A peculiar behaviour for cell death induced by weak carboxylic acids in the wine spoilage yeast *Zygosaccharomyces bailii*

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L. FERNANDES, M. CÔRTE-REAL AND C. LEÃO. 1999. In glucose-grown cells of *Zygosaccharomyces bailii*, ISA 1307 acetic acid and other carboxylic acids enhanced death. The effects were much lower than those described for *Saccharomyces cerevisiae*, being only detectable at higher acid concentrations. In *Z. bailii,* acetic acid and other weak acids also induced intracellular acidification, but this effect was less pronounced than that of death and no relationship was found with death enhancement. The results suggested that in *Z. bailii*, unlike *S. cerevisiae*, intracellular acidification induced by weak acids is less pronounced and appears not to have a significant role in death at the temperature range used.

INTRODUCTION

Zygosaccharomyces bailii is one of the most dangerous and frequently found yeasts in spoiled food and beverages. This is related to the high resistance/tolerance of *Z. bailii* towards stress environments, such as carboxylic acids, which are generally used as chemical preservatives in the food industry (Thomas and Davenport 1985; Malfeito-Ferreira *et al*. 1990; Fleet 1992). Attempts to elucidate the mechanisms which underlie this resistance are of considerable value for improving the quality control of different food products. In this context, *Z. bailii* ISA 1307 has been used extensively in studies focusing on the effects of weak acids on yeast metabolic activity, and some of the mechanisms underlying its resistance and/or adaptation have already been identified and published (Fernandes *et al*. 1997; Warth 1991; Sousa *et al*. 1996, 1997; Prudêncio *et al*. 1998). However, the responses of *Z. bailii* to temperature and weak acids, in terms of cell viability, remain to be clarified. This is of fundamental importance to the understanding of how the kinetics of cell death in *Z. bailii* depend on temperature and acid concentration. In *S. cerevisiae*, weak acids enhanced thermal death, causing a shift of the lethal temperatures to lower values (Pinto *et al*. 1989). Such weak lipophilic acids at intermediate and lower temperatures also induced a second type of death which could be considered to be a consequence of acidification of the cytoplasm (Cardoso and Leão 1992). In this paper, the effects of acetic acid and other weak carboxylic acids on the kinetics and activation parameters of death in *Z. bailii* ISA 1307 are reported and compared with death induced in *S. cerevisiae* under the same conditions. Furthermore, a possible correlation between intracellular acidification and death induced by weak acids at low and intermediate temperatures, is investigated.

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MATERIALS AND METHODS

Micro-organisms and culture conditions

Zygosaccharomyces bailii ISA 1307, originally isolated from a continuous production plant of sparkling wine, was used (Wium *et al*. 1990). *Saccharomyces cerevisiae* IGC 4072 was originally isolated from a sample of Fermivin, an industrial wine yeast distributed by Rapidase, Selin, France. Both species were maintained on slants of glucose (2% w/v), peptone (1% w/v), yeast extract (0·5% w/v) and agar (2% w/v). Cells were grown in 500 ml flasks containing 100 ml mineral medium (van Uden 1967) with vitamins and 2% (w/v) glucose (MGV). The cultures were grown to late-exponential phase, with mechanical shaking, at 25 °C. These cells were designated, in the death experiments, as non-adapted cells, as opposed to adapted cells which were grown in the same medium but in the presence of carboxylic acid.

Death experiments and calculation of death parameters

Loss of cell viability of *Z. bailii* ISA 1307 in the absence and presence of weak carboxylic acids, using *S. cerevisiae* IGC 4072 as a reference, was measured by methods described earlier (Pinto *et al*. 1989).

Measurement of intracellular pH

The intracellular pH was determined by the method described previously (Rottenberg 1979), measuring the relative distribution of [1–^{14}C]propionic acid. These assays were designed to reproduce the conditions in death experiments and were performed as reported earlier for acetic acid-induced intracellular acidification in *S. cerevisiae* (Cardoso and Leão 1992). The intracellular concentration of propionic acid was calculated using a value of $1{\cdot}1 \pm 0{\cdot}19$ μl intracellular water mg^{-1} dry weight of the yeast as previously described (Sousa *et al*. 1996).

