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Short Communication

Continuous malolactic fermentation in red wine using free *Oenococcus oeni*

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Summary

Malolactic fermentation (MLF) of wine in continuous culture was obtained by using *Oenococcus oeni* (formerly *Leuconostoc oenos*). The maximum malic acid degradation in our bioreactor system was reached at a dilution rate of 0.016 h^{-1}, and 92–95% of the malic acid (3.9–4.0 g/l) was converted to lactic acid and CO_2.

Introduction

The conversion of L-malic into L-lactic acid and CO_2 is known as the malolactic fermentation (MLF), and has been developed in wine by some lactic acid bacteria, mainly *Oenococcus oeni* (formerly *Leuconostoc oenos*) (Kunkee 1967; Wibowo *et al.* 1985). However, MLF is often difficult to conduct, even by inoculating with commercial strains of malolactic bacteria (Crapisi *et al.* 1987). Some alternatives to standard strain inoculation have been tried such as the use of cells entrapped in polyacrylamide (Rossi & Clementi 1984) or calcium alginate (Spettoli *et al.* 1987), but results did not improve those obtained with standard starter inoculation. Naouri *et al.* (1991) assayed a fluidized bed reactor but observed a short catalyst life due to depletion of NAD^+ or Mn^{2+}, two basic cofactors for malolactic enzyme activity, because of cessation of cellular growth. Similar findings have been recently reported using an enzymatic reactor (Formisyn *et al.* 1997). Here we describe for the first time the ability of free-growing cells of two selected *Oenococcus oeni* strains to perform an effective and rapid continuous MLF in a stirred tank reactor.

Materials and Methods

Strains and culture conditions

Oenococcus oeni MA4 and VV5 were isolated from Spanish wines as described by Pardo and Zúñiga (1992). Malolactic fermentation assays were performed by inoculating strains in Monastrell red wine containing (g/l): glucose, 0.9; fructose, 1.0; malic acid, 4.2. Initial pH of the wine was 3.5, and the ethanol content 11.1% (v/v). Precultures were prepared as follows: cells were grown in MLO broth (Medium for *Leuconostoc oenos*) (Caspritz & Radler 1983), containing (g/l): glucose, 10; fructose, 5; yeast extract, 5; tryptone, 10; $MgSO_4 \cdot 7\,H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.05; cysteine/HCl, 0.5; diammonium citrate, 3.5; Tween-80, 1, and tomato juice, 100 ml. The pH was adjusted to 4.8. Bacterial cultures were then transferred to preculture medium 10 (Pardo *et al.* 1992), containing Monastrell red wine; glucose, 5 g/l; yeast extract, 5 g/l; Tween-80, 5 mg/l; and tomato juice, 2.3%. Then the cells were incubated for 48–72 h at 28 °C and inoculated (10% v/v) in wine-filled 100 ml flasks and incubated for 2 weeks at 20 °C. Multifermenter 330 ml vessels (Biostat-Q Braun Instruments, Melsungen, Germany) were filled with 200 ml of sterile Monastrell red wine and inoculated 5% (v/v) with precultured bacteria (as described above). Fermentations were developed at 20 °C with agitation at 50 rev/min. After 10 days of batch operation, a continuous process with a dilution rate (D) of 0.016/h was applied. The pH was monitored but not controlled; T and stirring were controlled during the process.

Analytical methods

Periodically, 2 ml samples were aseptically collected through an in-line sampling port, and bacterial counts were determined by platting on MLO-agar. For quantification of organic acids, samples were centrifuged at 12,500 × g for 10 min. Supernatants were filtered through a 0.22 μm pore-size membrane filter (Whatman; Clifton, New Yersey) and 10 μl were injected in an HPLC system (Merck–Hitachi) with two coupled HPX-

87H Aminex columns (Bio-Rad Chemical Division, Richmond California) (Frayne 1986). External standards were used to quantify the compounds.

