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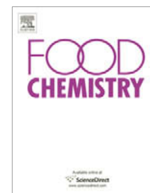

Short communication

Acetic acid removal by *Saccharomyces cerevisiae* during fermentation in oenological conditions. Metabolic consequences

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ABSTRACT

The commercial *Saccharomyces cerevisiae* strains used in champagne winemaking were tested for their ability to metabolise acetic acid during alcoholic fermentation. Fermentation tests were performed in conditions close to oenological ones using a Chardonnay grape juice supplemented with acetic acid. The amount of acetic acid metabolised by wine yeast increased with increasing initial acetic acid concentration and this elimination occurred during the second part of the exponential growth phase. When the initial acetic acid concentration exceeds 1 g/l, and whatever the yeast strain used, the concentration of acetic acid in the resulting wine cannot be reduced to an acceptable level according to the current legislation. Acetic acid removal modified yeast metabolism, since more acetaldehyde, less glycerol and less succinic acid were produced. Considering the reduction of the $NADPH/NADP^+$ ratio following acetic acid consumption, we propose, as a new hypothesis, that acetic acid could modify yeast metabolism by reducing the activity of the $NADP^+$ dependent aldehyde dehydrogenase Ald6p.

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1. Introduction

Acetic acid, which is the main component of volatile acidity, is critical for wine quality and can be a cause of incomplete alcoholic fermentation (Rasmussen, Schultz, Snyder, Jones, & Smith, 1995). Its concentration in wine usually ranges from 0.1 to 0.5 g/l (Whiting, 1976) and, according to current legislation, it must remain below 1.07 g/l for white wines and 1.2 g/l for red wines.

This acid is mainly produced before alcoholic fermentation by bacterial spoilage in *Botrytis cinerea* infected grapes or in Sour Rot infected grapes. The rupture of the grape berry skin caused by the infection allows acetic acid bacteria to access the inner part of the berry and to use, as a preferential carbon source, ethanol that is produced in small amounts by yeast. As a consequence, acetic acid concentration in the resulting musts can increase to over 1.5 g/l, while it is almost undetectable in the musts produced from "clean grapes" (Zoecklein, Williams, & Duncan, 2001).

As a normal end-product of the alcoholic fermentation, acetic acid can also be formed by *Saccharomyces cerevisiae* and additional amounts may be produced after alcoholic fermentation by lactic acid bacteria (Cogan, 1987) and/or acetic acid bacteria (Drysdale & Fleet, 1988). The concentration of acetic acid produced during alcoholic fermentation may vary with the species and strains of yeast (Shimazu & Watanabe, 1981), the composition of must (Delfini & Costa, 1993) and physical factors (Radler, 1983). Acetic acid produced by *S. cerevisiae* rapidly forms during the fermentation of the first 50–100 g/l of sugar but later some is metabolised.

The mechanism of the first step of acetic acid metabolism, namely its transport inside the cells, has been elucidated in *S. cerevisiae* IGC 4072 (Cardoso & Leao, 1992; Casal, Cardoso, & Leao, 1997, 1998). The anionic form is transported either by an acetate-proton symport or a more general monocarboxylate carrier that could also transport lactate, pyruvate and propionate. Both systems are inhibited by ethanol and subjected to glucose repression. The undissociated acetic acid enters the cell by a passive diffusion mechanism, which is mainly mediated by the open glycerol channel (Fps1 aquaglyceroporin) of the plasma membrane (Mollapour & Piper, 2007; Mollapour, Shepherd, & Piper, 2008). When *S. cerevisiae* is subjected to acetic acid stress, acetate transiently activates Hog1 MAP kinase, leading to the endocytosis and degradation of the Fps1 channel (Mollapour & Piper, 2007). Such Fps1 destabilisation, which does not occur when Hog1 is activated by hyperosmotic stress (Mollapour & Piper, 2006), is important at low pH. Glucose repressed the acquisition of resistance to acetic acid in *S. cerevisiae* cultures as it eliminates the channel implicated in the passive diffusional entry of this acid into cells.

