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Determining the effects of inositol supplementation and the *opi1* mutation on ethanol tolerance of *Saccharomyces cerevisiae*

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Abstract

The yeast *Saccharomyces cerevisiae* is an important microorganism for the ethanol fuel industry. As with many microorganisms, the production and accumulation of certain metabolites, such as ethanol, can have a detrimental effect on cell growth and productivity. Yeast cells containing a higher concentration of phosphatidylinositol (PI) in the cellular membrane, due to inositol supplementation in the growth media, have been shown to tolerate and produce higher concentrations of ethanol. The specific goal of our research was to assess the effects of inositol supplementation in the growth media as well as to compare the ethanol tolerance of the wild-type *S. cerevisiae* to a mutant, the *opi1* strain (*opi*=overproduction of inositol). The *OPI1* gene product is a negative regulatory factor that controls the transcription of the *INO1* structural gene, which encodes the enzyme catalyzing the limiting step in the biosynthesis of inositol, that is, the conversion of glucose-6-phosphate to inositol-3-phosphate. Upon the deletion of the *OPI1* gene, the cell will constitutively produce inositol, regardless of the extracellular inositol concentration. Inositol supplementation in cultures of wild-type cells increased ethanol tolerance in terms of cell viability. Cells grown in $-I$ media had a 20% higher specific death rate than cells grown in $+I$ media when exposed to 15% ethanol. The *opi1* strain, with the ability to constitutively produce inositol regardless of media composition, showed less inhibition of cell growth in the presence of ethanol than did the wild-type strain, particularly in inositol-free media. We conclude that the introduction of an *opi1* mutation in yeast results in an inherent increase in PI levels and constitutive biosynthesis of inositol that, in turn, will reduce the cost of supplementing inositol into the media to achieve a higher ethanol tolerance.

Introduction

The continued evolution of a domestic biofuels industry in the US hinges on expanding the understanding of microbial fermentation of sugars derived from starchy or lignocellulosic biomass. Countless microorganisms are capable of this task, and a handful of yeasts excel at the conversion of sugars into ethanol. The yeast *Saccharomyces cerevisiae* is the most widely used of all yeast species for the ethanol fuel industry. As with many microorganisms, the

production and accumulation of certain metabolites, such as ethanol, can have a detrimental effect on cell growth and productivity.

Of chief concern to the ethanol fuel industry is the maximum concentration of ethanol that *S. cerevisiae* and other microorganisms can tolerate while remaining productive and viable. Increasing this threshold concentration is critical for the economic feasibility of large-scale biofuel production facilities. Success in achieving higher ethanol concentrations during fermentation lessens the energy required to separate the ethanol and water fractions of the fermentation broth, a process that has an exponentially increasing energy requirement with decreasing ethanol concentration. Increasing the ethanol concentration in the fermentation product from 5% to 14% reduces the energy requirement for distillation by approximately 50%¹. Thus, expanding our understanding of the molecular mechanisms that confer ethanol tolerance for yeast and other microorganisms is an important goal of the industrial biotechnology community.

Assaying and defining ethanol tolerance has been a subject of debate due to the fact the ethanol toxicity and tolerance may vary depending on the fermentation regimen and the feeding rate of the carbon sources². In addition, it is clear that the toxic effects of ethanol vary depending on nutritional, environmental factors³.

The plasma membrane (the active barrier between the cytoplasm and the extracellular environment) is thought to be the primary target of ethanol damage for yeast and other microorganisms^{2,4–9}. Upon intercalation into the hydrophobic region of the cell membrane, ethanol opens the membrane structure, creating a more fluid environment and making the membrane more permeable to polar molecules. This process weakens hydrophobic interactions and affects the position and functionality of integral membrane proteins⁶. Intracellular membranes, most notably the mitochondrial membrane, are thought to be similarly affected by ethanol¹⁰. Through the increased permeability of these barriers, ethanol has the ability to directly or indirectly disrupt critical cellular processes such as nutrient transport, proton flux, and virtually every process that takes places within or across the membrane walls.

Changes in phospholipid composition clearly play a role in increasing the ability of yeast to tolerate ethanol^{10–14}. The composition of the major phospholipids in whole-membrane extracts of the yeast cell is, in general, well defined. Major phospholipids of yeast cells, depending on the growth regime, are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and lesser quantities of phosphatidylserine (PS), phosphatidylglycerol, and cardiolipin. The ionically charged hydrophilic head groups of phospholipids impart unique packing geometries to the membranes. Yeast cells are capable of altering the relative proportion of each phospholipid in response to environmental stressors¹⁵. It has been suggested that altering the ratio of charged head groups can affect ethanol tolerance. Clark and Beard¹⁶ found that decreasing the ratio of anionic-to-zwitterionic phospholipids in *E. coli*, by increasing PE content, rendered yeasts cells less tolerant to organic solvents. Additional reports find reduced ATPase inhibition by ethanol in cells containing higher proportions of PS⁹ or PI¹².

