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# Increasing stress tolerance in Saccharomyces cerevisiae using lipocalin genes

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Departmental Honors Thesis
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**ABSTRACT**

*Saccharomyces cerevisiae* is a microorganism that is commonly used in industries, but its productivity is significantly hindered by its inability to resist high stress levels found in industrial processes. By increasing stress tolerance in *S. cerevisiae*, its role in industrial processes could be maximized. The objective of this study is to determine if complementation of lipocalin genes into the wildtype and knockout strains of *S. cerevisiae* can increase stress tolerance against a variety of stressors. We identified the knockout yeast strains, *ALD3, ALD4, PDX3,* and *ILV1,* as being sensitive to various stressors including salinity, heat, oxidative, and osmotic stresses. *Arabidopsis thaliana At-TIL (AT5G58070)* and *Homo sapiens OBP2B (AY358981),* two identified lipocalin genes, were cloned into the yeast shuttle vector, p415 GPD, and transformed into the wildtype, *BY4743,* and knockout strains of yeast. Growth phenotypes of both transformants and non-transformant cells of the wild type and knockout strains were tested against heat, oxidative, and osmotic stresses, in addition to their alcohol tolerance levels. Compared to the wildtype and knockout strains, *ALD4::TIL* showed increased stress tolerance against heat when exposed to 50°C for 10 minutes. *BY4743::TIL* and *ALD4::TIL* showed increased stress tolerance against oxidative stress when exposed to 1mM $H_2O_2$ , but none of the transformants showed increased tolerance when exposed to 2mM $H_2O_2$. The cells showed no improvement in osmotic stress tolerance with either of the lipocalins, *At*-TIL or OBP2B, when exposed to 1.0M sorbitol. The *ALD4::TIL* and *ILV1::TIL* transformants showed increased stress tolerance against salinity when exposed to 0.8 M NaCl, while the OBP2B transformants seem to be irresponsive to the salinity stress.

## INTRODUCTION

*Saccharomyces cerevisiae* plays a significant role in industrial fermentation because it is able to metabolize sugars into alcohol. Nonetheless, it is faced with many stressors arising from operative conditions, so its productivity is significantly hindered. By transforming *S. cerevisiae* with lipocalin genes, stress tolerance in the yeast could increase, and its productivity could be maximized.

Lipocalins are a family of small extracellular proteins found in a variety of organisms that are able to bind to small hydrophobic compounds, making them critical for sequestration, detection, and transportation. Recent studies have further shown that lipocalins not only function as transporters, but also function in stress response in various organisms.[1,2] One such study has observed that when the gene encoding for the *Arabidopsis thaliana* temperature-induced lipocalin protein (*At*-TIL) is expressed in the wild type strain of *S. cerevisiae*, it shows a higher tolerance to stressors in comparison to the parental strain.[3] This finding suggests that lipocalin-mediated increased tolerance in *S. cerevisiae* could play a key role in industrial processes, particularly alcohol fermentation. Though, this is the case, *At*-TIL may not be the only lipocalin able to increase stress tolerance of the yeast cells to their full potential. Research has not shown whether human lipocalin proteins could maximize stress tolerance in yeast, as well. Due to the roles that lipocalins have shown to play in other organisms, we suspect that lipocalins from not only *Arabidopsis thaliana*, but also *Homo sapiens*, could show lipocalin-mediated increased stress tolerance in yeast cells through genetic complementation.

The productivity of yeast cells is limited by their own byproducts and the environmental conditions found in fermentative processes. By minimizing the effects of stress during fermentation, efficiency in yeast could be maximized. The results from our research could lead to increased efficiency in fermentation and the mutant strain's implementation into industrial processes. Here we consider the expression of two lipocalin protein genes, *At-TIL (AT5G58070)* and *Homo sapiens OBP2B (AY358981)*, in wildtype and knockout strains of *Saccharomyces cerevisia*e.

## BACKGROUND

### Role of Yeast in Fermentation

*Saccharomyces cerevisiae* is a workhorse in industrial fermentation due to its metabolism and innate traits. It is used for the production of alcoholic beverages, baked foods, biocontrol agents, enzymes, probiotics, chemical commodities, therapeutic proteins, biopharmaceuticals, and flavoring and coloring agents.[4] *S. cerevisiae* is a yeast essential in alcoholic fermentation because it is able to metabolize sugars into alcohol and carbon dioxide.[4] However, during industrial fermentation, *S. cerevisiae* is faced with various significant stressors caused by operative conditions such as fluctuations in temperature and product accumulation.[3] They are often limited in their productivity by their inability to withstand high levels of stress. Therefore, it is critical to optimize industrial *S. cerevisiae* strains to improve production efficiency of industrial fermentative processes.

### Industrial Fermentation

The market is continuously changing to be more sustainable, alter alcohol content, and increase company profit. Due to the market changing, new and more efficient yeast are required to solve the challenges that result from consumer demand. Yeast strain selection in alcoholic fermentation is important for both economic efficiency and product quality for the consumer. The use of genetically engineered strains could increase industrial productivity caused by the varying media conditions found across different technological processes.

During the process of fermentation, the yeast are exposed to several stress factors which include hyperosmotic stress, increase in temperature, cold stress, high carbon dioxide levels, high alcohol levels, and oxidative stress.[4] Yeast survival in alcoholic fermentation depends on the stress response mechanisms of the cells which involves sensor systems and signal transduction pathways that activate transcription factors. Yeast undergo fermentation in anaerobic conditions because they must produce ethanol for redox balancing and energy during sugar consumption.[4] Thus, the NAD+/NADH ratio is maintained, and intracellular electron balance can be accomplished.[4]

In alcohol production, *S. cerevisiae* undergoes glycolysis, converting glucose through sugar catabolism and producing 2 pyruvate, 2 ATP, and reduced NADH.[4] During anaerobic conditions, fermentation begins, and the pyruvic acid is converted to 2 carbon dioxide and then to 2 ethanol via acetaldehyde.[4] Finally, alcohol dehydrogenase catalyzes the regeneration of NAD+ to allow for glycolysis and ATP production to repeat.[4]

Osmotic and Salinity Stress

The main stress condition at the beginning of vinification is hyperosmotic stress caused by the high concentration of sugars and/or salt in the medium. As the sugars are converted, the

nutrients begin to deplete and the ethanol levels increase, both which affect the stress tolerance of the yeast cells and can cause deleterious impacts on alcoholic fermentation.[4] Osmotic stress can lead to the overproduction of glycerol, a stress-protectant molecule, during fermentation.[4] As a response to osmotic stress, yeast will maintain redox balance by production of glycerol through generation of NAD+ via dihydroxyacetone phosphate.[4] This mechanism occurs in response to compensate for water loss.[4]

Oxidative Stress

Oxidative stress occurs when there is an imbalance between prooxidant and antioxidants and the cell does not have proper cell antioxidant defenses to prevent reactive oxygen species (ROS) accumulation. This can lead to severe oxidative damage of important cell biomolecules, such as nucleic acids, proteins, lipids, and structures which compromise cell homeostatic functions and viability of the cells.[5,6,7,8] ROS accumulation can also be an important factor in programmed cell death or necrosis.[9,10,11] High exposure to hydrogen peroxide can cause necrosis to occur as a result of oxidative damage to cellular components.[9,12,13] This poses a big challenge in alcoholic fermentation because yeast cells should be able to respond to greater stress levels without viability loss.

