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The Effect of Ethanol on the Plasma Membrane Permeability of Spoilage Yeasts

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Summary

The effect of ethanol on the passive proton influx and on leakage of compounds absorbing at 260 nm, as representatives of intracellular content, was studied on food spoilage yeasts such as *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Pichia* sp. and *Debaryomyces hansenii*. For volume fraction below 10 %, the effect of ethanol on the proton influx was in general weak, but above a certain treshold of ethanol high values were observed. In *Z. bailii* ethanol had no effect up to 20 % (volume fraction). Previous growth of the cells in the presence of benzoic acid or ethanol did not affect the influx of protons in the presence of ethanol. Leakage of compounds absorbing at 260 nm was not observed at 25 °C and occurred at 30 °C only after a rather long incubation in high concentrations of ethanol, which induced cell death. This suggests that in the yeasts this process does not control the leakage of compounds in the presence of ethanol.

Key words: food spoilage yeasts, proton influx, plasma membrane permeability

Introduction

The yeast plasma membrane is one of the main targets for ethanol. It has been shown that ethanol inhibits processes of mediated transport *(1,2)* and stimulates the traffic of compounds that cross the membrane by simple diffusion *(3–5)*. This alteration in membrane permeability may be deleterious to the cells promoting the leakage of intracellular constituents or the entry of toxic extracellular substances which disturb the composition of the cytoplasm. Protons are usually in higher concentrations in the extracellular environment and yeast cells use a powerful H^+-pump ATPase that keeps the intracellular pH at physiological values, suitable for enzyme function, and generates a proton-motive force across the plasma membrane. Disturbing the homeostasis of the membrane to H^+, ethanol may induce intracellular acidification and degradation of ATP by the ATPase.

The objective of our work was to study the effect of ethanol on the leakage of protons and of intracellular compounds which absorb at 260 nm in yeasts involved in food spoilage. *Saccharomyces cerevisiae* was chosen for being resistant to ethanol; *Zygosaccharomyces bailii* and *Pichia* sp. were chosen for their resistance to weak acid preservatives and *Debaryomyces hansenii* was included as a spoilage yeast particularly resistant to salt. The effect of previous adaptation of cells to ethanol and to benzoic acid, which induce probable alterations in the composition of the plasma membrane, was also considered.

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Materials and Methods

Strains and culture conditions

Four strains representative of species associated with food spoilage were used: *Saccharomyces cerevisiae* IGC 3507, *Zygosaccharomyces bailii* IGC 4806, *Debaryomyces hansenii* IGC 2968 and *Pichia* sp. IGC 4595, kindly provided by the Portuguese Yeast Culture Collection. Inocula were prepared on agar YPD slants (2 % (w/v) glucose, 2 % (w/v) peptone, 0,5 % yeast extract, 2 % (w/v) agar), grown for 24 h at 25 °C. Cultures were prepared in 300–500 mL of mineral medium *(6)* with vitamins and 2 % (w/v) glucose, in 1 L Erlenmeyer flasks. Cultures were stirred magnetically and incubated aerobically at 26 °C in a water bath. For adaptation to benzoic acid, the acid was incorporated in the medium to a final concentration of 1 mM (*Sacch. cerevisiae, Z. bailii* and *Pichia* sp.) or 0.5 mM (*D. hansenii).* For adaptation to ethanol, the alcohol was added to the medium to a final volume fraction of 8 % (*Sacch. cerevisiae, Z. bailii* and *Pichia* sp.) or 2 % (*D. hansenii*). Exponential phase cells ($A_{260\ nm}$ 1) were harvested by centrifugation at 4 °C, washed twice with cold distilled water, for the passive proton influx assays, or with phosphate buffer (50 mM, pH=5) for measuring the leakage of compounds absorbing at 260 nm.

Passive proton influx

Cell suspensions were prepared in water at a concentration of 60–70 mg/mL and kept on ice. Proton movements were measured at 30 °C with a standard pH meter, Radiometer PHM62, connected to a recorder. In a water-jacked cell of 10 mL capacity, with magnetic stirring, 0.5 mL of cell suspension, distilled water and ethanol were mixed to obtain a final volume of 5 mL. The pH value of these suspensions was 5–6. The pH was quickly adjusted to 4.0 by the addition of HCl (100 mM) and the subsequent pH recovery was recorded, during 1 min. After each assay, the curve was calibrated using 10 mM HCl. The rate of proton influx was calculated as the rate of decrease of the concentration of extracellular protons *(3).* All the experiments were performed in the presence of 2-deoxy-D-glucose (1 mM) and antimycin (2 g/mL), to minimise H^+ movements created by the plasma membrane ATPase activity.