Yeast cells containing a higher PI concentration in the cellular membrane, due to inositol supplementation in the growth media, have been shown to tolerate and produce higher ethanol concentrations^{11,12}. One hypothesis that can be drawn from these studies is that increasing the proportion of PI in the cellular membrane of *S. cerevisiae* will enhance its ethanol tolerance. Inositol supplementation in growth media has reportedly caused an increase in the rate of PI synthesis as well as the cellular PI content in wild-type cells^{15,17–19}. The specific goal of our research was to assess the effects of inositol supplementation in the growth media as well as to compare the ethanol tolerance of the wild-type *S. cerevisiae* to a mutant, the *opi1* strain. The

OPII gene product is a negative regulatory factor that controls the transcription of the *INO1* structural gene, which encodes the enzyme catalyzing the limiting step in the biosynthesis of inositol (the conversion of glucose-6-phosphate to inositol-3-phosphate). Upon the deletion of the *OPII* gene, the cell will constitutively produce inositol, regardless of the extracellular inositol concentration. The *opiI* strain has been shown to accumulate higher levels of PI in the cellular membrane without media supplementation¹⁸. We report here on experimental studies designed to measure and compare the effects of inositol supplementation and the effects of the *opiI* mutation on ethanol tolerance of *S. cerevisiae* grown in the presence of exogenous ethanol.

Methods

STRAINS

S. cerevisiae wild-type diploid MVY#2008 in the W303 background and its mutant derivative MVY#2015 (*opiI-I* homozygous diploid) were used in all plating assays. *S. cerevisiae* wild-type haploid MVJY#19 in the S288C background and its mutant derivative MVJY#13 (*opiIΔ::KanMX4*) were used in all growth curve assays. The genotypes of these strains are presented in Table 1.

PREPARING THE INOCULUM

S. cerevisiae strains that had been stored at -80°C were plated onto YPD plates (1% yeast extract, 2% peptone, 2% dextrose [glucose], and 2% agar). Plates were incubated at 30°C for 48 hr (Low Temperature Incubator; Fisher Scientific, Pittsburgh, Pennsylvania). One colony from each plate was removed with a sterile loop and patched on a fresh YPD plate, incubated at 30°C for 48 hr, and stored at 4°C for further use. Small amounts of the single colony patch were removed from the plate with a sterile loop and suspended in 125 mL polycarbonate Erlenmeyer flasks containing 25 mL YPD (1% yeast extract, 2% peptone, and 2% glucose). The flasks were covered with screw caps and placed in an incubator-shaker (G24 Environmental Shaker; New Brunswick Scientific Company, Inc.; Edison, New Jersey) at 30°C and shaken at 250 rpm for 12 hr. Optical density (OD) at 600 nm was measured for each culture with a spectrophotometer (8453 UV-Vis Spectroscopy System; Agilent Technologies, Inc., Palo Alto, California). All further discussion of OD will imply measurement at 600 nm. The culture with an OD of 2–3 was selected for use in subsequent experiments.

ASSAY FOR OPI— PHENOTYPE

The Opi— phenotype was tested on both wild-type and *opiI* strains to ensure the absence/presence of this phenotype throughout all plating and growth curve experiments, as described by White et al²⁰. Composition of synthetic complete medium (which will be referred to as $-I$), is also described by White et al²⁰. Inositol-containing medium (which will be referred to as $+I$) and $+I$ plates (containing medium $+I$ plus 2% agar). The $+I$ liquid media and plates contain 75 μM *myo*-inositol.

A cell suspension of the tester strain, AID, was prepared by inoculating a single colony into a 250 mL Erlenmeyer flask containing 50 mL of YPD medium. The culture was incubated at 30°C and continuously shaken at 250 rpm for 24 hr. The cells were washed twice with sterile water and centrifuged at 4,500 rpm for 5 min. The supernatant was discarded after each washing. The cell pellet was resuspended in sterile water and stored at 4°C in a sterile glass sprayer. The diploid AID strain is homozygous for both the *ino1* and *ade1* markers (refer to Table 1 for genotype). Therefore, the AID strain exhibit a red color phenotype due to the *ade1* mutation and can only grow on inositol-containing media or $+I$ plates (inositol auxotrophy).

