

## Adaptation of Yeast Cell Membranes to Ethanol

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**A highly ethanol-tolerant *Saccharomyces* wine strain is able, after growth in the presence of ethanol, to efficiently improve the ethanol tolerance of its membrane. A less-tolerant *Saccharomyces* laboratory strain, however, is unable to adapt its membrane to ethanol. Furthermore, after growth in the presence of ethanol, the membrane of the latter strain becomes increasingly sensitive, although this is a reversible process. Reversion to a higher tolerance occurs only after the addition of an energy source and does not take place in the presence of cycloheximide.**

During growth in ethanol, *Saccharomyces* strains synthesize lipids enriched in C<sub>18:1</sub> fatty acyl residues to compensate for a decrease in palmityl residues (3). These changes, together with the reports that supplementation with unsaturated fatty acids favors alcohol tolerance by lowering membrane leakage (5), have led to the conclusion that the cell membrane is the main target for alcohol inhibition (6) and imply that membrane fatty acyl residues are important determinants of resistance to alcohol (5). Many studies propose that *Saccharomyces* spp. adapt to ethanol by changing the cell membrane composition (for reviews, see references 6 and 11). This assumption is consistent with the structural changes observed in the cell membranes of microorganisms tolerant to high concentrations of ethanol (6, 11).

However, trials to obtain more-tolerant strains by successive transfers of high- and low-tolerance yeast strains to media supplemented with ethanol have been unsuccessful, and no increase in tolerance has been attained (7). Furthermore, some strains become more sensitive after prolonged incubation in ethanol, despite substantial changes in their lipid composition (2). These changes detected in membrane composition may be due to enzymatic alterations produced by ethanol, as has been observed in *Escherichia coli* (6). Alternatively, *Saccharomyces* strains could positively adapt their membranes to ethanol by changing their composition, but this adaptation might not result in an enhancement of parameters such as growth and fermentation, because the overall kinetics of the inhibition of these processes by ethanol depends upon a number of underlying mechanisms, some of which are different from membrane-bound processes and any of which could be the rate-limiting step (1, 8, 11). This possibility has not yet been tested since no method has been described that specifically shows adaptation of the membranes.

Acidification curves have proved to be a reliable measurement of the degree of ethanol interference with cell membranes (9). In the present study, this method has been used to test the ability of the cell membrane to adapt to ethanol in a high- and a low-tolerance yeast strain.

The strains FSP6, a highly ethanol-tolerant *Saccharomyces pretoriensis* wine strain ( $K_i = 1.37$  M,  $K_i$  being the concentration of ethanol which reduces its growth rate by 50%) (8), and D517-4B (*MATa ade2 lys9*), a less-ethanol-tolerant *Saccharomyces cerevisiae* laboratory strain ( $K_i =$

0.95 M), generously provided by J. Conde, La Cruz del Campo, Sevilla, Spain, were used.

Cells were routinely grown in 500-ml flasks with 250 ml of either YPD medium (10) or YPD supplemented with 8% (vol/vol) ethanol (YPDE). Flasks were inoculated with 25 ml of a stationary-phase culture grown in YPD and incubated at 30°C with shaking (160 rpm) until the glucose was exhausted (stationary phase). Glucose was measured by injecting 25  $\mu$ l of the culture sample into a YSI-27 glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

To determine the ethanol tolerance of the cell membrane, acidification curves were measured by the method of Jiménez and van Uden (8). Cells were centrifuged, washed twice with distilled water, and finally suspended in 2 ml of distilled water to a cell density of about 0.1 g (dry weight) per ml. From the cell suspension, 0.5 ml was placed in a 20-ml glass vial together with 0.5 ml of a 20% glucose solution and distilled water with ethanol at the desired concentration (0, 2, 4, 6, and 8% [vol/vol]) to a total volume of 5 ml. The vials were screw-capped and incubated at 30°C with mechanical shaking (300 rpm) for 2 h. For the first 8 min, the pH was continuously registered with a standard recorder. After 2 h of incubation, the final pH ( $pH_f$ ) was determined with a pH meter (Crison Instruments, Madrid, Spain). To determine the dry weight, 0.1 ml of the cell suspension was taken and dried on aluminum foil at 105°C for 12 h.