Leakage of compounds absorbing at 260 nm

Cell suspensions (1–2 mg/mL) were prepared in phosphate buffer (50 mM, pH=5) with ethanol in the desired concentrations in tightly closed flasks. These suspensions were incubated in a shaking water bath at 30 °C for 4 h. At regular time intervals, samples (5 mL) were harvested, centrifuged for 5 min (6000 rpm) at 4 °C and the supernatants were separated immediately. Leakage was evaluated by measuring the absorbance of the supernatants at 260 nm in a spectrophotometer (Shimadzu UV-160-A). The content in compounds absorbing at 260 nm was estimated by using NAD^+ as a standard.

Evaluation of cell viability

The criterion of ability to form colonies was used to test viability of cells in which leakage of compounds absorbing at 260 nm was measured. Appropriate dilutions of cell suspensions, prepared at the beginning of experiments, were used in order to obtain a suitable number of colonies in plates. Cell suspensions were incubated as described in the previous paragraph. Samples (100 L) removed, when the loss of compounds absorbing at 260 nm was significantly high, were plated on YPD agar. Plates were incubated at 25 °C and colonies were counted after 8 days.

All experiments were performed at least three times.

Results

Passive proton influx

Fig. 1 illustrates a typical record of the extracellular pH of a yeast suspension when a pulse of acid was added. After a rapid drop in pH from 5–6 to 4, a steady increase was observed, corresponding to a net influx of H^+ into the cells. The net influx corresponds to the global movement of protons in both directions: protons entering the cells by passive diffusion and protons being pumped out by the ATPase. To test if the activity of the ATPase had any significant effect on the observed movement of protons, the cells were treated with 2-deoxi-D--glucose (1 mM) to decrease the level of ATP in the cytoplasm and with antimycin (2 g/mL) to block respiration. This treatment was shown to be necessary since particularly in the case of *Z. bailii*, the influx of protons was significantly higher, indicating that the ATPase was reducing its apparent value (results not shown). As shown in Fig. 1, the presence of ethanol in the assay resulted in a faster increase of pH, corresponding to a higher rate of passive influx of protons. From the slopes of these records, calibrated with 10 mM HCl, rates of passive H^+ influx were calculated for the strains involved at different concentrations of ethanol. Results are summarised in Fig. 2. Comparing the shape of the curves for each yeast, differences can be observed. In *Sacch. cerevisiae* (Fig. 2A) ethanol increased the rate of H^+ influx, although the effect was weaker than previously observed with a respiratory deficient mutant *(3). Z. bailii* (Fig. 2B) presented the remarkable behaviour of not be-

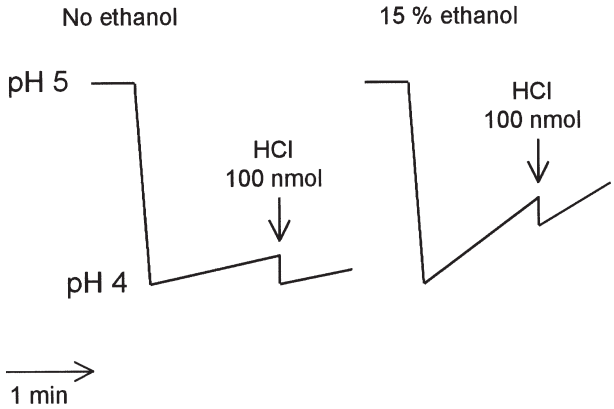

Fig. 1. Changes in pH of water suspension of *Sacch. cerevisiae* (70 mg/mL) triggered by a rapid acidification from pH=5 to 4, in the absence and in the presence of ethanol in volume fraction of 15 %.

