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The Effect of DL-Malic Acid on the Metabolism of L-Malic Acid during Wine Alcoholic Fermentation

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Summary

Insufficient wine acidity can affect wine quality and stability. To overcome this problem, DL-malic acid can be added to the grape juice prior to fermentation. We have investigated the effect of DL-malic acid on wine fermentations and its influence on the final concentration of L-malic acid, naturally present in grape juice. To this end yeast strains that metabolise L-malic acid in different ways were tested and compared; namely, *Schizosaccharomyces pombe* (efficient L-malic acid degrader), *Saccharomyces cerevisiae* (non-degrader), hybrid strain *S. cerevisiae* x *S. uvarum* (intermediate degrader) and *Saccharomyces uvarum* (promoting L-malic acid synthesis). In all cases, D-malic acid passively entered the yeast cells and did not undergo malo-alcoholic fermentation. However, its presence in the juice, as a component of the mixture of D- and L- malic acid (DL-malic acid), reduced the amount of L-malic acid that can be degraded or synthesised by yeasts during malo-alcoholic fermentation.

Key words: D-malic acid; DL-malic acid; L-malic acid; malo-alcoholic fermentation; yeast

Introduction

L-malic acid, together with tartaric acid, are the main organic acids of grapes and wine. During grape juice fermentation yeasts belonging to different species can affect the concentration of L-malic acid in different ways. *Schizosaccharomyces* yeasts can convert L-malic acid to ethanol (malo-alcoholic fermentation) very efficiently, using up all the compound available in the medium. The key enzyme of this reaction is the malic enzyme (EC 1.1.1.38). The high substrate affinity of this enzyme for L-malic acid, as well as the presence of an active transport system for L-malic acid, are responsible for the high metabolisation efficiency of L-malic acid in *Schizosaccharomyces* yeasts (*1,2*). *Saccharomyces cerevisiae* strains generally do not affect L-malic acid concentration (low metabolisation). Only some selected strains were found to degrade up to 50 % of the starting concentration of grape juice malic acid (*3*). The low metabolic efficiency of L-malic acid in *S. cerevisiae* strains is attributed to the low substrate affinity of the *S. cerevisiae* malic enzyme as well as to the absence of an active transport system for L-malic acid. L-malic acid, in fact, enters the cells of *S. cerevisiae* strains only by passive diffusion.

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Saccharomyces uvarum strains can synthesise L-malic acid depending on the starting concentration of the compound in grape juice (*4*). The mechanism of L-malic acid synthesis in *S. uvarum* has not yet been elucidated.

L-malic acid plays a fundamental role in wine acidity. Wines produced in hot countries often lack acidity. Low grape juice acidity affects the body, the colour as well as the microbial stability of the wine. According to the legislation of the country where the wine is produced, DL-malic acid can be added to grape juice to increase wine acidity. The choice of adding DL-malic acid is mainly driven by economic concern, since L-malic acid is an expensive compound.

Since L-malic acid is naturally present in grapes, as well as in most fruits, research concerning wine acidity has been focused on this isomeric form and little is known about the influence of D-malic acid on wine fermentations. D-malic acid, in fact, is not found in nature and it can only be chemically synthesised (*5*).

The aim of this work was to establish whether the addition of DL-malic acid affects the metabolism of L-malic acid, naturally present in grape juice, during wine fermentation.

Materials and Methods

The following yeast strains were used: *Schizosaccharomyces pombe* strain 4z (Istituto di Microbiologia agraria, Università di Firenze, Italy); *S. cerevisiae* strain DIPROVAL 6167 (Università di Bologna, Italy); *S. uvarum* strain DIPROVAL 12233; *S. cerevisiae* X *S. uvarum* interspecific hybrid strain DIPROVAL 35G2 X 12233. The effect of these strains on the metabolism of L-malic acid has been described in previous studies (*4,6–8*).

In order to test the activity of the malic enzyme in the metabolism of L-malic acid or D-malic acid independently from the presence or absence of a specific transport system, the yeast cells were permeabilised using the method described by Fuck *et al.* (*9*). The test was carried out in triplicate. Yeast cultures were grown at 25 °C in YPD containing DL-malic acid (yeast extract 2 % w/v, peptone 2 % w/v, glucose 10 % w/v, DL-malic acid 1.5 % w/v). After three days they were harvested and washed in a phosphate-potassium buffer 0.2 M, pH=7.4. The cells were then diluted in the phosphate--potassium buffer to a concentration of 5 mg/mL (dry weight). Two mL of this suspension were then transferred to test tubes and 50 mL of a solution of toluol-acetone 1:9 were added (*10*). The test tubes were mechanically shaken to favour permeabilisation. One mL of a 15 g/L L-malic or D-malic acid solution was then added to give a final concentration of 5 g/L. This would initiate the reaction catalysed by the malic enzyme. L- and D-malic acid concentrations were determined immediately after adding L- or D-malic (T0) and then after 30 min.

