JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Malolactic Fermentation in Wine with *Lactobacillus casei* Cells Immobilized on Delignified Cellulosic Material

NIKOLAOS AGOURIDIS,† ARGYRO BEKATOROU,† POONAM NIGAM,§ AND MARIA KANELLAKI*,†

Food Biotechnology Group, Department of Chemistry, University of Patras, GR-26500, Patras, Greece, and School of Biomedical Sciences, University of Ulster at Coleraine, BT52 1SA Coleraine, United Kingdom

In this work *Lactobacillus casei* ATCC 393 cells immobilized on delignified cellulosic material (DCM) were used for malolactic fermentation (MLF) of wine. Wine was produced using yeast cells immobilized on DCM at 20 °C, and after alcoholic fermentation, MLF at 27 °C followed using immobilized *L. casei* ATCC 393 cells. A total of 11 repeated alcoholic and subsequent MLF batches were performed within a period of 1 month. As the repeated MLF batches proceeded, the MLF activity of the immobilized biocatalyst was reduced. Malic acid degradation was reduced from 80 to 2%, pH was reduced by 0.5−0.1 unit, acetic acid concentrations were slightly reduced or remained stable (0.002 g/L), the higher alcohols 1-propanol, isobutyl alcohol, and amyl alcohol were decreased by 84, 23, and 11%, respectively, and ethyl acetate concentration was increased by ∼56%. Wine samples were analyzed by GC-MS before and after MLF, revealing some qualitative differences.

**KEYWORDS: Wine; malolactic fermentation; immobilization; delignified cellulosic material; *Lactobacillus casei*; volatiles**

## INTRODUCTION

Although winemaking is a very old practice and technology, it continues to attract research, development, and application of new technologies to produce wines of improved quality and organoleptic character. Malolactic bacteria such as *Oenococcus oeni*, *Pediococcus* sp., and *Lactobacillus* sp. during malolactic fermentation (MLF) are able to induce biological deacidification of wines, resulting in an increase of pH. In addition, MLF influences the microbiological stability and organoleptic quality of wine. MLF is usually desirable in wines of low pH produced in cold wine-producing areas. In warmer areas grapes tend to be less acidic, and a further decrease in acidity by MLF can be adverse to the sensory properties and biological stability of wine, by being susceptible to subsequent growth of microorganisms (*1*−*6*).

Malolactic bacteria are often located on grape skins or the wooden vats in which wine is stored and, therefore, spontaneous MLF may occur during the storage of new wines at slightly elevated temperatures over weeks or months, without always achieving satisfactory results (*6*−*8*). For better control of the timing and extent of MLF, immobilization of selected malolactic bacteria in suitable supports has been suggested. In addition, cell immobilization offers more advantages such as continuous operation, greater tolerance to high alcohol and sulfur dioxide concentration and low pH, improvement of cell separation and probable reuse of the immobilized biocatalyst, and increase of productivity due to a greater cell density (*6*, *8*−*12*).

Various materials have been proposed as supports for malolactic bacteria immobilization. Polyacrylamide gels have been used for immobilization of *Lactobacillus casei* cells (*13*); calcium alginate gels for immobilization of *Leuconostoc oenos* ML 34 cells (*14*); polyacrylamide gels for immobilization of *L. oenos* cells (*15*); κ-carrageenan gel for immobilization of various *L. oenos* strains (*9*); κ-carrageenan with and without addition of silica for immobilization of *L. oenos* ML 34 and *Lactobacillus* sp. 48 in a continuous reactor system (*16*); κ-carrageenan with and without the addition of bentonite for immobilization of *Lactobacillus* sp. 48 in a continuous flow bioreactor (*17*); calcium alginates for immobilization of *Lactobacillus* sp. 89 and *L. oenos* ATCC 23278 in batch and continuous systems (*18*); and cellulose sponge for immobilization of *O. oeni* (*12*).

The use of delignified cellulosic material (DCM) as a carrier for yeast immobilization was proved to be effective at ambient and low-temperature winemaking, exhibiting high operational stability, significant increase in fermentation rates, and improvement of organoleptic quality compared to free cells (*19*−*21*). DCM is a support of food grade purity, very cheap, abundant, easy to prepare industrially, and easily accepted by consumers. For beverages, cell immobilization that involves little preparation and no additional chemicals is ideal. Some authors have studied the suitability of immobilized *L. casei* for MLF process and

* Author to whom correspondence should be addressed (telephone +302610997104; fax +302610997105; e-mail m.kanellaki@upatras.gr).
† University of Patras.
§ University of Ulster at Coleraine.

10.1021/jf048736t CCC: $30.25 © 2005 American Chemical Society
Published on Web 03/05/2005

concluded that the application of the immobilized biocatalyst in winemaking is feasible (*10*, *11*, *13*). However, research on the use of immobilized *L. casei* cells for MLF is very limited but encouraging, so we decided to study the suitability of *L. casei* cells immobilized on DCM for use in MLF. Therefore, the aim of this work was to evaluate (a) the possibility of immobilization of *L. casei* cells on DCM for use in MLF, (b) the operational stability of the immobilized biocatalyst in repeated MLF batches, and (c) the effect of MLF using immobilized *L. casei* ATCC 393 cells on the contents of organic acids and volatile compounds.

