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Glucose respiration and fermentation in *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* express different sensitivity patterns to ethanol and acetic acid

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L. FERNANDES, M. CÔRTE-REAL, V. LOUREIRO, M.C. LOUREIRO-DIAS AND C. LEÃO. 1997. In the yeast *Zygosaccharomyces bailii* ISA 1307, respiration and fermentation of glucose were exponentially inhibited by ethanol, both processes displaying similar sensitivity to the alcohol. Moreover, the degree of inhibition on fermentation was of the same magnitude as that reported for *Saccharomyces cerevisiae*. Acetic acid also inhibited these two metabolic processes in *Z. bailii*, with the kinetics of inhibition again being exponential. However, inhibition of fermentation was much less pronounced than in *S. cerevisiae*. The values estimated with *Z. bailii* for the minimum inhibitory concentration of acetic acid ranged from 100 to 240 mmol l^{-1} total acetic acid compared with values of near zero reported for *S. cerevisiae*. The inhibitory effects of acetic acid on *Z. bailii* were not significantly potentiated by ethanol.

INTRODUCTION

Zygosaccharomyces bailii is considered to be one of the most dangerous yeasts in food technology due to its ability to survive in severe stress environments (Thomas and Davenport 1985). In the wine industry this yeast is responsible for important economic losses because of its capacity to re-ferment sweet wines and to grow and form sediments in dry wines, particularly white wines. In this latter case there is no residual sugar and the capacity of *Z. bailii* to grow is limited to the utilization of other respirable substrates and by the amount of dissolved oxygen in the wine (Malfeito-Ferreira *et al*. 1990).

Zygosaccharomyces bailii is rarely found during the alcoholic fermentation of grape, presumably because it is unable to compete with *Saccharomyces cerevisiae* due to its low growth rate. In contrast, it is frequently found in bottled wines, often dominating the yeast population, and may even appear as the sole yeast in pure culture, because of its high resistance to chemical preservatives such as SO_2 and sorbate and to its tolerance of the high stress environment of the wine itself (alcohol concentration, low pH, presence of toxic weak acids such as acetic acid, etc.) (Thomas and Davenport 1985; Malfeito-Ferreira *et al*. 1990; Loureiro and Malfeito-Ferreira 1993). Although some studies have pointed to the inhibitory effects of preservatives such as benzoic acid on fermentation by *Z. bailii* (Warth 1991), virtually no detailed study and certainly none comparable to those carried out on *S. cerevisiae* have been reported on the sensitivity of the metabolism of this organism to the presence of ethanol and acetic acid in the medium.

The aim of the present work was to compare possible mechanisms underlying the resistances displayed by *Z. bailii* and *S. cerevisiae* to acid media containing ethanol. In particular, the effects of acetic acid on the fermentation and respiration of glucose by *Z. bailii*, in the presence or absence of ethanol, were studied.

MATERIALS AND METHODS

Micro-organism, growth conditions and cell suspension preparation

The yeast *Z. bailii* strain ISA 1307, originally isolated from a continuous production plant of sparkling wine (Wium *et al*. 1990), was used. This strain was found to grow in the presence

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of sorbic acid (5·35 mmol l^{-1}) at pH 3·5 at 25°C. The strain was maintained in a medium containing glucose (2%, w/v), peptone (1%, w/v), yeast extract (0·5%, w/v) and agar (2%, w/v). For growth in liquid medium, a mineral medium with vitamins (Van Uden 1967) supplemented with glucose (2%, w/v) was used and cultures were incubated on a mechanical shaker at 25°C. Cells were harvested during the exponential growth phase, centrifuged, washed twice with ice-cold distilled water and suspended in 0·1 mol l^{-1} phosphate buffer at the desired pH to give a final suspension of about 1 mg dry weight ml^{-1}.

Measurement of the specific rates of fermentation and respiration of glucose

Cell suspensions, prepared as described above, were used to measure fermentation and respiration rates of glucose at various medium pH, either in the absence or in the presence of ethanol or acetic acid or both, using a Warburg apparatus (Umbreit *et al*. 1964). Appropriate concentrations of ethanol, acetic acid and cells in 0·1 mol l^{-1} phosphate buffer were achieved. The pH was adjusted with sodium hydroxide, all experiments were repeated at least three times, and the data reported represent the average values.

