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# The use of malolactic *Oenococcus oeni* (ATCC 39401) for deacidification of media containing glucose, malic acid and citric acid

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**Abstract** Malolactic fermentation is widely used to reduce the acidity of grape juices in wine production. However, application of the same technology to the production of wine from berries of the northern regions is not straightforward. Unlike grapes, these berries are rich in citric and malic acid while the sugar content is low. An ideal deacidification process for the northern berries would be the microbial degradation of these acids with minimal loss of sugars. Therefore, the co-metabolism of citric acid and glucose was studied under different conditions of malic acid degradation by *Oenococcus oeni*. At low pH values (pH $<4.5$) degradation of malic acid always proceeded first to completion with practically no consumption of glucose or citric acid. After the exhaustion of malic acid the degradation of both citric acid and glucose were initiated simultaneously. Following the exhaustion of malic acid and citric acid the remaining glucose remained nonfermentable. Thus, it is concluded that, by maintaining the culture in a resting state by the control of pH, selective degradation of acids can be achieved without subsequent loss of glucose.

**Key words** Berry wines · Deacidification · Malic acid · Citric acid · Malolactic fermentation

## Introduction

Malolactic fermentation (MLF) is an important secondary fermentation that occurs in many wines after completion of the alcoholic fermentation. The malolactic enzyme deacidifies the juice or wine by converting L-malic acid (a dicarboxylic acid) to L-lactic acid (a monocarboxylic acid) and carbon dioxide. In addition to deacidification, MLF is considered to contribute to the complexity of wine flavour [1–4] and to confer a degree of microbiological stability in wine [5–8]. MLF is conducted by lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Oenococcus*, *Lactobacillus* or *Pediococcus*. These bacteria are able to multiply in spite of high ethanol content (10% or higher), low pH (3.2 or lower) and added sulphur dioxide [9–11].

*Oenococcus oeni* (formerly *Leuconostoc oenos*) is the main species of lactic acid bacteria present in wine and one of the best adapted organisms to perform malolactic fermentation at the low pH of wine [11–13]. It is facultatively anaerobic and is tolerant to ethanol [14–17]. In addition to malic acid, some other organic acids [18, 19] and sugars [20, 21] may be utilised by this bacterium. This can lead to significant changes in the concentration of wine constituents and appearance of new metabolites that affect the sensory quality of wines [3, 22, 23].

The metabolism of sugars by *Oenococcus oeni* is reported to follow the heterolactic pathway (6-PG/PK pathway) [8, 24, 25]. Thus, it converts glucose into carbon dioxide, lactic acid, acetic acid and ethanol. The acetic acid/ethanol ratio depends on the redox potential of the system [26–28]. Diacetyl (2,3-butanedione) can also be formed by lactic acid bacteria in the presence of high concentrations of sugar, which affects wine quality negatively [29]. Sugar metabolism also has a positive influence on wine quality by forming aroma components such as acetaldehyde, acetic acid, diacetyl, acetoin, 2,3-butanediol, ethyl lactate, and higher alcohols [30–33]. The heterofermentative lactic acid bacteria can metabolise citric acid mainly to acetic acid, lactic acid and carbon dioxide [34–36].

The malolactic fermentation provides clear growth benefits for the bacterium. These include an increase in the pH of the growth environment and an increase in the intracellular pH of the bacterium [29, 37–39]. In addition, the cell can take advantage of the excess ATP formed in this proton motive force generating reaction. Thus, the malolactic fermentation is a chemiosmotic

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energy-yielding process for lactic acid bacteria adapted to the low pH of wine. The pathway includes the electrogenic (net negative) active transport of L-malic acid into the cell, the decarboxylation of L-malic acid to L-lactic acid and $CO_2$ by the malolactic enzyme, and the excretion of the end products, L-lactic acid and more than one proton, generating a proton gradient. The electrochemical proton gradient can then be used to drive tasks such as ATP synthesis by the ATPase, which is active at low pH [38, 40–44].