## MATERIALS AND METHODS

**Strains.** An alcohol-resistant and psychrotolerant *Saccharomyces cerevisiae* AXAZ-1 yeast strain isolated from the agricultural area of North Achaia, Greece (*22*), was used for dry, white winemaking. Cell growth was done at 28 °C in a liquid nutrient medium containing 20 g/L glucose, 4 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 5 g/L $MgSO_4$, and 1 g/L $KH_2PO_4$. Cells were harvested by centrifugation at 4000 rpm for 10 min. For MLF experiments, a *L. casei* ATCC 393 strain was used (DSMZ 20011, Germany). Cell growth was done at 37 °C in a liquid nutrient medium containing 37 g/L brain−heart, 0.5 g/L L-cys-HCl, 5 g/L yeast extract, 3 g/L $KH_2PO_4$, 5.8 g/L $MgSO_4 \cdot 7H_2O$, 0.12 g/L $MnSO_4 \cdot 2H_2O$, 0.034 g/L $FeSO_4 \cdot 7H_2O$, and 15 g/L glucose for 3 days without agitation. The pH was adjusted to 7.1−7.4. All media were sterilized at 130 °C for 15 min. Cells were harvested by centrifugation at 5000 rpm for 20 min at 20 °C.

**Cell Immobilization.** DCM was prepared from sawdust after boiling for 3 h in 1% NaOH solution for lignin removal (*19*). The delignified material was drained and sterilized at 130 °C for 15 min. Immobilization of yeast on DCM was performed by mixing 150 g of DCM, 800 mL of must (12 °Be density), and 16 g of yeast cells (on wet weight basis) in a 2-L glass cylinder. The system was allowed to ferment at 30 °C until 0−0.5 °Be density (6−8 h). After the end of fermentation, the immobilized biocatalyst (yeast cells immobilized on DCM) was filtered, washed with 400 mL of must, and used for winemaking experiments. *L. casei* ATCC 393 cells were immobilized on DCM following a similar procedure. Specifically, 250 g of DCM, 800 mL of liquid growth medium, and 35 g of *L. casei* ATCC 393 cells were used. The immobilized biocatalyst was filtered and washed gently with 400 mL of wine.

**Winemaking Experiments.** Concentrated grape must from white grapes was supplied by the G. Karelas S.A. winemaking company. It was diluted to 11.0−12.0 °Be density, sterilized at 120 °C for 15 min, and used without any nutrient or $SO_2$ addition. One hundred and fifty grams of biocatalyst (yeast cells immobilized on DCM) and 500 mL of must were introduced into a 1-L glass cylinder (total working volume was 720 mL), and the system was allowed to ferment at 20 °C. When the fermentation was completed (0 °Be density), the fermented liquid was decanted and the biocatalyst was washed twice with 100 mL of must and was used for the next fermentation batch. A total of 11 repeated alcoholic fermentation batches were performed. The dry, white wine samples produced were analyzed for pH, residual sugar, ethanol, volatiles, and organic acids and were subjected to MLF using immobilized *L. casei* ATCC 393 cells.

**Malolactic Fermentation Experiments.** After each winemaking batch, 400 mL of the produced wine was transferred into a 1-L glass cylinder containing 250 g of DCM with immobilized *L. casei* ATCC 393 cells. The system was kept at 27 °C for 3 days. The wine was then decanted, and the support was washed twice with 100 mL of commercial dry wine. The immobilized biocatalyst was then used for the next MLF batch. The wine samples after MLF were analyzed for pH, residual sugar, ethanol, volatiles, and organic acids. A total of 11 repeated MLF batches were performed.

**Electron Microscopy.** A piece of the immobilized biocatalyst (*L. casei* ATCC 393 cells immobilized on DCM) was washed with deionized water and dried overnight at 30 °C. The sample was coated with gold in a Balzers SCD 004 sputter coater and examined in a JEOL model JSM-6300 scanning electron microscope.

**Determination of Ethanol and Residual Sugar.** The fermentation kinetics of the winemaking experiments were performed by measuring the °Be density at various time intervals. Residual sugar and ethanol concentrations in the wine samples before and after MLF were determined on a Shimadzu LC-9A HPLC system consisting of a Shim-pack SCR-101N column, an LC-9A pump, an RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit. Three times distilled and filtered water was used as the mobile phase (0.8 mL/min), and 1-butanol (0.1% v/v) was used as the internal standard. Column temperature was 60 °C. Sample dilution was 1% v/v, and injection volume was 40 $\mu$L. Ethanol was also analyzed by means of GC on a Shimadzu GC-8A instrument connected with a Shimadzu CR-6A integrator and an FID. The column was packed with Porapac-S, and $N_2$ was used as carrier gas (40 mL/min). Column temperature was 140−180 °C (10 °C/min), and injection port and detector temperatures were 210 °C. 1-Butanol (0.1% v/v) was used as internal standard, and samples of 4 $\mu$L were injected directly in the column. Determinations were carried out using both standard curves and the internal standard method. The standard deviations for residual sugar were 0.2−0.5 and for ethanol, 0.3−0.5.

**Determination of Volatiles.** Volatiles (acetaldehyde, ethyl acetate, 1-propanol, isobutyl alcohol, and amyl alcohols) were determined by means of gas chromatography on a Shimadzu GC-8A gas−liquid chromatograph, with a stainless steel column packed with Escarto-5905 [consisting of 5% squalene, 90% Carbowax-300, and 5% bis(2-ethylhexyl sebacate)], with $N_2$ as the carrier gas (20 mL/min) and a FID. The injection port and detector temperatures were 210 °C, and the column temperature was 70 °C. The internal standard was 1-butanol (0.1% v/v). Samples of 4 $\mu$L of wine were injected directly onto the column, and the concentrations of the above compounds were determined using both standard curves and the internal standard method. Methanol was determined on the GC system described above for ethanol. The standard deviations before and after MLF were as follows: for acetaldehyde, 4.3−6.9 and 6.3−7.3; for ethyl acetate, 2.9−5.0 and 4.1−5.8; for 1-propanol, 1.0−2.1 and 0.2−0.4; for isobutyl alcohl, 2.1−3.4 and 0.9−2.1; for amyl alcohol, 4.2−5.8 and 4.7−5.2; and for methanol, 3.9−5.0 and 4.0−5.1, respectively.