RESULTS AND DISCUSSION

Effects of ethanol and acetic acid on the fermentation and respiration of *Z. bailii*

Zygosaccharomyces bailii ISA 1307 was able to respire and to ferment glucose. The specific rates of fermentation and respiration, at 25°C, expressed as rates of carbon dioxide production (μ_{CO_2}) and oxygen consumption (μ_{O_2}), were not significantly affected by extracellular pH over the range 3·5–5·5. The following average values were obtained: μ_{CO_2}, $1{\cdot}02 \pm 0{\cdot}1$ μl min^{-1} (mg dry weight)$^{-1}$; μ_{O_2}, $0{\cdot}99 \pm 0{\cdot}3$ μl min^{-1} (mg dry weight)$^{-1}$. Thus under the experimental conditions used, the relative contributions of fermentation and respiration to the catabolism of glucose were about 75% and 25%, respectively.

At pH 4·5 the presence of ethanol in the medium, above a minimum inhibitory concentration, decreased CO_2 production, the reduction increasing with alcohol concentration (Fig. 1). The semi-logarithmic plot of μ_{CO_2} against the concentration of ethanol yielded a shoulder followed by a straight line of negative slope (Fig. 1, insert). This plot fits the following equation (Van Uden 1989):

$$\mu^{X^{et}}_{CO_2} = \mu^{X^{et}_0}_{CO_2} e^{-k^{et}(X^{et} - X^{et}_{min})} \quad (1)$$

where $\mu^{X^{et}}_{CO_2}$ is the specific fermentation rate, under defined conditions, in the presence of concentration X^{et} of ethanol,

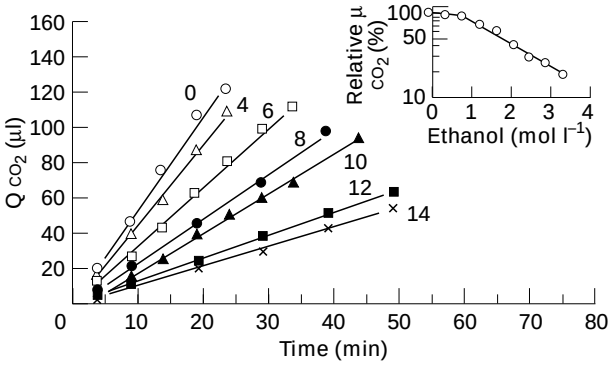

Fig. 1 Effects of ethanol on carbon dioxide evolution (Q_{CO_2}) by the yeast *Zygosaccharomyces bailii* ISA 1307, at pH 4·5 and 25°C. Numbers indicate ethanol concentrations (% v/v). Insert: semi-logarithmic plot of the relative specific rates (%) of fermentation (μ_{CO_2}), as a function of ethanol concentration

$\mu^{X^{et}}_{CO_2}$ is the specific fermentation rate in the absence of ethanol, X^{et}_{min} is the minimum inhibitory concentration of ethanol above which the effects were measurable, and K^{et} is the exponential constant of inhibition rate of ethanol on fermentation. From the results presented in Fig. 1 and from equation 1, the following values for the inhibition parameters were obtained k^{et}, 0·59 l mol^{-1}, and X^{et}_{min}, 1·0 mol l^{-1}.

The specific fermentation rate was also determined in the presence of different concentrations of acetic acid. At concentrations up to 120 mmol l^{-1}, at pH 4·5, fermentation was not affected but higher concentrations of acetic acid inhibited CO_2 production (data not shown), the specific fermentation rate ($\mu^{X^{aa}}_{CO_2}$) decreasing again exponentially with acid concentration (Fig. 2a) as described by equation 1. Similar behaviour was observed when the experiments were carried out at pH 3·5 and 5·5. The values for the exponential inhibition constants of acetic acid (k^{aa}) and the respective X^{aa}_{min} at several pH tested, are given in Table 1. The values of K^{aa} when expressed as the concentration of total acid increased with

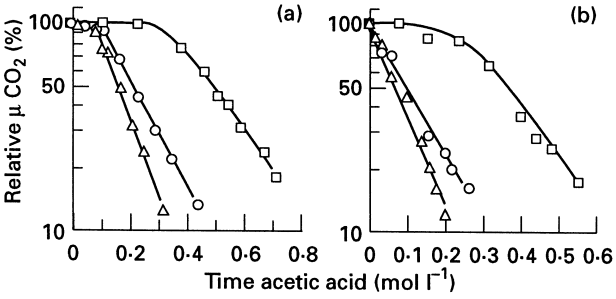

Fig. 2 Relative specific rates (%) of fermentation of glucose by *Zygosaccharomyces bailii* ISA 1307 (μ_{CO_2}), in the absence (a) and in the presence (b) of ethanol (1·47 mol l^{-1}), as a function of acetic acid concentration in reaction medium at pH 3·5 (Δ); pH 4·5 (○); pH 5·5 (□)

