The effect of ethanol on the growth of wild-type and *pdc*1 mutant *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae produces energy by alcohol fermentation, as well as oxidative phosphorylation. An important component of fermentation is ethanol as a product of pyruvate metabolism and a reactant for energy production. We investigated the effects that the addition of ethanol has on the growth rates of wild-type and *pdc*1 mutant *S. cerevisiae*. Three concentrations of ethanol were added to wild-type and mutant yeast media: 0, 2.5 and 5 g/L. The media were kept at 30ºC and cell concentrations were determined every 1.5 hours for 6 hours. Analysis of our results, by two-way ANOVA testing, suggests that the *pdc*1 mutation has a negative effect on the growth rate, ethanol does not have an effect on growth rate, and ethanol does not have different effects on the growth rates of wild type and *pdc*1 mutant *S. cerevisiae*. Our results also suggest that our experiment ended during log phase. Trends suggest that if we had continued our experiment past the six-hour time, we may have seen a significantly negative effect of ethanol on growth rates of *pdc*1 mutant but not wild-type *S. cerevisiae.*

Introduction

Saccharomyces cerevisiae is a unicellular eukaryotic organism that is used in baking, wine and beer making (Steensels *et al.* 2014). For at least 31% of yeast genes, there is a homologous mammalian gene, which makes it a useful organism for studying eukaryotic biological functions (Botstein, Chervitz & Cherry 1997). Ethanol, carbon dioxide, and energy are rapidly produced as a result of yeast pyruvate metabolism, through fermentation and oxidative phosphorylation (Figure 1) (Pronk *et al.* 1996). In anaerobic conditions, pyruvate is converted to acetaldehyde, which is then reduced to ethanol and acetate. Acetate is then converted to acetyl-CoA. In aerobic conditions, pyruvate is converted to more acetyl-CoA, which is metabolized to produce carbon dioxide and energy via the citric acid cycle (Pronk *et al.* 1996).

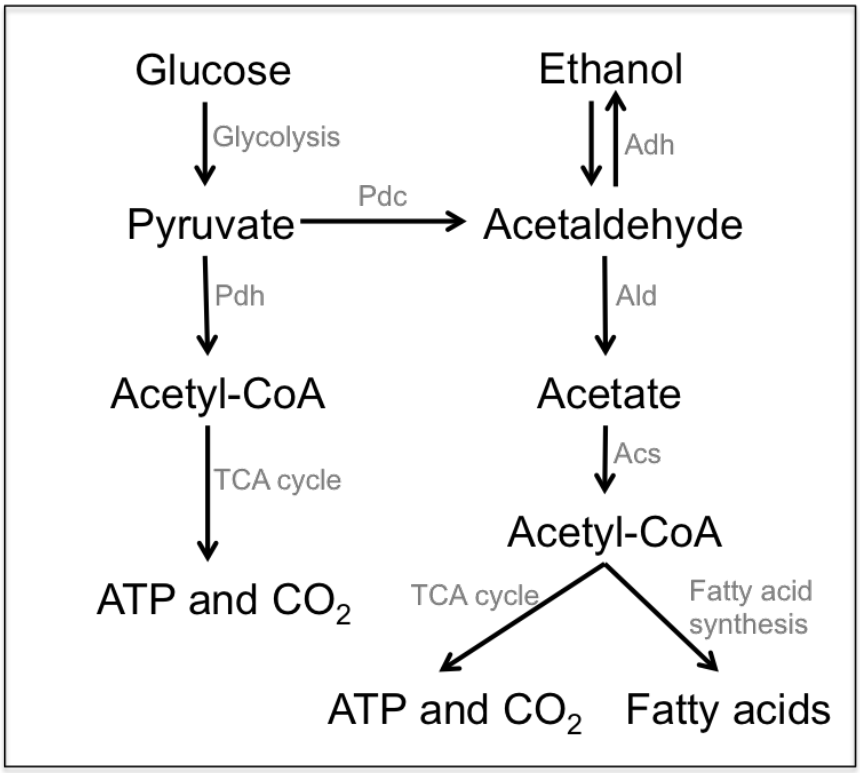


Figure 1. Pyruvate metabolism in *S. cerevisiae*. Pdh, pyruvate dehydrogenase; Pdc, pyruvate decarboxylase; Adh, alcohol dehydrogenase; Ald, acetaldehyde dehydrogenase; Acs, acetyl-coenzyme A synthetase.