**Determination of Organic Acids.** Organic acids (malic, lactic, acetic, succinic, and tartaric) were determined by ion-exchange liquid chromatography on a Shimadzu system consisting of a Shim-pack IC-A1 column, an LC-10AD pump, a CTO-10A oven, and a CDD-6A conductivity detector. A solution of 2.5 mM phthalic acid and 2.4 mM tris(hydroxymethyl)aminomethane (pH 4.0) was used as mobile phase (1.2 mL/min). The column temperature was 40 °C. The sample dilution was 5% v/v, and the injection volume was 60 $\mu$L. Determinations were carried out by means of standard curves. The standard deviations before and after MLF were as follows: for malic acid, 0.2−0.6; for lactic acid, 0.006−0.03; for tartaric acid, 0.03−0.07; for succinic acid, 0.04−0.08; and for acetic acid, 0.002−0.005.

**GC-MS Analysis.** Headspace analysis of volatiles in wine samples before and after malolactic fermentation was carried out by GC-MS analysis. A solid-phase microextraction (SPME) sampling method was used employing a DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA) for adsorption of volatiles (*23*, *24*). The conditions of headspace SPME sampling used were as follows: 5 mL of liquid sample and 1.5 g of NaCl were transferred into a 10-mL glass vial sealed with a rubber septum. The contents were magnetically stirred for 5 min at 50 °C, and the fiber was exposed to the headspace for 60 min. The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the gas chromatograph in splitless mode, at 240 °C for 5 min. Before each analysis, the fiber was exposed to the injection port for 10 min to remove any volatile contaminants. Separation of volatiles was performed on a Shimadzu GC-17A gas chromatograph (Supelco Wax-10 column), connected with a GCMS-QP505A mass spectrometer (70 eV ionization energy; *m*/*z* 29−400 mass range). Helium was used as carrier gas (2 mL/min). Oven temperature was programmed at 35 °C for 5 min, and then it was raised to 60, 200, and 250 °C at rates of 2.0, 5.0, and 25.0 °C/min, respectively. Identification was carried out by comparison of retention times and MS data with those of standard compounds and data obtained from NIST libraries.

## RESULTS AND DISCUSSION

The advantages of DCM, as presented in the Introduction, were the motive for the evaluation of DCM as a support for the immobilization of malolactic bacteria and its suitability for use in MLF of wine. Wine samples were prepared by alcoholic fermentation of must from white grapes using yeast cells immobilized on DCM. The evaluation of the immobilized *L. casei* ATCC 393 cells on DCM was made in terms of malic degradation, operational stability, and analysis of organic acids and volatiles in an effort to complete and optimize a winemaking technology based on immobilized cells.

Specifically, 11 repeated alcoholic fermentation batches of grape must (~11.5 °Be) were carried out at 20 °C with immobilized *S. cerevisiae* AXAZ-1 within a period of 1 month. Fermentation times during the first 3 batches were low (48−55 h) and decreased to a stable 40−45 h in the next 8 batches, showing operational stability of the system and fast adaptation to the fermentation process as also shown in previous studies (*19*, *20*). Residual sugar was low (1.4 ± 0.6 g/L), and ethanol concentrations were high (11.2 ± 0.7 g/L) in all of the produced wines. The wines had a fine clarity after the end of alcoholic fermentation.

After the end of each alcoholic fermentation batch, the wine produced was decanted and transferred to a second bioreactor containing *L. casei* ATCC 393 cells immobilized on DCM and was kept at 27 °C for 3 days for MLF to commence. In traditional MLF, which occurs under natural conditions, where the suitable bacteria for MLF (e.g., *O. oeni*) are in low concentrations, substantial malic acid consumption can be observed only after 3−4 weeks. In an immobilized system this period can be shortened because the probability of obtaining rapid and complete MLF is significantly enhanced by inoculating the wine with high levels of selected strains (*12*). Also, the MLF time required depends on several factors such as ambient temperature, pH, presence of nutrients and initial number of bacteria (*6*). For our experiments a period of 3 days was considered to be satisfactory for the completion of MLF by immobilized cells. The wine samples after this period were collected for further analysis, and the biocatalyst was used for the next MLF batch.

The first aim of this study, which was to examine if *L. casei* ATCC 393 strain can be immobilized on DCM, succeeded, as shown by the electron micrographs (**Figure 1**) and by the process and results of the repeated MLF batches.