Alcohol and Temperature Stress

High ethanol concentrations, a byproduct of alcoholic fermentation, and temperature can have deleterious impacts on the cell membrane structure and function of yeast cells and impair fermentation efficiency. Therefore, thermotolerance and ethanol tolerance are highly desirable characteristics for yeast involved in industrial fermentation. Though temperature during vinification is carefully controlled, small fluctuations are still possible, and some wineries use

different temperature due to the difference in the organoleptic properties of the product.[4] In response to ethanol and temperature stress, cells increase membrane integrity by synthesizing heat shock proteins and disaccharide trehalose which function as chaperones and cellular protectants, respectively.[4] With alcohol and temperature playing such an important role in membrane integrity and homeostatic functions, it is important to minimize the effects of the stressors.

The rate of fermentation is directly correlated with the rate of cellular reproduction during active cell growth.[4] Therefore, minimizing the effects of stressful conditions that compromise cell growth is critical. Genetically engineered yeasts can increase ethanol yields, increase stress tolerance, lower the levels of glycerol, and reduce the dependency on exogenous enzymes and nutrients, making the fermentation process significantly more efficient. Yeast cells must be able to withstand non-optimal stresses caused by fluctuating temperature, toxic compounds, and product accumulation. These fluctuations affect cellular function and viability, in turn affecting production yields.[4]

**Lipocalins**

Lipocalins are a diverse family of extracellular proteins found in plants, bacteria, protists, arthropods, and chordates.[2,3] There are more than thousand lipocalin genes among bacteria, plants, fungi, and animals.[14] Lipocalins have low amino acid sequence identity with three conserved structures that comprise a single eight-stranded antiparallel β-barrel that encloses a ligand-binding site.[3] In a study by Du et al. in 2015, human lipocalins were analyzed for protein sequence alignment and structure comparison. While the sequences were found to be very diverse, the tertiary structures are highly preserved and are very similar.[15] Lipocalins are usually

found in small concentrations in a variety of tissues, organs, and fluids, but their expression levels can increase as a response to stress caused by changes in physiological conditions, health, and developmental states. Therefore, they can function as biochemical markers of various disorders and diseases, such as cancer and inflammatory diseases.[16,17]

Lipocalins bind to small hydrophobic molecules, such as fatty acids, steroids, retinoids, pheromones, and odorants, and function in transportation and sequestration, among other specialized functions such as invertebrate cryptic coloration, prostaglandin synthesis, regulation of homoeostasis, and the modulation of the immune response.[1,3,18] Because lipocalins have great functional diversity and vary in the structure of the cavity and scaffold, they are able to bind to ligands of different size, shape, and chemical traits, allowing for great specificity.[3,19] Lipocalins can function as carrier proteins in clearance of endogenous or exogenous molecules.[1] The signaling activity of the lipocalins also correlates with other functions such as cell growth and metabolism, binding of cell surface receptors, membrane biogenesis and repair, induction of apoptosis, and maintenance of cell homeostasis.[3,17,20,21] Not only are the lipocalins transporting molecules, but they are also transferring extracellular signals into the cell and nucleus through protein-protein interactions.[15]

Initially, lipocalins were thought to be specifically a eukaryotic protein.[22] Later, new members of the lipocalin family were discovered in *E. coli* as outer membrane proteins expressed in response to environmental stress condition. It is believed that bacterial lipocalin proteins are involved in the dissemination of antibiotic resistance genes and activation of immunity.[23] However, lipocalins appear to be restricted to gram-negative bacteria and members of the Archea.[24,25]

**Plant Lipocalins, Temperature Induced Lipocalin (TIL)**

Plants have constitutive and inducible defense systems that protect them against fluctuating conditions and other environmental stressors. [2,3,17,20,26,27] Plant lipocalins are classified into two groups, temperature-induced lipocalins and chloroplastic lipocalins.[28] In addition, plants have violaxanthin de-epoxidases and zeaxanthin epoxidases which is classified as lipocalin like proteins such as, violaxanthin de-epoxidases and zeaxanthin epoxidases. These two lipocalin-like and the chloroplastic lipocalins possess signal peptide sequence that target them to the chloroplast. The temperature induced lipocalins do not show any signaling peptide but it is found in the plasm membrane.[28]

There are differences among plant species concerning TIL types. Contrary to *Arabidopsis thaliana* that contains only TIL-1 in chromosome 5, species such as *Oryza sativa* among a group of 12 plant species have TIL-1 and TIL-2 in chromosomes 2 and 8 respectively.[28]

Temperature induced lipocalin (TIL) is a lipocalin encoded by plant genes and has been found to give the plant greater stress tolerance. In preliminary studies, lipocalins in Arabidopsis showed an increase in *At*-TIL expression in response to low and high temperatures, and the TIL1 knockout showed accumulation of $H_2O_2$ and increased stress sensitivity against light, freezing temperatures, and Paraquat herbicide, and they accumulate more ROS.[2,17,26,27] In contrast, overexpression of TIL increased stress tolerance against those same stresses.[2,17,26,27]

*At*-TIL, localized in the cell membrane of *Arabidopsis thaliana*, has been shown to play a critical role in both basal tolerance as well as acquired thermotolerance by potentially preventing plasma membrane lipids from peroxidation and protecting the cells against oxidative stress caused by heat shock, freezing temperatures, paraquat, and light.[2,27] It has also been shown to

protect chloroplasts against salinity stress caused by high NaCl levels through ion homeostasis maintenance.[17,26,27,29,30] *At*-TIL also plays a role in nutrient reservoir activity, cellular response to hypoxia, hyperosmotic salinity response, response to reactive oxygen species, response to water deprivation, and seed maturation.[17,31] Recent studies have reported the association of *At*-TIL with other organelles, which suggests some intracellular role in stress responses, contrary to the initial thoughts that the *At*-TIL functions would be limited to the plasm membrane.[32]

**Odorant Binding Protein**

Odorant binding proteins (OBP) are eight-stranded beta barrel motifs that function in transportation. OBPs are expressed in the nasal mucosa, in addition to other locations in the body, and are very stable against temperature, organic solvents, and proteolytic digestion.[33] These soluble proteins mediate the transport of odorant molecules from the hydrophobic gas phase in the environment across the hydrophilic barrier of the aqueous mucus to the hydrophobic binding site of olfactory receptors of sensory neurons found in the nasal epithelia.[1,18] They also function as scavengers or as neutralizers of toxic volatile molecules.[18] More recent findings have suggested, though, that OBPs may not play an olfactory role, as the structural features are more similar to human tear lipocalin than OBPs of other mammals.[34] Specifically, odorant binding protein 2B (OBP2B) is primarily expressed in prostate and mammary glands which may indicate functional misidentification.[18]

**MATERIALS AND METHODS/EXPERIMENTAL**

Lipocalin proteins from *Arabidopsis thaliana* and *Homo sapiens* were distinguished by their known functions and potential to increase stress tolerance levels in yeast. The lipocalins chosen were *At*-TIL and OBP2B. These lipocalin genes were used for transformation and heterologous complementation of the yeast strains.

**Selection of Yeast Strains**

To gain a better understanding of the stress response in *S. cerevisiae*, several yeast cells carrying knockouts that rendered them sensitive to different stressors were identified. Preliminary data shows sensitivity in knockouts of *S. cerevisiae BY4743* wild type strains—*ALD3, ALD4, PDX3, and ILV1*—to salinity, osmotic, oxidative, and heat stresses.

**Yeast Growth Conditions**

The yeast strains--*BY4743, ALD3, ALD4, ILV1,* and *PDX3*--were grown in YPD medium [1% yeast extract, 2% peptone, and 2% dextrose] at 30ºC at 200 rpm. Following transformation of the clones, the yeast cells were grown on selective synthetic minimal media (SMM; leucine free) and synthetic complete media (SCM).