Apart from the use of a combination of reverse osmosis and ion exchange (Jones, 1997), there is no other technical possibility to selectively remove acetic acid from must and wine. Following the results published by Ventre (1937), an empirical practice, well known by winemakers, has been developed to remove acetic acid from wine. It consists of a wine refermentation after it has been mixed with healthy grape juice, and is based on the assumption

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that acetic acid present at the beginning of fermentation can be partially metabolised by fermentative yeasts at the middle-end of this process (during the fermentation of the first 50–100 g/l of sugars). This practice has been reported by Ussegliot-Tomasset (1995) and Ribéreau-Gayon, Dubourdieu, Donèche, and Lonvaud (2000) but in both textbooks the findings are not referenced and they do not contain any experimental data. Scientific papers have investigated acetic acid consumption during yeast fermentation, either by mutants of *S. cerevisiae* (Pampulha & Loureiro-Dias, 2000; Schneider, 1996) or by wild strains (Vilera-Moura, Schuller, Mendes-Faia, & Real, 2008). Nevertheless, with regard to the media and growth conditions used, none of these studies were performed under oenological conditions and none of them have investigated the metabolism of acetic acid by wine yeast during alcoholic fermentation.

In order to better understand the consequences and the oenological interest of alcoholic fermentation in the removal of volatile acidity and to improve our understanding of acetic acid metabolism by wine yeast, the present study investigates the capacity of oenological strains of *S. cerevisiae* to metabolise acetic acid during alcoholic fermentation in conditions close to oenological ones (unaerated and unshaken grape juice).

2. Materials and methods

2.1. Yeast strains

The commercial strains used in this study were *S. cerevisiae* DV10, (Station Oenotechnique de Champagne), *S. cerevisiae* IOC (Institut Oenologique de Champagne) and *S. cerevisiae* levuline CHP (Laboratoire Bolan). They were stored at −80 °C in YEG medium (glucose 1% w/v, yeast extract 0.5% w/v) supplemented with glycerol (30% w/v).

2.2. Media and yeast growth

A Chardonnay grape juice was used as fermentation medium with and without adding acetic acid. Before acetic acid addition juice was analysed (Institut Oenologique de Champagne) and its composition was: pH: 2.97, glucose: 88 g/l, fructose: 90 g/l, total SO_2: 46 mg/l, free SO_2: 20 mg/l. Acetic acid was added to grape juice at final concentrations of 0.25 g/l, 0.5 g/l, 0.75 g/l, 1 g/l and 2 g/l before the media were pasteurised at 100 °C for 15 min. Excessive concentrations, that is, 1 g/l and 2 g/l of acetic acid, were selected because they can be found in musts obtained from *Botrytis cinerea* infected grapes or from Sour Rot infected grapes (Zoecklein et al., 2001). Since some of the acetic acid added may be lost during the pasteurisation process, its concentration in the media before yeast inoculation, referred to in the tables as "initial concentration", was determined after pasteurisation. Media were inoculated at a concentration of 10^6 CFU/ml with a culture previously grown for 48 h at 24 °C in a pasteurised grape juice/water medium (50/50 v/v). Fermentations were carried out at 18 °C without any shaking, in 500-ml flasks filled to 95% of their volume and covered with a cotton cap. Growth was followed by measuring optical density at 600 nm and dry weight. All fermentations were performed in triplicate and for each acetic acid concentration tested, a flask filled with uninoculated medium was used as a control. Fermentations were considered as complete when less than 2 g/l residual sugars were left.

2.3. Evaluation of yeast growth

Yeast growth was monitored by periodic spectrophotometric measurements at 600 nm and calibrated against cell dry measurements. Cells were harvested by centrifugation, washed twice with distiled water and dried for 24 h at 103 °C.

2.4. Metabolite analyses

Concentrations of glucose, fructose, ethanol, acetaldehyde, glycerol, acetic acid, and succinic acid were measured using enzymatic test kits (Boehringer, Mannheim, Germany). The metabolite and biomass yields were calculated from the determined respective concentrations at the end of the exponential growth phase.

2.5. Determination of intracellular coenzymes contents

The intracellular contents of $NADP^+$ and NADPH were determined as described by Nissen, Anderlund, Nielsen, Villadsen, and Kielland-Brandt (2001).

2.6. Statistical analysis

A single-factor ANOVA (Excel, Microsoft) was used to evaluate the differences between the assays concerning acetic acid consumption and metabolite production.

3. Results and discussion

3.1. Evidence for acetic acid removal

For each medium, alcoholic fermentation was complete and although its duration gradually increased, it did not seem to be notably affected by acetic acid, even at 2 g/l (Table 1). As soon as acetic acid is added to grape juice, alcoholic fermentation leads to a decrease in the concentration of acetic acid relative to the starting juice for the treatment considered. The amount of acetic acid eliminated during alcoholic fermentation increases monotonically with increasing initial acetic acid concentrations. Nevertheless, when acetic acid is added at 2 g/l and whatever the strain used, its elimination during alcoholic fermentation is not sufficient to reach an acceptable level in the resulting wine according to the current legislation.