Reproducibility of the results

All the experiments were repeated at least three times and the data represent the average values.

RESULTS

Effects of weak carboxylic acids on activation parameters of death at high and intermediate temperatures in *Zygosaccharomyces bailii* ISA 1307

Loss of viability of glucose-grown cells of *Z. bailii* ISA 1307 induced by acetic, butyric, pentanoic and sorbic acids was measured at pH 3·0 and different temperatures. The combinations of temperature and concentrations of the acids were chosen in such a way that the experimental specific death rates were of the same order of magnitude. Typically, a deathless initial period was followed by a period of exponential death (results not shown). The absolute values of the slopes of the semilog survival plots (the specific death rates ^{k}d) obtained at pH 3·0 in the absence and the presence of the acids, were plotted as modified Arrhenius plots according to that described earlier (Pinto *et al.* 1989). Figure 1 shows representative results of these experiments. From these results it can be seen that, in the absence of acid, thermal death occurred (from 39–42 °C) which was expressed by a linear Arrhenius plot. The presence of acid in the external medium negatively affected the viability of *Z. bailii* ISA 1307, causing a shift of the lethal temperatures to lower values; the effect increased with the acid concentration. For all the acids tested, the Arrhenius plots constituted a family of straight lines whose slopes were about the same both in the absence and in the presence of the acid, a mean value of $97{\cdot}70 \pm 7{\cdot}30$ kcal mol^{-1} being obtained for activation enthalpy ($\Delta H^{\#}$). The results imply that these compounds affect thermal death in such a way that the toxic effects are reflected in the values of activation entropy ($\Delta S^{\#}$) but not on $\Delta H^{\#}$.

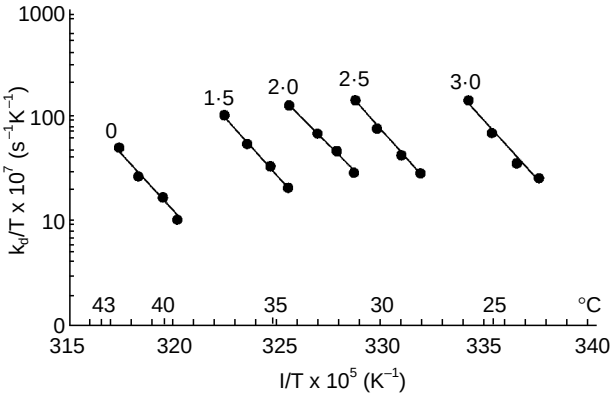

Fig. 1 Dependence of the specific death rates (k_d) of *Zygosaccharomyces bailii* ISA 1307 on the temperature at pH 3·0 in the absence and presence of acetic acid. Numbers indicate extracellular concentrations of acetic acid (%, v/v)

Effects of weak carboxylic acids on kinetics of death at constant temperature in *Z. bailii* ISA 1307

The specific death rates (k_d) for acetic, butyric and pentanoic acids (pH 3·0, 25 °C) and sorbic acid (pH 4·0, 30 °C) were also determined at constant temperature. Figure 2 shows how k_d typically depended on the concentration of acetic acid. For each of the acids, the effect increased with concentration in

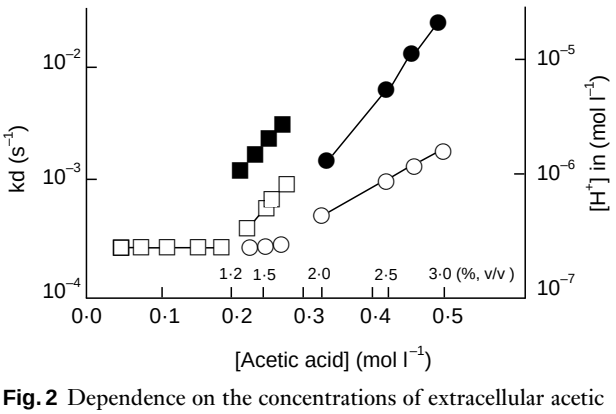

Fig. 2 Dependence on the concentrations of extracellular acetic acid of the specific death rates (k_d, closed symbols) and of the intracellular proton concentration ([H^+], open symbols) of *Zygosaccharomyces bailii* ISA 1307 (●, ○) and *Saccharomyces cerevisiae* IGC 4072 (■, □) at constant temperature, 25 °C, pH 3·0