In brief, the Opi test consisted of placing small aliquot of each culture or patching a small amount of the strain to be tested onto –I plates. The –I plates are incubated at 30°C for 48 hr. After this incubation, the plates were sprayed with a fine mist of a suspension of the AID tester strain. The plates were further incubated at 30°C for 48 hr. A red halo of growth indicates the strain to be tested overproduces and excretes inositol into the –I media (Opi— phenotype)¹⁹.

PLATING ASSAYS

The method for examining ethanol tolerance using a plating technique was adapted from Chi et al¹¹. Inocula of wild-type and *opi1* yeast strains were prepared as previously described. Cells were centrifuged at 4,500 rpm for 5 min at 4°C (Sorvall Legend RT; Kendro Laboratory Products, Asheville, North Carolina), and the supernatant was removed. Cells were washed twice with –I media, centrifuged at 4,500 rpm for 5 min.

The cell pellets were resuspended in inositol-free media and the OD was measured. Cells were inoculated into 110 mL of synthetic media either containing (+I) or lacking inositol (–I) at a final OD of 0.1. The cell cultures were placed in an incubator-shaker at 30°C and 250 rpm until the OD reached 0.5.

Two sterile 50 mL centrifuge tubes were prepared by adding to each tube a volumes of cell suspension containing 1×10^8 cells. The cell number was previously determined by cell counting using a hemacytometer (Bright-Line Hemacytometer; Reichert Scientific Instruments, Buffalo, New York). The cells were centrifuged at 4,500 rpm for 5 min at 4°C and the supernatant was removed. The cells were washed twice with either –I or +I media as previously described, and the resulting supernatants were discarded. Ten mL of ethanol (10, 15, or 18% [v/v]) was added to one of the 50 mL centrifuge tubes containing a washed cell pellet, and this represented the “shocked treated culture.” As a control, 10 mL of sterile water was added to the second 50 mL tube containing a washed cell pellet, or “control culture.” Two 25-μL aliquots of the control culture were removed and plated on YPD plates, representing time-zero of the experiment. Both shocked and control cell suspensions were transferred from the 50 mL centrifuge tubes to sterile 25 mL glass tubes. The glass tubes were placed in an incubator-shaker at 30°C and 250 rpm.

Samples of both the shocked and control cultures were taken every hour for 4 hr. Shocked cells samples were diluted 1:1000 in their corresponding ethanol solutions (10, 15, or 18%) before plating. Control cells were diluted in sterile water at the same dilution factor as above. Duplicate 25 μL aliquots of the dilutions were plated onto YPD plates for each shocked or control cell cultures over the 4 hr time course. All plates were incubated at 30°C. Colonies were counted on all plates after 48 and 72 hr. Duplicate plate counts were averaged. Ethanol tolerance of each strain, in terms of cell viability, in either +I or I media over a range of ethanol concentrations, was expressed as a ratio of the number of colonies counted in the shocked culture at 72 hr to the number of colonies counted in the control culture at 72 hr. Cell viability in the control culture at time-zero of the experiment was assumed to be 100%.

Ethanol tolerance of each strain was calculated, in terms of cell viability (CV), using the number of colony forming units (CFUs) in the shocked and control cultures after 72 hr of incubation using the following equation:

$$CV = \frac{CFU_s}{CFU_N} \times 100 \quad (1)$$

where

CFU_S = average number of CFUs counted in shocked culture

CFU_N = average number of CFUs counted in nonshocked culture

Cell viability in the control culture at time-zero of the experiment was assumed to be 100%. All ethanol concentrations are volume-volume percentages (v/v), while all glucose concentrations are in weight-volume percentages (w/v).

GROWTH CURVE ANALYSIS

The method for examining ethanol tolerance by profiling yeast growth was adapted from You et al.²¹. Inocula of wild-type and *opi1* yeast strains were prepared as previously described (see *Plating Assays*) except for the use of a Marathon 21K/BR bench top centrifuge (Fisher Scientific, Inc.; Hampton, New Hampshire). The cell pellet was suspended in –I media and the OD was measured by pipetting 200 µL of the cell suspension into a clean well of a 96-well plate and reading OD in a plate reader (Synergy HT Multidetector Microplate Reader; Bio-Tek Instruments, Inc., Winooski, Vermont).

From the cell suspensions above, new cell cultures were prepared inoculating at a final OD of 0.05, 50 mL of +I or –I containing either 2% or 12% glucose and 0 or 5% ethanol. Each media type was performed in triplicate for both yeast strains. Flasks were placed in an incubator-shaker at 30°C and 250 rpm (Gyratory Water bath Shaker; New Brunswick Scientific, Edison, New Jersey). The OD of each culture was measured over a period of at least 36 hr or until the cell density remained unchanged for a period of no less than 12 hr. Triplicate OD measurements were performed for each sample and measurements were averaged.