**Construction of Recombinant Plasmids**

The plasmid vector and lipocalin gene inserts were isolated by double digestion using BamHI and XhoI [8 µL DNA, 8 µL water, 1 µL BamHI, 1 µL XhoI, and 2 µL Cutsmart]. The digested DNA was run on an agarose gel and compared with a 1 kb ladder. DNA fragment sizes corresponding to the digested plasmid and the lipocalin fragment gene were excised from the gel and recovered using the geneJET gel extraction kit (Thermo Fisher Scientific)

To determine if the knockout yeast strains can recover stress tolerance by complementation, each lipocalin gene (*OBP2B* and *At-TIL*) was inserted into p415GPD to form a functional construct (figure 1). The recombinant plasmids were used to transform each knockout and the wild type cells.

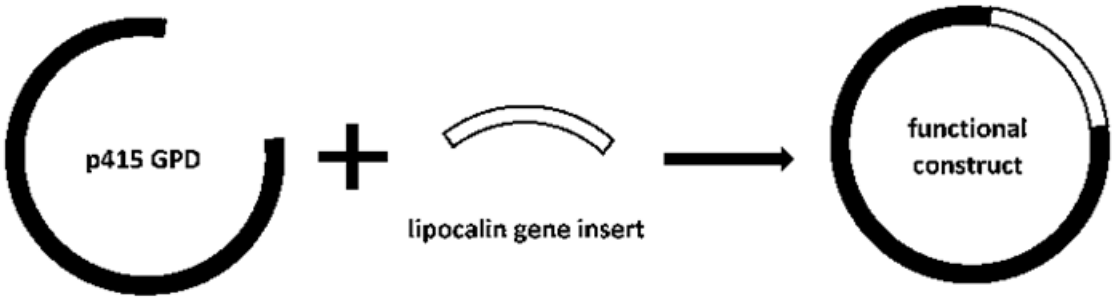



**Figure 1. Recombinant Functional Construct**

The ligation reaction [3 µL insert, 1 µL vector, 1 µL T4 ligase, 4 µL water, 1 µL buffer] was carried out and left at room temperature for 15 minutes, before placing in the refrigerator overnight for cohesive end ligation.

**Amplification of Clone**

Each clone was delivered into the *Escherichia coli* cells for amplification using heat shock. 75 µL of competent *E. coli* cells (C2987) were mixed with 5 µL DNA (p415GPD::TIL or p415GPD::OBP2B) and incubated on ice for 30 min.. The cells were then exposed to a 42°C heat bath for 90 seconds. The transformation mix was immediately removed and placed on ice for 3-5 minutes. 945µL of LB was added, and the mixture was incubated at 37°C for 45 min. 200 µL of the mixture was then transferred onto LB+Amp agar plates and incubated at 37°C for 24 hours.

**Isolation and Purification of Clones**

Transformant colonies were picked from the LB+Amp agar plates, and each was inoculated into LB broth [5mL LB + 5 µL Amp]. The cells were incubated on a shaker at 37°C for 24 hrs. The GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) was used to extract and purify the plasmid DNA.

**Verification of Clones**

To verify the presence of the clones, each of the samples, p415 GPD::*At-TIL* and p415 GPD::*OBP2B,* [3 µL DNA, 1 µL 6x dye, 6 µL water] were run on 1% agarose gel at 170V. A double digestion of the samples [8 µL DNA, 8 µL water, 1 µL BamHI, 1 µL XhoI, and 2 µL Cutsmart] was also carried out, and the samples [20 µL of DD sample, 1 µL 6x dye] were run on a 1% agarose gel at 170V for verification. Lastly, a polymerase chain reaction (PCR) [10 µL 2x LongAmp "Master Mix", 7.5 µL water, 1 µL forward primer, 1 µL reverse primer, 0.5 µL DNA] of the lipocalin gene inserts, *At-TIL* and *OBP2B,* was carried out under specific conditions. The PCR begins with exposure of the samples to 95°C for 3 minutes. During the first 10 cycles, the samples go through a denaturing step at 94°C for 30 seconds, followed by an annealing step at 60°C for 30 seconds, and an elongation step at 72°C for 45 seconds. This is followed by 35 cycles that include a denaturing step at 94°C for 30 seconds, an annealing step at 65°C for 30 seconds, and an elongation step at 72°C for 45 seconds. Then, the samples go through a final extension step at 72°C for 10 minutes. The samples were held at 4°C after completion of the PCR. The PCR samples were then run on 1% agarose gel [3 µL water, 1 µL 6x dye, 6 µL DNA] at 170V for verification by size.

**Transformation of Clones into Yeast**

Each clone was delivered into each of the wild type (WT), knockout (KO), and transformant yeast cells. Lithium acetate and polyethylene glycol according to the Gietz & Woods transformation method was used to allow for high efficiency transformation in the yeast cells.[35] The yeast cells were grown on selective minimal media (leucine-free) and synthetic complete media. Selected colony cells from the selective minimal media (transformed colonies) were tested against a variety of stressors.

**Stress Experiments**

To begin each stress experiment, an overnight culture of the WT, KO, and transformants were prepared in YPD [1% (w/v) yeast extract, 2% peptone, and 2% dextrose] and incubated on a shaker at 30°C at 200 rpm. The cells were set to $OD_{600}$ = 0.4 and were allowed to grow to $OD_{600}$ = 0.8 - 1.0. The cells were then centrifuge and resuspended in saline at $OD_{600}$ = 1.0. Treatments for each specific experiment described below were conducted in triplicate. Each data point represents the means of the three replicates and its respective standard error.

Heat Stress

As described previously cells were grown in an overnight culture and resuspended in saline. 300µL of the cells in saline was added into each of 6 microcentrifuge tubes that correspond to the strain. The experiment was carried out in triplicate, so three tubes were left on ice as the control, and the other three tubes were exposed to heat at 50°C in a water bath for 10 minutes. The tubes exposed to stress were immediately removed and placed on ice. A 10-fold serial dilution was carried out for each of the six tubes corresponding to each strain. 100µL of

each of the $10^{-3}$ and $10^{-4}$ dilutions were plated onto their corresponding YPD agar plates. The plates were incubated for 48 hours at 30°C. The colonies were counted.

Oxidative Stress

2 µL of each yeast strain (WT *BY4743*, KO, and transformants) was exposed to 98 µL of the control [1x SCM], 1.0 mM $H_2O_2$ [1.0 mM $H_2O_2$ + 1x SCM], and 2.0 mM $H_2O_2$ [2.0 mM $H_2O_2$ + 1x SCM] media in triplicate. 100 µL of each of the blanks [100 µL 1x SCM; 100 µL 1x SCM + 1.0 mM $H_2O_2$; 100 µL 1x SCM + 2.0 mM $H_2O_2$] were used. All of the samples for the growth experiment were conducted in a 96-well plate and placed in the Microplate reader under continuous shaking for up to 24 hours.

Osmotic Stress

2 µL of each yeast strain (WT *BY4743*, KO, and transformants) was exposed to 98 µL of the control [1x SCM] and 1.0 M sorbitol [1.0 M sorbitol + 1x SCM] media in triplicate. 100 µL of each of the blanks [100 µL 1x SCM; 100 µL 1x SCM + 1.0 M sorbitol] were used. All of the samples for the growth rate experiment were conducted in a 96-well plate and placed in the Microplate reader under continuous shaking for up to 24 hours.