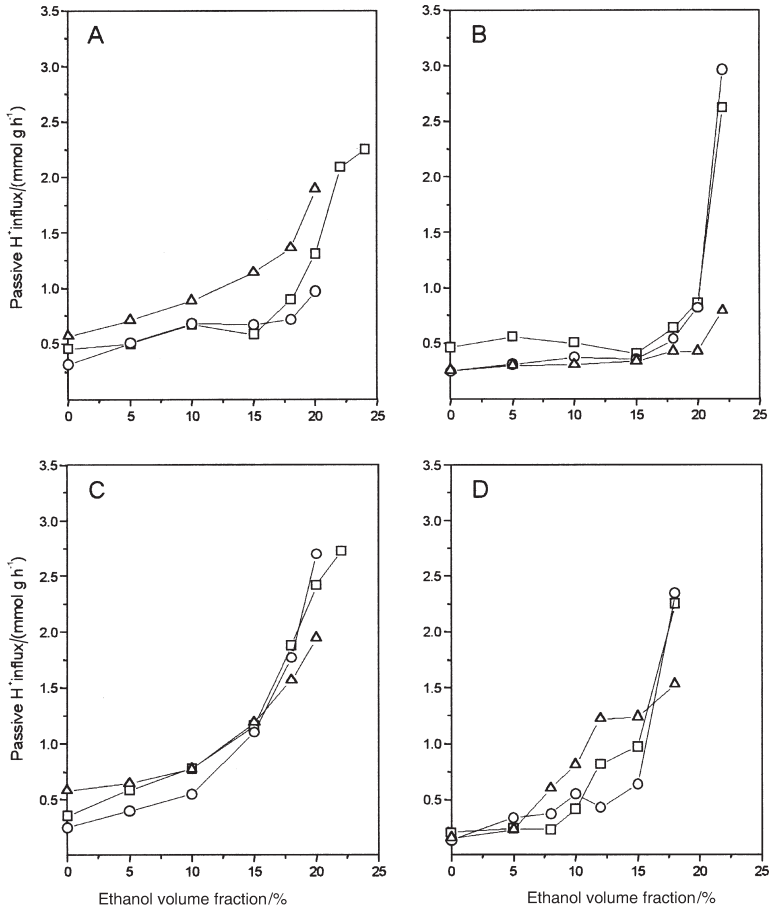

Fig. 2. Effect of ethanol on rates of passive proton influx in *Saccharomyces cerevisiae* (A), *Zygosaccharomyces bailii* (B), *Pichia* sp. (C) and *Debaryomyces hansenii* (D); different symbols refer to previous adaptation of the cells to ethanol (△), benzoic acid (o), control cells (□)

ing affected by ethanol for volume fractions up to 20 %. For higher volume fractions H^+ influx was very high. In the case of *Pichia* sp. (Fig. 2C), H^+ influx increased exponentially with ethanol in the range of volume fractions tested (not shown). For *D. hansenii* (Fig. 2D), the shape of the curve was similar to the other yeasts, but high values of H^+ influx were observed for volume fractions above 15 %. In all cases, for higher volume fractions of ethanol, the standard deviations of the measurements increased, suggesting some instability of the membranes. In the same figures, values are presented for cells grown in the presence of ethanol or benzoic acid. In general, the adaptation to these compounds did not affect significantly the results. It is interesting to notice that in the case of *Sacch. cerevisiae* cells grown in the presence of ethanol were significantly more leaky.

Leakage of compounds absorbing at 260 nm

The experiments whose results are shown in Fig. 3 were performed at 30 C. At this temperature, in the presence of rather high volume fractions of ethanol, an increase of volume fractions of compounds absorbing at 260 nm was observed after a few hours, indicating that the cells lost some of their constituents to the external environment. However, in similar experiments performed at 25 C, for all volume fractions tested, ethanol did not trigger almost any leakage. In Fig. 3 it is interesting to notice that even in the absence of ethanol, the extracellular absorbance at 260 nm increased to some extent, during incubation for a few hours in phosphate buffer. It is remarkable that incubation for 2 h of *Sacch. cerevisiae* with 17.5 % , *Z. bailii* with 18 %, *Pichia* sp. with 16 % and of *D. hansenii* with 12 % ethanol did not increase leakage of intracellular compounds. Fig. 3 indicates that *D. hansenii* was the most sensitive to ethanol, while *Z. bailii* was the most resistant. In all cases, when a significant increase in leakage was observed, cells did not form colonies after 8 days in plates incubated at 25 °C, indicating that most of the population was dead.

Discussion

Comparing the results obtained with H^+ influx and leakage of compounds absorbing at 260 nm, it is apparent that different mechanisms are involved in both processes. The influx of H^+ being much faster and detectable in a much shorter time interval. This is in agreement with the results obtained with liposomes, which