Pyruvate decarboxylase (*pdc*) is the enzyme responsible for converting pyruvate into acetaldehyde and carbon dioxide, primarily in anaerobic conditions (Pronk *et al.* 1996). The YLR044C mutation affects *pdc*1, which is a structural pyruvate decarboxylase gene (Seeboth, Bohnsack & Hollenberg 1990). *pdc*1 mutations decrease pyruvate decarboxylase activity to 60-70% compared to the wild type, suggesting that another gene homologous to the *pdc*1 gene is expressed at higher rates in such mutants. Sequencing lead to the identification of this compensating gene, *pdc*5 (Seeboth, Bohnsack & Hollenberg 1990).

The objective of our experiment was to test the effects of ethanol addition on growth rates of wild-type and mutant yeast. We proposed that ethanol addition would affect yeast growth rates through the following proposed mechanism: Alcohol dehydrogenase is an enzyme responsible for both the reduction of acetaldehyde to ethanol, and the reverse oxidation of ethanol to acetaldehyde (Abuin, Lissi & Leon 2008). By the mechanism in Figure 1, if the added

ethanol increases acetaldehyde production and ultimately leads to increased acetyl-CoA, growth rates will increase. However, ethanol can also serve as an inhibitor of growth, and even cause cell death at high concentrations (Stanley *et al.* 2010). If this occurs, growth rates are expected to decrease.

Research involving rapidly reproducing *S. cerevisiae* has been important in the medical field for studying tumor cell growth and vaccine production (Botstein & Fink 2011). Studying reduction-oxidation reactions between acetaldehyde and ethanol can also be useful for industrial processes involving the fermentation of yeast (Steensels *et al.* 2014).

Our first null hypothesis is that ethanol will have no effect on the growth rate of wild-type *S. cerevisiae*, and our first alternate hypothesis is that ethanol will have an effect on the growth rate of *S. cerevisiae*. We predict that the ethanol will have a positive effect on the growth rates of wild-type yeast due to the reaction that reduces ethanol to acetaldehyde by the enzyme alcohol dehydrogenase (Abuin, Lissi & Leon 2008). This will ultimately lead to acetyl-CoA production for the TCA cycle (and energy production).

Our second null hypothesis is that loss of function of the *pdc*1 gene will have no effect on the growth rate of *S. cerevisiae*. Our second alternate hypothesis is that the loss of function of the *pdc*1 gene will have an effect on growth rate. We predict that the loss of function of the *pdc*1 gene will have a negative effect on growth rate. Previous research by Seeboth, Bohnsack and Hollenberg (1990) shows that *pdc*1 mutations decrease pyruvate decarboxylase activity to 60-70% to that of wild-type *S. cerevisiae*, and this leads to blocking the conversion of pyruvate to acetaldehyde. We predict that this will cause a build-up of pyruvate which will stunt energy production through glucose metabolism.

Our third null hypothesis is that ethanol will not have differing effects on the growth rates of wild-type and *pdc*1 mutant *S. cerevisiae*. Our third alternate hypothesis is that ethanol will have differing effects on the growth rates of *pdc*1 mutant and wild-type *S. cerevisiae*. We predict ethanol will have a more positive effect on growth rates of *pdc*1 mutant than that on wild-type *S. cerevisiae*. Because of the 60-70% decrease in pyruvate decarboxylase activity of *pdc*1 mutants identified by Seeboth, Bohnsack, and Hollenberg (1990), we predict *pdc*1 mutant *S. cerevisiae* will benefit more from a mechanism that bypasses the pyruvate decarboxylase reaction to produce energy.

