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Chapter 2

Quantitative analysis of the modes of growth inhibition by weak organic acids in yeast

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Abstract

Weak organic acids are naturally occurring compounds that are commercially used as preservatives in food and beverage industries. They extend shelf life of food products by inhibiting microbial growth. There are a number of theories that explain the antifungal properties of these weak acids, but the exact mechanism is still unknown. We set out to quantitatively determine the contributions of various mechanisms of antifungal activity of these weak acids, as well as the mechanisms that yeast uses to counteract their effects. We analyzed the effects of four weak organic acids differing in lipophilicity (sorbic, benzoic, propionic and acetic acid) on growth and intracellular pH (pHi) in *Saccharomyces cerevisiae*. Although lipophilicity of the acids correlated with the rate of acidification of the cytosol, our data confirmed that not initial acidification, but rather the cell’s ability to restore pHi was a determinant for growth inhibition. This pHi recovery in turn depended on the nature of the organic anion. We identified long term acidification as the major cause of growth inhibition in acetic acid stress. Restoration of pHi, and consequently growth rate, in the presence of this weak acid required the full activity of the plasma membrane ATPase Pma1p. Surprisingly, the proposed anion export pump Pdr12p was shown to play an important role in the ability of yeast cells to restore pHi upon lipophilic (sorbic and benzoic) acid stress, probably through a charge interaction of anion and proton transport.
Introduction

Almost 40% of the food produced for human consumption is spoiled by microbes, an important issue because of considerable loss of resources and economics [1]. Because of consumer safety, only few preservatives are approved for food [2, 3]. In recent years, consumer demands have shifted towards fewer and reduced amounts of food additives, creating a need for better mechanistic understanding of traditional preservatives in order to find alternative preventive measures or improve efficacy of existing ones. A major group of traditional preservatives that are allowed are weak organic acids [4]. Weak acid preservatives such as sorbic, benzoic, propionic, and acetic acid are widely used antimicrobial compounds in food and beverage industry to prevent microbial spoilage [5]. The specific inhibitory effect of these preservatives depends on pH, causing them to be effective mostly in foods with low pH [6, 7].

At low extracellular pH, weak acids are protonated and uncharged. With a rate that depends on membrane solubility of the molecule, the protonated form of the acids can diffuse over the plasma membrane, and enter the cell. At the near to neutral cytosolic pH during exponential growth on glucose [8], the acid dissociates to generate protons and anions, which are charged and therefore cannot simply diffuse back out. This causes intracellular acidification and anion accumulation, both of which impede metabolic function [4, 9]. Indirect consequences of weak acid stress are oxidative damage [10] and inferred perturbation of the plasma membrane [11, 12].

Yeast can overcome many of the hurdles put up to prevent the microbial spoilage associated with their growth. *Saccharomyces cerevisiae*, commonly known as “baker’s yeast” or “brewer’s yeast” is an ordinary spoilage yeast which is able to withstand the presence of many weak acids in the concentrations maximally allowed in food [13]. During weak acid stress *S. cerevisiae* uses the plasma membrane $\text{H}^+$-ATPase Pma1p to export protons, and induces the ATP-binding cassette (ABC) anion efflux pump Pdr12p which exports the remaining anions out of cell. Cells with reduced Pma1p and Pdr12p membrane proteins or activity show weak acid hypersensitivity [14-16]. However, induction of Pdr12p alone is not sufficient to acquire generic weak acid resistance [3], and other pumps may contribute to anion efflux [17].

Another major cause of growth inhibition by weak acids could be energy depletion. Krebs *et al*. concluded that weak acids likely inhibit glycolysis by
acidification, because a key enzyme of glycolysis, phosphofructokinase, is sensitive to low pH \textit{in vitro} [4]. Inhibition of glycolysis should eventually lead to ATP depletion. However, while depletion of ATP during weak acid stress was observed, this appeared not to be due to an inhibition of ATP generating capacity. Rather, the energy demand of pH\textsubscript{i} restoration and anion export was proposed as the major cause of the depletion of the intracellular ATP pool [15].

Toxicity of weak acid preservatives also depends on their structure; Acetic acid and sorbic acid have different inhibitory effects, despite their identical pK\textsubscript{a} values. The toxicity of weak acids increases with their carbon chain length, affecting lipophilicity. Lipophilicity of a weak acid is reflected by its partitioning between an organic solvent and water. Toxicity of weak acids strongly correlates with such partition coefficients, suggesting that the weak acids’ plasma membrane mediated entry into the yeast cell also is an important determinant of the efficacy of growth inhibition. Therefore, compared to the lipophilic sorbic acid, a much higher concentration of the hydrophilic acetic acid is required to cause similar growth inhibition [11, 18].

At present, the exact mechanisms that yeast uses to adapt to weak acids are still unknown. In order to optimize the use of these well-known safe preservatives it is important to prevent such adaptation. Interfering with adaptation is best done knowledge-based, so that new, optimal preservation strategies can be formulated whilst improving robustness and maintaining required safety. In order to disentangle the quantitative contributions of the various inhibitory mechanisms of weak acid preservatives, we compared the effects of four weak acids, sorbic acid (HS), acetic acid (HA), benzoic acid (HB), and propionic acid (HP) on pH\textsubscript{i} and growth of \textit{S. cerevisiae}. Using mutants with reduced proton or anion excreting capacity we systematically disrupted specific aspects of the mechanisms counteracting the stress. We established that acidification is the main cause of growth inhibition by acetic acid, while the specific anions contribute much to growth inhibition by lipophilic acids. Our data clearly show that acid and anion expulsion are interdependent.
Materials and Methods

Yeast strains and culture condition

Strains used in this study are listed in Table 1. *Saccharomyces cerevisiae* BY4741 and three isogenic derivatives *pma1-007, pdr12Δ* and *haa1Δ* were transformed with plasmid pYES-ACT-pHluorin [8].