To determine whether changes in membrane ethanol tolerance in the cells grown in ethanol were reversible, 1,000 ml of stationary-phase culture grown in YPDE (8% ethanol) medium was divided into four 250-ml subcultures. In one (the control), the cells were directly used to measure acidification curves as described above. The cells from the other three subcultures were centrifuged, washed as described above, and suspended in 100 ml of (i) distilled water, (ii) synthetic medium (YNB without amino acids or ammonium sulfate) (10) with 0.5 g of glucose, or (iii) solution ii but with the addition of 20 mg of cycloheximide. These three suspensions were incubated for 4 h at 30°C and then centrifuged, washed, and suspended in 2 ml of distilled water to determine the acidification curves as described above.

When yeast cells grown in YPD were suspended in water containing glucose, protons were extruded and the extracellular pH dropped to a final value ( $pH_f$ ) after following a characteristic acidification curve (Fig. 1). At this  $pH_f$ , the active extrusion of protons is balanced by passive proton influx (9). Ethanol enhances the diffusion constant of the proton influx and consequently increases the  $pH_f$ , the rela-

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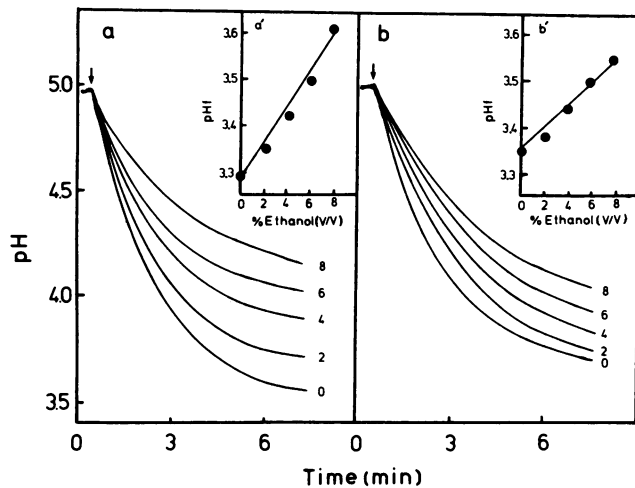


FIG. 1. Effects of ethanol on the acidification curves in aqueous suspensions of the strain D517-4B (a) and the ethanol-tolerant strain FSP6 (b) when glucose (2% [wt/vol] final concentration) was added (arrows). Each curve corresponds to the ethanol concentration (% [vol/vol]) indicated by the number beside it. Insets a' and b' represent the  $\text{pH}_f$  values reached after 2 h of acidification at the different ethanol concentrations tested in the same strains.

tionship between ethanol concentrations and the  $\text{pH}_f$ , being linear (8, 9). Additionally, a correlation between the ethanol tolerance of a strain (defined by its  $K_i$ ) and the  $\text{pH}_f$  was found, so that the less tolerant a strain, the steeper the linear relationship between ethanol concentrations and  $\text{pH}_f$  values (8). Figure 1 (insets) confirms these findings.

When acidification curves were measured in cells grown in medium with 8% ethanol (YPDE), two different behaviors were observed according to the strain under study. Strain D517-4B was unable to adapt to ethanol during its growth in YPDE (Fig. 2a). Furthermore, this strain became more sensitive to ethanol, since the  $\text{pH}_f$  values were much higher and the slope was steeper than those of the cells grown in medium without ethanol (YPD) (Fig. 2a). This increase in ethanol sensitivity is a reversible effect since, when the cells grown in YPDE were incubated for 4 h in synthetic medium with a carbon source (glucose) to allow some minimal metabolism but without a nitrogen source to prevent changes due specifically to cell growth, the cells recovered their ethanol tolerance (Fig. 2b). Under these conditions, the  $\text{pH}_f$  values obtained were the same as those measured when the cells were grown in YPD (Fig. 2a). This reversion, however, did not take place when cells were incubated either in synthetic medium plus cycloheximide or in distilled water, since their acidification curves were the same as those of the control with cells grown in YPDE and directly used to determine the  $\text{pH}_f$  values.

The highly ethanol-tolerant strain FSP6 behaved differently. The slope of the linear relationship between ethanol concentrations and  $\text{pH}_f$  values was the same for cells grown in YPDE as for cells grown in YPD. Moreover, the  $\text{pH}_f$  absolute values were much lower for cells grown in YPDE than for those grown in YPD (Fig. 2a), indicating that a true adaptative process had taken place in the cell membranes during growth in YPDE.