Northern berries have high acidity, which causes difficulties in berry wine production. In contrast to grapes, the main acids are citric and malic acid. Before fermentation the juices are commonly diluted and sugar is added. This causes significant weakening of the aroma and body of the wine. Information concerning the degradation of malic acid and citric acid by malolactic bacteria and the requirement for sugar and acid co-metabolism has led to varying hypotheses. Therefore, to facilitate the processing and to improve the quality of berry wines, the co-metabolism of glucose, citric acid and malic acid by the lactic acid bacterium *Oenococcus oeni* was studied. The effects of pH on the acid and glucose metabolism were also evaluated with the aim of altering and controlling the acid concentrations without loss of sugars.

## Materials and methods

*Microorganism.* The organism used was a commercial strain of *Oenococcus oeni* (ATCC 39401) (formerly *Leuconostoc oenos*) (Oregon State University Er1a) and was obtained from the American Type Culture Collection (Maryland, USA). The strain was stored in 10% (v/v) glycerol at –60 °C.

*Culture media and growth conditions.* The inoculum was prepared by first growing the thawed bacteria in de Man-Rogosa-Sharpe (MRS) medium in 10-ml filled tubes. The pH was preadjusted to 5.0 with 2 mol $l^{-1}$ HCl. The strain was grown at 30 °C without shaking for 4–6 days or to the stationary phase of growth.

The experiments were carried out in a modified MRS medium and depending on the experiment citric acid (5–8.5 g $l^{-1}$) or L(–)malic acid (1–7 g $l^{-1}$) was added. The acid concentrations were chosen in accordance with those commonly present in northern berries and berry wines. Glucose was added at 2.5–13.5 g $l^{-1}$. In some experiments glucose was excluded in order to eliminate sugar interference. To examine the effect of pH, the growth medium was preadjusted to pH 4.9 with 1 mol $l^{-1}$ NaOH prior to sterilization by heating for 15 min at 121 °C. All ingredients were made up in Milli-Q water.

Klett flasks with a volume of 250 ml (150 ml of medium) were used. After inoculation the flasks were closed with aluminium foils and incubated without shaking to obtain microaerophilic conditions. The experiments were carried out at 25 °C. Two or three parallel inoculated samples and two uninoculated samples (as control) were used for each combination. The growth was monitored with a Klett-Summerson (New York, USA) colorimeter (filter no. 66).

*Analytical procedures.* The content of glucose, citric acid, malic acid, lactic acid and acetic acid was determined by high pressure liquid chromatography analysis (HPLC), with a Waters (Waters Corporation, USA) chromatograph equipped with an Aminex HPX-87 $H^+$ column (300 × 7.8 mm) (Bio-Rad Laboratories, USA), Waters 410 refractometer, and 486 UV-detector in series. The column was eluted with 0.0015 mol $l^{-1}$ sulphuric acid at 40 °C, at a flow rate of 0.6 ml $min^{-1}$. Before injection the samples were centrifuged (6000 rpm, 10 min) and filtered through a membrane (0.2 μm pore size). Identification was based on relative retention times that were determined by injection of standards. Quantitative measurements were performed by applying external standards. Each assay was duplicated.

## Results and discussion

Berries of the northern regions are rich in organic acids. Therefore berry juices have very low pH (pH 2.7–3.6) and, due to the low sugar content, the flavour is also very acidic. Much of this acidity results from the presence of malic acid and citric acid. There are reports concluding that malic acid degradation occurs only by co-fermentation with sufficient concentration of a fermentable carbohydrate [25, 45, 46]. To elucidate this, *Oenococcus oeni* was incubated in the absence of sugar in a medium containing malic acid and citric acid at concentrations (7 g $l^{-1}$ and 7.5 g $l^{-1}$ respectively) that yielded an initial pH of 3.5 (Fig. 1). As shown in Fig. 1, even in the absence of sugar L-malic acid was readily fermented into lactic acid while citric acid remained unattacked. This result, conflicting with the literature, may indicate that the strain, pH of the medium or cultural conditions are not the same. However, no indication of differences in experimental conditions was given in the above-mentioned literature.