Salinity

2 µL of each yeast strain (WT *BY4743*, KO, and transformants) was exposed to 98 µL of the control [1x SCM] and 0.8 M $H_2O_2$ [0.8 M NaCl + 1x SCM] media in triplicate. 100 µL of each of the blanks [100 µL 1x SCM; 100 µL 1x SCM + 0.8 M NaCl] were used. All of the samples for the growth rate experiment were conducted in a 96-well plate and placed in the Microplate reader under continuous shaking for up to 24 hours.

**Statistical Analysis**

Using statistical analysis of the survival/growth rates, the phenotypes of the transformant cells were compared to the wildtype and knockout cells to determine any differences in stress response. For the heat stress, % survival of the transformant strains was determined based on 100% growth of the control (no heat exposure) of the corresponding knockout cell. A histogram was created that indicates the percentage of cells survival rate and the values are the means of three replicates. For the oxidative, salinity, and osmotic stress, a growth curve carried out over the course of 17-18 hours was analyzed. A line graph was created, and the data point represents the means of three replicates. A P-value of 0.05 was used to determine statistical significance based on a Student's t-test. Thus, a P-value $\leq 0.05$ means that there is a significant difference between the knockout/wildtype and the transformant strains exposed to the stressor.

**RESULTS**

The WT *BY4743,* transformant, and knockout cells were tested for their responses to a variety of stresses including 0.8 M NaCl, 1.0 M Sorbitol, 1.0 mM and 2.0 mM $H_2O_2$, and 50°C for salinity, osmotic, oxidative, and heat stresses, respectively. Yeast cells responses were analyzed by growth rate and/or the survival rates of each strain determined by the growth curve over 17-18 hours or the number of colonies between the undisturbed samples and the samples exposed to the stress, respectively.

## Heat

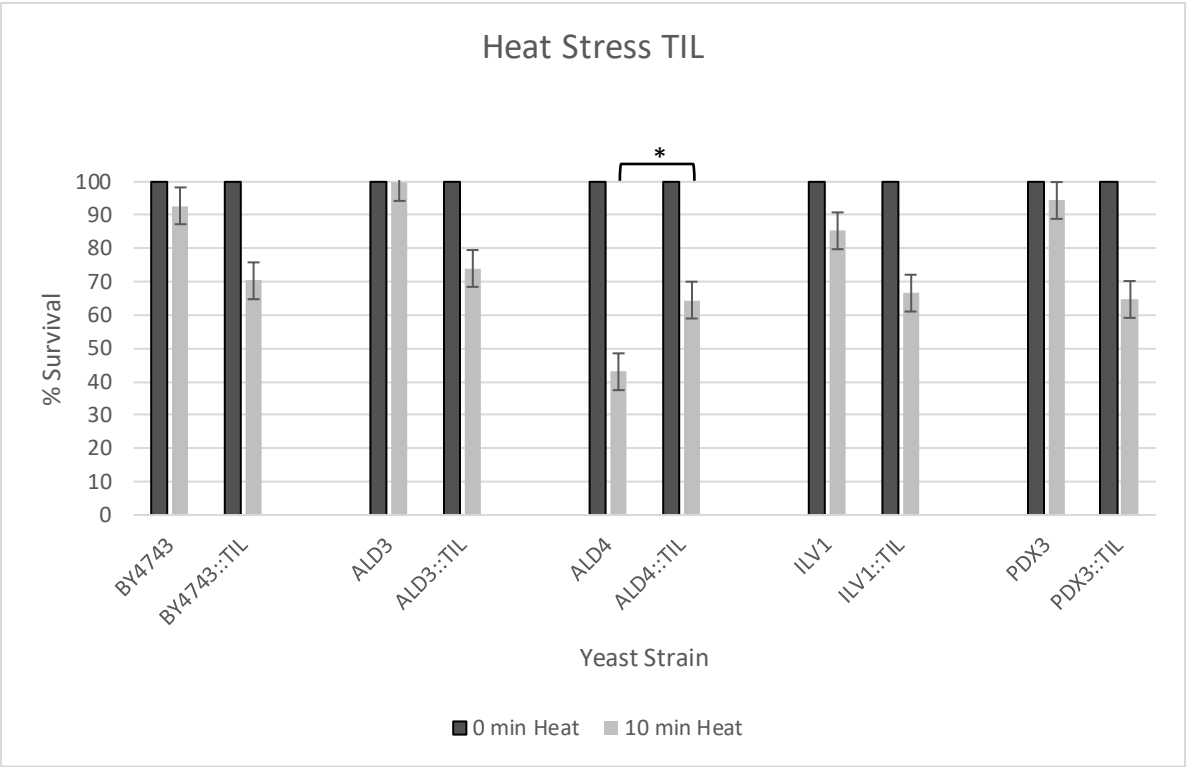



**Figure 2. Heat Stress on *S. cerevisiae* (*At*-TIL)**

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::TIL*, *ALD3::TIL*, *ALD4::TIL*, *ILV1::TIL*, and *PDX3::TIL*) cells were exposed to heat at 50°C in a water bath for 10 minutes. The plates were incubated for 48 hours at 30°C. The colonies were counted for each plate, and the average of the 3 replicate plates was determined for each experimental and control group. The % survival was calculated in comparison with 100% growth of the control (no heat exposure) of the corresponding knockout cell. It was determined that only the *ALD3::TIL* transformant showed an increase in stress tolerance when exposed to 50°C for 10 minutes as compared to the knockout strain. The histogram indicates the percentage of cells survival rate and the values shown are the means of three replicates (*$P < 0.05$ Student's *t*-test).

