production which helps maintain basic cell function and proper cell membrane structure (Uschner and Klipp 2014, Gonzalez-Hernandez *et al.* 2005). Additionally, the Hog pathway activates transporters that help expel Na+ ions from the cytosol back into the medium (Li *et al.* 2012). These changes are coupled with genetic alterations that translate to reduced reproductive rates as energy is allocated to the stress response (Hirasawa *et al.* 2006). Taking this into account, we expected to find increased cell concentrations in all treatments, but a reduced growth rate in cultures exposed to NaCl. Similarly, we predicted that

higher concentrations of salt would result in reduced growth rates compared with lower or no salt presence.

Methods

Yeast strains, media, and culture conditions

We obtained *Saccharomyces cerevisiae* wild type strain from the BIOL 342 lab at the University of British Columbia, Vancouver. The original cell culture was grown in standard medium (YPD). Standard medium without cells and standard medium with 120g/L NaCl concentration were also provided by this lab.

Preparation of our cultures

The method used for preparing cell counts is depicted in Figure 2. First, the cell solution was vortexed enough to resuspend any solids found in the test tube. A 100μL sample was then taken from the cell solution and placed into a microcentrifuge tube along with 10μL of the fixative, Prefer. These two were thoroughly mixed using the micropipette. We then placed a 10μL sample of the fixed cells in a haemocytometer and counted the cells as viewed in the microscope. All subsequent cell counts throughout this experiment were conducted in the same manner.
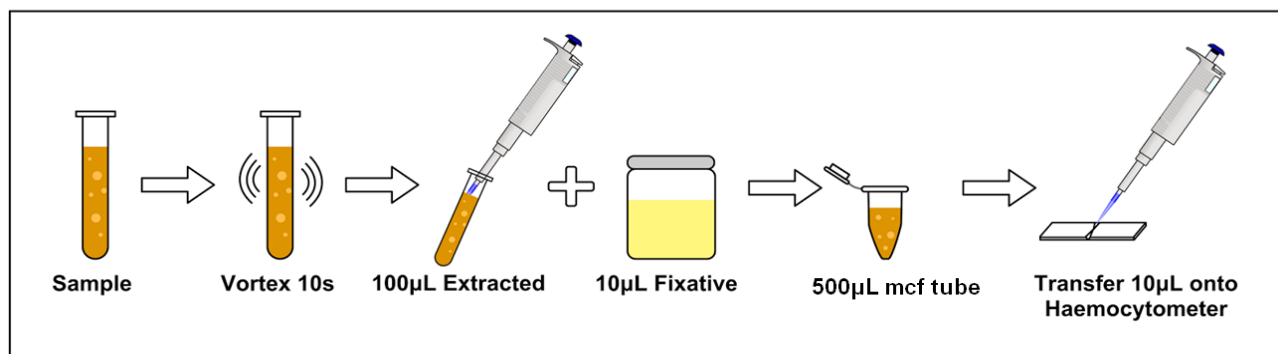

Figure 2. The procedural steps followed to count the cells of *S. cerevisiae* in samples to determine the cell concentration.

The concentration of the stock solution was 1.7×10^7 cells/mL. Our experiment required an initial cell concentration in our samples of approximately 2×10^5 cells/mL; to obtain this concentration, we diluted 174.4μL of the stock solution in 49.5mL of YPD.