the external medium and k_d values increased exponentially as a function of the acid concentration according to equation:

$$\ln k_d^X = \ln k_d^{X_m} + k_1(X - X_m) \quad (1)$$

where $k^x{}_d$ and $k^x{}_d{}^m$ are the specific death rates in the presence of X and X_m acid concentration, X_m being the minimum concentration above which the enhancement of death was measurable under the experimental conditions and k_1, the exponential enhancement death constant characteristic for each acid. The values of k_1 calculated according to equation 1 are listed in Table 1. In the case of *S. cerevisiae*, the value of k_1 for acetic acid was also estimated ($15{\cdot}81\,mol^{-1}$) and was of the same order of magnitude of that previously reported (Cardoso and Leão 1992).

The specific death rates (k_d) with regard to acetic and sorbic acids,were also determined, at constant temperature, in adapted cells (see material and methods). The dependence of k_d values on acid concentration was again expressed by exponential kinetics according to equation 1. The values of k_1 estimated for each acid are also listed in Table 1.

Assessing intracellular acidification associated with death at constant temperature in *Z. bailii* ISA 1307

To evaluate whether the correlation between death and intracellular acidification described previously for *S. cerevisiae* was present in *Z. bailii*, the effects of acetic acid on intracellular pH were studied under isothermic conditions at 25 °C, pH 3·0. The internal pH was about 6·8 in the absence of the acid. The presence of acetic acid in the extracellular medium at concentrations below 2% (v/v) did not affect the intracellular pH (Fig. 2). Only above this acid concentration was it possible to detect a decrease in the intracellular pH, the intracellular proton concentration being an exponential function of the extracellular acid concentration according to the equation:

$$\ln [H^+]_{in}^X = \ln [H^+]_{in}^{X_m} + k_2(X - X_m) \quad (2)$$

where X is the acid concentration, X_m is the concentration of the acid above which the enhancement of intracellular acidification was measurable under the experimental conditions, and k_2 is the exponential enhancement acidification constant characteristic for the acid. From the results depicted in Fig. 2, and according to equation 2, the value of $7{\cdot}91\,mol^{-1}$ was estimated for the constant k_2.

Table 1 Exponential enhancement constants of death (k_1) induced by weak carboxylic acids at constant temperatures, in non-adapted and adapted cells of *Zygosaccharomyces bailii* ISA 1307

		k_1* ($l\,mol^{-1}$)	
Carboxylic acid	pH	Non-adapted cells	Adapted cells
Acetic acid	3·0	19·33	14·44
Butyric acid	3·0	36·20	nd
Pentanoic acid	3·0	106·10	nd
Sorbic acid	4·0	330·00	209·63

* Values of k_1, expressed as undissociated carbocylic acid, estimated from the results presented in Fig. 2 and according to Equation 1.
nd, Not determined.