By the end of the fermentations, acetic acid elimination is never complete and the media in which the concentration of acetic acid were the highest before alcoholic fermentation were still the most concentrated in acetic acid after alcoholic fermentation. Moreover, when starting acetic acid concentrations did not exceed 1 g/l, the amounts of acetic acid removed (i.e., the difference between the initial acetic acid content and the final one) are strongly correlated with the initial acetic acid content. Linear regression indicates that about 50% of the initial acetic acid content is eliminated by the end of the alcoholic fermentation ($r^2 = 0.99$).

3.2. Evolution of acetic acid in relation with yeast growth

The evolution of acetic acid during yeast growth was followed using either unsupplemented grape juice or grape juice supplemented with 1 g/l of acetic acid (Fig. 1). For both fermentations, three steps in acetic acid evolution can be observed. In unsupplemented grape juice, acetic acid concentration increases from the beginning of the alcoholic fermentation to the middle exponential growth phase, then it decreases until yeast growth stops and finally increases slightly until the end of the fermentation. In supplemented grape juice, the first step where acetic acid concentration increased disappears. Acetic acid concentration seems to remain almost unchanged until the middle of the exponential growth phase and then it decreases, this decrease being more pronounced, as compared to the unsupplemented grape juice. The yield factor

Table 1
Evolution of acetic acid, ethanol and sugars following alcoholic fermentation.

Added acetic acid (g/l)	Acetic acid concentration (g/l)			Time (h)	Ethanol (g/l)	Residual hexose (g/l)	
	Initial	Final	Variation			Glucose	Fructose
S. cerevisiae DV10							
0	0.06 ± 0.04	0.12	+0.06 ± 0.04 a	119	82.7 ± 1.2 a	0	0.03
0.25	0.31	0.21	−0.10 b	119	82 ± 1 a	0	0.04
0.50	0.56 ± 0.01	0.31 ± 0.03	−0.25 ± 0.04 c	119	82.2 ± 0.9 a	0	0.04
0.75	0.82 ± 0.02	0.49	−0.33 ± 0.02 d	130	82.4 ± 1.3 a	0	0.1
1	1.05	0.61 ± 0.01	−0.44 ± 0.01 e	140	82.7 ± 0.4 a	0	0.21 ± 0.09
2	2.07	1.52 ± 0.1	−0.55 ± 0.1 f	165	82.3 ± 0.8 a	0	0.47 ± 0.04
S. cerevisiae IOC							
2	2.07	1.45 ± 0.1	−0.62 ± 0.1 f	165	82.5 ± 0.5 a	0	0.58 ± 0.1
S. cerevisiae levuline CHP							
2	2.03 ± 0.01	1.49 ± 0.07	−0.54 ± 0.08 f	165	82.9 ± 0.5 a	0	0.60 ± 0.04

Different letters within the same column show significant difference at $p_{value} = 0.05$.

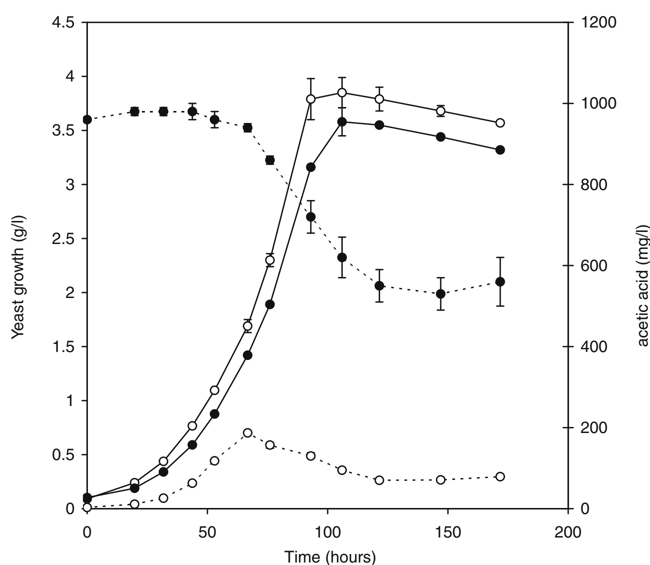

Fig. 1. Growth kinetics (—) and variation of acetic acid concentration (—) during fermentations with *S. cerevisiae* DV10. Fermentations were performed in must without adding acetic acid (○) or supplemented with 1 g/l of acetic acid (●).

calculated from the acetic acid removed and the biomass produced between the middle of the exponential growth phase and the end of the yeast growth, increases from 0.088 g/g biomass to 0.127 g/g biomass following grape juice supplementation.