**MLF Activity.** With regard to the MLF activity of the immobilized biocatalyst, 11 repeated MLF batches were performed with immobilized cells in wine of initial pH 3.5−3.8 in a period of 1 month. After each MLF batch, the pH value was increased, but this change was decreased from 0.5 to 0.1 as batches proceeded from the 1st to the 11th. Residual sugar after alcoholic fermentation (average = 1.43 g/L) was further converted by ~40% after MLF (average = 0.75 g/L), whereas ethanol concentrations remained unaltered (**Table 1**). The initial MLF activity for the first batch was high (80% degradation of malic to lactic acid). After that, the MLF activity declined gradually up to the 6th batch (14.7%) and increased gradually up to the 9th batch (29.5%). For the 10th and 11th batches the MLF activity was very small (~2%), and so it was meaningless. Generally, we can tell that the MLF activity after the 1st batch declined with fluctuating malic acid degradation values toward the end of the MLF batches (batches 6−9) (**Table 2**). This decline is probably attributed to the leakage of immobilized cells during the repeated washings of the immobilized biocatalyst after the end of each MLF batch. The degradation of malic acid through MLF by *L. casei* immobilized on DCM did not quantitatively yield lactic acid (1 mol of degraded malic acid → 1 mol of produced lactic acid in the optimum case). In MLF, the degradation of malic acid and therefore the lowering of total acidity are a major demand. A high content of lactic acid in wine does not contribute positively to wine aroma, because lactic acid contributes to volatile acidity, which is also used as an indicator of wine spoilage (*2*). The stability and/or very small increase of acetic acid (**Table 2**) and the small concentration of the produced lactic acid after MLF by *L. casei* immobilized on DCM compose a very good advantage for use of the above immobilized biocatalyst for MLF. Maicas et al. (*12*) performed subsequent MLF batches with immobilized cells on cellulose sponge. After the first batch, where the malic acid was almost completely metabolized, a strong drop in the MLF activity was also observed. This was attributed more to cell mortality than leakage, as indicated by the low level of cells found in suspension. Also, attempts to deacidify wine using the malolactic enzyme immobilized on gel have not been successful, probably because of the inactivity of the enzyme at wine pH and because the required cofactor NAD is particularly unstable in wine (*1*, *7*, *8*, *10*). Other authors have reported that ethanol inhibits the growth of lactic acid bacteria and MLF (*1*, *15*, *16*, *25*).

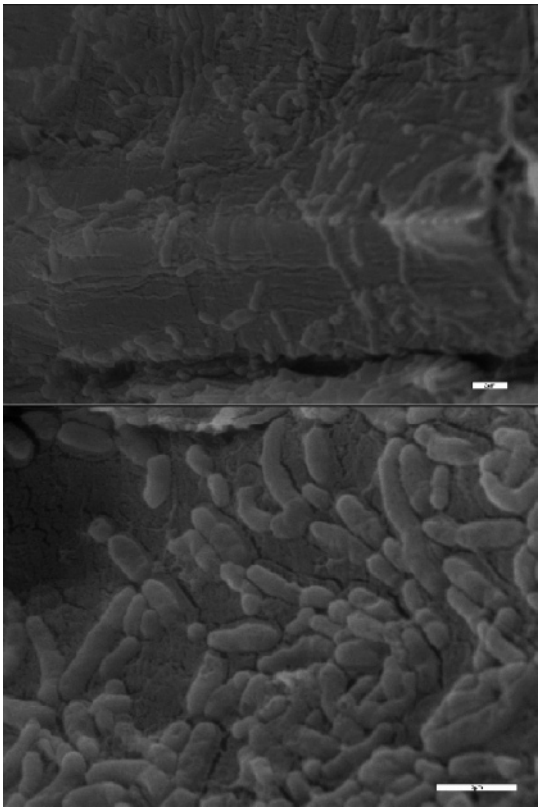

**Figure 1.** Electron scanning micrographs of *L. casei* ATCC 393 cells immobilized on DCM.

With regard to the MLF operational stability, Spettoli et al. (*14*) reported that ~40−60% of the malic acid in wine was transformed into L-lactic acid by immobilized *L. oenos* cells in calcium alginate gels. Immobilized cells showed gradually decreasing activity after several experiments. In another study Spettoli et al. (*16*) reported that the operational stability of *L. oenos* immobilized in κ-carrageenan with silica gel was increased to 4 days from 2 days, with a 58% conversion ratio of malic acid to lactic acid in a continuous system. *Lactobacillus* cells immobilized in κ-carrageenan containing silica showed a higher conversion ratio, that is, 66%, and operational stability

**Table 1.** Residual Sugar and Ethanol Concentrations in Wines after Alcoholic (AF) and Malolactic (MLF) Fermentation with Immobilized Cells of *S. cerevisiae* AXAZ-1 and *L. casei* ATCC 393, Respectively

| | residual sucrose (g/L) | | residual glucose (g/L) | | residual fructose (g/L) | | total (g/L) | conversion (%) | ethanol (% v/v) | ethanol (%v/v) |
|---|---|---|---|---|---|---|---|---|---|---|
| batch | AF | MLF | AF | MLF | AF | MLF | MLF | MLF | AF | MLF |
| 1 | 0.25 | 0.19 | 0.21 | 0.16 | 0.99 | 0.13 | 0.48 | 35.9 | 9.7 | 9.8 |
| 2 | 0.28 | 0.19 | 0.32 | 0.22 | 1.14 | 0.56 | 0.97 | 44.3 | 10.5 | 10.5 |
| 3 | 0.22 | 0.15 | 0.19 | 0.07 | 0.55 | 0.26 | 0.48 | 50.0 | 11.4 | 11.5 |
| 4 | 0.30 | 0.20 | 0.21 | 0.17 | 0.58 | 0.19 | 0.56 | 48.6 | 11.6 | 11.5 |
| 5 | 0.28 | 0.18 | 0.29 | 0.19 | 0.58 | 0.23 | 0.6 | 47.8 | 11.4 | 11.3 |
| 6 | 0.34 | 0.25 | 0.35 | 0.22 | 2.12 | 0.85 | 1.32 | 53.0 | 11.1 | 11.1 |
| 7 | 0.24 | 0.18 | 0.11 | 0.08 | 0.84 | 0.61 | 0.87 | 26.9 | 11.4 | 11.4 |
| 9 | 0.30 | 0.23 | 0.26 | 0.20 | 0.44 | 0.25 | 0.68 | 32.0 | 11.7 | 11.8 |
| av ± SD[1−9] | 0.28 ± 0.04 | 0.20 ± 0.03 | 0.24 ± 0.08 | 0.16 ± 0.06 | 0.91 ± 0.55 | 0.39 ± 0.26 | 0.75 ± 0.29 | 40.3 ± 9.5 | 11.1 ± 0.7 | 11.1 ± 0.7 |