Strains were cultivated in defined mineral medium [19] using 2 % glucose (w/v) as the only carbon source. This medium contains ammonium sulphate as a nitrogen source, and full supplements of vitamins, minerals, and trace elements, but is low in fluorescence. Precultures were generated in Erlenmeyer flasks on a rotary shaker at 200 rpm at 30 °C. To buffer external pH, potassium citrate was added to a final concentration of 25 mM. All experiments were done at an external pH of 5.0. For pH controlled growth experiments strains were cultivated in 500 ml batch fermentors with a steady airflow (500 ml/min) and stirring rate (600 rpm). The pH was controlled at values indicated by automatic titration with 0.2 M KOH using an Applikon ADI 1030 Controller (Applikon, Schiedam, The Netherlands). Cultures were inoculated to an OD$_{600}$ of approximately 0.2 in batch fermentors, and grown to an OD$_{600}$ of 0.8, corresponding to $\sim 2.4 \times 10^7$ cells/ml. For microplate assays cells were transferred to CELLSTAR black polystyrene clear bottom 96 well microtiter plates (BMG Labtechnologies, Germany) for pH$_i$ and growth measurements.

Measurement of pH$_i$

pH$_i$ was registered using the pH sensitive green fluorescent protein ratiometric pHluorin as described before [8, 20]. Briefly, cultures expressing cytosolic pHluorin were excited with 390 or 470 nm light. Emission was registered at 510 nm in a FLUOstar Optima (BMG Labtechnologies, Germany). Background fluorescence of untransformed cultures was subtracted from both signals independently, before the ratio of the two signals was determined. The pH$_i$ signal was calibrated using cultures of the same yeast strain, grown in defined mineral medium with glucose. The cells were permeabilised with digitonin (100 µg/ml in PBS), for 10 minutes at room temperature. Cells were collected by centrifugation (5 min at 4000 r.p.m.) and culture aliquots were resuspended in phosphate-citrate buffers at a range of pH values between 4.5 and 8.5. The emission ratios at 510 nm upon excitation at 390 and 470 nm were determined.
GFP ratiometric pHluorin fluorescence ratios can be accurately transformed to pH\(_i\) values between 5.1 and 8.1 (r\(^2 = 0.99\)).

**Weak acid stress**

Stock solutions of all four weak acids were prepared in water (Sigma-Aldrich, Germany). The pH of the stock solutions was adjusted to a value of 5.0 with potassium hydroxide or hydrochloric acid. Stress experiments were initiated after registration of baseline pH\(_i\). In short term experiments, such as acidification rate assays, pH\(_i\) was registered in seconds after acid pulse. For these experiments cells were grown in batch fermenters to an OD\(_{600}\) 1.0 and then transferred to microtiter plates. For long term assays in the plate reader, exponentially growing cells were used and the medium was buffered at pH 5.0 with 25 mM potassium citrate. In these experiments growth (OD\(_{600}\)) and fluorescence were measured every 10 minutes for 16 hours.

**Table 1. Yeast strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>pma1-007</td>
<td>BY4741 ygl007w::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
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<td>EUROSCARF</td>
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<td>EUROSCARF</td>
</tr>
<tr>
<td>tpo2Δ</td>
<td>BY4741 ygr138c::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
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<td>BY4741 + pYES-ACT-pHluorin (URA3)</td>
<td>This study</td>
</tr>
<tr>
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<td>pma1-007 + pYES-ACT-pHluorin (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>AK003</td>
<td>pdr12Δ + pYES-ACT-pHluorin (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>AK004</td>
<td>haa1Δ + pYES-ACT-pHluorin (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>AK005</td>
<td>tpo2Δ + pYES-ACT-pHluorin (URA3)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Propidium iodide staining**

The effect of weak acid stress on the membrane integrity of yeast cells was assessed using propidium iodide (PI) staining (Sigma-Aldrich, Germany). Growing cultures of *S. cerevisiae* wild-type were exposed to concentrations of weak acids and ethanol (EtOH) inhibiting growth by 50% (1.0 mM HS, 42 mM HA, 0.6 mM HB, 10 mM HP, 12.3% EtOH). Cells were harvested 4 and 24 hours after exposure to the stress by centrifugation (5000 rpm, 5 min), washed in H\(_2\)O. The pellet was incubated with 10 µg/ml PI in H\(_2\)O for 10 min at room temperature in the dark. After staining, cells were washed twice to remove
Weak organic acid stress

excess dye [21]. Stained cells were visualized with a fluorescence microscope (Axiovert 40 CFL, Carl Zeiss) using appropriate filters. Image acquisition was done with a Canon Power Shot A640 camera using Canon remote capture (v. 2.7.5.27) software and analyzed with ImageJ v. 1.45s (National Institutes of Health) software. For each sample, approximately 500 cells from 5 random fields of a slide were counted and the percentage of PI positive cells was calculated.

