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Effects of ethanol and acetic acid on the transport of malic acid and glucose in the yeast *Schizosaccharomyces pombe*: implications in wine deacidification

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Abstract

Ethanol and acetic acid, at concentrations which may occur during wine-making, inhibited the transport of L-malic acid in *Schizosaccharomyces pombe*. The inhibition was non-competitive, the decrease of the maximum initial velocity following exponential kinetics. Glucose transport was not significantly affected either by ethanol (up to 13%, w/v) or by acetic acid (up to 1.5%, w/v). The uptake of labelled acetic acid followed simple diffusion kinetics, indicating that a carrier was not involved in its transport. Therefore, the undissociated acid appears to be the only form that enters the cells and is probably responsible for the toxic effects. Accordingly, deacidification by *Ss. pombe* during wine fermentation should take place before, rather than after, the main alcoholic fermentation by *Saccharomyces cerevisiae*.

Keywords: *Schizosaccharomyces*; Ethanol inhibition; Acetic acid inhibition; Carboxylic acid transport; Glucose transport; Wine deacidification

1. Introduction

Malic acid is one of the principal organic acids in grape must and wine. Microbiological deacidification, when desirable, may include the use of yeasts that are able to degrade this acid under conditions similar to those employed in wine-making. *Schizosaccharomyces (Ss.) pombe*, a yeast frequently proposed for such deacidification, transports malic acid by a proton-dicarboxylate symport which is not subject to glucose repression, being active even in the presence of high glucose concentrations, like those present during vinification [1]. Under these conditions, although the intracellular steps of the acid metabolism are not completely clarified, the yeast is certainly able to use and convert L-malic acid to ethanol and carbon dioxide (the so-called malo-alcoholic fermentation) simultaneously with the utilization of glucose.

Acetic acid is a normal end-product of fermentation by *Saccharomyces cerevisiae* and additional amounts may be produced in vinification by contaminating lactic and/or acetic acid bacteria [2]. Although relevant research has been carried out on the

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toxic effect of acetic acid, alone or in combination with ethanol, on the activity of the fermentative yeast *S. cerevisiae*, virtually nothing is known about its effects on the yeast *Ss. pombe* and, in particular, on the transport of malic acid across the plasma membrane, the first step of the acid metabolism. This information may help to clarify the still controversial question of whether deacidification by this yeast must be conducted before or after the main fermentation [3,4]. Here we report on the effects of ethanol and acetic acid on the transport of malic acid and glucose in the strain G2 of *Ss. pombe*. Furthermore, possible implications of these effects on the deacidification activity of the yeast during wine fermentation are also discussed.

2. Materials and methods

2.1. Microorganism, growth conditions and cell suspension preparation

The yeast *Schizosaccharomyces pombe* G2, isolated at the Institut Coopératif du Vin (Montpellier, France), was maintained on a medium containing glucose (2%, w/v), peptone (1%, w/v), yeast extract (0.5%, w/v), and agar (2%, w/v). For growth, a liquid mineral medium with vitamins [5] supplemented with the carbon sources, indicated in Results, and mechanical shaking at 25°C, were used. At mid-exponential growth phase, cells were harvested, centrifuged, washed twice with ice-cold distilled water and suspended in distilled water to a final concentration of about 80 mg dry weight ml^{-1}.