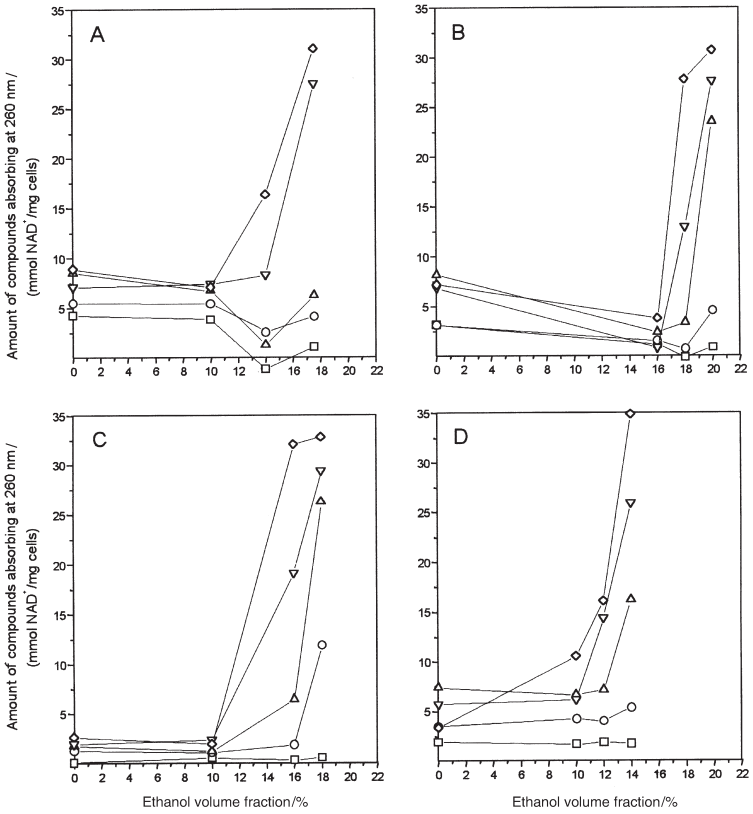

Fig. 3. Effect of ethanol on leakage of compounds absorbing at 260 nm in *Saccharomyces cerevisiae* (A), *Zygosaccharomyces balii* (B), *Pichia* sp. (C) and *Debaryomyces hansenii* (D); cell suspensions were incubated at 30 °C for 1 h (□), 2 h (o), 3 h (△), 4 h ( ) and 5 h (✧)

indicate that while most compounds cross the lipids by partition in the lipids followed by diffusion, H^+ utilise transient pores *(7)*. In general, the shape of the curves in Fig. 2 suggests that the effect of ethanol on H^+ influx was negligible or small for low volume fractions while for the higher volume fractions tested, the cells became suddenly very leaky and the data less reproducible. This was evident, in particular, in the case of *Z. bailii*, in which volume fractions up to 20 % had no effect and above this value a dramatic increase was observed. The same type of result was obtained in studies of H^+ permeability performed with L- -dipalmitoylphosphatidylcholine vesicles *(8)*. These results were explained assuming that ethanol would induce interdigitation of the phospholipids in the membrane creating an abrupt change in permeability *(8)*.

The results obtained with leakage of compounds, absorbing at 260 nm, suggest that this is not a process relevant to the effect of ethanol on yeasts performance. The fact that no leakage was observed at 25 C and that such high concentrations of ethanol had to be used at 30 C is indicative that the source of leakage observed were non-viable cells that had lost their integrity. This was confirmed by the loss of ability to form colonies. Probably ethanol promotes significant leakage by potentiating thermal death, as was described for *Sacch. cerevisiae (9,10)*.

In previous researches, performed with a deficient respiratory mutant of *Sacch. cerevisiae*, for H^+ permeability *(3)* and leakage of intracellular constituents *(4)* higher diffusion rates were measured. We have no explanation for the different behaviour presented by the strains examined in this paper as compared with the mutant used in those studies.

Many researches were previously carried out on the performance of *Sacch. cerevisiae* in the presence of ethanol which showed its remarkable resistance to ethanol. Our results indicate that other yeasts, as *Z. bailii*, famous for their performance as food spoilers and resistance to weak acids, exhibit also a very good performance in the presence of ethanol. Probably both types of resistance are associated with similar membrane properties. On the other hand, *D. hansenii*, highly resistant to NaCl and frequently found in salt preserved food, presents a relatively poor behaviour in the presence of ethanol.

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Utjecaj etanola na propusnost membrane plazme kvasaca uzročnika kvarenja

Sažetak

Saccharomyces cerevisiae, Zygosaccharomyces bailii, Pichia sp. i *Debaryomyces hansenii* odabrani su, kao kvasci uzročnici kvarenja, da bi se proučio utjecaj etanola na pasivni ulaz protona i na otpuštanje spojeva koji apsorbiraju pri 260 nm, kao predstavnika intracelularnog sastava stanice. Utjecaj etanola na ulaz protona bio je općenito slab pri volumnom udjelu ispod 10 %. Iznad određene razine etanola utvrđen je veći ulaz protona i pojačan izlaz spojeva koji apsorbiraju pri 260 nm. U *Z. bailii* etanol nije utjecao do volumnog udjela od 20 %. Prethodni rast stanica u prisutnosti benzojeve kiseline ili etanola nije utjecao na ulaz protona u prisutnosti etanola. Otpuštanje spojeva, koji apsorbiraju pri 260 nm, nije opažen pri 25, a dogodio se pri 30 °C, ali tek nakon duge inkubacije pri velikom udjelu etanola, koji je uzrokovao smrt stanica. To pokazuje da u tim kvascima navedeni proces ne kontrolira otpuštanje spojeva u prisutnosti etanola.