Data analysis

Calculation of growth inhibition

Raw data was exported from Optima data analysis software to Microsoft Excel 2010 for analysis. We corrected the OD\textsubscript{600} data to fix the non-linearity of OD and cell density as described earlier [22]: Background was subtracted, and non-linearity was corrected by measuring OD\textsubscript{600} values of samples of a range of ODs in plate reader and adequately diluted in a spectrophotometer (Pharmacia LKB Biochrom). This resulted in a calibration formula \(1.1252*(\text{OD}_{600})^2 + 0.6808*(\text{OD}_{600}) - 0.0002\). The corrected \text{OD}_{600} was normalized to \(t=0\) and log transformed [23]. Maximum specific growth rates (\(\mu_{\text{max}}\)) were calculated from transformed growth curves as described previously [24]. Briefly, the slope of the growth curve was determined by linear regression of seven consecutive time points (corresponding to one hour of growth), using a sliding window over the entire growth curve. The maximum two values were removed, and the 3rd highest values were averaged over at least 3 biological replicates. Growth inhibition was calculated as percentage of the maximal specific growth rate of non-stressed control cultures:

\[
\text{Growth inhibition (\%) } = 100 \times \left(1 - \frac{\mu_{\text{max stress}}}{\mu_{\text{max control}}}\right)
\]

In all experiments non-stressed control cultures were analyzed simultaneously.

\(pH_i\) analysis

Initial acidification was considered as the minimum \(pH_i\) reached within 2 minutes upon exposure to the acids, while recovered \(pH_i\) represented the \(pH_i\) that cells reached when they were growing at the maximum specific growth rate after acid exposure. Weak acid entry rates were measured by approximation by measuring acidification in 1-second intervals upon exposure to the acids. As
acid-base equilibria settle almost instantaneously, measurement of acidification is an accurate approximation of measurement of entry of the protonated acid. For analysis, only fluorescence ratios corresponding to pH\textsubscript{i} values higher than 5.0 were used.

**Calculation of the undissociated fractions of weak acids**

The concentrations of undissociated weak organic acid at pH 5.0 were calculated using the Henderson-Hasselbalch equation

\[
\text{pH} = pK_a + \log \frac{[A^-]}{[HA]}
\]

using pK\textsubscript{a} values of 4.76, 4.21, 4.88 and 4.76 for sorbic, benzoic, propionic, and acetic acid, respectively [25].

**Statistics**

All comparative analyses were analyzed for significance using the student’s T-test with a p-value cut-off of 0.05, unless mentioned otherwise in the text. The correlation of variables was assessed by Pearson correlation (R) and significance of the relation is expressed by p-values.

**Results**

The growth inhibitory effect of weak acids relates to the extent of pH\textsubscript{i} restoration after stress

We quantitatively investigated the inhibitory effect of a wide range of concentrations of four commonly used weak acids (acetic, propionic, sorbic, and benzoic acid) on the growth of *S. cerevisiae* BY4741. Weak acids were added to exponentially growing cells of strain BY4741 at pH 5.0. Growth (OD\textsubscript{600}) was recorded for 16 hours after acid addition, during which time control strains went from lag phase through exponential growth to the post diauxic phase, and we determined the maximum growth rate, compared to that of unstressed cells (Fig. 1A-B, Fig S1). The effects of four different weak acids on growth are presented as a function of the concentration of the undissociated form of the acid, which is known to be the main contributor to the growth inhibitory effect [26-28].

As observed before [18], HB and HS caused growth inhibition at concentrations one to two orders of magnitude lower than HP and HA, with 50%
Figure 1. The effect of weak acid preservatives on growth and pHi of *Saccharomyces cerevisiae* BY4741.

Exponentially growing cultures were stressed with increasing concentrations of sorbic acid (♦, C), acetic acid (■, D), benzoic acid (●, E), propionic acid (▲, F). Addition of weak acid stress caused growth inhibition (A, B) and reduced pHi (C-F). (A, B) Growth inhibition was determined as described in Materials and Methods. (C-F) Open bars represent the minimum pHi reached within one minute after acid exposure, grey bars represent recovered pHi after adaptation to stress. Intracellular pH is presented in fluorescence ratios (R\textsubscript{390/470}), after subtracting background, and dotted lines represent the corresponding pHi values. Data represent mean ± SD of at least three biological replicates.
growth inhibition at 0.6 mM, 1.0 mM, 10 mM and 42 mM, respectively. We hypothesized that the main cause of growth inhibition would be intracellular acidification.

Therefore, we recorded pH$_i$ along with growth during 16 hours following addition of a range of concentrations of the four weak acids (Fig. 1C-F). The initial decrease in pH$_i$ is the combined outcome of dissociation of newly entered acid, protons absorbed by cellular buffers, and protons extruded by the extrusion activities present in the unstressed cells. Specific anion extrusion might affect the acid-base equilibria, but anion extrusion activity is negligible before prior exposure to the acids [29]. The pH$_i$ that is eventually reached after an adaptation phase depends on these same aspects, but may additionally be affected by newly expressed or activated mechanisms that assist in recovery, such as anion extrusion pumps, increased proton extrusion capacity, or changes in cellular buffering. The initial decrease in pH$_i$ caused by weak acid addition (measured after 1 minute) varied strongly between the acids, where concentrations of HS that completely inhibited growth caused the pH$_i$ to drop to only just below 6, while concentrations of HA leading to much stronger pH reduction did not yet cause any growth inhibition (Fig. 1C-F, white bars, Fig. S2).