We then set up three 30-mL stock preparations for our treatments with 0 g/L NaCl (control), 30g/L NaCl (treatment 1) and 60g/L NaCl (treatment 2). These concentrations were chosen based on previous results that showed that *S. cerevisiae* (strain NRRL Y-977), grown in potato dextrose agar medium at room temperature, exhibited decreased growth between 20-60 g/L NaCl and reduced survivability at higher concentrations (Marshal and Odame-Darkwah 1995). We placed 15 mL of the diluted cell solution in each of the three stock preparation flasks followed by the addition of 15 mL and 7.5 mL YPD to flasks 1 and 2. Finally, 7.5 mL and 15 mL of the 120 g/L NaCl YPD were added to flasks 2 and 3, respectively (Figure 3).
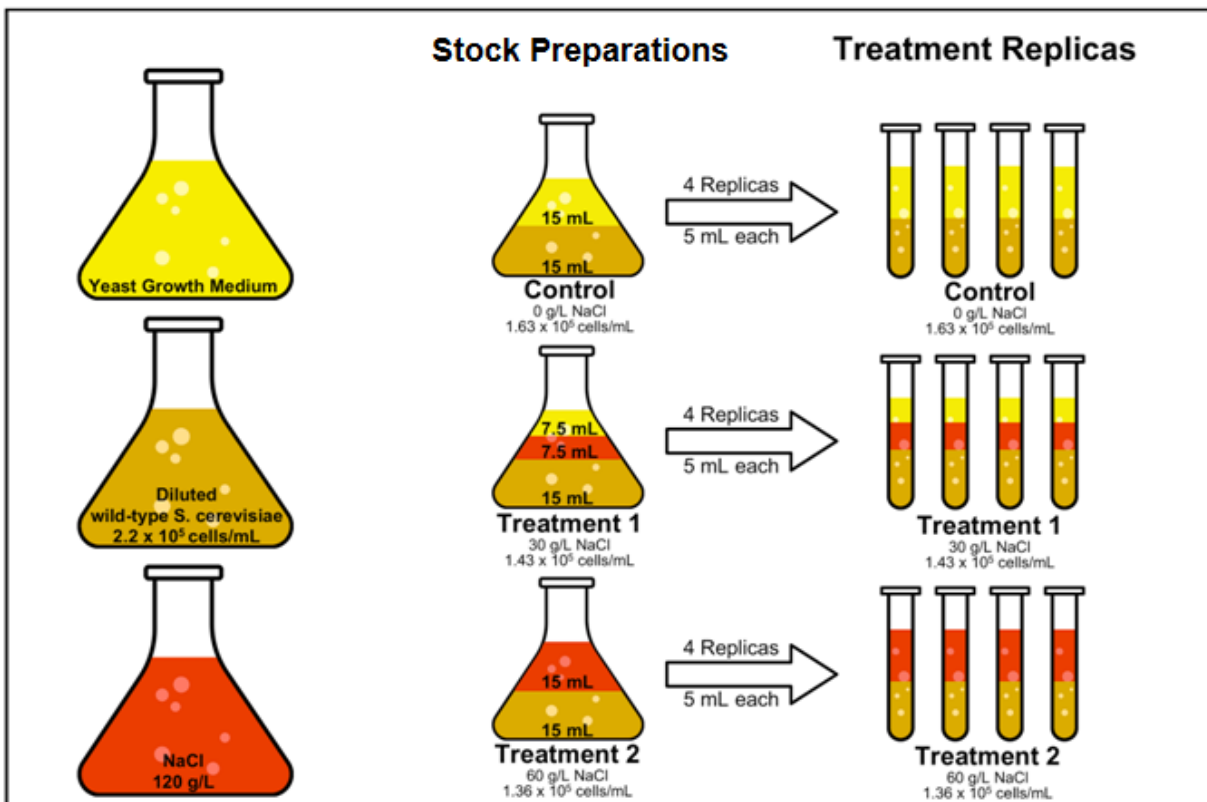

Figure 3. Preparation of our stock preparations and the four replicates for each treatment.

Cell counts for each of the treatment stock preparations yielded a cell concentration of 1.63×10^5 cells/mL for control, 1.43×10^5 cells/mL for treatment 1 and 1.36×10^5 cells/mL for treatment 2. These values represented our cell concentration in each culture at t=0. Four replicates of each treatment were prepared by placing 5 mL of each stock preparation in four test tubes (Figure 3). All test tubes were placed in the incubator at 30˚C.