DISCUSSION

The influence of acetic, propionic, butyric and pentanoic acids on temperature-dependent death in glucose-grown cells of *S. cerevisiae* was previously reported (Pinto *et al*. 1989). In summary, the main finding was that in *S. cerevisiae*, the presence of the acids induced two types of death: (i) high enthalpy thermal death (HED) predominating at high temperatures and low acid concentrations; and (ii) low enthalpy death (LED) predominating at lower temperatures and higher acid concentrations. This could be of practical importance, specially in the case of acetic acid, an important normal end-product of alcoholic fermentation by *S. cerevisiae*. High enthalpy thermal death could contribute to the so called 'heat-sticking' of alcoholic fermentations, particularly of red wine and fuel ethanol fermentations in warm countries, in the absence of efficient temperature control. In addition, at intermediate and low temperatures, the loss of viability of cells induced by the acid could worsen because of LED. The results presented in this work suggested a different death sensitivity pattern to weak acids in the spoilage yeast *Z. bailii*. The acids also enhanced death of glucose-grown cells of *Z. bailii* ISA 1307 but, unlike *S. cerevisiae*, the presence of the acids only induced death characterized by high enthalpy (HED), no LED being measurable over the same acid concentration and temperature ranges. If death occurs at all, it will be at much higher acid concentrations and lower temperatures. In fact, as shown in Fig. 1, the values of $\Delta H^{\#}$ calculated from the Arrhenius plots in the presence of different acetic acid concentrations were about the same as those for thermal death in the absence of the acid, either at high or intermediate/low temperatures. Moreover, at these lower temperatures, the effects were only measurable at much higher acid concentration than in *S. cerevisiae*. It must also be noted that, under isothermic conditions, the kinetic profile of death induced by weak acids kept its main features in both species. However, in *Z. bailii* ISA 1307, the acid concentration above which the enhancement effects began to be measurable

was higher than in *S. cerevisiae*. Figure 2 exemplifies the different patterns of behaviour exhibited by both yeast species.

The high resistance of *Z. bailii* to acidic media containing ethanol compared with that of *S. cerevisiae*, like other resistance phenomena in organisms, is complex and it is not possible to reduce it to one or two mechanisms/processes. However, underlying the inhibitory effects of weak acids on yeast performance, intracellular acidification has been suggested as one of the most important mechanisms for explaining the different acid sensitivity patterns in yeasts (Pampulha and Loureiro-Dias 1989). In the case of *S. cerevisiae*, it was found that intracellular acidification underlies the induction of LED by weak monocarboxylic acids at low and intermediate temperatures (Cardoso and Leão 1992). The results now presented for *Z. bailii* ISA 1307 show that, under the same experimental conditions, at those temperatures (i) the acidification enhancement induced by acids was much lower than that of death, no relation being found with death enhancement, and (ii) the death induced by acids was significantly lower than that described for *S. cerevisiae*, being only detectable at higher acid concentration; specifically, for acetic acid (Fig. 2) in *S. cerevisiae*, concentrations of the acid above 0·2 mol l^{-1} (1·2%, v/v) began to induce intracellular acidification whereas in *Z. bailii*, this effect was only observed in the presence of acetic acid concentrations above 0·33 mol l^{-1} (2·0%, v/v). Furthermore in *Z. bailii*, the value of k_2 was lower than in *S. cerevisiae*. These results suggest that while in *S. cerevisiae* the enhancement of death by weak acids at intermediate temperatures could be considered a consequence of the acidification of the cytoplasm, in *Z. bailii*, the intracellular acidification induced by weak acids is less pronounced and appears not to have a significant role in death at such a temperature range. This reinforces the idea that in *Z. bailii* ISA 1307, as opposed to *S. cerevisiae*, weak acids in general and acetic acid in particular only enhance thermal death and not LED, which may occur at lower temperatures. Furthermore, significant HED at these lower temperatures requires rather high concentrations of the toxic compounds which, at least in the case of acetic acid, are much less realistic for alcoholic fermentations than the ones that induced significant death of this type in *S. cerevisiae*. As a consequence, it could be postulated that cell viability of *Z. bailii* ISA 1307 will not be significantly affected, even at the end of the normal alcoholic fermentation processes. Specifically in wine, this property of *Z. bailii* ISA 1307 may be associated with its presence at the end of the process where the environmental conditions are too severe to allow survival of *S. cerevisiae*. Contributing to these different patterns of behaviour between the two species is probably the fact that *Z. bailii* ISA 1307 is able to use acetic acid simultaneously with glucose, even at low pH values such as 3·5. Under these conditions, in the latter species both the membrane transport flux and the intracellular metabolic flux of the acid seem to be regulated in such a way that the intracellular free acetic acid is maintained at levels below which negative effects may occur while under the same conditions, *S. cerevisiae* is unable to metabolize acetic acid which in turn could be toxic for the yeast (Sousa *et al*. 1997). In contrast to *S. cerevisiae,* the intracellular acidification induced by acetic acid at lower temperatures in *Z. bailii* ISA 1307 was mildest and not related to death. This could be explained by the co-ordination of membrane transport and intracellular metabolic fluxes of the acid.