Figure 2. Acidification rate caused by weak acids. After the addition of increasing concentration of A, sorbic (♦) and benzoic acid (●), and B, propionic (▲) and acetic acid (■, secondary axis) pH$_i$ was measured in 1-second intervals. The initial rate of acidification is plotted against weak acid concentration. Notice that the x-axis in panels A and B have different scales.

Since in non-stressed cells the buffering and proton extrusion activities present are a given, not dependent of the acid to which the cells are exposed, the
difference in initial acidification between different acids at identical dosages must be caused by differences in entry rates. Since dissociation is extremely rapid, different entry rates also lead to different rates of acidification of the cytosol. We recorded pH$_i$ every second for the first minute after acid exposure (Fig. S3). The initial slope of these curves represents the rate of acidification (Fig. 2) and reflects the rate of acid entry (zero trans-influx). Indeed, the rate of acidification was highest for benzoic and sorbic acid (Fig. 2A) and much lower for propionic and acetic acid (Fig. 2B, note the difference in acid concentration range). Interestingly, the relation between weak acid concentration and acidification rate was linear for HB, HS and HP ($R^2 > 0.99$, $p < 10^{-4}$), which substantiates an entry mode of simple diffusion. Entry of HA was not linear with acid concentration, suggesting a combination of entry modes with different kinetics. The curve could however not be fit with known mechanisms such as combination of for instance diffusion and a Michaelis-Menten term.

**Figure 3. Correlation of growth and pH$_i$ upon weak acid exposure.**

Growth inhibition data of (wild-type) is compared with initial acidification (A) and recovered pH$_i$ (B). Data represent the mean ± SD of at least three biological replicates. The relation of growth inhibition with initial and recovered pH$_i$ was calculated as mentioned in materials and methods. The initial acidification correlated significantly with the growth inhibition of lipophilic weak acids (correlation coefficients $r$ of -0.94, -0.97, and -0.99 ($p < 10^{-7}$) for HB (○), HS (♦), and HP (▲), respectively) while for HA (□) no significant correlation could be observed. The stable pH$_i$ that the cells were able to restore after adaptation to the initial stress (Fig. 1C-F) correlated better overall with the growth behavior (correlation coefficients of -0.97, -0.98, -0.99, and -0.94, for HB, HS, HP, and HA, respectively and p values were $< 0.005$ for all acids).
To assess whether the drop in pHi as a consequence of acid entry is likely to be the cause of growth inhibition [30], we assessed the correlation of the two parameters. While initial acidification correlated relatively well with growth inhibition for each individual acid (Fig. 3A), marked differences can be observed between the hydrophilic HA and the more lipophilic HB, HS and HP. Overall, growth inhibition correlated better with the pHᵢ value that the cells were able to restore after an adaptation period (overall correlation of growth inhibition with pHᵢ for all acids increasing from an r of -0.79 to -0.92 mostly because acetic acid now agreed with the other three acids, Fig. 3B). We therefore decided to investigate the role of molecular mechanisms for proton and acid extrusion.

**Figure 4. Maintenance of a high pHᵢ upon acetic acid stress depends on an energy consuming process.**
Exponentially growing cells of *S. cerevisiae* wild-type were harvested and starved for 30 minutes in mineral medium without glucose. Cultures with and without glucose were exposed to medium (black bars), sorbic acid (grey bars), or acetic acid (white bars) and intracellular acidification was measured after one minute. Data represent mean ± SD of at least three biological replicates.

**The role of proton efflux pumping in weak acid stress**
Since pHᵢ correlated with growth, we tested the role of proton pumping during weak acid stress. First, we show that the pHᵢ that is reached upon acid exposure indeed depends on a combination of intracellular buffering capacity and an energy consuming process. We showed in the previous part that identical dosages of different acids cause a different extent of intracellular acidification. In Figures 1C and 1D we see for instance that a 2 mM concentration of HS causes a pHᵢ drop to 5.4, while an identical concentration of HA causes no apparent drop in pHᵢ, even although the acids have an identical pKₐ. We assessed the effect of depletion of cellular energy, by starving the cells for glucose for 30 minutes, a time which is not sufficient for glucose derepression [31, 32]. In these glucose starved cells, proton extrusion activity is abolished (Fig. S4). In the absence of the proton pumping activity, 1 mM of 50
either HA or HS causes an identical decrease of pH$_i$ after one minute (Figure 4). This suggests that the pH$_i$ that is observed in the presence of glucose depends on the balance of acid entry and proton pumping. Pma1p is responsible for this activity in S. cerevisiae [15, 33], pumping protons at the expense of ATP. In the absence of glucose ATP cannot be replenished. Pma1p is the most abundant plasma membrane protein of yeast, and PMA1 is an essential gene. However, a disruption of an ORF (ygl007w) in the promoter region results in a reduction of PMA1 mRNA, protein, and activity [34, 35] to approximately 50%. We used this hypomorphic allele of PMA1 (pma1-007) to see the contribution of proton pumping activity to growth inhibition. We analyzed the resistance of pma1-007 towards the same four acids, and found that the reduction of Pma1p did not enhance the growth inhibitory effect of HS, HB or HP (and in fact the mutant

![Figure 5. The effect of weak acids on the growth of S. cerevisiae.](image)

Wild-type (black symbol), pma1-007 (open symbol) and pdr12Δ mutants (grey symbol) were stressed with increasing concentration of weak acids (A) sorbic, (B) acetic, (C) benzoic, and (D) propionic acid. Growth inhibition was determined as described in the Materials and Methods section. Data represent mean ± SD of at least three biological replicates. (∗ p < 0.05 pdr12Δ vs. wild-type; ∗ p < 0.05 pma1-007 vs. wild-type).
was less sensitive to HP). It did cause a severe increase in the growth inhibition caused by HA, reducing the 50% inhibitory concentration from 42 mM to 24 mM (Fig. 5A-D).