**Table 2.** Organic Acid Concentrations (Grams per Liter) in Wines before (AF) and after (MLF) Malolactic Fermentation with Immobilized Cells of *S. cerevisiae* AXAZ-1 and *L. casei* ATCC 393, Respectively

| | malic acid | | | lactic acid | | tartaric acid | | succinic acid | | acetic acid | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| batch[a] | AF | MLF | % reduction | AF | MLF | AF | MLF | AF | MLF | AF | MLF |
| 1 | 1.33 | 0.27 | 79.7 | 0.035 | 0.108 | 0.54 | 0.10 | 0.34 | 0.43 | 0.03 | 0.01 |
| 2 | 2.04 | 1.38 | 32.4 | 0.039 | 0.115 | 0.60 | 0.40 | 0.34 | 0.43 | 0.03 | 0.02 |
| 4 | 1.32 | 1.02 | 22.7 | 0.056 | 0.124 | 0.52 | 0.45 | 0.32 | 0.41 | 0.03 | 0.03 |
| 6 | 1.50 | 1.28 | 14.7 | 0.047 | 0.101 | 0.55 | 0.46 | 0.30 | 0.25 | 0.03 | 0.02 |
| 7 | 1.71 | 1.35 | 21.1 | 0.035 | 0.060 | 0.58 | 0.47 | 0.32 | 0.29 | 0.03 | 0.02 |
| 9 | 1.32 | 0.93 | 29.5 | 0.015 | 0.017 | 0.45 | 0.29 | 0.29 | 0.22 | 0.03 | 0.02 |
| av ± SD[1−9] | 1.54 ± 0.29 | 1.04 ± 0.42 | 33.4 ± 23.6 | 0.04 ± 0.01 | 0.09 ± 0.04 | 0.54 ± 0.05 | 0.36 ± 0.14 | 0.32 ± 0.02 | 0.34 ± 0.09 | 0.03 ± 0.00 | 0.02 ± 0.01 |

[a] The data for batches 3 and 5 are not included.

reached ∼8 days. Kosseva et al. (*11*) reported that ∼23% of malic acid degradation was observed when calcium pectate gel and chitopearls SH-5010 were used as supports for *L. casei* cells for MLF after alcoholic fermentation for wine samples with and without $SO_2$. Operational stability was 6 months after eight fermentations and 2 months after five fermentations, respectively. Maicas et al. (*12*) reported the possibility of using cells of *O. oeni* immobilized by absorption to positively charged cellulose sponge (DE or DEAE) for MLF of wine in a semicontinuous system. Satisfactory results were recorded in the first 24 h, where the initial 3.5 g/L of malic acid almost completely metabolized. However, subsequent batches with immobilized cells showed a strong drop in malolactic activity.

**Other Organic Acids.** *Tartaric Acid.* Wine lactic bacteria can perform total or partial degradation of tartaric acid, which lowers the fixed acidity and is accompanied by an increase in the volatile acidity. Tartaric acid is an essential acid in wine, and its degradation lowers wine quality (*26*). Also, calcium tartrate precipitation is highly pH dependent. The higher the pH, the lower the degree of solubility. Any activity that increases pH, including MLF, will significantly increase the possibility of tartrate precipitation (*2*, *27*). With regard to our experiments, tartaric acid analysis showed a drop after MLF ranging from 0.47 to 0.01 g/L (**Table 2**), without any increase of acetic acid, which mainly contributes to the volatile activity. Therefore, it is more probable that the decrease of tartrate was due to precipitation because of a change of pH caused by MLF or metabolism to succinic acid and not to acetic acid according to Radler and Yannissis (*28*).

*Succinic Acid.* During the first four MLF batches, where the activity of the biocatalyst was relatively high (80−23% degradation of malic acid), the concentrations of succinic acid after the completion of MLF (0.41−0.43 g/L) were higher than those before MLF commenced (0.32−0.34 g/L) (**Table 2**). For the remaining seven repeated batches the succinic acid concentrations showed a tendency to decrease (by ∼15%).

*Acetic Acid.* After MLF, the acetic acid concentrations were slightly reduced or remained stable at low values (<0.03 g/L) (**Table 2**). This is a very interesting result because the acetic acid content highly affects wine quality.

**Major Volatiles in Wine.** *Acetaldehyde.* After MLF, the acetaldehyde content of the produced wines (65.3−80.3 mg/L) was higher than that after alcoholic fermentation (39.9−50.6 mg/L), but it did not exceed the maximum acceptable value for wines (100−125 mg/L), which is the sensory threshold range (**Table 3**). So, because acetaldehyde concentrations are <100 mg/L, they are not sufficient to affect negatively the sensory character of the wines after MLF. Immediately after alcoholic fermentation, table wines generally have an acetaldehyde concentration of <75 mg/L. As wine ages, acetaldehyde levels are expected to increase (*2*).