Methods

Determining Original Cell Concentrations of Stock Solution

First, we determined the original concentrations of our wild-type and mutant samples by fixing with glutaraldehye and counting using a haemocytometer. . We calculated the cell concentration of wild-type stock to be 8.72×10^7 cells/mL and 3.68×10^7 cells/mL for the mutant stock.

Dilutions

Since our experiment required a cell concentration of 2.5×10^5 cells/mL for our treatments, we diluted the wild-type stock by adding 68.8 µl into 29.93 mL YPD (yeast extract peptone dextrose) medium. We chose this cell concentration because it was used by previous work in which similar experiments to ours were done (Shokoohi *et al.* 2016). For the mutant stock solution, we diluted the cells by adding 163µl into 29.84 mL YPD solution. We used YPD medium because it provides the proper conditions, such as glucose and amino acids, for yeast growth (Maresova *et al.* 2006).

Treatments

Using the diluted stocks of yeast, we prepared three secondary stocks solutions with volume of 30 mL for each of our wild-type treatments (wild-type control, wild type with 2.5 g/L ethanol, wild type with 5 g/L ethanol). We prepared another three secondary stock solutions with similar volumes for each of our mutant treatments (mutant control, mutant with 2.5 g/L and mutant with 5 g/L ethanol). For wild-type and mutant treatments with 2.5 g/L ethanol, we added 100 µl of ethanol to 30 mL of cell solution. For wild-type and mutant treatments with 5 g/L ethanol, we added 200 µl of ethanol to 30 mL of cell solution. We chose these ethanol concentrations because they were used by previous work (Flikweert *et al.* 1996). Since the volumes of ethanol additions relative to the cell solutions were relatively quite low (0.33 % of cell solutions for 2.5 g/L and 0.66 % of cell solutions for 5 g/L), we considered them negligible in terms of volume changes. Consequently, we did not add any liquid to the control treatments. In the above treatments, we used 95% ethanol rather than 100% because addition of 100% ethanol leads to protein coagulation, disrupts protein denaturation, and is toxic to yeast cells. The overall impact on cells is that this results in interruption of cell metabolism and cell lysis (Block 2001).

Replicates

We set up four replicates from each of our secondary stock solutions by adding 5 mL individually to 24 test tubes (Figure 2). We used four replicates because they would give us greater confidence in our results by increasing statistical power, ensure differences between treatments are due to independent variables and not by chance, and inform us of more representative values of cell numbers in every treatment (Vaux *et al.* 2012). We placed them in two test tube racks and set in water bath at 30^{o}C.