2.2. Measurement of initial uptake rates

The uptake rates of malic acid, acetic acid and glucose were measured by the use of L-[U-^{14}C]malic acid, [U-^{14}C]acetic acid, and D-[1-^{3}H]glucose, respectively. To estimate the uptake rates, 10-μl amounts of the yeast suspension were mixed in 10-ml conical tubes with 30 μl of 0.1 M phosphate buffer at the desired pH value. After 2 min of incubation in a water bath (25°C), the reaction was started by the addition of 10 μl of an aqueous solution of the radiolabelled compound at the desired concentration and stopped by dilution with 5 ml of ice-cold water. When the uptakes of glucose and malic acid were measured in the presence of ethanol, acetic acid, or an uncoupler (carbonyl cyanide-*m*-chlorophenyl-hydrazone, CCCP), the cells were preincubated for 5 min with the inhibitor before adding the labelled substrate. Sampling times were 0, 5 and 10 s for malic acid or glucose uptake and 0, 30 and 60 s for acetic acid uptake. After stopping the reaction, the mixtures were immediately filtered through GF/C filters (Whatman, Clifton, NJ), the filters washed with 10 ml of ice-cold water, and counted in the scintillation fluid OptiPhase HiSafe II (LKB Scintillation Products). Radioactivity was measured with a Packard Tri-Carb 2200 CA liquid scintillation counter, with correction for disintegrations per minute (Packard Instruments, Rockville, MD).

2.3. Calculation of concentrations

Concentrations of the ionization forms of acetic acid were calculated by the Henderson-Hasselbach equation using a pK value of 4.76.

2.4. Chemicals

Radioactively labelled malic acid, acetic acid and glucose were obtained from Radiochemical Center (Amersham, Buckinghamshire, UK) and had the following specific activities: L-[U-^{14}C]malic acid, 51.4 mCi $mmol^{-1}$; [U-^{14}C]acetic acid, 56 mCi $mmol^{-1}$; D-[1-^{3}H]glucose, 8.3 mCi $mmol^{-1}$. All other chemicals were reagent grade and were obtained from commercial sources.

2.5. Reproducibility of the results

All the experiments were repeated at least three times, and the data reported here are the average values.

3. Results

3.1. Effect of ethanol and acetic acid on transport of L-malic acid

Cells of *Schizosaccharomyces pombe* grown in a medium with either glucose or glucose plus L-malic

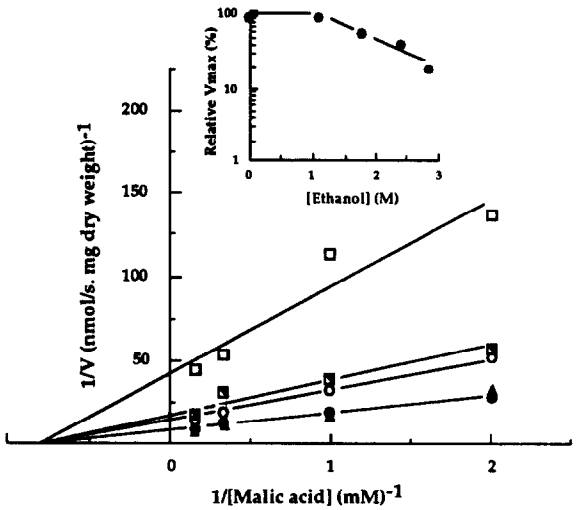

Fig. 1. Double reciprocal plots of the initial uptake rates of labelled malic acid by *Ss. pombe* G2 in the absence (●) or in the presence of ethanol: △, 1.09 M; ○, 1.74 M; ◹, 2.39 M; □, 2.82 M. Insert: Semilog plots of the relative (%) maximum uptake rates of malic acid by *Ss. pombe* G2 as a function of the ethanol concentration.

acid as carbon and energy sources were used to measure the transport of L-malic acid in the absence and presence of ethanol or acetic acid. Double reciprocal plots of the initial uptake rates of labelled L-malic acid (pH 3.0), as a function of its concentration, showed that the affinity of the transport system for malic acid was not affected by ethanol (Fig. 1), the average value of K_m, with and without ethanol, being 1.2 ± 0.1 mM total acid. The effect of the alcohol appeared to be essentially on V_{max}, the maximum initial uptake rate, indicating that the observed inhibition was of a noncompetitive type. Furthermore, the V_{max} values, obtained by extrapolating the double reciprocal plots, decreased exponentially with increasing ethanol concentration, above the minimum inhibitory concentration, in accordance with the following Eq. [6]:

$$V_{max}^{X} = V_{max}^{0}\, e^{-k(X - X_{min})} \quad (1)$$

where V_{max}^{X} is the maximum initial uptake rate under defined conditions, V_{max}^{0} is the maximum uptake rate under these conditions in the absence of ethanol, X is the external ethanol concentration, X_{min} is the minimum inhibitory concentration of ethanol and k is the exponential inhibition constant. From the data