## Oxidative

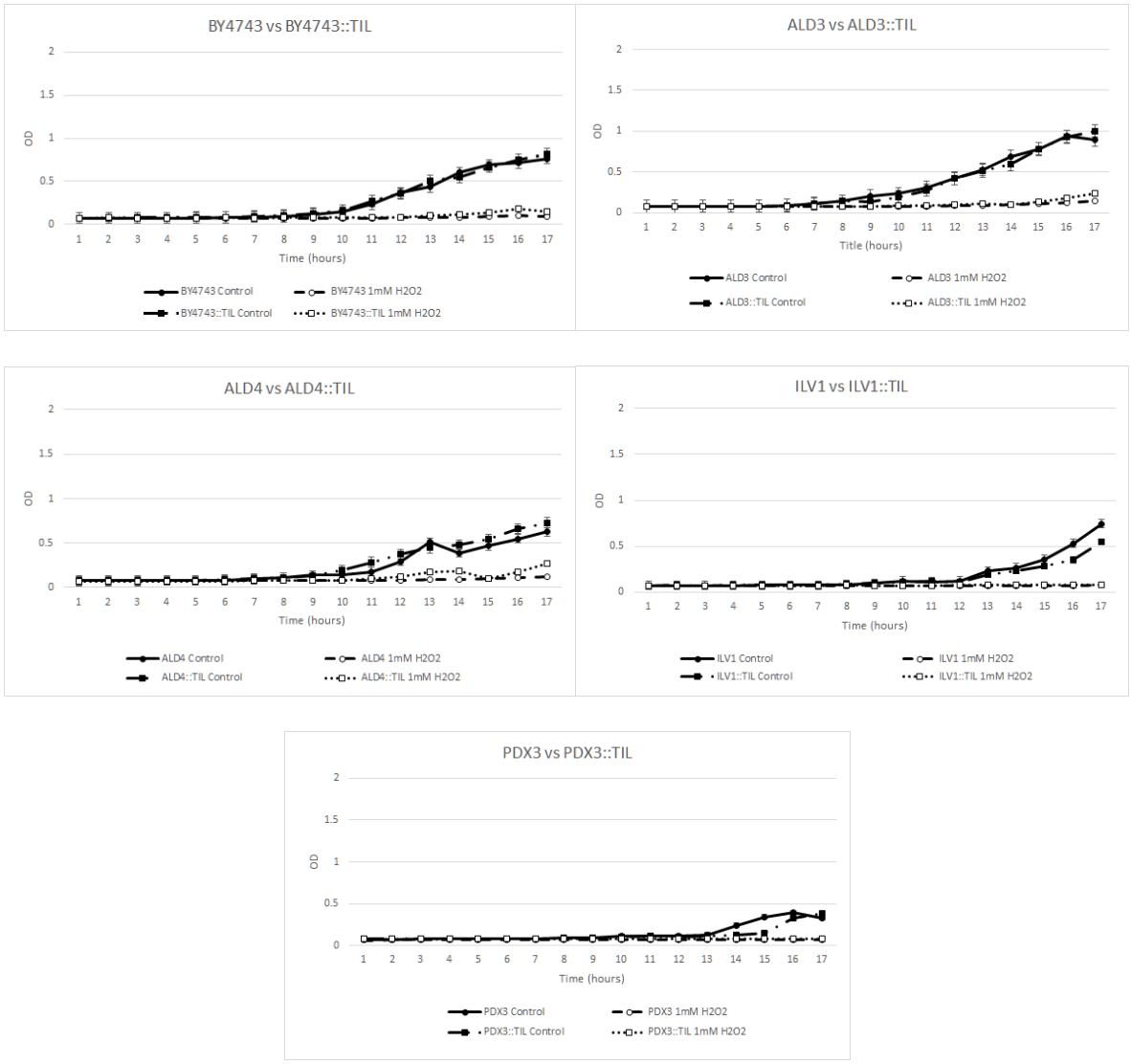



**Figure 3. 1mM $H_2O_2$ Oxidative Stress on *S. cerevisiae (At*-TIL*)***

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3, ALD4, ILV1*, and *PDX3*), and transformant (*BY4743::TIL, ALD3::TIL, ALD4::TIL, ILV1::TIL*, and *PDX3::TIL*) cells were grown in either synthetic complete media (SCM) or SCM+1 mM $H_2O_2$ in a 96-well plate and placed in the Microplate reader under continuous shaking for 17 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that the *ALD4::TIL* showed an increase in stress tolerance beginning at about 12 hours when exposed to 1 mM $H_2O_2$ as compared to the knockout strain. The *BY4743::TIL* and *ALD3::TIL* also show some increase in stress tolerance beginning at about 15 hours. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).

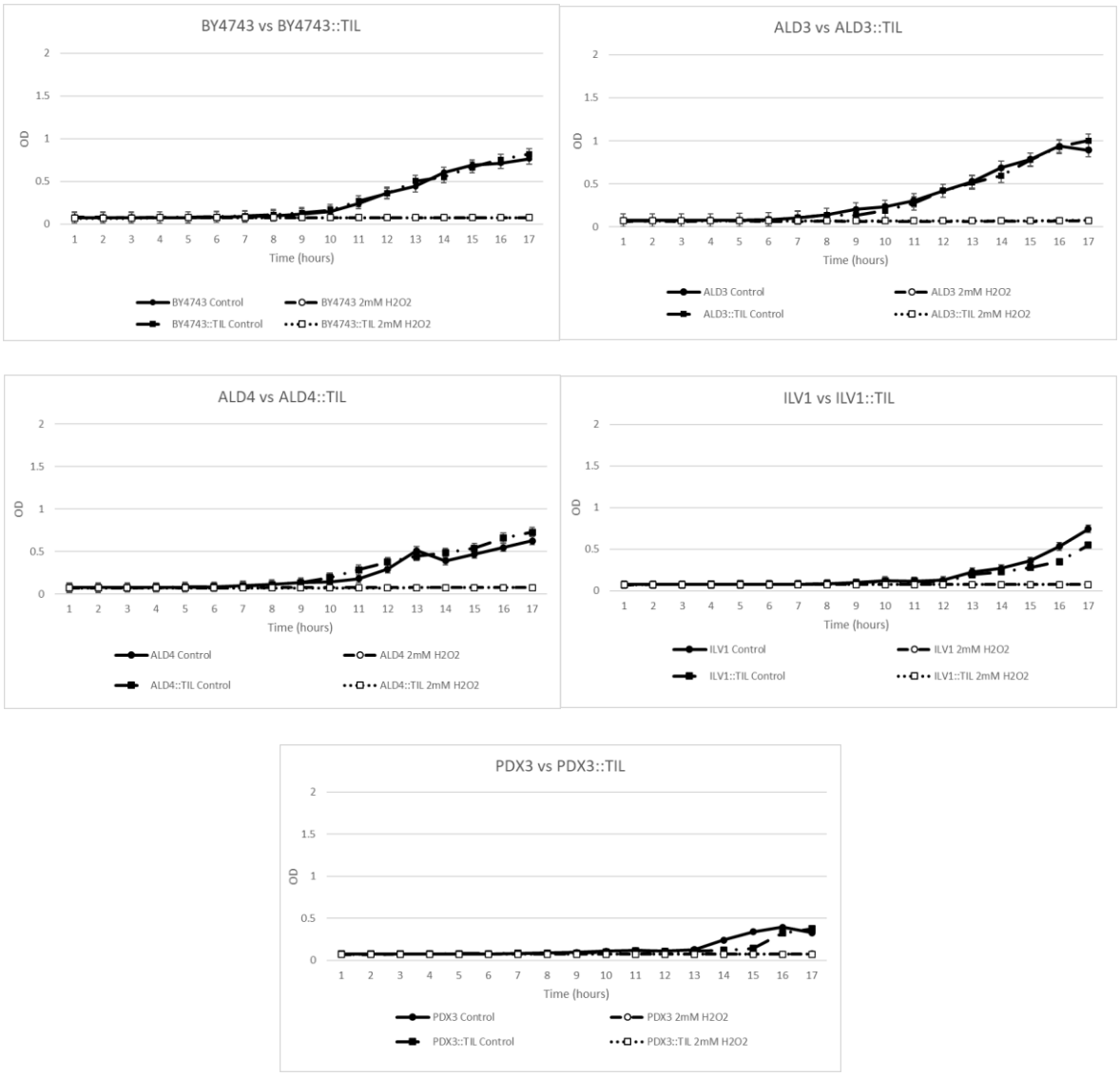



**Figure 4. 2mM $H_2O_2$ Oxidative Stress on *S. cerevisiae* (*At*-TIL)**

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::TIL*, *ALD3::TIL*, *ALD4::TIL*, *ILV1::TIL*, and *PDX3::TIL*) cells were grown in either synthetic complete media (SCM) or SCM+2 mM $H_2O_2$ in a 96-well plate and placed in the Microplate reader under continuous shaking for 17 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that none of the strains exposed to the stress showed any growth, so none of the transformants showed increase stress tolerance against 2 mM $H_2O_2$. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).