*Ethyl Acetate.* Ethyl acetate content was generally increased after MLF but did not give a vinegar aroma in the produced wine. Ethyl acetate concentrations in the produced wines after MLF were mainly in the range of 43.5−62.4 mg/L, and only in one case was concentration of ∼107 mg/L observed (**Table 3**). In sound wines, ethyl acetate concentrations are generally in the range of 50−100 mg/L, whereas concentrations >150 mg/L are likely to contribute a sour-vinegar off-odor (*1*). Increase of ethyl acetate after MLF has also been reported (*1*, *29*).

*Higher Alcohols.* After MLF, the higher alcohol concentrations were decreased, and in the case of 1-propanol a drastic drop (∼84%) was reported as it occurs in MLF with immobilized cells (*1*). 1-Propanol concentrations were in the range of 1.5−2.5 mg/L, isobutyl alcohol, 18.7−30.7 mg/L, and amyl alcohol, 88.7−119.0 mg/L (**Table 3**). The sum of the maximum values of undesirable higher alcohols was ∼150 mg/L, and so

**Table 3.** Volatiles (Milligrams per Liter) in Wines before (AF) and after (MLF) Malolactic Fermentation with Immobilized Cells of *S. cerevisiae* AXAZ-1 and *L. casei* ATCC 393, Respectively

| | acetaldehydes | | ethyl acetate | | 1-propanol | | isobutyl alcohol | | amyl alcohols | | methanol | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| batch | AF | MLF | AF | MLF | AF | MLF | AF | MLF | AF | MLF | AF | MLF |
| 1 | 42.5 | 72.6 | 34.5 | 43.5 | 8.4 | 2.2 | 22.9 | 25.8 | 130.6 | 119.0 | 51.0 | 97.2 |
| 2 | 42.0 | 69.7 | 34.6 | 58.0 | 10.1 | 1.5 | 23.4 | 18.7 | 123.9 | 104.3 | 87.3 | 92.5 |
| 3 | 50.3 | 70.2 | 36.8 | 55.2 | 9.9 | 1.9 | 29.6 | 24.9 | 110.3 | 96.3 | 76.7 | 80.2 |
| 4 | 45.2 | 80.3 | 30.4 | 44.4 | 11.5 | 2.3 | 28.3 | 27.2 | 105.4 | 109.9 | 69.4 | 113.9 |
| 5 | 50.6 | 65.3 | 40.1 | 49.9 | 13.3 | 1.6 | 30.1 | 25.3 | 111.1 | 98.6 | 70.6 | 88.6 |
| 6 | 39.9 | 69.3 | 42.3 | 52.6 | 10.2 | 2.5 | 35.0 | 26.3 | 120.6 | 100.5 | 75.4 | 86.7 |
| 7 | 44.4 | 73.8 | 33.5 | 106.5 | 16.6 | 1.6 | 42.4 | 19.5 | 111.4 | 88.7 | 89.5 | 96.5 |
| 9 | 43.7 | 66.9 | 50.8 | 62.4 | 15.2 | 1.9 | 44.6 | 30.7 | 123.4 | 116.3 | 91.1 | 111.5 |
| av ± SD[1−9] | 44.8 ± 3.8 | 71.0 ± 4.7 | 37.9 ± 6.4 | 59.1 ± 20.2 | 11.9 ± 2.9 | 1.9 ± 0.4 | 32.0 ± 8.1 | 24.8 ± 3.9 | 117.1 ± 8.7 | 104.2 ± 10.3 | 76.4 ± 13.3 | 95.9 ± 11.7 |
| % change | | +58 | | +56 | | −84 | | −23 | | −11 | | +26 |

**Table 4.** Volatile Compounds Identified by GC-MS Analysis in Grape Must and in Wine Samples (Batch 1) Produced before (A) and after MLF (M) with Immobilized Cells of *S. cerevisiae* AXAZ-1 and *L. casei* ATCC 393, Respectively

| time (min) | compound | must | A1 | M1 | time (min) | compound | must | A1 | M1 |
|---|---|---|---|---|---|---|---|---|---|
| 5.008 | ethyl acetate | − | + | + | 36.025 | nonanol | − | − | − |
| 6.592 | ethanol | + | + | + | 36.092 | 3-methylbutyl octanoate | − | − | + |
| 11.942 | 2-methyl-1-propanol | + | − | − | 36.175 | heptanal | − | − | − |
| 12.808 | 1-butanol, 3-methyl acetate | − | + | + | 36.558 | diethyl succinate | − | − | + |
| 19.950 | 3-methylbutanol | + | + | + | 36.617 | dodecanal | − | − | − |
| 20.258 | ethyl hexanoate | − | + | − | 37.004 | ethyl 9-decenoate | − | − | − |
| 27.467 | acetic acid, 2-ethylhexyl | − | + | + | 37.058 | α-terpineol | − | + | + |
| 27.692 | nonanal | − | + | + | 37.358 | cyclohexane, 1,5-diisopropyl, 2,3-dimethyl | − | + | + |
| 29.542 | ethyl octanoate | + | + | − | 38.183 | 4-decanoic acid, ethyl ester | − | − | + |
| 30.175 | isopentyl hexanoate | − | + | + | 38.558 | isooctanol | − | − | − |
| 30.592 | furfural | + | − | − | 38.625 | 1-decanol | − | − | + |
| 31.133 | 2-ethyl hexanol | − | − | + | 40.133 | 2-acetic acid, 2-phenylethyl ester | + | + | + |
| 31.433 | decanal | − | + | − | 40.667 | ethyl dodecanoate | − | + | + |
| 32.175 | benzaldehyde | − | − | + | 40.975 | hexanoic acid | − | + | − |
| 32.283 | 1-octanol, 3,7-dimethyl | − | − | + | 41.092 | *N*-(3-methylbutyl)acetamide | − | + | + |
| 32.617 | ethyl nonanoate | − | + | + | 41.117 | lauric acid, 2-methylbutyl ester | − | + | + |
| 32.875 | 2-nonanal | − | − | + | 41.883 | ethyl 9-hexadecenoate | − | + | + |
| 33.200 | 1-octanol | − | + | + | 42.333 | phenylethyl alcohol | + | + | + |
| 33.617 | 1-heptanol, 2,4-dimethyl | − | − | + | 43.367 | 1-decanol | − | + | − |
| 33.900 | 2-heptanol, 5-ethyl | − | − | + | 43.367 | tridecanal | − | − | + |
| 34.058 | 1-nonadecanol | − | − | + | 45.575 | octanoic acid | − | + | + |
| 34.342 | 1-octanol, 2-butyl | − | − | + | 45.975 | oleic acid | − | + | + |
| 34.617 | undecanol | − | − | + | 46.183 | 6,10,14-trimethyl-2-pentadecanoate | − | − | + |
| 34.833 | isooctanol | − | − | + | 46.425 | undecanal | + | − | − |
| 35.100 | 5-hexadecanol | − | − | + | 47.383 | nonanoic acid | − | + | − |
| 35.200 | 6-methyl-1-octene | − | − | + | 48.475 | ethyl hexadecanoate | − | + | + |
| 35.592 | ethyl decanoate | − | + | + | 48.817 | decanoic acid | − | + | + |