Along with ODs, ethanol and glucose concentrations were determined for samples taken from both *opi1* and wild-type cells grown in I media containing 12% glucose and 5% ethanol. One 2-mL sample was removed from the flasks periodically, tested for OD, and immediately passed through 0.22 µm filters (Millex-GP Syringe Driven Filter Unit; Millipore Corporation, Billerica, Massachusetts) to remove all cells, thereby stopping any further consumption of substrate, nutrients, and/or products in the supernatant. Samples were stored at –20°C for further analysis. Glucose and ethanol concentrations were determined using a YSI 2700 Select™ Biochemistry Analyzer (Giargallo Scientific Company; Pittsburg, Pennsylvania).

Results & discussion

PLATING ASSAYS

Values for CV are plotted versus time in Figure 1 for the *opi1* and the wild-type strains after growth in –I media. For ethanol exposure of 10 and 15%, the *opi1* cells had higher CV values, and the rate of decrease in CV with time was less than that observed with wild-type cells. In 18% ethanol, CV values of *opi1* cells fell to near 0% in the first hour after growth in –I (results not shown). Similar results were obtained for the *opi1* and the wild-type strains after growth in +I media (Figure 2). In addition, for +I, *opi1* showed higher tolerance to 18% ethanol than the wild-type did in 15% ethanol after growth in +I.

To model the data presented in Figures 1 and 2 and to estimate specific death rates, a simple first-order kinetic model was fitted to the data using the nonlinear parameter estimation tool in KaleidaGraph (Synergy Software; Reading, Pennsylvania):

$$y = a \times e^{-dt} \quad (2)$$

where

a = initial percent cell viability at time zero

d = specific death rate (hr^{-1})

t = time (hr)

The resulting curve-fits obtained for this model and the experimental data are shown in Figures 1 and 2, and the estimates of the d values and the error associated with these estimates for all media types and strains are summarized in Table 2.

Estimates for d and the plots in Figures 1 and 2 clearly show the effects of ethanol on yeast cell viability. In 10% ethanol, d values were less than 0.1 hr^{-1} for all strains, regardless of whether inositol was present in the growth media or not. While the R^2 values reported in Table 2 for the 10% ethanol curve fits are very low (most likely due to the use of an exponential model, which is consistent with standard fermentation kinetics modeling, when a linear model might yield a better fit), the d values predicted by this model are consistent with a general trend of declining CV values after exposure to 10% ethanol. In 15 and 18% ethanol, estimated d values were lower for cells grown in the presence of inositol, +I, than those grown without inositol, -I, indicating that inositol supplementation in wild-type and *opi1* strains increased ethanol tolerance.

Cells carrying the *opi1* mutation had a clear advantage over the wild-type cells when exposed to 15% ethanol after growth in -I media. The value for d of wild-type cells was nearly four times that of *opi1* cells experiencing the same growth conditions and ethanol treatment (3.14 hr^{-1} for wild-type and 0.79 hr^{-1} for *opi1*). The difference in d values for the two strains after growth in +I media and exposure to 15% ethanol was nearly as sizeable: wild-type cells died at a rate just over 3.5 times that of *opi1* cells (2.61 hr^{-1} for wild-type and 0.71 hr^{-1} for *opi1*). It is interesting that the only incidence of significant survival in 18% ethanol occurred in *opi1* cells after growth in +I media. In fact, d was 38% lower than that of wild-type cells grown in +I media and exposed to 15% ethanol (1.61 hr^{-1} in *opi1* in 18% ethanol and 2.61 hr^{-1} for wild-type in 15% ethanol).

Of particular interest is the small variability in d values of *opi1* cells grown in +I and -I media. During exposure to 15% ethanol, the d estimated for *opi1* increased 11% after growth in -I media, in comparison to +I media (0.79 hr^{-1} after growth in -I, 0.71 hr^{-1} for +I). For the wild-type strain, however, the d value after growth in -I media was 20% higher than the d value after growth in +I (2.61 hr^{-1} after growth in -I, 3.14 hr^{-1} for +I).

The main distinction between these two strains of yeast is the inherent ability of *opi1* to produce inositol constitutively, due to the lack of negative regulation of the *INO1* gene. This trait allows the *opi1* mutant to maintain a higher level of both intracellular inositol and membrane PI content¹⁸. Assuming that this is the principal difference between the wild-type and *opi1* strains used in this study, one can infer that this alteration in inositol and PI content is responsible for the varied response to ethanol as demonstrated here. Furthermore, the results of the plating assays presented here support conclusions of two other reports on the influence of inositol on the ethanol tolerance of yeast: supplying inositol to yeast cells increases cell viability in the presence of added ethanol^{11,12}.