Table 1 Values for the minimum inhibitory concentration (X_{min}^{aa+et}) and the exponential inhibition constants (k^{aa+et}) of acetic acid on glucose respiration and fermentation, in the absence or presence of ethanol, at different pH by the yeast *Zygosaccharomyces bailii* ISA 1307 compared to previously reported data on fermentation by the yeast *Saccharomyces cerevisiae* IGC 3507-III

		Z. bailii				*S. cerevisiae*†	
		Respiration		Fermentation		Fermentation	
pH	Ethanol (mol l^{-1})	X_{min}^{aa+et} (mmol l^{-1})	K^{aa+et}* (l mol^{-1})	X_{min}^{aa+et} (mmol l^{-1})	k^{aa+et}* (l mol^{-1})	X_{min}^{aa+et} (mmol l^{-1})	k^{aa+et} (l mol^{-1})
	0	150	11	100	9·2	Near zero	32
3·5	0·87	120	13	100	9·4	Near zero	42
	1·74	10	14	10	11	Near zero	61
	0	180	9·1	120	6·0	Near zero	31
4·5	0·87	140	9·8	100	6·8	Near zero	38
	1·74	45	11	20	7·2	Near zero	55
	0	300	5·9	240	4·4	Near zero	10
5·5	0·87	270	6·2	220	4·7	Near zero	13
	1·74	200	7·1	160	5·2	Near zero	16

* Values estimated from equation 1 expressed as total acid.
† Data on fermentation by *S. cerevisiae* expressed as total acid, from Pampulha and Loureiro (1989).

the decrease in extracellular pH, indicating that most probably the undissociated form of the acid is the active inhibitor. However, if the values were expressed as the concentration of the undissociated form of the acid, they increased slightly with pH (data not shown), suggesting that other factors are probably involved in inhibition.

The effects of acetic acid in the presence of ethanol on fermentation were also investigated at different pH. Again, exponential inhibition kinetics conforming to equation 1 were observed. Figure 2b shows the effects induced by acetic acid in the presence of ethanol (1·47 mol l^{-1}) as representative of this behaviour. The values of the exponential inhibition constant of acetic acid were also an exponential function of ethanol concentration (Fig. 3a), which could be expressed by the following equation (Pampulha and Loureiro 1989):

$$\mu_{CO_2}^{X^{aa}+X^{et}} = \mu_{CO_2}^{X_0^{aa}+X_0^{et}} e^{-k^{et}X^{et}-k^{aa}X^{aa}} e^{+k^{aa+et}X^{et}} \quad (2)$$

where $\mu_{CO_2}^{X^{aa}+X^{et}}$ is the fermentation rate in the presence of concentration X^{aa} of total acetic acid and X^{et} of ethanol, and k^{aa+et} is the exponential enhancement constant that expresses the enhanced inhibition induced by ethanol over the inhibition of fermentation by acetic acid. The values for k^{aa+et} estimated from the slopes of the lines presented in Fig. 3a were very low and very similar for all pH tested (Table 2).

The cell suspensions prepared for the fermentation assays were used, in parallel, to study the effects of ethanol, or acetic acid or both on the respiration of glucose. Measurements of the consumption of O_2, at 25°C, at pH 4·5, showed that

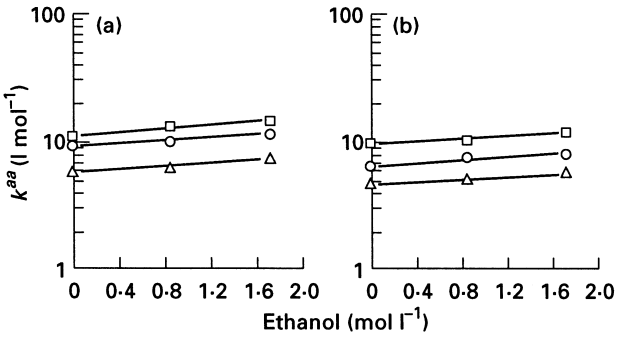

Fig. 3 Effect of ethanol on the exponential inhibition constant of acetic acid (k^{aa}) on the specific rates of fermentation (a) and respiration (b) in the yeast *Zygosaccharomyces bailii* ISA 1307 in reaction medium at pH 3·5 (□); pH 4·5 (○); pH 5·5 (△)

ethanol inhibited the respiration rate of glucose. As in fermentation, the inhibition conformed to equation 1 and thus obeyed exponential kinetics (data not shown). The following values for the inhibition parameters were obtained: k^{et}, 0·9 l mol^{-1}, and X_{min}^{et}, 1·0 mol l^{-1}. Similarly, the inhibitory effects of acetic acid on respiration at different pH values obeyed exponential inhibition kinetics (data not shown). Table 1 shows data for the inhibitory effects of acetic acid on glucose respiration. There was a correlation between the exponential inhibition constants of acetic acid and ethanol, at the different pH values (Fig. 3b), which conformed to equation 2. The inhibition of respiration by acetic acid was not significantly