Contrary to expectations, this reduction of plasma membrane proton pumping capacity did not lead to a larger drop in pH<sub>i</sub> upon acid exposure compared to wild-type, even in the case of acetic acid (Fig. 6, white bars). Also, the rate of acidification measured in the first seconds after acid exposure was not significantly affected for any of the acids compared to wild-type (Fig. S5). This suggests that the initial pH<sub>i</sub> is primarily determined by the intrinsic cellular buffer capacity rather than the by direct ATP coupled extrusion of protons. However, the restoration of pH<sub>i</sub> after HA exposure was impaired in the pma1-007 hypomorph (Fig. 6B) when compared with wild-type (Fig.1D). This suggests that for the three more lipophilic acids

<table>
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<tr>
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<th>24 hours</th>
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<tbody>
<tr>
<td>Control (no stress)</td>
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<td>0.68</td>
</tr>
<tr>
<td>Ethanol</td>
<td>18.33</td>
<td>40.64</td>
</tr>
<tr>
<td>Sorbic acid</td>
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<td>1.21</td>
</tr>
<tr>
<td>Acetic acid</td>
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<td>Benzoic acid</td>
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<td>1.44</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.97</td>
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</tr>
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</table>
the range of the efflux capacity of Pma1p, and a reduction of Pma1p activity now affects the balance between influx and efflux, causing a larger pH\textsubscript{i} drop and stronger growth inhibition.

**Anion efflux affects pH\textsubscript{i} homeostatic capacity**

If pH\textsubscript{i} homeostasis is not the major cause of growth inhibition for lipophilic acids then what could be the reason for this growth inhibition? Although an effect of the lipophilic acids on membrane integrity has been suggested before [18, 36], we found no strong increase in propidium iodide (PI) permeability of cell

![Figure 6. Plasma membrane ATPase Pma1p is crucial for pH\textsubscript{i} recovery in acetic acid stress.](image)

Growing cultures of strain *pma1-007* were stressed with increasing concentration of (A) sorbic acid, (B) acetic acid, (C) benzoic acid and (D) propionic acid, to assess the role of Pma1p during stress. Open bar represent the acidification after 2 minutes acid exposure and grey bars represent recovered pH\textsubscript{i} after adaptation to stress. Intracellular pH is presented in fluorescence ratios (R\textsubscript{390/470}), after subtracting background, and dotted lines represent the corresponding pH\textsubscript{i} values. Data represent mean ± SD of at least three biological replicates. To allow direct comparisons of these results with wild-type (Fig 1C-F), all experiments were done in identical conditions.
exposed to 50% growth inhibitory concentrations of the four acids for four or even 24 hours (Table 2). A concentration of 12.3% ethanol, also leading to 50% inhibition of growth, did result in a significant destabilization of the plasma membrane in this assay. A contribution of the weak acid anions to growth inhibition has also been suggested, and we used a \( pdr12\Delta \) mutant to test this.

Pdr12p is a plasma membrane ATP-binding cassette (ABC) efflux pump that is believed to have a major role in the resistance of \( S.\ cerevisiae \) to lipophilic acids, likely because it catalyzes the export of the anions.

Figure 7. Pdr12p ABC transporter is essential for pH\(_i\) recovery upon hydrophobic acid stress.

Growing cultures of the \( pdr12\Delta \) mutant were stressed with increasing concentrations of (A) sorbic, (B) acetic, (C) benzoic, and (D) propionic acid. Open bars shows the pH\(_i\), after 2 minutes stress, while grey bars represent recovered pH\(_i\) after adaptation to stress. Intracellular pH is presented in fluorescence ratios (R\(_{390/470}\)), after subtracting background, and dotted lines represent the corresponding pH\(_i\) values. Data represent mean ± SD of at least three biological replicates. To allow direct comparisons of these results with wild-type (Fig 1C-F), all experiments were done in identical conditions.
Deletion of *PDR12* was shown to lead to hypersensitivity to weak organic acids [16, 29, 37], although only for the more lipophilic acids. Its affinity appears highest for HB and HS, which also lead to induction of expression of the protein [16].

We investigated the role of the Pdr12p transporter on growth and pH$_i$ recovery during weak acid stress. Indeed, deletion of *PDR12* increased sensitivity toward the more lipophilic acids HB, HS, and also HP, while it did not sensitize towards HA (Fig. 5). Benzoic and propionic acid became more potent growth inhibitors than HS in the absence of ABC transporter, suggesting that Pdr12p is important to eliminate HB and HP from the cell interior. As with *pma1-007*, deletion of *PDR12* did not lead to a bigger initial drop in pH$_i$ (Fig. 7). In the case of acetic acid stress, the cells restored their pH$_i$ similar to the parental strain and the *pdr12Δ* mutant showed no acetic acid hypersensitivity. However, in the cases of HP, HS and HB the recovery of pH$_i$ was strongly impaired.