GROWTH CURVE ASSAYS

Cell growth, using OD as a surrogate for cell mass, versus time is plot in Figures 3–6 for wild type and *opi1* in +I and -I media containing 2% or 12% glucose and 0% or 5% ethanol. Error bars are also plotted for all data, representing the standard deviation of the triplicate measurements at each time point. A lower ethanol concentration than that used in the plating

assays was employed because of the increased sensitivity of cell growth to ethanol and glucose⁵. Previous studies employing this method of evaluating ethanol tolerance used media containing various concentrations of glucose: 2%²¹, 5%²², and 20%⁴. Osmotic pressure due to high glucose concentrations (generally above 5%) has been shown to inhibit growth rate². However, industrial ethanol production requires high glucose feedstocks in order to minimize energy requirements for distillation of fermentation products¹. In this study, growth curve assays for both strains were performed in a low glucose concentration (2%) to ensure the absence of osmotic effects from glucose, and in a high glucose concentration (12%) to more accurately portray the conditions in an industrial process.

The differences in growth between wild-type and *opi1* yeasts in growth media without ethanol is illustrated in Figure 3. In +I, with 2% and 12% glucose concentrations, the wild-type growth appears to surpass that of *opi1*, if only slightly. Reduced lag times and higher final cell concentrations were also observed for wild-type cells in 12% glucose (Figure 3). The enhanced growth of wild type cells in +I is possibly due to the de novo synthesis of inositol in *opi1* cells, requiring energy that could otherwise be used for cellular growth.

The differences in growth between wild-type and *opi1* yeasts in growth media containing 5% ethanol in +I media are shown in Figure 4. Wild-type and *opi1* had very similar growth curves in 2% glucose. In 12% glucose, the two strains appear to grow at a similar growth rate, but wild-type cells had a shorter lag phase.

It is interesting to note that while wild-type had a slight advantage over *opi1* in +I media containing 2% glucose without ethanol, the growth rates appear to be virtually equal when ethanol was present. This difference in the comparative profiles of the two yeast strains exposes a distinction in the *opi1* strain with regard to inositol production. This distinction is best investigated by performing experiments in -I media. The *opi1* strain has been shown to accumulate higher levels of PI in the cellular membrane in -I media than the wild-type yeast¹⁸. If PI is indeed responsible for increased ethanol tolerance, *opi1* cells should have an advantage over wild-type cells when grown in the presence of ethanol.

The growth curves for wild-type and *opi1* for growth in -I without ethanol can be seen in Figure 5. In 2% and 12% glucose, the growth profiles of the two strains appear to be virtually equal. It has been shown that the wild-type and *opi1* yeasts show similar growth trends in -I media containing 2% glucose¹⁸. Therefore these results are consistent with the literature. Addition of ethanol in -I media (Figure 6) created an environment in which the *opi1* strain had a clear advantage over the wild-type strain in both 2% and 12% glucose concentrations. While the lag phases of these strains are similar in both 2% and 12% glucose, growth rates and final cell concentration of the *opi1* mutant exceeded those of the wild-type.

The difficulty in discerning differences in lag phase, growth rate, and the extent of growth from simply looking at these plots is obvious from the preceding discussion. Specific growth rate, or the growth rate over time per unit of biomass, was calculated in order to provide a quantitative assessment of the impact of experimental conditions on growth rate. Specific growth rates were estimated for both yeast strains for all of the conditions mentioned using the following first-order kinetic equation:

$$y=a \times e^{\mu t} \quad (3)$$

where

a = initial cell concentration, in terms of OD

μ = specific growth rate (hr^{-1})

t = time (hr)

This equation was fit to the data presented in Figures 3 through 6 using the nonlinear parameter estimation tool in KaleidaGraph. The estimated values for μ for both strains in all growth conditions are summarized in Table 3.

It is typical for yeasts to experience a lag phase, during which there is no observable increase in cell concentration, when inoculated into fresh medium. In light of the fact that lag phase is dependent on many variables, and that lag phase in response to a given condition is not consistent among all strains of yeast, it is very difficult to model. While all cultures in this study were of similar age, multiple types of growth media were used, and each strain responded differently to each media type. Hence, lag phase data has been omitted for this analysis, and only exponential growth data has been used for the purpose of quantitative comparison of the wild-type and *opi1* strains.