they contribute to the wine aroma without off-odors. In table wines, the total higher alcohol concentrations reported were in the range of 140−420 mg/L (*2*). Maicas et al. (*29*) reported significant increases of higher alcohols in all wines that had undergone the MLF by three different *O. oeni* strains and a *Lactobacillus* sp. strain.

Lactic acid bacteria cannot grow with L-malic acid as a unique carbon source; therefore, these microorganisms need an additional energy source such as residual fermentable sugars (*30*) or amino acids (*31*) to allow cell growth. Therefore, a probable explanation may be that malolactic bacteria utilize amino acids, which are therefore not converted to higher alcohols.

*Methanol.* Methanol concentrations were increased after MLF but were <114 mg/L. Within the usual range of 0.1−0.2 g/L, methanol has no direct sensory effect (*1*).

**GC-MS Analysis.** The identification of volatiles by GC-MS analysis was carried out to evaluate the qualitative differences between the wines produced before and after the completion of MLF (**Table 4**). A SPME was employed, which gives more peaks than the common headspace technique. After the completion of the first alcoholic fermentation batch, the wine sample (A1) was found to contain many more compounds than the unfermented must, mainly esters, alcohols, and acids. After the completion of MLF, this wine (M1) contained more alcohols and carbonyl compounds compared to A1. Further analysis of wines after the ninth batch showed some qualitative changes regarding the absence of some compounds that were identified in the wines produced in the first batches, which may be related to the reduction of malolactic activity beyond that point.

From the above results one can conclude that DCM is a promising support for MLF, but more research is necessary to improve some parameters such as operational stability and yield in order to industrialize the method. Operational stability of the immobilized biocatalyst in 11 repeated MLF batches was decreased as the MLF batches continued over a period of 1 month. This is a general problem in MLF. Malic acid degradation did not give the stoichiometric quantity of lactic acid, but a much lower concentration. This question must be examined. However, the industrial experience proves that high lactic acid content in alcohol and wine does not contribute to fine aroma because lactic acid contributes to volatile acidity (*2*, *32*). The pH value of the wine after MLF was increased by 0.5−0.1 unit

as the MLF batches proceeded. The quality of the produced wines is encouraging continued research, as it is the main consumer demand. After the MLF batches, the ethyl acetate content was increased, giving aroma to wines, whereas higher alcohols (1-propanol, isobutyl alcohol, and amyl alcohols) were decreased, contributing in this way to the quality of wines (**Table 3**). From the examination of organic acids (**Table 2**) it is noteworthy that after MLF, the acetic acid, which is the main component of volatile acidity and an indicator of wine spoilage, remained at low levels (*2*).

## LITERATURE CITED


(1) Jackson, R. S. In *Wine Science*; Taylor, S. L., Ed.; Academic Press: New York, 1994; pp 184, 201, 259, 262, 265, 268.

(2) Zoecklein, W. B.; Fugelsang, C. K.; Gump, H. B.; Nury, S. F. In *Wine Analysis and Production*; Chapman and Hall: New York, 1995; pp 101, 192, 221−222, 292, 240.

(3) Davis, C. R.; Wibowo, D.; Fleet, G. H.; Lee, T. H. Properties of wine lactic acid bacteria: Their potential enological significance. *Am. J. Enol. Vitic*. **1988**, *39*, 137−42.

(4) Kunkee, R. E. Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiol. Rev.* **1991**, *88*, 55−72.

(5) Versari, A.; Parpinello, G. P.; Cattaneo, M. *Leuconostoc oenos* and malolactic fermentation in wine: A review. *J. Ind. Microb. Biotechnol*. **1999**, *23*, 447−455

(6) Maicas, S. The use of alternative technologies to develop malolactic fermentation in wine. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 35−39.

(7) Colagrande, O.; Silva, A.; Fumi, M. D. Recent applications of biotechnology in wine production. Review. *Biotechnol. Prog.* **1994**, *10*, 2−18.

