World Journal of Microbiology & Biotechnology **16**: 805–810, 2000.

Malolactic fermentation in wine with high densities of non-proliferating *Oenococcus oeni*

Sergi Maicas[1,2,*], Àngels Natividad[1,3], Sergi Ferrer[1] and Isabel Pardo[1]
[1]*Departament de Microbiologia i Ecologia, Facultat de Biologia, Universitat de València, Burjassot, Spain*
Present addresses: [2]*Departament de Biotecnologia, Institut d'Agroquímica i Tecnologia d'Aliments (CSIC), Paterna, Spain*
[3]*Greendale Laboratories Ltd. Knaphill, Woking, Surrey, UK*
**Author for correspondence: Sergi Maicas, IATA (CSIC), P.O. Box 73, 46100 Burjassot, Spain*
Tel.: 34-6-3900022, Fax: 34-6-3636301, E-mail: sergi.maicas@iata.csic.es

Received 21 April 2000; accepted 11 October 2000

Keywords: Lactic acid bacteria, *Leuconostoc oenos*, malic acid, malolactic fermentation, wine production

Summary

Five strains of *Oenococcus oeni* (syn. *Leuconostoc oenos*) under non-proliferating conditions were assessed for the performance of the malolactic fermentation in wine at various initial pH values, malic acid concentration and densities of cells. We succeeded in inducing the malolactic fermentation after inoculation of high densities of *O. oeni* G6 even in recalcitrant wines where the traditional malolactic fermentation was inhibited by adverse environmental conditions (low pH and high concentration of malic acid). Optimal degrading conditions in wine, under different physico-chemical environments, were determined in order to achieve rapid depletion of malic acid in red wine. Off-odour compounds were not formed under these conditions, suggesting an attractive alternative for wine production.

Introduction

The malolactic fermentation (MLF) is the microbial process which allows some lactic acid bacteria present in wine to perform the bioconversion of L-malic acid to L-lactic acid and CO_2 (Kunkee 1967; Liu & Gallander 1983; Wibowo *et al.* 1985). In addition to deacidification, the MLF is considered to contribute to the complexity of wine flavour and to confer a degree of microbiological stability to the wine (Davis *et al.* 1985; Henick-Kling 1995; Maicas *et al.* 1999a). *Oenococcus oeni* is the best-adapted lactic acid bacterium for the induction of this enological process, although its growth in the wine to be deacidified is usually a pre-requisite. The growth of *O. oeni* in wine is complex and consists of distinct phases that occur during alcoholic fermentation, malolactic fermentation and conservation after malolactic fermentation (Davis *et al.* 1986). The natural conditions of the malolactic conversion are unfavourable, and several weeks or months are required to obtain an appropriate number of cells able to degrade the malic acid present in red wines efficiently. The inoculation of wines or musts with optimal numbers of cells that perform the MLF requires the previous production of high quantities of biomass (Hayman & Monk 1982; Henick-Kling 1991). Growth rate, biomass yield and ability to conduct the MLF in wine can be optimized by using an adequate combination of media and culture conditions. As part of a programme to produce *O. oeni* starter cultures to induce MLF in red wines, we described the optimal conditions for the efficient production of high densities of cells (Maicas *et al.* 1999b).

In previous work in our laboratory, the inefficiency of some *O. oeni* strains to induce MLF in wine under natural conditions was assessed (Pardo & Zúñiga 1992). Here, we show the capability of high-density inocula of such strains to perform MLF in a short period of time. Moreover, we have shown the importance of characterizing several strains in order to choose the best-adapted malolactic strain for each type of wine.