The responses of the yeast to stress conditions could be considered using both non-adapted and adapted cells. In the present work, although essentially the first type of cells were used, it was also tested whether adaptation of cells to acetic and sorbic acids in the growth medium modified the cell death sensitivity pattern to acid environments. The results showed that in the case of acetic acid, the negative effects induced in cell viability, expressed by the exponential death enhancement constant (k_1), were slightly lower in adapted than in non-adapted cells. This is consistent with the fact that in *Z. bailii* ISA 1307, as mentioned above, transport and intracellular acetic acid metabolism operate independently of the presence of glucose in the growth medium. Meanwhile, adaptation to sorbic acid in the growth medium was expressed by a higher increase in yeast resistance to the acid, evaluated by the enhancement effects on cell death, a decrease of about 37% in the value of k_1 being observed. We found that sorbic acid, unlike acetic acid, is not used by *Z. bailii* ISA 1307 as the only carbon and energy source, but it remains to be clarified and identified which are the mechanisms underlying such adaptation to the acids in the presence of glucose.

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REFERENCES

Cardoso, H. and Leão, C. (1992) Mechanisms underlying low and high enthalpy death induced by short-chain monocarboxylic acids in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **38**, 388–392.

Fernandes, L., Côrte-Real, M., Loureiro, V., Loureiro-Dias, M.C. and Leão, C. (1997) Glucose respiration and fermentation in *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* express different sensitivity patterns to ethanol and acetic acid. *Letters in Applied Microbiology* **25**, 249–253.

Fleet, G. (1992) Spoilage yeasts. *Critical Reviews in Biotechnology* **12**, 1–44.

Malfeito-Ferreira, M., Lopes, I. and Loureiro, V. (1990) Characterization of spoilage yeasts in Portuguese bottled dry white wines. In *Actualités Oenologiques* Vol. 89. Paris: Dunod.

Pampulha, M.E. and Loureiro-Dias, M.C. (1989) Combined effect

of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Applied Microbiology and Biotechnology* **31,** 547–550.

Pinto, I., Cardoso, H. and Leão, C. (1989) High and low enthalpy death in *Saccharomyces cerevisiae* induced by acetic acid. *Biotechnology and Bioengineering* **33,** 1350–1352.

Prudêncio, C., Sansonetty, F. and Côrte-Real, M. (1998) Flow cytometric assessment of cell structural and functional changes induced by acetic acid in the yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. *Cytometry* **31,** 307–313.

Rottenberg, H. (1979) The measurement of membrane potential and pH in cells, organelles and vesicles. *Methods in Enzymology* **5,** 547–569.

Sousa, M.J., Miranda, L., Côrte-Real, M. and Leão, C. (1996) Transport of acetic acid in *Zygosaccharomyces bailii*: effects of ethanol and their implications on the resistance of the yeast to acid environments. *Applied and Environmental Microbiology* **62,** 3152–3157.

Sousa, M.J., Rodrigues, F., Côrte-real, M. and Leão, C. (1997) Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose by the yeast *Zygosaccharomyces bailii*. *Microbiology* **144,** 665–670.

Thomas, D.S. and Davenport, R. (1985) *Zygosaccharomyces bailii*: a profile of characteristics and spoilage activities. *Food Microbiology* **2,** 157–169.

van Uden, N. (1967) Transport-limited fermentation and growth of *Saccharomyces cerevisiae* and its competitive inhibition. *Archives in Microbiology* **5,** 155–168.

Warth, A.D. (1991) Mechanism of action of benzoic acid on *Zygosaccharomyces bailii*: effects on glycolytic metabolite levels, energy production and intracellular pH. *Applied and Environmental Microbiology*. **57,** 3415–3417.

Wium, H., Malfeito-Ferreira, M., Loureiro, V. and St. Aubyn, A. (1990) A rapid characterization of yeast contaminants associated with sparkling wine production. *Industrie Delle Bevande* **19,** 504–506.