The quantitative analysis of the μ values of the wild-type and *opi1* strains in all growth conditions is, in general, in agreement with the qualitative comparison of the complete growth profiles discussed above. As seen in Figures 3 through 6, both strains can have similar growth rates during the phase of exponential growth, but exhibit different lengths of lag phase and extent of fermentation, resulting in different final cell concentrations. Lag phase reflects the cells' ability to adapt to new media. Moreover, since lag phase and extent of fermentation are not included in this model, the specific growth rates here do not completely describe the cells' ability to deal with the effects of ethanol. While R^2 values for these curve fits were all above 0.90, a minimal number of data points in the exponential growth phase for some of the cultures resulted in μ values that did not necessarily agree with the qualitative assessment of the above plots.

A very indicative trend seen in Table 3 is that in 5% ethanol, μ values of *opi1* always exceeded those of the wild-type, regardless of inositol or glucose concentration, whereas growth rates in the same medium without ethanol were virtually equal. Although the increase in μ value in 5% ethanol for *opi1* was small in +I media (5% higher in 2% glucose and 16% higher in 12% glucose), it was quite significant in -I media (57% higher in 2% glucose and 33% higher in 12% glucose).

These results indicate that *opi1*, with the ability to constitutively produce inositol regardless of media composition, has a marked advantage over the wild-type yeast in environments where ethanol is present and inositol is not. As previously discussed, it is most likely the composition of the cellular membrane that imparts this advantage for the *opi1* yeast. The *opi1* strain has been shown to accumulate higher levels of PI in the cellular membrane without media supplementation of inositol¹⁸. Furukawa et al¹² showed that in the presence of ethanol, ATPase activity was inhibited to a lesser extent in cells having a higher proportion of inositol-containing phospholipids. The functionality of many cellular transport systems is largely dependent on the activity of the ATPase enzyme²³. Inhibition of the enzyme decreases the transport of sugar substrates and nutrients across the cell membrane and thus affects the growth and fermentation ability of the cell⁵. On this basis, it stands to reason that cells with an increased content of PI, as in the case of *opi1*, would have a higher resistance to ethanol. Additionally, altering the ratio of charged head groups has been discussed as a possible mechanism for increased ethanol tolerance¹⁶. Since PI is the major anionic phospholipid in yeast, it is possible that alterations in membrane charge are responsible for the increased tolerance in PI-enriched cells.

Evaluating growth in the presence of exogenous ethanol has been used extensively as a means of determining ethanol tolerance^{21,22,24}. Although the response of a given yeast strain to

exogenously imposed ethanol may not mirror the response of the same yeast to endogenously produced ethanol, this method provides a relatively quick means of gauging the ethanol tolerance of a strain. This method of adding ethanol into the media is especially useful when dealing with ethanol concentrations that are higher than the concentration naturally produced by the organism when, for example, investigating the effects of ethanol on the cell.

Conclusions

The following conclusions can be made regarding the effect of the *opi1* mutation on ethanol tolerance. Cells containing the *opi1* mutation exhibited higher tolerance to exogenous ethanol, in terms of cell viability, at ethanol concentrations that drastically affect the viability of the wild-type cell. The tolerance of the *opi1* strain in 15% and 18% ethanol is further increased when the yeast is grown in the presence of inositol. The *opi1* strain, with the ability to constitutively produce inositol regardless of media composition, showed less inhibition of cell growth in the presence of ethanol than did the wild-type strain, particularly in inositol-free media.

Inositol, PI, and phosphorylated forms of PI are essential components of many cellular processes. Phosphoinositides play an important role in membrane transport and lipid signaling. Species of phosphoinositides are specifically localized in the cell, providing signaling functions for various organelles, including the plasma membrane²⁵. Inositol synthesis is meticulously controlled in the yeast cell¹⁵.

Yeast cells containing a higher concentration of PI in the cellular membrane, due to inositol supplementation in the growth media, have been shown to tolerate and produce higher concentrations of ethanol^{11,12}. In these reports, increasing cellular inositol content through media supplementation had the following effects on yeast cells: increased cell viability, decreased nutrient leakage, higher PI concentrations, and subsequent higher ATPase activity when compared to cultures with limited inositol availability^{11,12}. Ethanol production has also been increased in some strains by inositol supplementation¹¹. These reports, in addition to the evidence presented here, clearly demonstrate the importance of cellular inositol and/or PI content in conferring ethanol tolerance in yeast. Moreover, the introduction of an *opi1* mutation in yeast results in an inherent increased in PI levels and constitutive biosynthesis of inositol that in turn will reduce the cost of supplementing inositol into the media to achieve a higher ethanol tolerance.