Materials and Methods

Bacterial strains and culture conditions

Oenococcus oeni strains (M42, G6, N172, T46 and M41) were collected from Requena (Eastern Spain), isolated and identified by Pardo & Zúñiga (1992). High cellular densities were achieved by growing strains in MLO broth (Caspritz & Radler 1983), containing (g l^{-1}): glucose, 10; fructose, 5; yeast extract, 5; tryptone, 10; $MgSO_4 \cdot 7H_2O$, 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. Then cells were washed twice in distilled water previous to

inoculation in red wine containing (g l^{-1}): glucose, 0.9; fructose, 1.0 and ethanol, 11.1% (v/v). Malic acid concentrations were adjusted to 3.5–7.0 g l^{-1}. The pH of wine was adjusted to 3.1 or 3.5 with 0.1 M HCl or 0.1 M KOH, respectively, allowed to stabilize overnight and then sterilized by filtration (0.45 μm). The pH and malic acid concentrations were significant for those found in wine.

Assay of malolactic activity in wine

Cells of *O. oeni* were inoculated into 10.0 ml screw tubes filled with red wine to give densities ranging from 10^6 to 10^8 c.f.u. ml^{-1}. Fermentations were carried out at 20 °C avoiding light incidence for a period of 3 weeks. At different phases of incubation a sterile sample was taken, the cells were removed by membrane filtration (0.45 μm) and sugars, acid and ethanol concentrations were monitored by HPLC. To act as a control, non-inoculated wines prepared as described were also included in each experiment.

Analytical methods

Sugars, organic acids and ethanol were quantified by HPLC as previously described (Maicas *et al*. 1999c). Bacterial growth was checked by periodical plating onto MLO as previously described (Maicas *et al*. 1999b). Wine pH was determined with a MicropH 2001 pH-meter (Crison Instruments, Spain). Calibrations were done with reference solutions at pH 2 and 4.

Results

The percentages of L-malic degradation at different stages of incubation in wine by non-proliferating suspensions of cells are shown in Tables 1–4. The five strains used in this study were able to metabolize some quantities of malic acid. However, some differences were detected under different environmental conditions of pH or malic acid concentration.

Fermentations at pH 3.1 with 7.0 g l^{-1} of malic acid

None of the five *O. oeni* strains gave complete degradation of malic acid, even with the addition of the higher cell density, about 1×10^8 c.f.u. ml^{-1} (Table 1). As a consequence of the strain variability, different malolactic activities were recorded. Malic acid degradation was negligible when approximately 1×10^6 c.f.u. ml^{-1} were inoculated in wine, irrespectively of the strain used. G6 was an exception to this rule provided that more than 10% of the malic acid was metabolized in the first week of assay (Figure 1a). Nevertheless, the viability of the cells dropped dramatically and no more malic acid consumption was recorded after this stage. Hence, the pH increment was only of 0.02 units. Survival rates after 3 weeks were not highly increased for the higher inocula, 1×10^7–1×10^8 c.f.u. ml^{-1}, provided that only 1×10^4 c.f.u. ml^{-1} were recorded with *O. oeni* N172 (Table 1). However, as the number of surviving cells during the first week of experimentation was high, the percentages of malic acid metabolized were around 43–69%. The final pH was also recorded and values ranged from 3.12 to 3.26 depending on the strain (Table 1).

Fermentations at pH 3.1 with 3.5 g l^{-1} of malic acid

The results obtained under these conditions are shown in Table 2. The strong conditions for the development of cells in a low pH environment only allowed the detection of 1×10^5 c.f.u. ml^{-1} after 3 weeks for the

Table 1. Malolactic fermentation in red wine at pH 3.1 following inoculation with *Oenococcus oeni* (the initial malic acid concentration was 7.0 g l^{-1}).