## Osmotic

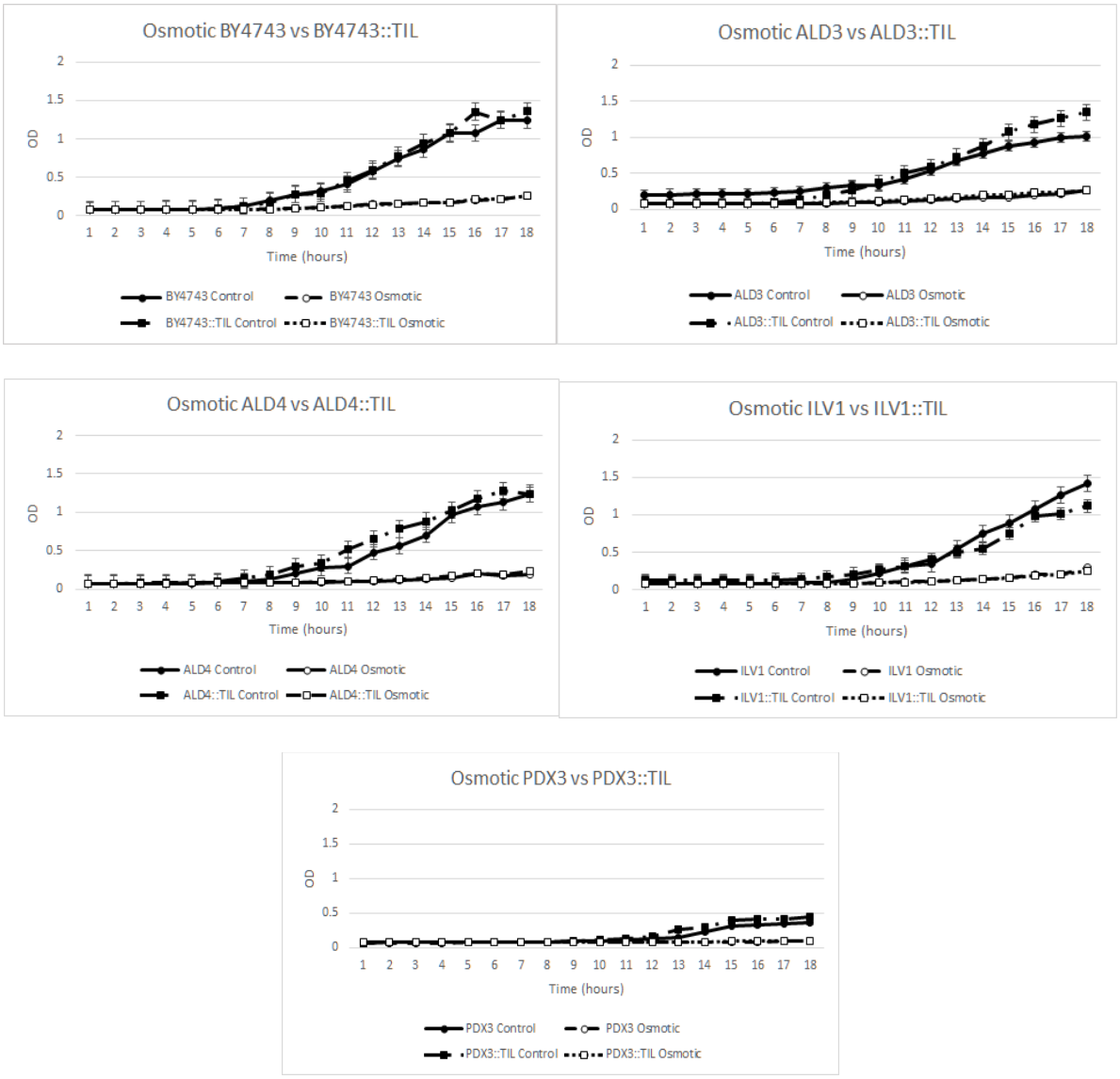
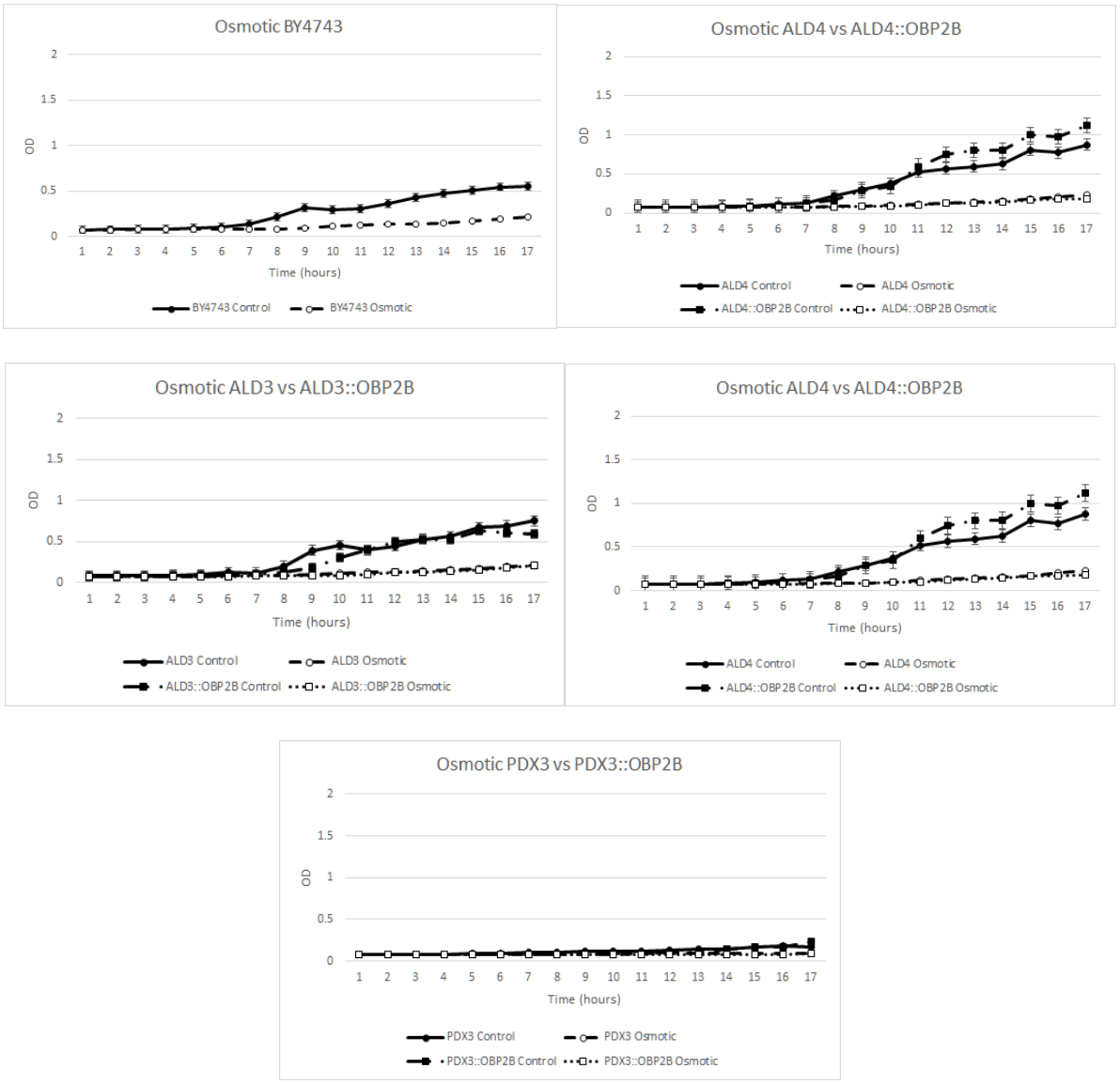



**Figure 5. Osmotic Stress on *S. cerevisiae* (*At*-TIL)**

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::TIL*, *ALD3::TIL*, *ALD4::TIL*, *ILV1::TIL*, and *PDX3::TIL*) cells were grown in either synthetic complete media (SCM) or SCM+1.0 M sorbitol in a 96-well plate and placed in the Microplate reader under continuous shaking for 18 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that the transformants showed no increase in stress tolerance when exposed to 1.0 M sorbitol. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).