Data collection

Every hour from t=1 to t=6, the test tubes were removed from the incubator in order to obtain counting samples. According to Altmann *et al.* (2007), the generation time of *S. cerevisiae* is about 120 minutes. We vortexed all test tubes for 10 seconds before preparing the haemocytometers using the cell count method previously described. The fixative was preloaded into all microcentrifuge tubes, so after adding the sample from each tube, the mix was vortexed to distribute the fixative throughout the entire sample.

Statistical Analysis

We first averaged the cell count obtained for each of the four replicates in each treatment at each time. Those values were graphed to show the general growth trend of each treatment over time. We then conducted a two-way ANOVA test using the counts obtained from each sample. The *p*-values obtained allowed us to analyze the statistical significance of our data.

Results

Cultures grown in 60g/L NaCl had a decreased cell number compared to those in the other treatments. We noted that test tubes became slightly more turbid as time progressed with the biggest change seen at the end of 6 hours.

The general growth patterns of each treatment are shown in Figure 4. There is a clear trend between the three treatments where growth rate decreases as the salt concentration

increases. The control treatment with 0 g/L of NaCl had a mean initial cell concentration of 1.63 x 10^5 cells/mL and a mean final concentration of 3.14 x 10^6 cells/mL. The 30 g/L NaCl treatment had a mean initial concentration of 1.43 x 10^5 cells/mL and a mean final concentration of 1.05 x 10^6 cells/mL. The 60 g/L NaCl treatment had a mean initial concentration of 1.36 x 10^5 cells/mL and a mean final concentration of 4.21 x 10^5 cells/mL. Error bars showing the 95% confidence intervals were included in the graph, but are undetectable in most data points due to the minimal variability among the replicates for our cell counts.
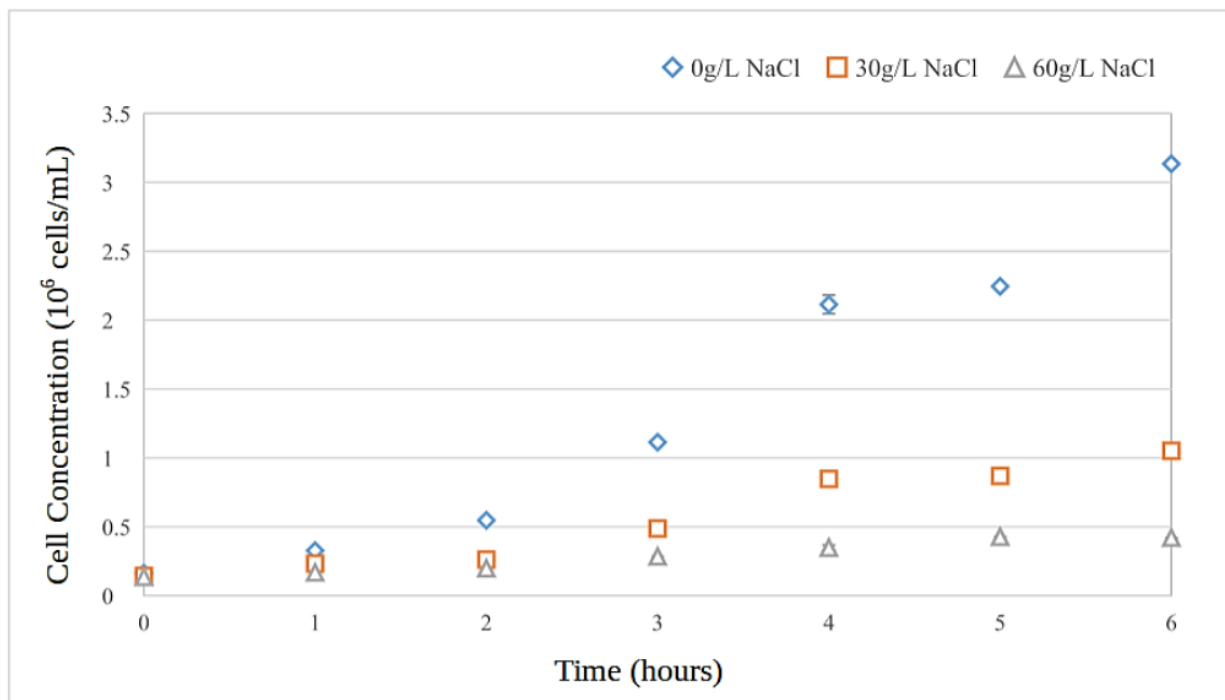