Strain	Initial bacteria in wine (c.f.u. ml^{-1})	Bacteria in wine after 3 weeks (c.f.u. ml^{-1})	Malic acid degradation (%)				pH	
			1 day	1 week	2 weeks	3 weeks	Initial	Final
Control	ND	ND	0	0	0	0	3.10	3.10
M42	6×10^5	3×10^2	0	0	0	0	3.10	3.10
M42	1×10^7	1×10^3	0	17	17	17	3.10	3.24
M42	6×10^7	6×10^2	34	63	69	69	3.10	3.25
M41	1×10^6	1×10^2	0	0	0	0	3.10	3.10
M41	1×10^7	2×10^2	3	5	5	5	3.10	3.16
M41	7×10^7	7×10^1	33	47	47	47	3.10	3.19
N172	2×10^6	3×10^2	0	0	0	0	3.10	3.10
N172	1×10^7	2×10^2	0	0	27	27	3.10	3.14
N172	7×10^7	1×10^4	34	63	69	69	3.10	3.26
G6	8×10^5	9×10^2	8	12	12	12	3.10	3.12
G6	2×10^7	5×10^3	41	55	55	55	3.10	3.24
G6	6×10^7	3×10^3	53	64	64	64	3.10	3.25
T46	1×10^6	7×10^2	1	1	1	1	3.10	3.10
T46	7×10^6	5×10^1	6	20	22	27	3.10	3.11
T46	6×10^7	7×10^1	30	43	43	43	3.10	3.12

Control: without cell addition; ND: not detected.

Table 2. Malolactic fermentation in red wine at pH 3.1 following inoculation with *Oenococcus oeni* (the initial malic acid concentration was 3.5 g l^{-1}).

Strain	Initial bacteria in wine (c.f.u. ml^{-1})	Bacteria in wine after 3 weeks (c.f.u. ml^{-1})	Malic acid degradation (%)				pH	
			1 day	1 week	2 weeks	3 weeks	Initial	Final
Control	ND	ND	0	0	0	0	3.10	3.10
M42	7×10^5	1×10^2	0	0	0	0	3.10	3.10
M42	1×10^7	3×10^1	14	38	42	43	3.10	3.20
M42	8×10^7	1×10^3	59	77	77	77	3.10	3.21
M41	1×10^6	1×10^2	0	0	0	0	3.10	3.10
M41	8×10^6	1×10^2	0	19	19	19	3.10	3.12
M41	9×10^7	2×10^3	39	66	66	67	3.10	3.20
N172	2×10^6	6×10^2	0	0	11	11	3.10	3.12
N172	1×10^7	4×10^2	0	38	42	42	3.10	3.16
N172	8×10^7	1×10^5	59	93	98	98	3.10	3.26
G6	1×10^6	4×10^3	31	32	32	32	3.10	3.15
G6	1×10^7	3×10^1	66	91	91	92	3.10	3.25
G6	6×10^7	5×10^4	50	72	74	77	3.10	3.25
T46	7×10^5	1×10^1	2	2	2	2	3.10	3.13
T46	8×10^6	5×10^2	9	14	14	14	3.10	3.12
T46	7×10^7	7×10^2	37	41	41	41	3.10	3.19

Control: without cell addition; ND: not detected.

Table 3. Malolactic fermentation in red wine at pH 3.5 following inoculation with *Oenococcus oeni* (the initial malic acid concentration was 7.0 g l^{-1}).

Strain	Initial bacteria in wine (c.f.u. ml^{-1})	Bacteria in wine after 3 weeks (c.f.u. ml^{-1})	Malic acid degradation (%)				pH	
			1 day	1 week	2 weeks	3 weeks	Initial	Final
Control	ND	ND	0	0	0	0	3.50	3.50
M42	8×10^5	3×10^4	6	6	32	47	3.50	3.52
M42	1×10^7	1×10^5	0	32	49	67	3.50	3.56
M42	8×10^7	4×10^6	36	73	99	100	3.50	3.75
M41	1×10^6	1×10^6	4	5	11	41	3.50	3.52
M41	9×10^6	4×10^6	15	25	26	30	3.50	3.58
M41	6×10^7	3×10^5	28	84	90	94	3.50	3.73
N172	3×10^6	4×10^4	6	6	32	32	3.50	3.55
N172	1×10^7	2×10^5	15	26	46	62	3.50	3.58
N172	8×10^7	1×10^5	40	81	99	100	3.50	3.77
G6	8×10^5	1×10^5	3	13	23	36	3.50	3.56
G6	2×10^7	2×10^6	54	93	98	99	3.50	3.73
G6	9×10^7	2×10^6	35	81	88	93	3.50	3.68
T46	9×10^5	3×10^5	1	1	1	10	3.50	3.52
T46	8×10^6	7×10^4	6	20	22	27	3.50	3.56
T46	6×10^7	1×10^5	30	43	43	43	3.50	3.59