![Figure 8. Anion accumulation perturbs the pH$_i$ recovery.](image)

**Figure 8. Anion accumulation perturbs the pH$_i$ recovery.** Effect of increasing concentrations of acetic acid on the growth (A) and pH$_i$ (B) of growing cultures of *S. cerevisiae* wild-type (black) and *haa1Δ* mutant (grey). pH$_i$ data represent the recovered pH$_i$ after stress. Intracellular pH is presented in fluorescence ratios (R$_{390/470}$), after subtracting background, and dotted lines represent the corresponding pH$_i$ values. Data represent mean ± SD of at least three biological replicates. (*) p < 0.05 vs. wild-type.

We conclude that in *Saccharomyces cerevisiae* Pdr12p is involved in HP, HS and HB stress resistance but not in HA stress resistance as observed earlier [37]. Remarkably, it appears that Pdr12p mediated anion efflux effects proton homeostasis. To see if indeed anion efflux in general is important for pH$_i$ restoration we decided to test whether acetate efflux also interacts with pH$_i$.
homeostasis. To do so, we analyzed pH\textsubscript{i} responses of a haa1\textDelta\ mutant. Haa1p is a transcriptional activator of multidrug transporters Tpo2p and Tpo3p, which are known to be required for acetate efflux. Cells lacking the Haa1p transcription factor accumulate higher concentrations of acetate than the parental strain [17, 38]. Growth analysis of the haa1\textDelta strain in the presence of HA (Fig. 8A) is consistent with previous findings where haa1\textDelta showed enhanced sensitivity to acetic acid [38]. Interestingly, we found that the strain lacking Haa1p displayed significantly reduced recovery of pH\textsubscript{i} (Fig. 8B, Fig. S6), which likely reflects its transcriptional activation of Tpo2p, as a tpo2\textDelta strain also had a reduced recovery of pH\textsubscript{i} (Fig. S7). This shows that acetate efflux, like benzoate, sorbate and propionate efflux, is directly relevant for proton extrusion and therefore pH\textsubscript{i} homeostasis.

**Discussion**
A number of theories have been presented to explain the antifungal properties of weak acids, including acidification of cytoplasm, anion accumulation, membrane perturbation and ATP depletion [33, 36, 39]. To what extent these various mechanisms contribute to the antifungal activity of weak acids is still unresolved. We addressed this issue by systematic quantitative analysis of growth and pH\textsubscript{i} dynamics of *Saccharomyces cerevisiae*. In wild-type yeast, growth inhibition correlated with the lipophilicity of weak acids, fitting the notion that the acids need to diffuse over the membrane to cause growth inhibition [18, 40]. Indeed, for the three lipophilic acids diffusion appeared to be the main mode of entry. Acetic acid appeared to enter by a (combination of) mechanisms, which did not fit with diffusion. Since the cells were growing on glucose, it is unlikely that the glucose repressed H\textsuperscript{+}-acetate symporter (Ady2p) contributes to the entry [41]. Entry through for instance the Fps1p aquaglyceroporin [42] cannot be excluded, but this should theoretically appear as diffusion kinetics, and therefore does not satisfactorily explain the profile observed.

**Acidification as a mode of growth inhibition**
Our first approach was to determine whether the intracellular acidification caused by the four weak acids could be considered the main mechanism of growth inhibition, as pH\textsubscript{i} was shown to be in control of growth rate in non-
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stressed cells [30]. Addition of weak acids to growing cells instantly acidified the cells, after which pH_i was recovered to varying extent. Both initial and recovered intracellular pH data correlated with the growth inhibition for each acid individually. However, different acids at concentrations causing the same level of acidification led to a different extent of growth inhibition: at any specific pH_i, lipophilic acids caused stronger inhibition than hydrophobic acids. A direct comparison of pH_i and growth inhibition indeed showed that initial acidification cannot be the only or major growth inhibitory mechanism of the lipophilic weak acids. Pma1p activity is a key determinant of acetic acid resistance. This strongly contrasts with the other three acids, for which the decrease in Pma1p content did not affect growth inhibition. This strongly suggests that the capacity of Pma1p to de-acidify the cytosol is required fully for pH_i recovery in the case of acetic acid, and that acidification is the major cause of growth inhibition by this acid. Conversely, we can conclude that the other mechanisms do not contribute much, and therefore that HA does not cause membrane damage, and the anion is not (very) toxic, even at high concentrations. The latter does make sense, as yeast cells can produce and metabolize acetate at relatively high concentrations [43, 44], and therefore metabolism must not be strongly negatively affected by the compound. Benzoic and propionic acid had a very comparable relation between pH_i and growth inhibition, while sorbic acid more effectively inhibited growth at the same pH_i, suggesting the involvement of growth inhibitory mechanisms other than acidification. Lowering the capacity of Pma1p affected the rate of recovery of pH_i in sorbic and benzoic acid stress, but the pH_i that was reached was similar to wild-type, and the effect on growth was negligible. The combined data for these acids suggests that acidification does not contribute strongly to the growth inhibitory effect of lipophilic acids, and that the other proposed antifungal mechanisms of lipophilic acids are the primary cause of growth inhibition. As we did not observe an apparent effect on membrane integrity, we first explored the hypotheses that energy limitation and anion toxicity are important contributors to the growth inhibitory potential of weak acids.