Our observation that the degradation of citric acid was arrested until malic acid was completely used up was reproduced when different relative concentrations of the two acids were used and sugar was added (Figs. 2–4). During the period of malic acid utilization, lactic acid was detected as the only product, supporting the conclusion that malic acid degradation is always pri-

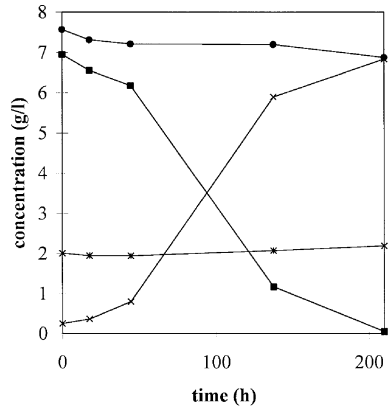


**Fig. 1.** Degradation of L-malic acid in MRS medium in the absence of glucose (pH 3.5). ■ Malic acid, ● citric acid, x lactic acid, ∗ acetic acid

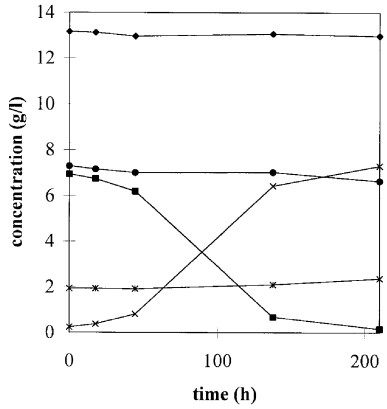


**Fig. 2.** The effect of glucose on malolactic degradation (pH 3.5). ◆ Glucose, ■ malic acid, ● citric acid, x lactic acid, * acetic acid

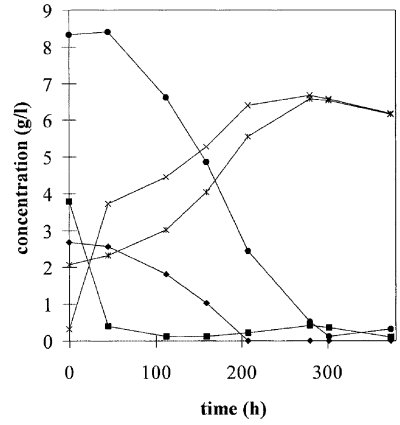


**Fig. 4.** Degradation of citric acid remaining as the only fermentation substrate (pH 3.5). ◆ Glucose, ■ malic acid, ● citric acid, x lactic acid, * acetic acid

oritized, at least in the pH range 3.5–3.7. Further, in the present experiments malic acid degradation always proceeded to completion and the pH increased during the malolactic fermentation by about 0.2 units. Thus, the organism can be used to reduce malic acid content without conversion of citric acid into acetic acid. This result disagrees with those of Martineau and Henick-Kling [36] who tested the same organism in three different wine types and observed that malic acid and citric acid were co-metabolised and the highest rate of citric acid utilization was observed in the presence of malic acid. The pH in their studies was also low (pH 3.0–3.5).

Several authors have observed that the malolactic reaction occurs in the absence of cell growth [3, 47–50]. Henick-Kling et al. [39] reported that lactic acid bacteria with the malolactic enzyme system require carbohydrates as carbon source for cell maintenance and growth. Our results support such a conclusion as in our experiments the degradation of malic acid also occurred without an increment in cell mass, indicating that malic acid was not used as the sole growth substrate.