Table 1
Parameters of the inhibition of malic acid and glucose transport in *Ss. pombe* G2, at pH 3.0, by ethanol and acetic acid

Inhibitor	Exponential inhibition constant (M^{-1})		Minimum inhibitory concentration (M)	
	Malic acid	Glucose	Malic acid	Glucose
Ethanol	0.85	– [a]	1.2	– [b]
Acetic acid	98.5	3.29	near zero	0.02

[a] No inhibition was detected.
[b] Not applicable.

depicted in the insert of Fig. 1, the values of the inhibition constant for ethanol and the respective minimum inhibitory concentration could be calculated and are shown in Table 1.

Like ethanol, at pH 3.0, acetic acid inhibited L-malic acid transport non-competitively and exponentially (Fig. 2). The average value of K_m for L-malic acid, with and without acetic acid, was 1.0 ± 0.1 mM total acid, whereas the maximum transport capacity, V_{max}, decreased exponentially with the external acetic acid concentration. The exponential inhibition constant for acetic acid was calculated (Table 1) from the data depicted in the insert of Fig. 2 and according to equation 1. Moreover, under our

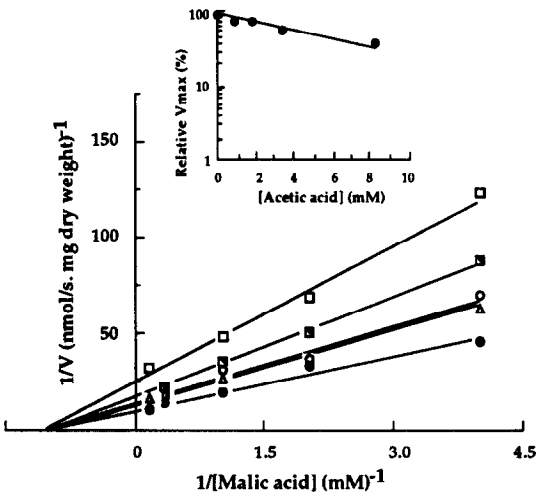

Fig. 2. Double reciprocal plots of the initial uptake rates of labelled malic acid by *Ss. pombe* G2 in the absence (●) or in the presence of acetic acid: △, 0.83 mM; ○, 1.67 mM; ◹, 3.33 mM; □, 8.33 mM. Insert: Semilog plots of the relative (%) maximum uptake rates of malic acid by *Ss. pombe* G2 as a function of the acetic acid concentration.

experimental conditions the minimum inhibitory acid concentration was practically zero.

3.2. *Effect of ethanol and acetic acid on transport of glucose*

Cells of *Ss. pombe* G2 grown in a medium with either glucose, fructose or glucose plus L-malic acid as carbon sources, transported glucose across the plasma membrane by a proton symport system which also accepted 2-deoxyglucose (results not shown). These observations are in accordance with previous studies on the transport of those sugars in another strain of *Ss. pombe* [7]. The uptake of labelled glucose was measured at pH 3.0 and by extrapolating the double reciprocal plot of its initial uptake rates, as a function of its concentration, the following kinetic parameters were obtained: K_m, 50 ± 5 mM glucose and V_{max}, 1.2 ± 0.1 nmol of glucose s^{-1} (mg dry weight)$^{-1}$. When the uptake of labelled glucose was measured in the presence of either ethanol or acetic acid, the effects of both compounds on glucose transport were much less pronounced than those described above for the transport of L-malic acid in cells grown under the same experimental conditions. Double reciprocal plots of the initial uptake rates of glucose, at pH 3.0, were practically coincident, with and without ethanol, up to 13% (w/v) (not shown) indicating that, under these experimental conditions, ethanol had no significant effect on glucose transport. On the other hand, following the same methodology, at pH 3.0, the affinity of the transport system for glucose was not affected by acetic acid (up to 1.5%, w/v) and the maximum transport capacity (V_{max}) was only slightly affected. The values of the respective exponential inhibition constant and the minimum inhibitory concentration are shown in Table 1.