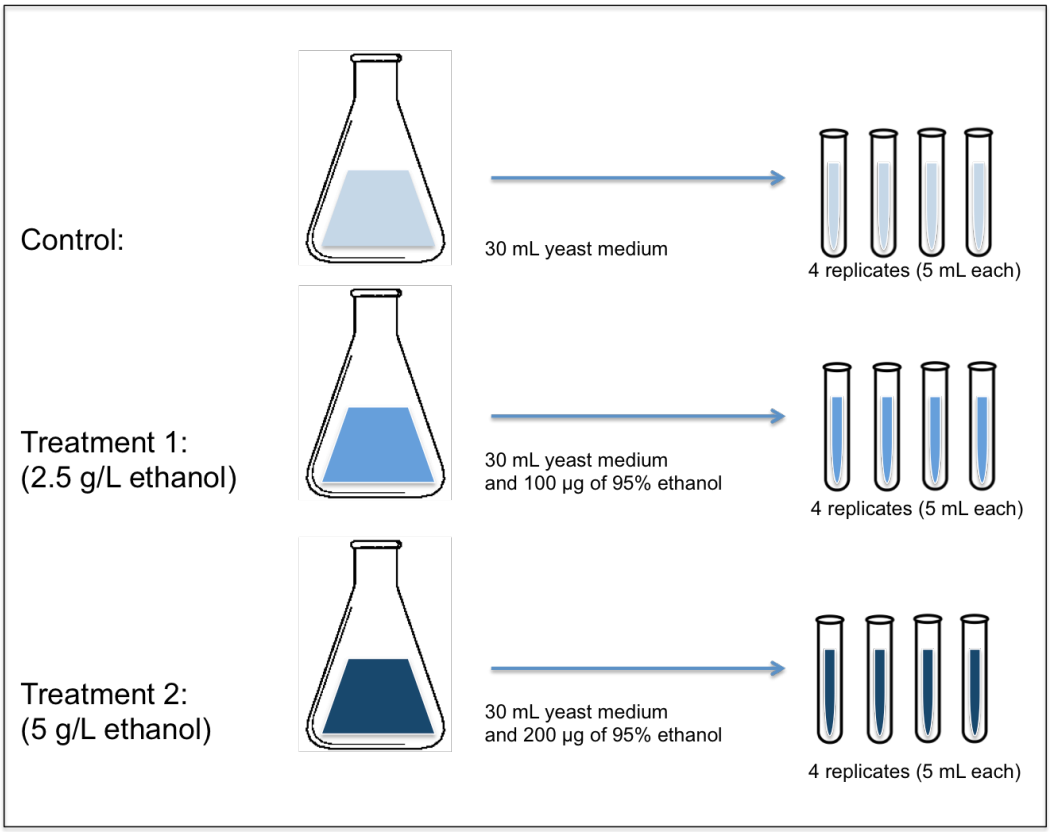


Figure 2. Replicate preparation procedure. This procedure was implemented for both wild-type and mutant yeast medium.

Data Collection

Over the course of six hours, we counted the number of cells present every 1.5 hours (time (t)= 0 h, 1.5 h, 3 h, 4.5 h, 6 h) in each of tubes for our treatments. We used 1.5 hour intervals because the optimum doubling time for yeast is 90 minutes (Bergman 2001). After each interval, we removed 100 µl aliquots from each of these replicates, fixed and counted the *S. cerevisiae* cells using an Axio Microscope and hand tally clicker.

For our experiment, we ensured that the temperature of the water bath was at 30^{o}C

Analysis of Data

To analyze our raw data, we calculated growth rates for each of our replicates. To calculate growth rates, we used the formula: (final cell concentration – initial cell concentration) / 6h. We chose this method of calculation because our data did not have a high amount of

variation among replicates. The growth rate was averaged for each treatment. We then used the two-way ANOVA statistical test to determine the statistical significance in the mean difference in growth rates between wild-type and mutant *S. cerevisiae* in media with different concentrations of ethanol.

Results

The two-way ANOVA test on yeast growth rates resulted in *p*-values of 0.878, 0.000198, and 0.408. The *p*-value 0.878 applies to hypothesis one: ethanol will have an effect on the growth rate of wild-type *S. cerevisiae*. The *p*-value 0.000198 applies to hypothesis two: loss of function of the *pdc*1 gene will have an effect on the growth rate of *S. cerevisiae*. Moreover, the *p*-value 0.408 applies to hypothesis three: ethanol will have differing effects on the growth rates of wild-type and *pdc*1 mutant *S. cerevisiae*. Only the *p*-value from the second hypothesis is statistically significant, indicating that there is a significant difference in growth rates between *pdc*1 mutant and wild-type *S. cerevisiae*.

S. cerevisiae cultures grown in ethanol displayed no significant difference in growth rates compared to wild-type or mutant controls with no ethanol. As time progressed, growth occurred in both wild-type and mutant yeast.

The growth rate of wild-type yeast compared to mutant can be seen in Figure 3. Based on Figure 3, we see a difference between mutant and wild type yeast growth over time. Although no significant trend can be seen between wild-type growth rate in different concentrations of ethanol, we see that mutant growth rates decreased from 1.47×10^6 in 0g/L ethanol to 5.3×10^5 in 5g/L ethanol. Throughout all treatments, mutant growth rates were lower than those of wild-type

S. cerevisiae. This visual representation in Figure 3, is consistent with our statistical analysis from the two-way ANOVA indicated above, suggesting this is statistically significant.