Figure 4. The growth rate of wild-type *Saccharomyces cerevisiae* in varying salt concentrations of 0 g/L NaCl (control), 30 g/L NaCl, and 60 g/L NaCl over 6 hours. N=4 for each treatment; 95% CI shown in graph.

The two-way ANOVA test resulted in *p*-values of 3.1 x 10^{-45}, 2.6 x 10^{-44} and 5.9 x 10^{-36} for our first, second and third sets of hypotheses, respectively. All *p*-values are significant, i.e., less than *p* = 0.05.

Discussion

Based on the two-way ANOVA test conducted with our data, we rejected all the null hypotheses proposed and provided support for our alternate hypotheses. Our results support that a) time in NaCl has an effect on the growth rate of *S. cerevisiae*, b) NaCl concentration has an effect on the growth rate of *S. cerevisiae* and c) there is an interaction between these two factors on the growth rate of *S. cerevisiae*. We predicted that there would be an increase in cell growth as time elapsed and that cultures exposed to higher concentrations of NaCl would have slower growth rates than those exposed to less or no NaCl. The growth pattern we observed confirmed our prediction; when *S. cerevisiae* was grown in medium without NaCl growth rate was higher than cultures grown in NaCl-containing medium. In addition, *S. cerevisiae* in the 30g/L treatment grew slower than our control, but faster than *S. cerevisiae* grown in medium containing 60g/L.

These results are consistent with those found in previous studies using both laboratory and industrial strains of this organism (Marshall and Odame-Darkwah 1995; Trainotti and Stambuk 2001; González-Hernández *et al.* 2005; Hirasawa *et al.* 2006; Jamnik *et al.* 2006). Despite having similar results, different *S. cerevisiae* strains employ different mechanisms for osmotic tolerance. Gonzalez-Hernandez *et al.* (2005), for example, found that the commercial strain tested increased its Na+ intracellular concentration by more than twice when exposed to 1M NaCl. While the active dry yeast strain used also increased its Na+ uptake, it did so less than the commercial strain (Gonzalez-Hernandez *et al.* 2005). Hirasawa *et al.* (2006) found that the laboratory strain they tested was unable to carry out protein synthesis after the addition of 0.5M NaCl while a brewing strain remained fully functional at this NaCl level.

One mechanism that explains *S. cerevisiae's* ability to cope with NaCl relies in its ability to regulate ion concentrations. Yeast cells will increase their uptake of salt cations in the

presence of high salt concentrations (Li *et al.* 2012). Gonzalez-Hernandez *et al.* (2005) explained that, on some occasions, the intracellular concentration of salts will equal that of the environment, but yeast cells will reduce cell salinity by introducing compatible solutes. Hamilton *et al.* (2002) added that accumulation of potassium ions and production of glycerol help protect the cells from NaCl stress by dissipating the driving force for Na+ uptake through the plasma membrane. Ren *et al.* (2012) found that *S. cerevisiae* increased its intracellular concentration of potassium ions in order to maintain equal electrical charge without the presence of toxic concentrations of sodium ions inside the cell. Although there are many components that contribute to ion intake in yeast cells, Hamilton *et al.* (2002) suggested that it is V-ATPase that seems to have the greatest effect on the stress response to salinity. They reported an increase in this enzyme immediately after addition of small concentrations of NaCl and a continued increased presence in all concentrations tested compared to baseline levels (Hamilton *et al.* 2002). The mechanism they proposed, consistent with the mechanism later proposed by Gonzalez-Hernandez *et al.* (2005) was the creation of a proton motive force that helped the Na+/H+ antiporter move Na+ cations into vacuoles to reduce salt concentration in the cytoplasm (Hamilton *et al.* 2002). Maintaining a constant cytoplasmic salt concentration was also important in order to avoid disrupting homeostasis of other cations such as K+ and Ca2+ and to promote Cl- uptake through the chloride channel via an electrical potential gradient (Hamilton *et al.* 2002).