The results obtained here indicate that both laboratory and wine yeast strains seem to alter their membrane tolerance after growth in ethanol (Fig. 2a). However, whereas the membranes of the laboratory strain become more sensitive,

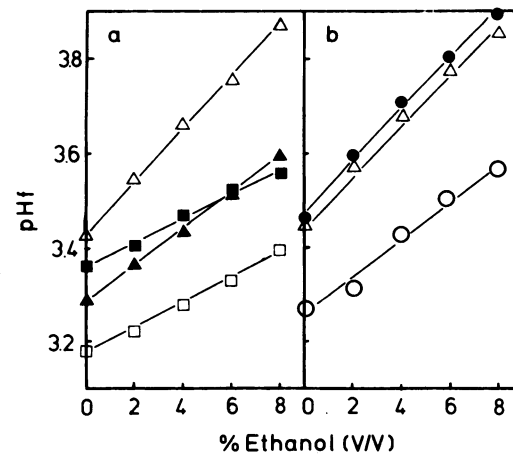


FIG. 2. Relationship between ethanol concentrations and the  $\text{pH}_f$  values from the acidification curves of cells obtained from (a) strain D517-4B grown in YPD (▲) or in YPDE (8% ethanol) (Δ) and ethanol-tolerant strain FSP6 grown in YPD (■) or in YPDE (8% ethanol) (□) and (b) strain D517-4B grown in YPDE (8% ethanol) and incubated for 4 h in distilled water (Δ), in synthetic medium without a nitrogen source (YNB) and with 0.5% glucose (○), and in this last solution plus 200  $\mu\text{g}$  of cycloheximide per ml (final concentration) (●). Results are the average of two to five experiments.

those of the wine strain improve their ethanol tolerance, which could indicate that the capacity to adapt cell membranes to ethanol depends upon the strain, as happens with ethanol-tolerant growth (7).

The increase in sensitivity observed in the laboratory strain grown in YPDE can be reverted, but the process requires an energy source and does not occur in the presence of cycloheximide, which inhibits protein synthesis (Fig. 2b). Dombek and Ingram (4) suggest that in *Escherichia coli* a decrease in the lipid/protein ratio could be essential for ethanol tolerance. In *Saccharomyces* spp. it has also been found that the lipid/protein ratio of cells incubated in ethanol-containing medium falls as the concentration of ethanol rises (2). In addition, the increase in  $\text{pH}_f$  caused by ethanol is due to an increase in passive diffusion of protons through the cell membrane (9). Since proteins are barriers to ion movement (5), these changes observed in the membrane tolerance after incubation in media with ethanol might reflect changes in the protein composition of the cell membrane. In fact, *Saccharomyces* laboratory strain D517-4B does not recover its membrane tolerance in the presence of cycloheximide (Fig. 2b). Therefore these results, together with other analytical data (2, 4), suggest that the protein components of the cell membranes play an important role in the ethanol tolerance of yeast strains.

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#### LITERATURE CITED

1. Aguilera, A., and T. Benítez. 1985. Role of mitochondria in ethanol tolerance of *Saccharomyces cerevisiae*. Arch. Microbiol. 142:389-392.
2. Aguilera, A., and T. Benítez. 1986. Ethanol-sensitive mutants of *Saccharomyces cerevisiae*. Arch. Microbiol. 143:337-344.
3. Beaven, M. J., C. Charpentier, and A. H. Rose. 1982. Production

- and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *J. Gen. Microbiol.* **128**:1447-1455.
4. **Dombeck, K. M., and L. O. Ingram.** 1984. Effects of ethanol on the *Escherichia coli* plasma membrane. *J. Bacteriol.* **157**:233-239.
  5. **Hayashida, S., and K. Ohta.** 1978. Cell structure of yeasts grown anaerobically in *Aspergillus oryzae*-proteolipid-supplemented media. *Agric. Biol. Chem.* **42**:1139-1145.
  6. **Ingram, L. O., and T. M. Buttke.** 1984. Effects of alcohols on microorganisms. *Adv. Microb. Physiol.* **25**:253-300.
  7. **Ismail, A. A., and M. M. Ali.** 1971. Selection of high ethanol-yielding *Saccharomyces*. I. Ethanol tolerance and the effect of training in *Saccharomyces cerevisiae* Hansen. *Folia Microbiol.* **16**:346-349.
  8. **Jiménez, J., and N. van Uden.** 1985. Use of extracellular acidification for the rapid testing of ethanol tolerance in yeasts. *Biotechnol. Bioeng.* **27**:1596-1598.
  9. **Leao, C., and N. van Uden.** 1984. Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **774**:43-48.
  10. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1979. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  11. **van Uden, N.** 1985. Ethanol toxicity and ethanol tolerance in yeasts. *Annu. Rep. Ferment. Proc.* **8**:11-58.