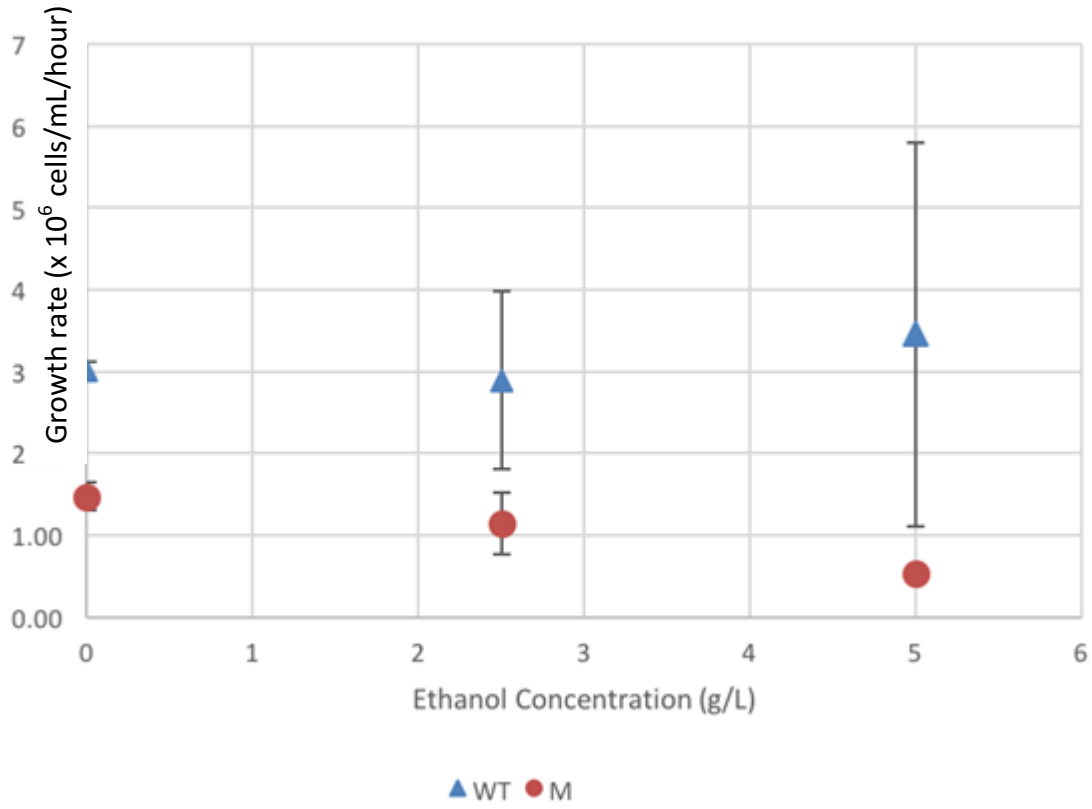


Figure 3. Growth rates of wild-type and *pdc*1 mutant *S. cerevisiae* in ethanol concentrations 0g/L, 2.5g/L and 5g/L. Error bars represent the 95% confidence intervals. WT, wild-type; M, mutant.

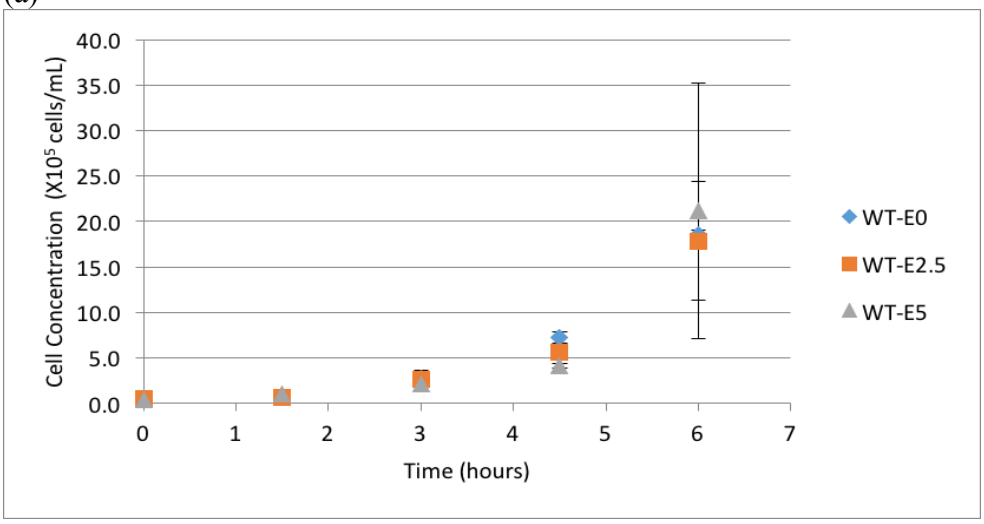
The two- way ANOVA resulted in a *p*-value 0.878 for our hypothesis one. Both strains of yeast are able to continue grow in the presence of ethanol, however, there is no significant difference of growth rates between ethanol and the control; no ethanol.