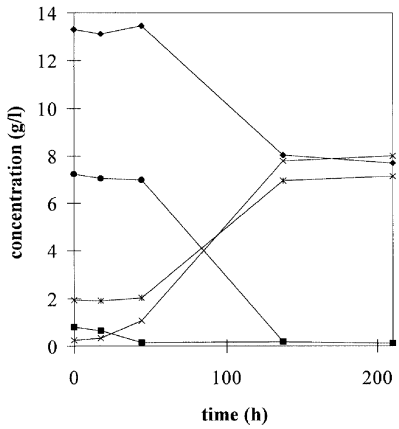


**Fig. 3.** Correlation between the degradation of glucose and citric acid at a low pH value (pH 3.9). ◆ Glucose, ■ malic acid, ● citric acid, x lactic acid, * acetic acid

As malolactic degradation of malic acid can occur without glucose, a question of practical significance with low-sugar berries arises as to whether glucose is degraded during the malolactic process. To clarify this the organism was incubated in a mixture of malic acid and citric acid in the presence of 13 g/l glucose and at pH 3.5. As shown in Fig. 2, the degradation of malic acid proceeded first to completion with practically no consumption of glucose or citric acid. Thus, acetic acid was not formed. Accordingly, the malolactic degradation of L-malic acid is a glucose- and citric acid-independent process. Henick-Kling et al. [39] have also noted that glucose catabolism does not affect the rate of malic acid catabolism, although with the prerequisite that citric acid is not present.

Vaillant and Formisyn [51] and Naouri et al. [52] observed that citric acid is a competitive inhibitor of malic acid for the malolactic enzyme. Our results did not show such an effect at any of the citric acid concentrations used. Moreover, Miranda et al. [53] noticed that malolactic fermentation by some strains of *Oenococcus oeni* at pH 3.5 was inhibited by glucose and the addition of citric acid completely relieved the glucose-induced inhibition. Our results did not support this view either. In contrast, the organism that we used was able to degrade selectively malic acid and arrest the degradation of glucose and citric acid at least as long as the malolactic process was ongoing. Similarly, Henick-Kling [3] and Henick-Kling et al. [39] noticed that at low pH malic acid catabolism inhibits glucose utilization. Consequently, the organism seems promising for use in low-pH media, such as berry juices and wines, to

reduce selectively the acidity by degradation of malic acid while maintaining the sugar content.

A question can be raised as to what happens to other fermentable compounds upon the exhaustion of malic acid and whether it explains some of the controversy between the present and the above-mentioned literature data. Therefore low initial malic acid content (1 g $l^{-1}$) was chosen for a fermentation mixture containing 7.5 g $l^{-1}$ citric acid and 13.5 g $l^{-1}$ glucose and having a low pH (pH 3.9). Figure 3 shows that malic acid was again degraded first to lactic acid, and during malic acid degradation glucose and citric acid remained unattacked. Malic acid degradation seems to be prioritized over glucose and citric acid degradation even at low initial malic acid content. However, after the exhaustion of malic acid the pH reached a value of 4.1 and the degradation of both citric acid and glucose were initiated simultaneously.

Figure 3 also shows that when citric acid was exhausted before glucose due to its lower initial concentration, the degradation of glucose was arrested. Thus, in contrast to malic acid, citric acid triggered glucose utilization and growth and the presence of citric acid was necessary during the whole period of glucose utilization. According to Saguir and Manca de Nadra [54], glucose utilization is dependent on the presence of citric acid provided that the pH is below 4.5. On the other hand, Pimentel et al. [21] noted that the addition of citric acid had no influence on bacterial growth of the same lactic acid bacterium used in the present study at pH values 3.5–4.5. However, their studies were carried out in a tomato glucose broth supplemented with appropriate acids.

The present results suggest that the organism would be applicable for deacidification of berry juices and wines via fermentation of malic and citric acids. By monitoring the progress of fermentation, malic acid can be removed quantitatively without any loss of glucose. Further, after the degradation of both malic and citric acid the remainder glucose remaining nonfermentable.