## Figure 6. Osmotic Stress on *S. cerevisiae* (OBP2B)

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::OBP2B, ALD3::OBP2B, ALD4::OBP2B, ILV1::OBP2B*, and *PDX3::OBP2B*) cells were grown in either synthetic complete media (SCM) or SCM+1.0 M sorbitol in a 96-well plate and placed in the Microplate reader under continuous shaking for 18 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that the transformants showed no increase in stress tolerance when exposed to 1.0 M sorbitol. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).

## Salinity

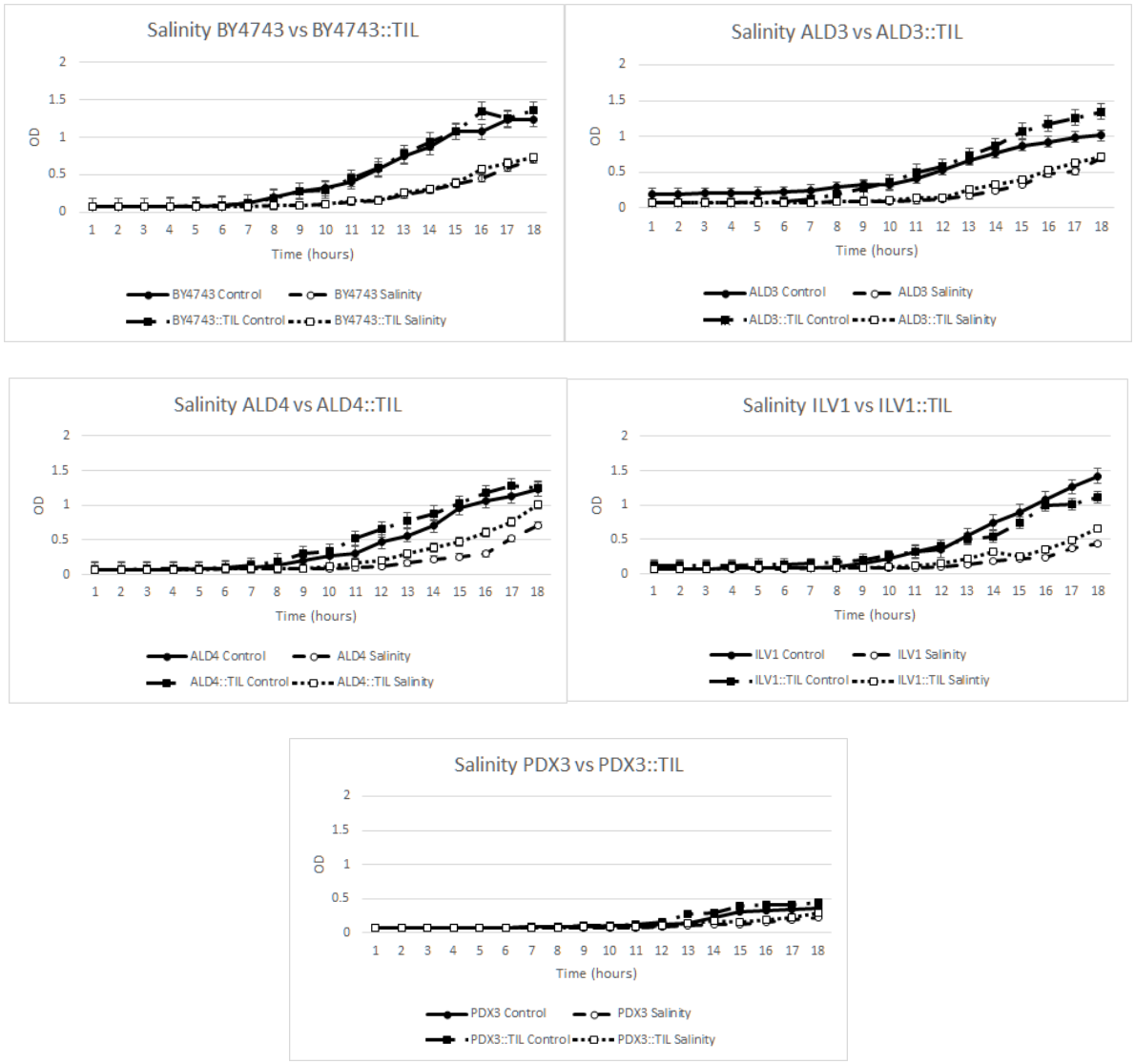
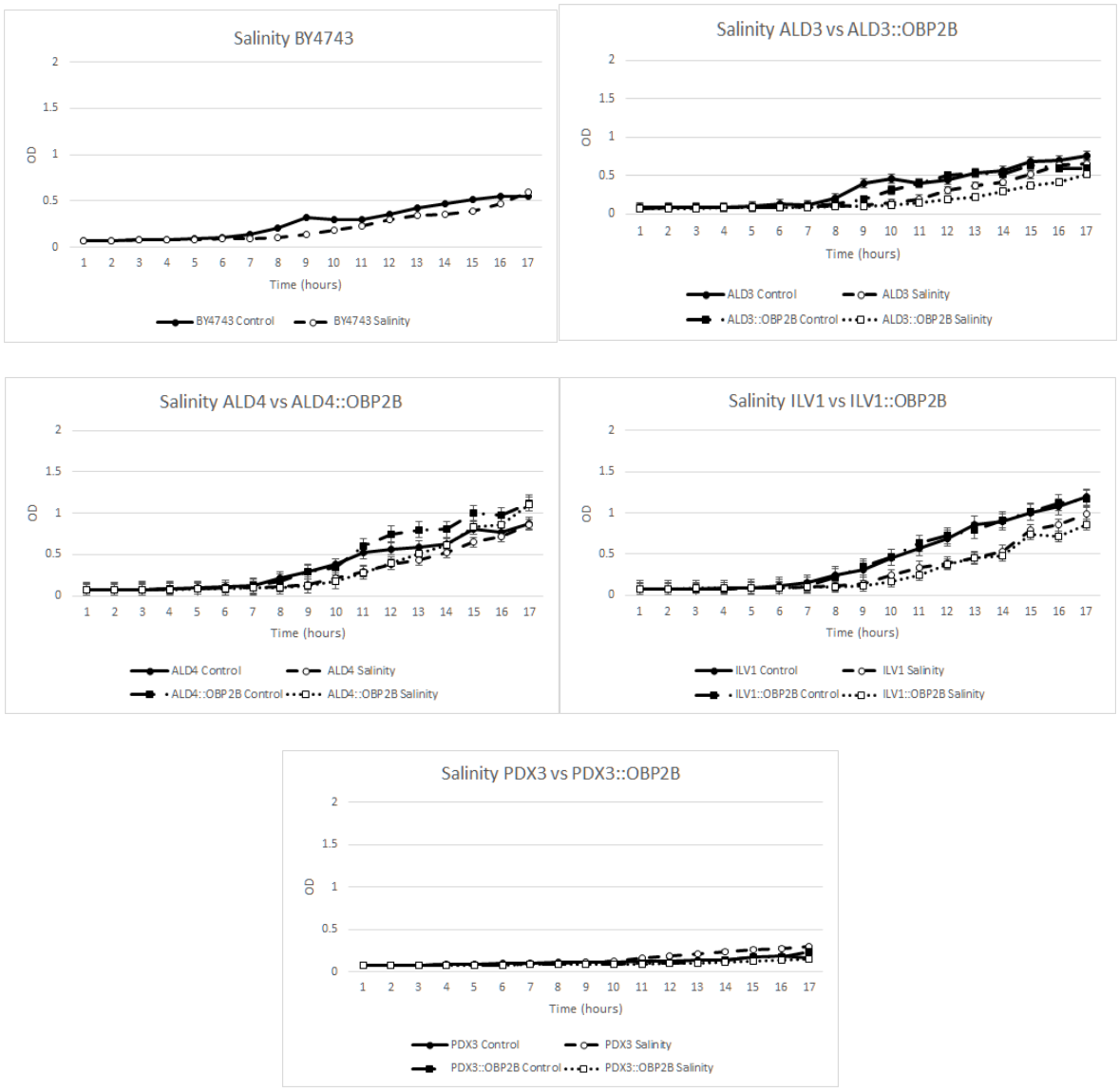