Another factor important for industrial production of ethanol for food or biofuels is the fact that the yeast is generally fermenting in high-concentration and perhaps high-solute feedstocks. As evident in Figure 5 in this report, a mutation in the *OPI1* gene will greatly ameliorate the growth defect observed in wild-type strains when growing in media with high osmolarity (12% glucose) and lacking inositol.

Despite the continued attention that this area of research has received over the past few decades, many questions still plague researchers concerning the physiological limits to which microorganisms can produce and tolerate high concentrations of ethanol. These questions must be addressed in order to fully exploit the true potential of microbial biofuel production.

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References

1. Jacques, K.; Lyons, TP.; Kelsall, DR. The Alcohol Textbook. Vol. 3. Nottingham University Press; Nottingham, United Kingdom: 1999.
2. Casey GP, Ingledew WM. Ethanol tolerance in yeasts. *CRC Crit Rev Microbiol* 1986;13(3):219–280.
3. Alper H, Moxley J, Nevoigt E, Fink G, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science* 2006;314:1565–1568. [PubMed: 17158319]
4. Beaven M, Charpentier C, Rose AH. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *J Gen Microbiol* 1982;128:1447–1455.
5. D'Amore T, Panchal CJ, Russell I, Stewart GG. A study of ethanol tolerance in yeast. *Crit Rev Biotechnol* 1990;9:287–304. [PubMed: 2178780]
6. Ingram LO, Buttke TM. Effects of alcohols on micro-organisms. *Adv Microb Physiol* 1984;25:253–300. [PubMed: 6398622]
7. Mansure JJC, Panek A, Crowe LM, Crowe JH. Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochim Biophys Acta* 1994;1191:309–316. [PubMed: 8172916]
8. Mishra P, Prasad R. Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. *J Gen Microbiol* 1988;134:3205–3211. [PubMed: 3077635]
9. Thomas DS, Rose AH. Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Arch Microbiol* 1979;122:49–55. [PubMed: 391174]
10. Chi Z, Arneborg N. *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance exhibit different adaptive responses to produced ethanol. *J Ind Microbiol Biotechnol* 2000;24:75–78.
11. Chi Z, Kohlwein SD, Paltauf F. Role of phosphatidylinositol (PI) in ethanol production and ethanol tolerance by a high ethanol producing yeast. *J Ind Microbiol Biotechnol* 1999;22:58–63.
12. Furukawa K, Kitano H, Mizoguchi H, Hara S. Effect of cellular inositol content on ethanol tolerance of *Saccharomyces cerevisiae* in sake brewing. *J Biosci Bioeng* 2004;98:107–113. [PubMed: 16233674]
13. Jones RP, Greenfield PF. Ethanol and the fluidity of the yeast plasma membrane. *Yeast* 1987;3:223–232. [PubMed: 3332975]
14. Kajiwarra S, Shirai A, Fujii T, Toguri T, Nakamura K, Ohtaguchi K. Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae*: Expression of ethanol tolerance and the *FAD2* gene from *Arabidopsis thaliana*. *Appl Environ Microbiol* 1996;62:4309–4313. [PubMed: 8953702]
15. Greenberg ML, Lopes JM. Genetic regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol Rev* 1996;60:1–20. [PubMed: 8852893]
16. Clark DP, Beard JP. Altered phospholipids composition in mutants of *Escherichia coli* sensitive or resistant to organic solvents. *J Gen Microbiol* 1979;113:267–274. [PubMed: 92527]
17. Gaspar ML, Aregullin MA, Jesch SA, Henry SA. Inositol induces a profound alteration in the pattern and rate of synthesis and turnover of membrane lipids in *Saccharomyces cerevisiae*. *J Biol Chem* 2006;281:22773–22785. [PubMed: 16777854]
18. Jiranek V, Graves JA, Henry SA. Pleiotropic effects of the *opi1* regulatory mutation of yeast: Its effects on growth and on phospholipid and inositol metabolism. *Microbiol* 1998;144:2739–2748.
19. White MJ, Lopes JM, Henry SA. Inositol metabolism in yeasts. *Adv Microb Physiol* 1991;32:1–51. [PubMed: 1882726]
20. White MJ, Hirsch JP, Henry SA. The *OPI1* gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. *J Biol Chem* 1991;266:863–872. [PubMed: 1985968]
21. You KM, Rosenfield C, Knipple DC. Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Appl Environ Microbiol* 2003;69:1499–1503. [PubMed: 12620835]
22. Aguilera F, Peinado RA, Millán C, Ortega JM, Mauricio JC. Relationship between ethanol tolerance, H⁺-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *Int J Food Microbiol* 2006;110:34–42. [PubMed: 16690148]