Based on the above observations, one would expect that, if the initial concentration of citric acid was chosen to exceed clearly those of malic acid and glucose and the pH was kept low (pH 3.5), a situation would result where citric acid remains as the only substrate. Figure 4 illustrates the progress of such a fermentation. As malic acid and then glucose were utilized, citric acid was still left but its consumption continued to completion. Thus, citric acid can be metabolised without glucose although glucose could not be metabolised without the presence of citric acid as shown in Fig. 3. Consequently, in complex highly acidic substrate mixtures undergoing the malolactic fermentation, citric acid degradation can be inhibited only by the presence of malic acid. Again, contradictory conclusions have been reached. Saguir and Manca de Nadra [54] and Cogan [55] reported that citric acid was metabolised only if glucose was present. However, the conditions of the experiments were not sufficiently described, i.e. whether the discrepancies were due to differences in strains or medium composition. The pH also has a great influence on the results. Saguir and Manca de Nadra [54] carried out the experiments at a relatively high pH (pH 4.8).

All the above experiments were carried out at low pH values (pH 3.5–3.9). In order to elucidate whether the degradation of citric acid was pH-dependent or due to the inhibition of citrate lyase by malic acid as suggested by McCord and Ryu [48] and Subramanian and SivaRaman [35], the initial pH was raised to 4.9 in a fermentation medium containing equal initial levels of malic acid and citric acid and a high glucose concentration (Fig. 5). Again, utilization of malic acid started most rapidly but simultaneous consumption of the two other compounds was also evident. Even though after the exhaustion of malic acid the degradation rate of the two other substrates was increased, the higher initial pH seemed to reduce the selectivity observed under more acidic conditions in the order of degradation of the substrates. An explanation for this may come from the suggestion of Cox and Henick-Kling [56] and Henick-Kling [3] that, as the pH increases, the efficiency of ATP production from malic acid catabolism decreases. This may be compensated by initiating simultaneously the degradation of glucose which in turn requires the coordinated degradation of citric acid. Consequently, among the fermentation parameters, pH seems to be an effective controlling factor influencing the order of consumption of glucose, malic acid and citric acid and the relative amounts of the end products. However, from the practical point of view, the consumption of sugar seems not to be rapid within a quite broad pH region.

The fact that the efficient degradation of malic acid occurs by nongrowing cells, and under conditions where glucose is not attacked, gives a promising opportunity for deacidification of low-pH berry juices and

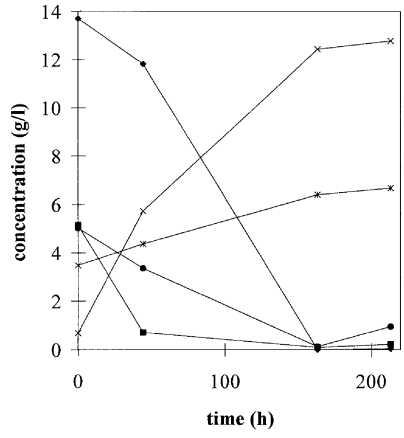


**Fig. 5.** Degradation of malic acid and glucose at initial pH of 4.9. ◆ Glucose, ■ malic acid, ● citric acid, x lactic acid, ∗ acetic acid

berry wines without loss of sugars and possibly with minimal interference with other berry constituents. It also seems evident that the degradation of citric acid can be avoided only as far as the medium also contains malic acid and the pH of the fermentation media is low, probably below pH 4.5, as is the case in all berry juices and wines. The present results point to the importance of process monitoring during malolactic fermentation. For deacidification purposes the critical monitoring points are the initial concentrations of different acids and sugar, pH of the medium and the progress of degradation of malic acid and to lesser extent that of citric acid. With this monitoring the malolactic fermentation represents a promising means for deacidification of, for example, northern berry juices and wines without a significant loss of their natural sugar content.

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