Energy limitation as a cause of growth reduction
The Pma1p ATPase activity to recover pH_i requires a lot of ATP [45], and already consumes some 20% of the ATP generated during normal growth on
glucose [46]. At the same time, acidification decreases ATP production through the inhibition of glycolysis [4]. Together, this is thought to lead to depletion of the cellular ATP pool, which may well contribute to growth inhibition. Indeed, to conserve energy, Pma1p activity can be downregulated by Hsp30p [47]. While deletion of Hsp30 did not lead to increased sensitivity to different stresses but rather extended the adaptation period, this is still indication of the need for energy conservation during adaptation [47]. Also, upon exposure to glucose Pma1p is phosphorylated with activating phosphates [48], leading to an increased substrate affinity and activity [49]. One reason for the apparent resistance to propionic acid and lack of sensitivity to HB and HS of the pma1-007 mutant could be a lower use of ATP for proton pumping in this mutant, saving energy for an improved adaptive response. Interestingly, in the absence of glucose, sorbic acid and acetic acid caused similar acidification (at the same concentration of acid), while in the presence of glucose acetic acid reduced pH higher much less than sorbic acid at the same concentration. This shows that the high pH maintained during HA stress is dependent on an ATP driven process. However, even though the Pma1p mediated proton extrusion is responsible for this process both in case of the hydrophilic and the lipophilic acids [50], we showed that in wild-type Pma1p capacity, depending on both expression and ATP availability, is still far from limiting with the lipophilic acids. Wild-type cells exposed to high dosages of WOA leading to complete growth arrest did not deplete the glucose in the medium during the time course of our experiments (our unpublished data), This suggest that the futile cycle of energy consuming proton and anion export, followed by extracellular reassociation and influx, is not sustained by glucose consumption. Whether the cells cannot sustain the cycle, or whether mechanisms are in place that prevent the futile cycle, remains to be established.

The contribution of the anion to growth inhibition

The third contribution of weak acid stress to growth inhibition may be the specific anion itself. Both the dosages and the nature of the anion are quite different for the four different acids, varying with carbon chain length and structure. Unlike some (spoilage) yeasts, S. cerevisiae does not metabolize these anions [39] in the presence of glucose [51]. To counteract anion stress, S. cerevisiae induces the Pdr12p ABC transporter, which exports anions at the
expense of energy [29, 52]. Indeed, while a \textit{pdr12}\Delta mutant did not display increased sensitivity to acetic acid, it was hypersensitive to benzoic and propionic acid [29, 37, 39]. Cells deleted for the Pdr12 pump (\textit{pdr12}\Delta) showed little sensitivity to sorbic acid, and the induction of this pump was previously shown to be insufficient for the acquisition of resistance against sorbic acid [3]. Others indeed showed a stronger sensitivity of the mutant toward benzoic acid than to sorbic acid [16], and the exact extent of sensitivity may well depend on for instance the use of the medium and the pH, as rich media at low pH result in some weak acid stress because of weak organic acid components of the medium itself [8].

We have argued that acidification is not the major antifungal mechanism of the lipophilic acids, but that anions might also play a role. We cannot measure the anion concentrations, but using our data it is possible to make estimations. Weak acids acidify the cytosol in seconds, depending on the lipophilicity of acid. If we assume that the concentrations of undissociated acid in and outside the plasma membrane reach equilibrium during this time [36] we can estimate the intracellular anion concentration using our pH\textsubscript{i} determinations and the Henderson-Hasselbalch equation. Interestingly, such an analysis would suggest that the anion concentration in \textit{pdr12}\Delta mutant was lower than that in the WT-strain upon HS exposure (data not shown). This is not consistent with the observation that the \textit{pdr12}\Delta mutant is hypersensitive to HS. We believe therefore that the assumption of equilibrium of internal and external sorbic acid is incorrect, which implies that the proton and possibly also anion export fluxes are high with respect to the influx, so that such a simple equilibrium cannot establish.

Interestingly, we revealed an interaction between anion extrusion capacity and the ability to restore pH\textsubscript{i}; our pH\textsubscript{i} data showed that upon acetic acid stress the \textit{pdr12}\Delta mutant behaves like wild-type and can restore its pH\textsubscript{i}. However, in case of sorbic, benzoic and propionic acid we clearly observed that the \textit{pdr12}\Delta mutant could not efficiently restore pH\textsubscript{i}. While previous reports have shown that Pdr12p exports different kinds of molecules, such as water-soluble carboxylate anions, fluorescein [16], caffeine [53] and fusel acids [54], no data suggest how Pdr12p might be involved in pH\textsubscript{i} regulation itself. It is apparent from our data that the Pdr12p protein is not interfering with pH\textsubscript{i} regulation directly, as the mutant did not show defects in pH\textsubscript{i} recovery under HA stress. This suggests a
relation with its function as an anion exporter. It could be that the protons are co-transported with anions, but since Pma1p activity is easily high enough there is no reason to assume that the absence of such symport would cause a major defect in deacidification. Another possible explanation could be that these specific anions hamper the cells’ ability to generate ATP from glucose [55]. However, the fact that a \textit{haa1}\Delta mutant, that cannot express the acetate transporters Tpo2p and Tpo3p and was shown to accumulate acetate, has a similar defect in \(pH_i\) restoration, but now upon acetic acid stress, suggests that rather than anion specific, there may be an aspecific effect of anion accumulation on proton pumping. This might for instance be an effect on membrane potential through a defect in charge balancing, which in turn affects Pma1p or other proton translocating activities [12, 56] but this cannot be confirmed yet. Formally, we cannot exclude that, rather than a consequence, the defect in \(pH_i\) restoration is the primary effect, and that this defect is a \textit{cause} of the increased anion accumulation in \textit{pdr12}\Delta and \textit{haa1}\Delta strains [17, 38]. Simply the low \(pH_i\) values do not explain this, as at equilibrium a low \(pH_i\) should lead to a \textit{decrease} in anion concentration. However, such a reversal of cause and consequence would imply a different type of function for these anion pumps, for which currently no evidence is at hand.