Figure 3 shows that mutant growth rate decreases as ethanol concentration increases. However, this trend is not significant since our *p*-value from our third hypothesis is 0.408. This graph also indicates a wide range of variation in values, particularly for wild-type yeast.

Figure 4 represents the results from our growth rate calculations for wild-type and mutant yeast. This figure also shows that ethanol does not have a significant impact on the growth rates

of either wild type or mutant yeast, with more temporal detail than included in Figure 3. The data in Figure 4 were summarised and used to analyze the differences between mutant and wild-type growth rates over time, which are shown in Figure 3. 95% Confidence Intervals are included in the figures but are only detectable on the graph at the sixth hour.

(a)



(b)

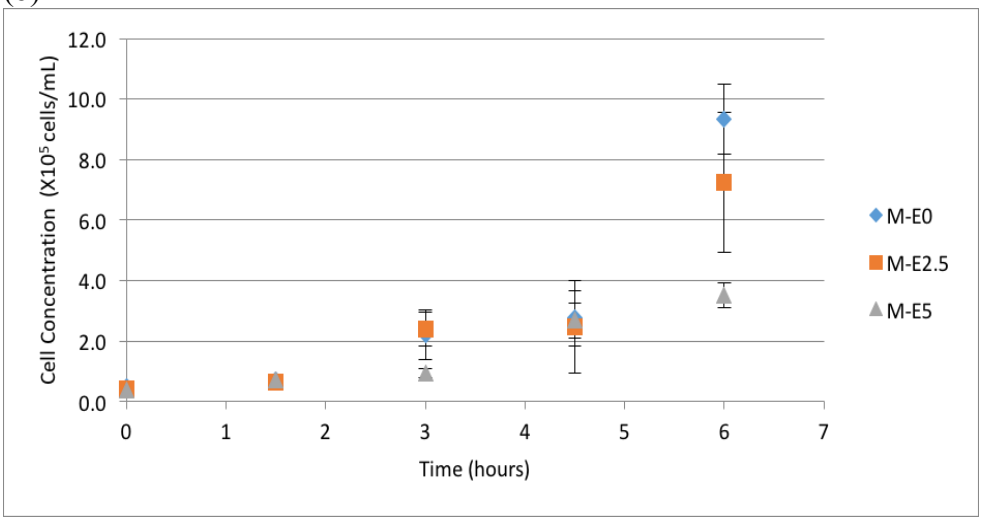


Figure 4. (a) Growth curve of wild-type *S. cerevisiae* at 0, 2.5, and 5 g/L ethanol over a time period of 6 hours. Error bars show 95% confidence intervals. (b) Growth curve of *pdc*1 mutant *S. cerevisiae* at 0, 2.5, and 5 g/L of ethanol over the time period of 6 hours. Error bars show 95% confidence intervals. WT, wild-type; M, mutant.