Table 2 Exponential enhancement constants of ethanol on the inhibition of fermentation and respiration by acetic acid (k^{aa+et}), at different pH, in the yeast *Zygosaccharomyces bailii* ISA 1307. Values of k^{aa+et} previously reported, calculated from data from the yeast *Saccharomyces cerevisiae* IGC 3507-III, are included

	k^{aa+et}* (l mol^{-1}) for:		
	Z. bailii		*S. cerevisiae*†
pH	Fermentation	Respiration	Fermentation
3·5	0·12	0·13	0·33
4·5	0·11	0·11	0·33
5·5	0·10	0·10	0·33

* Values estimated from equation 2.
† Data from Pampulha and Loureiro (1989).

stimulated by ethanol at any pH tested between 3·5 and 5·5 (Table 2).

Comparison with *S. cerevisiae*

The results presented in the previous section show that ethanol inhibited fermentation and respiration of glucose in *Z. bailii*, both metabolic processes displaying similar sensitivities to the effects of alcohol as evidenced by the values of the inhibition parameters, k^{et} and X^{et}_{min}. The values obtained for these two parameters on fermentation were not significantly different from those reported for *S. cerevisiae* (Leão and Van Uden 1985). Acetic acid also inhibited respiration and fermentation of glucose in *Z. bailii*. Although the kinetics of inhibition of fermentation were very similar to those described for *S. cerevisiae*, the differences between the values obtained for parameters k^{aa} and X^{aa}_{min} in both yeasts are noteworthy (Table 1). For *Z. bailii*, the values for k^{aa} at pH between 3·5 and 5·5 were, on average, 6–15 times lower than for *S. cerevisiae*. In turn, the values of X^{aa}_{min} over the same pH range were near zero for *S. cerevisiae*, whereas for *Z. bailii* they were very high, ranging from 100 to 240 mmol l^{-1} total acetic acid. Furthermore, in *Z. bailii* ethanol did not significantly potentiate the inhibitory effects of acetic acid, the values of the exponential enhancement constant k^{aa+et} being very low and similar at all the pH tested, and about one-third of the values reported for *S. cerevisiae* (Table 2). In contrast, it is noteworthy that both fermentation and respiration were affected to the same extent by acetic acid in *Z. bailii*, which is probably an indication that metabolism is affected in a global way. As for *S. cerevisiae*, in *Z. bailii* previous data indicated that the respiratory flux is relatively constant and changes in glycolytic flux are reflected mainly in changes in fermentation (Sousa-Dias *et al*. 1996). The fact that acetic acid affects both pathways suggests that the response is not a simple inhibition of glycolysis, but that mitochondrial activity is also disturbed.

In industrial alcoholic fermentations, acetic acid is often present in concentrations of about 10 mmol l^{-1} as a result of the metabolic activity of yeasts and lactic and/or acetic bacteria (Pampulha and Loureiro 1989). The present results indicate that the inhibitory effects induced by acetic acid alone or combined with ethanol on the respiration of glucose by *Z. bailii* were only measurable at total acid concentrations above 150 or 10 mmol l^{-1}, respectively (Table 1). Therefore respiration of glucose will not be significantly affected by either ethanol or acetic acid at the concentrations in which these are present in wine, and this may explain the capacity of *Z. bailii* to grow and form sediments in wine. *Zygosaccharomyces bailii* is also characterized by its ability to re-ferment wines, particularly sweet wines. The results obtained suggest that this ability cannot be due simply to its higher resistance to the effects of ethanol on fermentation than *S. cerevisiae*. However, a different picture emerges in relation to the effects of acetic acid on fermentation. Here, the inhibitory effects of acetic acid either alone or combined with ethanol on the fermentation of *Z. bailii* were only measurable at total acid concentrations above 100 or 10 mmol l^{-1}, respectively (Table 1). As a consequence, one could postulate that the capacity of *Z. bailii* to ferment glucose will not be significantly inhibited even at the end of the normal alcoholic fermentation process in wine, even though the acid and ethanol concentrations are too high to allow glucose fermentation by *S. cerevisiae*. This property of *Z. bailii* may thus be associated with its ability to re-ferment sugars in wines.

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