Hirasawa *et al.* (2006) speculated that free water diffused from cells immediately after the addition of NaCl, which dehydrated cells and disrupted basic cell processes such as transcriptional machinery. Jamnik *et al.* (2006) agreed and added that cells experienced loss of turgor. This dehydration effect was retarded by the production of trehalose and glycerol, the two

main carbohydrate storage sources in yeast (Hirasawa *et al.* 2006). Ren *et al.* (2012) also reported this mechanism and explained that cells introduce signalling pathways, such as the Hog pathway presented in our initial model, that lead to high production of glycerol, which also help restore turgor and cell function. Gonzalez-Hernandez *et al.* (2005) found an increase in glycerol production with increasing concentrations of salt and a decrease in trehalose concentrations with increasing NaCl exposure, most likely due to the use of trehalose in stabilizing membranes and preserving enzyme activity (Gonzalez-Hernandez *et al.* 2005). Glycerol participates in osmoregulation and protection of enzymatic activities under salt stress (Gonzalez-Hernandez *et al.* 2005). Jamnik *et al.* (2006) also reported that glutathione, an antioxidant, is recycled into its reduced form and used to recruit reactive oxygen species that help the cell combat stress contributing to the osmotic stress response.

Both of these responses can be explained by changes seen in gene expression during NaCl exposure. Hirasawa *et al.* (2006) demonstrated that the addition of NaCl resulted in gene-expression adaptations within the first hour that allowed the cells to respond appropriately. The major gene changes observed related to those controlling for carbohydrate metabolism, electron transport, energy metabolism, response to stress, RNA metabolism, protein biosynthesis and ribosome assembly and biogenesis; the first four were upregulated and the last three were downregulated (Hirasawa *et al.* 2006).

All these findings support the model presented in our introduction and offer a more detailed view of the mechanisms in place. Our yeast strain, like many others tested before, may have responded to NaCl exposure by shutting down certain processes and increasing defense mechanisms against the salt. Cells relocate their energy from basic functionalities such as reproduction to the activation of these pathways that consume most of the energy available

(Marshall and Odame-Darkwah 1994; Nevitt *et al.* 1990). This increased expenditure in the stress response translates to slower reproduction and hence, reduced growth rates consistent with the results obtained in our experiment. Since no decrease in cell concentration was seen, we could suggest that cells were able to respond to stress and prevent cell death at the cost of reduced reproduction.

Despite having very clear results, there were some sources of error that should be addressed if this experiment is used as a model for future studies. Our experiment was set up as an open experiment meaning cell counts may have been biased. Our data would have been more accurate if we had blinded the observers from the sample they were counting. Similarly, the fact that several researchers were involved in the production of our tubes and in the counting of samples may have allowed for errors in individual differences when measuring. It was later brought to our attention that our initial cell concentration was too low to provide accurate counts in the haemocytometer. It is possible that because of this, our cell concentrations are inaccurate with an error range in the hundreds of thousands of cells. However, our results would still have been significant given the small *p*-values obtained.

Future studies should explore the genetic relationship between gene expression and NaCl tolerance. It is clear that different yeast strains respond differently to NaCl; a clear image of the genes at work would allow for the creation of a tolerant yeast strain that would highly benefit the industries that employ yeasts. This could in turn lead to future studies and improvements in yeast use.

Conclusion

We rejected all three null hypotheses and were able to support the alternate hypotheses with *p*-values<0.5. The significant differences in cell concentrations among the treatments over

six hours indicate that the growth rate of wild-type *Saccharomyces cerevisiae* decreases as NaCl concentration increases. This was likely due to the increasing stress placed upon the organism which negatively interfered with its basic cell functioning and growth.

Acknowledgements

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