3.3. Effects of acetic acid on growth parameters and metabolite production

Yeast growth is affected by acetic acid, with the most obvious effects being the elongation of the lag phase, the reduction of the specific growth rate (from 0.074 h^{-1} to 0.061 h^{-1}) and the reduction of the maximum biomass production. Acetic acid supplementation also leads to a decreased biomass yield (mg biomass/g hexose) and to an increased specific hexose consumption rate (Table 2). These results are consistent with literature data showing that acetic acid activates glycolytic activity in *S. cerevisiae*, through the reduction of the biomass yield on ATP (Pampulha & Loureiro-Dias, 2000; Taherzadeh, Niklasson, & Liden, 1997).

Metabolite production and intracellular coenzyme ratio are also affected by acetic acid (Table 2). The acetaldehyde yield is increased while both the glycerol and the succinic acid yields, together with the NADPH/$NADP^+$ ratio are significantly decreased. According to Ribéreau-Gayon et al. (2000), increase of the acetaldehyde yield could result from the reduction of assimilated acetate assimilated. Unfortunately there are no data supporting the idea that *S. cerevisiae* is able to reduce acetate to acetaldehyde. Considering the reduction of the NADPH/$NADP^+$ ratio, one can assume, as another hypothesis, that the increase in the acetaldehyde yield following acetic acid addition, could result from a lower expression level of the $NADP^+$ dependent aldehyde dehydrogenase Ald6p, which is the main aldehyde dehydrogenase responsible for acetaldehyde oxidation in glucose-grown cells (Meaden, Dickinson, & Mifsud, 1997).

The decrease of the glycerol yield following acetic acid addition has been reported for a respiratory-deficient mutant of *S. cerevisiae* (Pampulha & Loureiro-Dias, 2000) and could partially be explained by the decrease of the biomass yield. Nevertheless, since the glycerol yield on biomass is also significantly decreased following acetic acid addition, there is at least another explanation for the changes observed in the case of glycerol. In *S. cerevisiae*, glycerol is produced from dihydroxyacetone phosphate (DHAP) in two steps, that is, reduction to glycerol 3-phosphate by a glycerol 3-phosphate dehydrogenase followed by dephosphorylation by a glycerol phosphatase. During grape juice fermentation, this glycerol production helps yeast cells to maintain the cytosolic redox balance, since SO_2, which is added to must as an antimicrobial agent, binds with acetaldehyde (Straford & Rose, 1986) and then prevents NADH oxidation from acetaldehyde reduction to ethanol.

Keeping this in mind, the decrease of glycerol production following acetic acid addition can also be explained by the fact that

Table 2
Intracellular coenzyme ratio, product yields (mg/g hexose), and specific productivity (g/g biomass/h). The values were estimated during the exponential growth phase.

Acetic acid (g/l)	R_{NADPH}	$Y_{Biomass}$ (mg/g)	$Q_{Hexoses}$ (g/g/h)	Y_{Ald} (mg/g)	Q_{Eth} (g/g/h)	Y_{Succ} (mg/g)	Y_{Gly} (mg/g)	Q_{Gly} (mg/g/h)
0	4.92 ± 0.2	64.7 ± 1.03	1.14 ± 0,02	1.50 ± 0.06	0.41	15.7 ± 0.2	42.6 ± 1.7	48.6 ± 1.2
1	3.54 ± 0.52	50.8 ± 1,02	1.20 ± 0.02	1.81 ± 0.09	0.52 ± 0.01	13.4 ± 0.1	36.2 ± 1.1	43.4 ± 0.4
Signif.[a]	***	****	*	**	****	**	**	***

[a], *, **, ***, ****: significant at $p = 0.1$, 0.05, 0.01 and 0.001, respectively.
R_{NADPH} = ratio of NADPH/$NADP^+$, $Y_{Biomass}$ = biomass yield, Y_{Ald} = acetaldehyde yield, Y_{Succ} = succinic acid yield, Y_{Gly} = glycerol yield, $Q_{Hexoses}$ = hexoses specific productivity, Q_{Eth} = ethanol spécific productivity, Q_{Gly} = glycerol specific productivity.

there is a lower necessity for NADH oxidation *via* DHAP reduction. Indeed, since acetaldehyde yield and ethanol-specific productivity are increased, it can be hypothesised, that following acetic acid addition, more acetaldehyde can be reduced in ethanol, leading to a higher NADH oxidation rate.

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