Discussion:

Our first hypothesis was regarding the effect of ethanol on wild-type *S. cerevisiae*. The statistical analysis testing this hypothesis yielded a *p*-value of 0.878. Since this value is greater than 0.05, we fail to reject our null hypothesis that ethanol will not have an effect on the growth rate of wild-type *S. cerevisiae*. The data do not support our prediction that ethanol should have a positive effect on the growth rate of wild-type *S. cerevisiae*. This was surprising due to our previously proposed mechanism involving the uptake and conversion of ethanol to acetaldehyde, which can be used in the TCA cycle to increase growth rates (Figure 1). The wild-type data (Figure 4a) show that there was no apparent effect of ethanol addition on the growth rate of wild-type *S. cerevisiae*. There are several possible explanations for this observation. First, it is possible that the amount of ethanol that was added to our samples (2.5 or 5g/L) was not enough to elicit an effect on the growth rate of wild-type *S. cerevisiae*. Previous work has reported changes in growth rates with similar ethanol concentrations to those used in our study (Flikweert *et al.* 1996). However, the only growth rates reported in this work were that of triple-*pdc* knockout mutants and the culture used for their growth contained only ethanol. Thus, the discrepancy between our data and this work may be occurring because we included ethanol and glucose in the media of ethanol treatments. We also assumed that ethanol concentrations that elicited a change in growth rates in *pdc* mutants would be enough to also elicit changes in wild-type growth rates. However, other reports show that ethanol concentrations of 5 to 15% of the culture solution are needed to cause varying physiological effects to wild-type *S. cerevisiae* such as changes in fatty acid concentrations and enzyme dysfunction (Nagodawithana and Steinkraus 1976; You *et al.* 2003). Furthermore, from the wild-type data (Figure 4a), it may be that our experiment had ended when the cells were in log phase, a period of rapid growth in which cells

use up most of their nutrients until they plateau and reach stationary phase (Bergman 2001). If our experiment had continued for a longer period of time, we may have seen a difference between the growth rates of wild-type *S. cerevisiae* in differing concentrations of ethanol as the cells approached or arrived at stationary phase. A mechanistic explanation for this could be that because the *S. cerevisiae* were growing in glucose-containing media, they preferentially used glucose as their sole carbon source for energy production and did not need an alternative source of carbon, such as ethanol, until they had run out. Such an event would likely have occurred towards the end of log phase or the beginning of stationary phase. At stationary phase, the cells which were growing in ethanol-added media could have used ethanol as an additional carbon source and thus grown further as a result. Support for this prediction comes from reports which identify transcription factors that are activated by dephosphorylation only when glucose is depleted and cells switch to growth using ethanol (Weinhandl *et al.* 2014; Walther and Schuller 2001).

Our second hypothesis addressed the difference in growth rates between *pdc*1 mutant and wild-type *S. cerevisiae*. The statistical analysis testing this hypothesis yielded a *p*-value of 0.000198. Since this value is less than 0.05, it allows us to reject the null hypothesis that the loss of function of *pdc*1 will have no effect on growth rates. This also provides support for the alternative hypothesis that the presence of the *pdc*1 mutation has an effect on the growth rate of *S. cerevisiae*. Our results show that in all of our treatments mutant *S. cerevisiae* have slower growth rates than wild-type (Figure 3). These findings support our prediction that the *pdc*1 mutation will have a negative effect on the growth rate of *S. cerevisiae*. The rationale for this prediction is that the loss of function of pyruvate decarboxylase (which *pdc*1 encodes for) results in a buildup of pyruvate and slows down the production of energy through glucose breakdown

(Schmitt and Zimmermann 1982). These findings are confirmed by previous work showing that when grown in similar conditions to our experiments, *pdc*1 mutants exhibit decreased growth rates and increased pyruvate secretion compared to wild-type *S. cerevisiae* (Seeboth *et al.* 1990; Schaaff *et al.* 1989).