Fermentation tests were carried out in triplicate on grape juice from *Vitis vinifera* cv. Trebbiano containing 0.4 g/L of L-malic acid. 1.5 g/L of L-malic acid or D-malic acid or 3 g/L of DL-malic acid were subsequently added to three separate samples of grape juice. Grape juice samples were then poured into 200 mL flasks and pasteurised at 60 °C for 30 min. Overnight cultures of each strain, grown aerobically in natural grape juice, were used as inoculum at 1 x 10^6 cell/mL. Flasks were incubated at 25 °C and the fermentation progress was monitored by measuring the weight loss caused by CO_2 release. The wines obtained were cooled at 4 °C and clarified by filtration using porous filters (pore size 0.45 μm). L-and D-malic acid and DL-malic acid concentrations were measured enzymatically using specific kits following the instructions of the manufacturer (Boehringer, Mannheim, Germany).

Data relating to the malic enzyme activity and to the malic acid concentration at the end of fermentation were subjected to statistical analysis using SPSS 6.1 for Winows 3.11 Base System (SPSS Inc., USA).

Results and Discussion

The assessment of the malic enzyme activity in permeabilised yeast cells was carried out using three yeasts with different L-malic acid degrading abilities: *Schiz. pombe* strain 4z (efficient degrader), *S. cerevisiae* strain DIPROVAL 6167 (non-degrader) and the interspecific hybrid strain DIPROVAL 35G2 X 12233 (intermediate degrader) (Table 1). All the yeast cultures tested gave equal values of degradation for L-malic acid. This demonstrates that the ability of these yeasts to perform efficient malo-alcoholic fermentation could be attributed mainly to the presence or absence of a transport system. *Schizosaccharomyces pombe* is the only yeast, among the tested ones, provided with an active transport system for malic acid and also the most efficient malic acid degrading strain. This yeast, however, is not suitable for vinification because it produces undesirable off-flavours. Recently, recombinant *S. cerevisiae* wine strains expressing the *Schiz. pombe* gene encoding for malate permease have been constructed (*11,12*). These strains can degrade L-malic acid more efficiently than ordinary wine strains, confirming the essential role of an active transporter on malic acid metabolism.

Table 1. Activity of malic acid enzyme
Yeast cells from three different strains were permeabilised and suspended in a phosphate-potassium buffer added to the toluene-acetone solution (buffer mix). Five g/L of D-malic or L-malic acid were then added to start the reaction carried out by the malic enzyme. Concentration and percentage variations of D-malic and L-malic acids were determined in the centrifuged supernatant of the buffer mix at the moment malic acid was added (t_0), as well as 30 minutes later. The values represent the mean of three independent replications. Within the rows, means followed by the same letter are not significantly different ($p<0.01$).

Yeast strain	γ(L-malic acid)/(g/L)			γ(D-malic acid)/(g/L)		
	t_0	30 min	var./%	t_0	30 min	var./%
Schiz. pombe	5.00 a	4.06 b	–21	4.97 a	4.91 a	0
Hybrid	5.00 a	3.98 b	–23	4.99 a	4.89 a	0
S. cerevisiae	4.98 a	4.14 b	–20	4.98 a	4.89 a	0
Negative control*	5.00 a	4.98 a	0	4.98 a	4.98 a	0

* Buffer mix without addition of yeast culture.
var. = variation

Table 2. Concentration and percentage variation of L-malic acid and D-malic acid content of grape juice supplemented with 1.5 g/L of L-malic acid, 3 g/L of DL-malic acid or 1.5 g/L of D-malic acid and fermented with four different yeast strains. The values represent the mean of three independent replications. Within the columns, means followed by the same letter are not significantly different ($p<0.01$).