(8) Gestrelius, S. Potential application of immobilized viable cells in the food industry: Malolactic fermentation of wine. *Enzyme Eng.* **1982**, *6*, 245−250.

(9) McCord, J. D.; Ryu, D. D. Y. Development of malolactic fermentation process using immobilized whole cells and enzymes. *Am. J. Enol. Vitic*. **1985**, *36*, 214−218.

(10) Divies, C.; Cachon, R.; Cavin, J. F.; Prevost, H. Theme-4−Immobilized cell technology in wine production. *Crit. Rev. Biotechnol*. **1994**, *14*, 135−153.

(11) Kosseva, M.; Beschkov, V.; Kennedy, J. F.; Lloyd, L. L. Malolactic fermentation in chardonnay wine by immobilized *Lactobacillus casei* cells. *Process Biochem*. **1998**, *33*, 793−797.

(12) Maicas, S.; Pardo, I.; Ferrer, S. The potential of positively-charged cellulose sponge for malolactic fermentation of wine, using *Oenococcus oeni*. *Enzyme Microb. Technol*. **2001**, *28*, 415−419.

(13) Divies, C.; Siess, M. H. Study on L-malic acid catabolism by *Lactobacillus casei* cells immobilized into polyacrylamide-gel lattice. *Ann. Microbiol.* **1976**, *B127* (4), 525−539.

(14) Spettoli, P.; Bottacin, A.; Nuti, M. P.; Zamorani, A. Immobilization of *Leuconostoc oenos* ML 34 in calcium alginate gels and application to wine technology. *Am. J. Enol. Vitic.* **1982**, *33* (1), 1−5.

(15) Rossi, J.; Clementi, F. L-Malic acid catabolism by polyacrylamide gel entrapped *Leuconostoc oenos*. *Am. J. Enol. Vitic*. **1984**, *35*, 100−102.

(16) Spettoli, P.; Nuti, M. P.; Crapisi, A.; Zamorani, A. Technological improvement of malolactic fermentation in wine by immobilized cells in a continuous flow reactor. *Ann. N. Y. Acad. Sci.* **1987**, *501*, 386−389.

(17) Crapisi, A.; Nuti, M. P.; Zamorani, A.; Spettoli, P. Improved stability of immobilized *Lactobacillus sp* cells for the control of malolactic fermentation in wine. *Am. J. Enol. Vitic*. **1987**, *38*, 310−312.

(18) Naouri, P.; Bernet, N.; Chagnaud, P.; Arnaud, A.; Galzy, P.; Rios, G. Bioconversion of L-malic acid into L-lactic acid using a high compacting multiphasic reactor (HCMR). *J. Chem. Technol. Biotechnol*. **1991**, *51*, 81−95.

(19) Bardi, E. P.; Koutinas, A. A. Immobilization of yeast on delignified cellulosic material for room temperature and low-temperature wine making. *J. Agric. Food Chem.* **1994**, *42*, 221−226.

(20) Iconomopoulou, M.; Kanellaki, M.; Psarianos, C.; Koutinas, A. A. Delignified cellulosic material supported biocatalyst as freeze-dried product in alcoholic fermentation. *J. Agric. Food Chem.* **2000**, *48*, 958−961.

(21) Bardi, E.; Koutinas, A. A.; Psarianos, C.; Kanellaki, M. Volatile by-products formed in low-temperature wine-making using immobilized yeast cells. *Process Biochem*. **1997**, *32*, 579−584.

(22) Argiriou, T.; Kaliafas, A.; Psarianos, K.; Kanellaki, M.; Voliotis, S.; Koutinas, A. A. Psychrotolerant *Saccharomyces cerevisiae* strains after an adaptation treatment for low-temperature wine making. *Process Biochem.* **1996**, *31*, 639−643.

(23) Mallouchos, A.; Komaitis, M.; Koutinas, A. A.; Kanellaki, M. Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products. *Food Chem.* **2002**, *80*, 109−113.

(24) Tsakiris, A.; Sipsas, V.; Bekatorou, A.; Mallouchos, A.; Koutinas, A. A. Red wine making by immobilized cells and influence on volatile composition. *J. Agric. Food Chem.* **2004**, *52*, 1357−1363.

(25) Lonvaud-Funel, A. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek* **1999**, *76*, 317−331.

(26) Riberau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. In *Handbook of Enology*. *The Chemistry of Wine Stabilization and Treatments*; Wiley: Chichester, U.K., 2000; Vol. 2, p 140.

(27) McKinnon, T. Some aspects of calcium tartrate precipitation. *Aust. Grapegrower Winemaker* **1993**, Annu. Tech. Issue, 89−91.

(28) Radler, F.; Yannissi, C. Decomposition of tartrate by *Lactobacilli*. *Arc. Mikrobiol.* **1972**, *82*, 219.

(29) Maicas, S.; Gil, J. V.; Pardo, I.; Ferrer, S. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Res. Int*. **1999**, *32*, 491−496.

(30) Liu, S. Q.; Pritchard, G. G.; Hardman, M. J.; Pilone, G. J. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic-acid bacteria. *Appl. Environ. Microbiol.* **1995**, *61*, 310−316.

(31) Liu, S. Q.; Pilone, G. J. A review: Arginine metabolism in wine lactic acid bacteria and its practical significance. *J. Appl. Microbiol*. **1998**, *84*, 315−327.

(32) Koutinas, A. A.; Pefanis, S. In *Biotechnology of Foods and Drinks;* University of Patras: Patras, Greece, 1996; 168 pp.


**Received for review July 28, 2004. Revised manuscript received January 26, 2005. Accepted January 27, 2005.**

JF048736T