In addition, at the same pH, the value of the V_{max} decreased by about 60% in the presence of 45 μM CCCP, giving evidence of the dependence of glucose transport on the ΔpH.

To clarify whether the transport of glucose was more sensitive to the toxic effects of ethanol and acetic acid at higher pH values, the uptake of labelled glucose was measured at pH 5.0, in the absence and presence of those compounds. The value of V_{max} for glucose transport at this pH, estimated by extrapolating the experimental double reciprocal plot of its initial uptake rates (not shown), was 1.3 ± 0.2 nmol of glucose s^{-1} (mg dry weight)$^{-1}$. The inhibition induced by both compounds became more pronounced than at pH 3.0, a decrease of about 55% on the V_{max} being observed in the presence of 13% (w/v) ethanol or 1.5% (w/v) acetic acid. A similar inhibition value (60%) was found when the

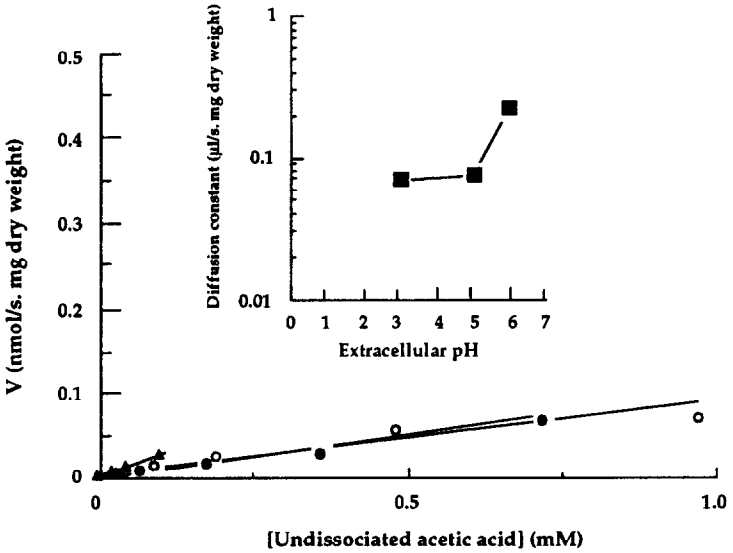

Fig. 3. Initial uptake rates of undissociated acetic acid as a function of its concentration by glucose grown cells of *Ss. pombe* G2. ○, pH 3.0; ●, pH 5.0; ▲, pH 6.0. Insert: dependence on the extracellular pH of the diffusion constants calculated from the slopes.

glucose uptake was estimated at pH 5.0, after incubation with 45 μM CCCP.

3.3. Transport of acetic acid across the plasma membrane

In order to better understand the mechanisms underlying the toxic effects of acetic acid, we set out to elucidate the mechanism of membrane transport of the acid in cells grown in a medium containing either glucose or a mixture of glucose and malic acid. The initial uptake rates of labelled acetic acid were measured at various extracellular pH values (from pH 3.0 to 6.0) at concentrations up to 2.0 mM. At pH 3.0, when the acid is nearly 100% undissociated, the plot of the initial velocities against the concentration of the undissociated acid was linear (Fig. 3). As also shown in Fig. 3, similar results were obtained at pH 5.0 and 6.0. These data suggest that a carrier-mediated transport was not involved and that the uncharged acetic acid entered the cells by simple diffusion. The values of the diffusion constant (k_d) could be calculated from the slopes of the linear plots at the various pH values. The permeability of the plasma membrane for the undissociated acid did not appear to be significantly affected by the extracellular pH, with the diffusion constant increasing slightly only between pH 5.0 and 6.0. This is shown in the insert of Fig. 3 in which the value of k_d is plotted against the respective extracellular pH value.