It is important to note that recovery of \(pH_i\) to neutral in the absence of mechanisms to prevent acid entry would only lead to more acid influx, more intracellular dissociation of acid and eventually higher accumulation of the anion. Additionally, since the ratio of undissociated and dissociated acid in the cell depends on \(pH_i\), it is quite obvious that increasing \(pH_i\) without a matching export of anions would lead to tremendous accumulation of anions in the cell, to within the molar range. It might be that a low \(pH_i\) is preferable to the accumulation of high concentrations of organic anions, and or energy loss for the cell. Therefore, it might be beneficial for the cell not to export protons if anions are abundantly present, and thus prevent an energy consuming futile cycle and anion accumulation. Pdr12p dependent anion extrusion reduces the negative charge present in the form of weak acid anions, which should facilitate Pma1p mediated extrusion of positively charged protons, as electrostatic charge across the plasma is required for Pma1p activity [29, 57]. Also, it is well possible that a mechanism has evolved that regulates Pma1p activity in response to the intracellular concentration of weak acid anions. The failure of
the \( pdr12\Delta \) mutant to restore \( pH_i \) upon lipophilic acid exposure, and of the \( haa1\Delta \) mutant defective in expression of acetate exporters to do so upon acetic acid stress, fits with the existence of such a mechanism. Taken together, our data strongly suggest that proton extrusion is repressed by anion accumulation.

**Abbreviations**

HA, Acetic acid; HB, benzoic acid; HP, propionic acid; HS, sorbic acid; \( pH_i \), intracellular pH; PI, propidium iodide.

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**References**

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Supplementary Materials

Quantitative analysis of the modes of growth inhibition by weak organic acids in yeast

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Figure S1. Effect of different weak acids on the growth of baker’s yeast.

Exponentially growing cultures of S. cerevisiae wild-type were exposed to increasing concentration of (A) sorbic, (B) acetic, (C) benzoic, and (D) propionic acid. Each growth curve represents the natural logarithm of the OD$_{600}$ over time. Growth (OD$_{600}$) was measured in microtiter plate reader as described in Materials and Methods. The experiments were repeated as three independent biological replicates. One representative experiment is shown here.
Supplementary Material

Figure S2. Effect of different weak acids on the intracellular pH$_i$ of baker's yeast.

Exponentially growing cultures of S. cerevisiae wild-type were exposed to increasing concentration of (A) sorbic acid, (B) acetic acid, (C) benzoic acid, (D) propionic acid. Intracellular pH of cells was measured in microtiter plate reader as described in Materials and Methods. Intracellular pH is presented in fluorescence ratios ($R_{390/470}$), after subtracting background, and dotted lines represent corresponding pH$_i$ values. The experiments were repeated for three independent biological replicates. One representative experiment is shown here.
Figure S3. Determination of acidification rates upon weak acid exposure. Exponentially growing cultures of *S. cerevisiae* wild-type were pulsed (at 0 time point) to increasing concentration of (A) sorbic acid, (B) acetic acid, (C) benzoic acid, and (D) propionic acid. Initial acidification were measured by registration of $pH_i$ in second time scale for one minute, and determination of the slope of the linear part of these curves using the initial couple of seconds. No pH values below 5 (dashed line) were used in any of the subsequent analyses. The experiments were repeated in three independent biological replicates. One representative experiment is shown here.
Figure S4. Glucose is required for proton pumping activity in the presence of acetic acid.

$p_{\text{Hex}}$ was monitored during the incubation of wild-type cells washed in water with 0.5 mM of acetic acid. At $t=0$ 10 mM of glucose was added. Traces of three replicate experiments with independent yeast cultures are shown.
Figure S5. Comparison of acidification rates of WT, pma1-007 and pdr12Δ.
Comparison of initial acidification of S. cerevisiae WT (closed circle), pma1-007 (gray circles) and pdr12Δ (dark triangles) upon exposure to (A), sorbic, (B), acetic, (C), benzoic, and (D), propionic acid. Intracellular acidification was measured in 1-second intervals upon acid exposure.
Figure S6. Effect of acetic acid on growth and pH$_i$ of *S. cerevisiae* wild-type and *haa1Δ* mutant.

Effect of acetic acid on the growth (A, C) and pH$_i$ (B, D) of *S. cerevisiae* wild-type (A, B) and *haa1Δ* (C, D). Growing cultures were stressed with increasing concentration acetic acid. Each growth curve represents the natural logarithm of the OD$_{600}$ over time (A, C). Intracellular pH is presented in fluorescence ratios ($R_{390/470}$), after subtracting background, and dotted lines represent corresponding pH$_i$ values. The experiments were repeated for three independent biological replicates. One representative experiment is shown here.
Figure S7. Deletion of TPO2 largely recapitulates the effect of deletion of HAA1.

The effect of exposure of wild-type, haa1Δ and tpo2Δ to 40 mM of acetic acid on (A) growth inhibition and (B) $pH_i$ at the time of maximum growth after acid adaptation.