Our third hypothesis focused on the possibility of a differential effect of ethanol on the growth rate of *pdc*1 mutant compared to wild-type *S. cerevisiae*. The statistical analysis testing this hypothesis yielded a *p*-value of 0.408. Since this value is greater than 0.05, we fail to reject the null hypothesis that ethanol will not have differing effects on the growth rate of wild-type compared to *pdc*1 mutant *S. cerevisiae*. Although not statistically significant, a trend can be seen in the mutant data (Figure 4b) that is not seen in the wild-type data (Figure 4a). The mutant cell counts appear similar in every treatment until hour six. At this time, we see a noticeable decrease in cell numbers as ethanol concentration increases. We predict that this trend would have become more apparent had our experiment continued for a longer period of time. Our statistical analysis and this trend (Figure 4b) do not support our prediction that ethanol will have a more positive effect on the growth rate of *pdc*1 mutant than that of the wild-type *S. cerevisiae*; the trend seen in our mutant data suggest a negative effect on *pdc*1 mutant but not on wild-type *S. cerevisiae*. These findings suggest that the previously proposed mechanism, through which we predicted a rescue of growth rate deficits in *pdc*1 mutants (Figure 1), did not occur in our experiment. A biological explanation for the observed, but not statistically significant, negative effect of ethanol on the growth rate of *pdc*1 mutants may be due to lower concentrations of fatty acids available to cells. As previously outlined (Figure 1), acetate can be used to produce acetyl-CoA which can then be used for fatty acid synthesis (Pronk 1996). Previous work has demonstrated an important role for fatty acids, such as oleic acid, on the ability of *S. cerevisiae* to tolerate ethanol (You *et*

al. 2003; Alexandre *et al.* 1994). Thus, a decreased amount of acetyl-CoA available for lipid biosynthesis, a downstream effect of lower pyruvate decarboxylase levels (due to the *pdc*1 mutation), may cause a reduction in fatty acids that are important to the yeast's ability to tolerate ethanol (You *et al.* 2003). Moreover, the lack of rescue of these growth rate deficits can be explained by reports showing that the expression of *adh*2, the gene encoding for alcohol dehydrogenase 2, is downregulated in high-glucose conditions (Piskur and Compagno 2014; Gancedo 1998). *adh*2 is the isozyme of alcohol dehydrogenase that catalyzes the conversion of ethanol to acetaldehyde, a key step in our proposed mechanism (Figure 1). Thus, due to the fact that the culture medium used in our experimental setup contained a rich supply of glucose, we expect that not much acetaldehyde was produced in yeast exposed to ethanol due to the decreased expression of *adh*2. We predict to have continued seeing the negative effect of ethanol on mutant growth rates had our experiment continued, possibly due to lower fatty acid concentrations in mutant yeast. However, a reversal of this trend may have occurred when yeast populations reach stationary phase. At this phase, glucose would be depleted from the culture medium thus allowing the upregulation of *adh*2 and leading to an increased conversion of ethanol to acetaldehyde (Piskur and Compagno 2014; Gancedo 1998).

There are sources of variation and uncertainty observed in our data. We noticed that the amount of error and variation accumulated in our experiment for example, the proper mixing of a sample before its extraction, the precise depth within the sample at which extraction occurs, the precise location on the haemocytometer that counting is done, and most importantly a subjective difference in haemocytometer cell counts. The most variable data point is the sixth hour of WT-E5, and we believe that the variation was introduced when the samples were fixed. For example, it is possible that the last replicate, which is very different from the others, was collected without

proper mixing or from an area in the test tube that happened to be less representative of the entire test tube. We made an effort in our experimental design to minimize these sources of uncertainty and variation by collecting and counting four replicates from all of our samples and enforcing a strictly followed, check-list type procedure for the collection of every replicate.

Conclusion

Following statistical analysis by two-way ANOVA testing, we are able to reject our second null hypothesis (loss of function of the *pdc*1 gene will have no effect on growth rates of *S. cerevisiae*) and support our second alternate hypothesis (loss of function of the *pdc*1 gene will have an effect on the growth rate of *S. cerevisiae*). This finding supported our prediction that *pdc*1 mutants should have a negative effect on *S. cerevisiae* growth rates due to decreased pyruvate decarboxylase activity. However, we are unable to reject our first (ethanol will not have an effect on the growth rate of *S. cerevisiae*) and third null hypotheses (there will not be differing effects between *pdc*1 mutant and wild-type *S. cerevisiae*). These findings did not support our predictions that although ethanol addition to the culture media of *S. cerevisiae* will have a positive effect on the growth rate of both *pdc*1 mutant and wild-type strains, it will have a more positive effect on the growth rate of *pdc*1 mutants. This work may contain useful information for industrial processes involving the fermentation of yeast.

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