**Figure 7. Salinity Stress on *S. cerevisiae* (*At*-TIL)**

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::TIL*, *ALD3::TIL*, *ALD4::TIL*, *ILV1::TIL*, and *PDX3::TIL*) cells were grown in either synthetic complete media (SCM) or SCM+0.8 M NaCl in a 96-well plate and placed in the Microplate reader under continuous shaking for 18 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that *ALD4::TIL* and *ILV1::TIL* showed an increase in stress tolerance when exposed to 0.8 M NaCl. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).




## Figure 8. Salinity Stress on *S. cerevisiae* (OBP2B)

This experiment was carried out in triplicate, with three samples for each of the controls and three samples for each of the yeast strains exposed to the stress. The wildtype (*BY4743*), knockout (*ALD3*, *ALD4*, *ILV1*, and *PDX3*), and transformant (*BY4743::OBP2B*, *ALD3::OBP2B*, *ALD4::OBP2B*, *ILV1::OBP2B*, and *PDX3::OBP2B*) cells were grown in either synthetic complete media (SCM) or SCM+0.8 M NaCl in a 96-well plate and placed in the Microplate reader under continuous shaking for 17 hours. Averages of each of the triplicates was determined for each hour, and a growth curve was produced. It was determined that the transformants are irresponsive to the salinity stress. Furthermore, *ALD3::OBP2B*, *ILV1::OBP2B*, and *PDX3::OBP2B* show a decrease in stress tolerance when exposed to 0.8 M NaCl as compared to the respective knockouts. Each data point represents the means of three replicates (the standard error $P<0.05$ Students t-test).

## DISCUSSION

### Heat Stress

Compared to the wildtype and knockout strains, *ALD4::TIL* showed increased stress tolerance against heat when exposed to 50°C for 10 minutes (figure 2). *ALD4* is the gene that codes for mitochondrial aldehyde dehydrogenase (ALD4). ALD4 is an enzyme that plays a role in scavenging in response to oxidative stress.[36] Because the TIL also functions in stress response against heat and oxidative stress, we see an increase in stress tolerance against those stressors because TIL becomes a source of relief for the transformant cells in the absence of ALD4. Two of the most important products of these heat shock response (HSR) are the synthesis of trehalose and heat shock proteins.[37] Heat shock proteins are involved in many routine cell functions. They assist in the folding, protecting and movement of newly synthesized proteins. Fungi and invertebrates only posse one form of the HSF. Although it is not clear what role lipocalins may play in heat shock response, we suspect that their ability to cluster and binding to other molecules may provide the protective effect to heat stress. Furthermore, heat stress may disturb the normal cell metabolic process, which could lead to synthesis of compounds similar to those caused by the oxidative stress and consequently triggering similar stress response.

### Oxidative Stress

*BY4743::TIL*, *ALD3::TIL*, and *ALD4::TIL* showed increased stress tolerance against oxidative stress when exposed to 1 mM $H_2O_2$ (figure 3), but none of the transformants showed increased tolerance when exposed to 2 mM $H_2O_2$ (figure 4). It is believed that *At*-TIL participates in the scavenging of reactive oxygen intermediates (ROIs) either exogenously applied or generated after abiotic stress conditions. ROIs are able to damage DNA leading to sugar damage,

single strand breaks, and DNA-protein crosslinking.[38] ROIs also have the ability to damage lipids, leading to impairment of the structure integrity of the membrane and increases membrane fluidity and damage proteins leading to protein crosslinking.[38] TIL may act in preserving cell viability and restoring the integrity of cell membranes, a mechanism previously described in plants.[17, 39, 40]

**Osmotic Stress**

The cells showed no improvement in osmotic stress tolerance with either of the lipocalins, *At*-TIL or OBP2B, when exposed to 1.0 M sorbitol (figure 5 & figure 6). TIL has been implicated in a myriad of stress responses which may protect yeast cells from stress induced damages that can occur during industrial processes.[3] However, most lipocalin proteins seem to have just a transient protective effect to most stresses, particularly those whose protective effect depend on the binding of lipocalins to specific stressor agents.[3] Such protective model mechanism could be explained if considering the possibility of saturation of lipocalins binding site by the stressor agents. These findings could explain the lack of osmotic stress alleviation by either of the lipocalins.

**Salinity Stress**

The *ALD4::TIL* and *ILV1::TIL* transformants showed increased stress tolerance against salinity when exposed to 0.8 M NaCl (figure 7), while the OBP2B transformants seem to be irresponsive to the salinity stress. The odorant binding proteins are notable for their ability to bind to volatile compounds[1,18]. They mediate the transport of odorant molecules from the hydrophobic gas phase in the environment across the hydrophilic barrier of the aqueous mucus to the hydrophobic binding site of olfactory receptors of sensory neurons found in the nasal

epithelia.[1,18] There are no indication in the literature that they may play any role in stress alleviation. They specifically bind to the organic volatile compounds and not to inorganic molecules such NaCl. Moreover, they function as scavengers or as neutralizers of toxic volatile molecules.[18] Their putative involvement in stress alleviation may be tissue and stresses specific, considering that OBP2B is primarily expressed in prostate and mammary.[18] Additional studies of function of this protein, isolated or combined with other lipocalins under a myriad of stresses, may deem necessary for better understanding and characterize the function of OBP2B.

**Limitations**

Because our experimentation was conducted at a small scale, the 96 well microplate reader, we are faced with some limitations. For the oxidative, osmotic, and salinity stress, the cells were grown in 100 µL on a 96-well plate for 17-18 hours. One approach to combat this is to widen the time frame of the growth curves to determine if the stress tolerance results are maintained or if there is actually a significant difference between the knockout and transformant strains. Additionally, the small volume of the medium poses a stress on the cells as the nutrients begin running out. A smaller ratio of inoculum to media would be needed to combat this limitation. The growth rate experiments could be run at larger volumes in culture tubes to provide a greater amount of nutrients to a smaller inoculum of yeast. Particularly, this was a broad study, which included four yeast knockouts and the wild-type strain and two different lipocalins (the OBP2B and the *At*-TIL). This approach creates some level of difficulty for specific stress response analysis. Most research available in the literature just analyzes specific effects of the lipocalin without relating it to a specific stress mechanism created in a gene knockout.

## CONCLUSION

Understanding how some lipocalins may be inhibiting or causing a reduction in stress resistance is important for determining which lipocalins function in stress tolerance when transformed into yeast cells and which ones cause cell viability loss.

Further experimentation with different genes and/or strains would give us a greater understanding of their potential roles in industrial fermentation as well as an in-depth knowledge of the underlying mechanisms employed by the yeast in response to a variety of stressors to further improve fermentation yeasts. Future work would involve engineering a genetic construct, where several lipocalins are placed in tandem and transformed into the wild type *S. cerevisiae*. The transformant yeast cells could potentially withstand abnormal levels of industrial stressors from various factors.

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