4. Discussion

Schizosaccharomyces pombe cannot use externally added L-malic acid as the only carbon and energy source. However, this species is able to use L-malic acid if glucose is present in the culture medium. This ability makes *Ss. pombe* a potential candidate for a microbiological wine deacidification process which, in principle, should be conducted under conditions that favour malic acid removal. Earlier studies have been reported on the influence of fermentation conditions on the kinetics of deacidification by *Ss. pombe* G2 [3]. Among the factors that may contribute to the optimization of such a process, possible interactions must be considered of ethanol, and other by-products of alcoholic fermentation, with the membrane transport of those substrates, malic acid and glucose. In accordance with our results, ethanol or acetic acid inhibited the transport of L-malic acid while the transport of glucose, at pH 3.0, was not significantly affected by any of the compounds at the same concentration range.

In our experimental conditions, only the undissociated form of acetic acid appears to enter the cells by simple diffusion. The transport of both glucose and L-malic acid is mediated by proton symport [1,7]. Thus, ethanol or acetic acid could, in principle, affect the transport of these substrates by acting on the transport protein and/or as uncouplers and dissipate either the ΔpH or the membrane potential, or both. Concerning L-malic acid transport, the observed inhibition by ethanol or acetic acid, was consistent with dependence on the ΔpH [1]. In the case of glucose transport, a significant inhibitory effect by any of the compounds was only observed at higher pH (5.0). At this pH, inhibition by the uncoupler CCCP indicated that the transport of glucose depended on the proton-motive force. Hence, at pH 5.0, ethanol and acetic acid decreased the capacity of sugar transport system probably by dissipating either the ΔpH, the $\Delta\Psi$ or both. On the other hand, at lower pH values (pH 3.0), at which the membrane potential is almost absent, although the inhibitory effect induced by CCCP was consistent with the dependence of the glucose transport on the ΔpH, apparently both ethanol and acetic acid are not able to produce a decrease of the ΔpH sufficient to inhibit transport.

From an enological point of view, and taking into account that in a grape must fermentation the acetic acid is frequently present at concentrations around 0.06% (w/v), the results presented show that the effect of ethanol (up to 13%, w/v) and of acetic acid (up to 1.5%, w/v) on glucose transport, at pH 3.0, could be negligible. Measurable inhibitory effect on this transport system will require higher and less realistic concentrations of the toxic metabolites. On the other hand, according to our results, L-malic acid maximum transport capacity was reduced by about 70% in the presence of 0.05% (w/v) acetic acid. Hence at the end of the fermentation, the consumption of L-malic acid, compared with that of glucose, may be significantly inhibited by acetic acid. Furthermore, these effects may become greater because

of increasing ethanol concentration. As a consequence, the conditions present at the end of the fermentation would not favour the consumption of malic acid, the objective of a deacidification process. Thus, our results give support to the proposal that deacidification by *Ss. pombe* should take place by adding an appropriate inoculum to the grape must before, rather than after, the traditional alcoholic fermentation by *S. cerevisiae*. Since the values of ethanol yield (expressed as grams of ethanol formed per grams of glucose consumed) of *Ss. pombe* are lower than those described for *S. cerevisiae* [3], in such a process, the first step would be the deacidification of grape must by *Ss. pombe* lasting long enough only to reduce malic acid to the desired concentration without consuming too much glucose. In the next step, after removing *Ss. pombe*, the main alcoholic fermentation by *S. cerevisiae* would follow.

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