	Grape juice + L-malic acid		Grape juice + DL-malic acid				Grape juice + D-malic acid			
	γ(L-malic acid)/(g/L)	var/%	γ(L-malic acid)/(g/L)	var/%	γ(D-malic acid)/(g/L)	var/%	γ(L-malic acid)/(g/L)	var/%	γ(D-malic acid)/(g/L)	var/%
Grape juice	1.76	0	1.85	0	1.44	0	0.40	0	1.50	0
Schiz. pombe	0.07 a	–96	0.63 a	–66	1.42 a	–1	0.16 a	–59	1.47 a	–2
S. cerevisiae	1.48 b	–16	1.66 b	–10	1.36 a	–6	0.40 b	0	1.20 a	–20
S. uvarum	3.45 c	+96	2.03 c	+10	1.33 a	–8	0.40 b	0	1.33 a	–11
Hybrid	0.99 d	–42	1.44 b	–22	1.22 a	–15	0.34 a	–13	1.20	–20

Consistently with what had already been pointed out by Baranowski and Radler (*13*), the permeabilised cells proved to be entirely inactive on D-malic acid (Table 1).

Fermentation tests were carried out on *Schiz. pombe*, *S. cerevisiae* and hybrid strains as well as on *Sacch. uvarum* strain DIPROVAL 12233, able to synthesise L-malic acid. The addition of L-malic, D-malic or DL-malic acid to grape juice before the beginning of fermentation did not affect the fermentation progress for any of the yeast strains employed (results not shown). Strains of *S. cerevisiae*, *S. uvarum* and the interspecific hybrid completed the fermentation in seven days in control grape juice as well as in grape juice with added D-malic, L-malic or DL-malic acid. *Schiz. pombe* strain did not complete the fermentation process, leaving a concentration of unfermented sugars of 10–20 g/L (results not shown).

When L-malic acid was added to the grape juice, *Schiz. pombe* degraded the compound almost to exhaustion, *S. cerevisiae* and the interspecific hybrid degraded the compound causing a reduction of the starting concentration of 16 and 42 %, respectively, whereas *S. uvarum* synthesised the compound increasing its concentration (Table 2).

When D-malic or DL-malic acid were added to the grape juice, *Schiz. pombe*, *S. cerevisiae* and the hybrid strains showed a drastically reduced ability to degrade L-malic acid. When *S. uvarum* strain was employed, the synthesis of L-malic acid was also drastically reduced (Table 2).

The results obtained indicate that D-malic acid is able to enter the yeast cell only by passive diffusion and its presence affects the transport system for L-malic acid in *Schiz. pombe*, resulting in the reduction of the ability to degrade L-malic acid. In *S. cerevisiae* and *S. uvarum* D-malic and L-malic acid enter the cell by passive diffusion and seem to compete with one another at metabolic level. D-malic acid (not susceptible to malo-alcoholic fermentation) presumably accumulates in the cells reducing the activity of the yeast on L-malic acid. We suppose that the same happens when DL-malic acid is added to the grape juice. According to our findings, the final acidity of a wine obtained from grape juice supplemented with DL-malic acid will not correspond to the addition of the starting concentration of the natural L-malic acid of the grape juice and the amount supplemented as DL-malic acid. The final acidity will be higher than expected when *S. cerevisiae* or the hybrid culture *S. cerevisiae* x *S. uvarum* are used as starter cultures and lower than expected when *S. uvarum* is used as starter culture.

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Utjecaj DL-jabučne kiseline na metabolizam L-jabučne kiseline tijekom alkoholnog vrenja

Sažetak

Nedovoljna kiselost vina može utjecati na njegovu kakvoću i stabilnost. Da bi se riješio taj problem, može se moštu prije vrenja dodati DL-jabučna kiselina. Ispitan je utjecaj DL-jabučne kiseline na fermentaciju vina i njezin utjecaj na konačnu koncentraciju L-jabučne kiseline koja se nalazi u moštu. Stoga su ispitani i uspoređeni sojevi kvasca koji metaboliziraju L-jabučnu kiselinu, i to *Schizosaccharomyces pombe* (djelotvoran razgrađivač L-jabučne kiseline), *S. cerevisiae* (ne razgrađuje), hibridni soj *S. cerevisiae* x *S. uvarum* (djelomično razgrađuje) i *S. uvarum* (promotor sinteze jabučne kiseline). U svim slučajevima D-jabučna kiselina pasivno ulazi u stanice kvasca i ne provodi jabučno-alkoholno vrenje. Međutim, njezina prisutnost u grožđanom soku, kao dio smjese D- i L-jabučne kiseline (DL-jabučna kiselina) snizuje količinu jabučne kiseline koja se može razgraditi ili sintetizirati s kvascima tijekom jabučno-alkoholnog vrenja.