23. Cartwright CP, Veazey FJ, Rose AH. Effect of ethanol on activity of the plasma-membrane ATPase in, and accumulation of glycine by, *Saccharomyces cerevisiae*. J Gen Microbiol 1987;133:857–865. [PubMed: 2958598]
24. Kalmokoff ML, Ingledew WM. Evaluation of ethanol tolerance in selected *Saccharomyces* strains. ASBC J 1985;43:189–196.
25. Jesch, SA.; Henry, SA. Yeast inositol phospholipids: Synthesis, regulation, and involvement in membrane trafficking and lipid signaling. In: Daum, G., editor. Cell Biology and Dynamics of Yeast Lipids. Vol. 37/661. Res Signpost; Kerala, India: 2005. p. 105-131.

Figure 1.
Cell viability of wild-type (WT) and *opi1* yeasts after growth in -I media and exposure to 10 and 15% ethanol

Figure 2.
Cell viabilities of wild type (WT) and *opi1* yeasts after growth in +I media and exposure to 10, 15, and 18% ethanol concentrations

Figure 3.
OD of wild-type (WT) and *opi1* yeast grown in +I media containing 2% or 12% glucose and 0% ethanol

Figure 4.
OD of wild-type (WT) and *opi1* yeast grown in +I media containing 2% or 12% glucose and 5% ethanol

Figure 5.
OD of wild-type (WT) and *opi1* yeast grown in -I media containing either 2% or 12% glucose and 0% ethanol

Figure 6.
OD of wild-type (WT) and *opi1* yeast grown in $-I$ media containing either 2% or 12% glucose and 5% ethanol

Table 1*S. cerevisiae* strains used in this study

STRAIN	GENOTYPE	SOURCE
AID	<i>ade1/ade1 ino1/ino1 MATa/α</i>	S.A. Henry
MVY#2008	<i>ade2-1/ADE2 his3-11/HIS3 leu2-3/LEU2 lys2/LYS2 trp1-1/TRP1 ura3-1/URA3 opi1-1/</i> <i>opi1-1 Mat α/Mat α</i>	M. Villa
MVY#2015	<i>ADE2/ADE2 HIS3/HIS3 l LEU2/LEU2 LYS2/LYS2 TRP1/TRP1 URA3/URA3 OPI1/</i> <i>OPI1 Mat α/Mat α</i>	M. Villa
MVJY#19	<i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Mat α</i>	M. Villa
MVJY#13	<i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 opi1Δ::KanMX4 Mat α</i>	M. Villa

Table 2

Specific death rates of wild-type (WT) and *opi1* yeast cultures shocked in 10, 15, or 18% ethanol. Cells marked “NA” indicated specific death rates that could not be determined by this method.

MEDIA TYPE	SPECIFIC DEATH RATES IN 2% GLUCOSE (d, hr^{-1})			
	WT	R ²	<i>opi1</i>	R ²
+I				
10% Ethanol	0.084±0.032	0.70	0.036±0.035	0.26
15% Ethanol	2.61±0.022	1	0.71±0.174	0.93
18% Ethanol	NA	NA	1.61±0.111	1
−I				
10% Ethanol	0.049±0.021	0.65	0.006±0.051	0.005
15% Ethanol	3.14±0.025	1	0.79±0.065	0.99
18% Ethanol	NA	NA	NA	NA

Table 3

Specific growth rates of wild-type (WT) and *opi1* yeast cultures grown in either −I or +I media containing 2% (a) or 12% (b) glucose and 0% or 5% ethanol concentrations (v/v).

a. MEDIA TYPE	SPECIFIC GROWTH RATES IN 2% GLUCOSE (μ , hr ^{−1})			
	WT	R ²	<i>opi1</i>	R ²
+I				
0% Ethanol	0.153±0.001	1	0.125±0.036	0.934
5% Ethanol	0.111±0.007	0.988	0.118±0.012	0.973
−I				
0% Ethanol	0.102±0.008	0.988	0.100±0.010	0.973
5% Ethanol	0.065±0.004	0.988	0.102±0.011	0.976
b. MEDIA TYPE	SPECIFIC GROWTH RATES IN 12% GLUCOSE (μ , hr ^{−1})			
	WT	R ²	<i>opi1</i>	R ²
+I				
0% Ethanol	0.102±0.016	0.959	0.142±0.012	0.981
5% Ethanol	0.109±0.010	0.986	0.127±0.007	0.993
−I				
0% Ethanol	0.100±0.007	0.995	0.102±0.013	0.954
5% Ethanol	0.057±0.007	0.964	0.076±0.010	0.975