Results and Discussion

Figure 1 shows the bacterial population dynamics and remaining malic acid content in the time course of continuous MLF by growing *Oenococcus oeni* strains MA4 and VV5. The pH of the wines increased from 3.5 to 3.7 for both strains after MLF. The bioreactors were operated for two (MA4) and three (VV5) weeks without bacterial contamination. This exceeds half-life values reported for reactors based on cells that were either gel-entrapped (Spettoli *et al.* 1987; Naouri *et al.* 1991) or adsorbed onto oak chips (Janssen *et al.* 1993). For accelerating the fermentation rate, several authors have designed different bioreactors with immobilized cells, obtaining adequate performances (Cuenat & Villetaz 1984; Crapisi *et al.* 1987). In these studies, malolactic activity was lost within 2–7 days due to the chemical stresses of cells in wine. Similar results were obtained by Gao & Fleet (1995) using a 300 ml cell-recycle membrane bioreactor system with a flow rate of 6 ml/min for up to 56 h. Greater than 95% degradation of malic acid occurred in this experiment.

In our system, operation at $D = 0.016$/h improved these previous reports: 92–95% of the malic acid (4.2 g/l) was stoichiometrically converted to lactic acid by continuously growing cells. Working with 200 ml of wine, 76.8 ml were processed per day (0.38 volumes), yielding final levels of malic acid lower than 0.3 g/l from 4.2 g/l. These production rates were 3- to 4-fold higher than those in a conventional batch process, where cell growth must be reached previous to malic acid consumption, and avoid the NAD^+ and Mn^{2+} problems associated with enzymatic reactors (Formisyn *et al.* 1997). Our system was efficiently operated over 2–3 weeks, and high cell populations were established (3.0×10^8 c.f.u./ml, VV5; 3.0×10^7 c.f.u./ml, MA4) (Figure 1). At these cell densities, complete MLF of wines was observed throughout all the continuous processes. The different behaviour of VV5 and MA4 strains in these cultures can be explained because of different characteristics of each strain: different growth-carrying capacities and growth taxes, different number of cells per chain, and different malolactic activity of each strain. Population dynamics were maintained in equilibrium until contaminating bacterial numbers were high. This caused an imbalance in cell populations, and a dilution of *O. oeni* cells was observed (Figure 1). Even under those last conditions, consumption rates represented more than 75% of the initial malic acid concentration in wine (4.2 g/l).

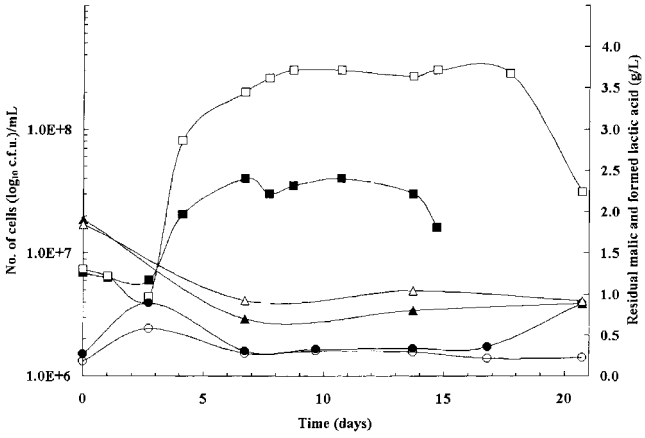

Figure 1. Time course of continuous malolactic fermentation in wine with *Oenococcus oeni* strains MA4 or VV5 at a dilution rate of 0.016/h, after batch operation. Time 0 = start of continuous operation. Malic acid concentration in flow-in wine was 4.2 g/l. (■), c.f.u./ml (MA4); (□) c.f.u./ml (VV5); (●) residual malic acid (MA4); (○) residual malic acid (VV5); (▲) lactic acid formed (MA4); (△) lactic acid formed (VV5).

In conclusion, the success of the continuous cultivation of *O. oeni* strains MA4 and VV5 was due to the preculture conditions that enabled their adaptation and active growth in wine, where low pH levels existed and minimum levels of nutrients were available. This fermentation system enabled continuous growing to replace outflow of cells and MLF was successfully performed under controlled conditions. Further research is being conducted to scale up such a fermentation system for industrial wine deacidification.

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