Control: without cell addition; ND: not detected.

strain N172 when 8×10^7 c.f.u. ml^{-1} were inoculated. The final numbers of cells in the other batches with the other strains and quantities of inoculum almost mirrored results reported for batches including 7 g l^{-1} of malic acid. However, as the amount of malic acid was half in the present series of assays, better results were recorded by using some of these strains. Therefore, more than 77% of the malic acid was metabolized when levels of cells higher than 1×10^6 c.f.u. ml^{-1} were added to wine. Even 98% of the malic acid was metabolized in a period of 2 weeks when *O. oeni* N172 was inoculated at a concentration of 8×10^7 c.f.u. ml^{-1}. Malic acid degradation was usually negligible if only about 1×10^6 c.f.u. ml^{-1} were added to wine. Only *O. oeni* G6 was able to degrade almost a third of the malic acid in 24 h (Figure 1b). The proportion of L-malic acid degraded did not increase progressively with time given that cells quickly died. The change in pH was coupled to the malic acid consumption (Table 2).

Fermentations at pH 3.5 with 7.0 g l^{-1} of malic acid

When a favourable pH was provided, all the strains used in this study were able to degrade some L-malic acid to a minor or a major extent (Table 3). The amount of L-malic acid degraded increased progressively with time, as can be observed for *O. oeni* G6 (Figure 1c). In this respect, should be outlined the elevated efficiency of high densities of malolactic strains, with the exception of T46, in degrading important amounts of L-malic acid. The

Table 4. Malolactic fermentation in red wine at pH 3.5 following inoculation with *Oenococcus oeni* (the initial malic acid concentration was 3.5 g l^{-1}).

Strain	Initial bacteria in wine (c.f.u. ml^{-1})	Bacteria in wine after 3 weeks (c.f.u. ml^{-1})	Malic acid degradation (%)				pH	
			1 day	1 week	2 weeks	3 weeks	Initial	Final
Control	ND	ND	0	0	0	0	3.50	3.50
M42	7×10^5	1×10^4	9	9	36	54	3.50	3.52
M42	1×10^7	1×10^5	17	51	81	81	3.50	3.56
M42	1×10^8	3×10^6	56	94	98	100	3.50	3.65
M41	1×10^6	2×10^6	5	6	14	25	3.50	3.55
M41	8×10^6	1×10^6	14	28	37	43	3.50	3.64
M41	1×10^8	1×10^7	48	99	99	100	3.50	3.66
N172	2×10^6	4×10^4	30	30	54	54	3.50	3.54
N172	2×10^7	1×10^5	45	61	81	81	3.50	3.65
N172	7×10^7	1×10^6	56	94	98	100	3.50	3.70
G6	9×10^5	3×10^5	5	22	30	48	3.50	3.55
G6	2×10^7	4×10^6	69	94	100	100	3.50	3.73
G6	7×10^7	3×10^6	48	85	90	99	3.50	3.76
T46	8×10^5	6×10^5	4	4	8	10	3.50	3.53
T46	6×10^6	6×10^5	10	26	28	34	3.50	3.55
T46	6×10^7	3×10^5	44	59	61	61	3.50	3.60

Control: without cell addition; ND: not detected.

use of lower densities of cells at about 1×10^6 c.f.u. ml^{-1} only permitted the degradation of 10–47% of the initial level of L-malic acid. The pH increased concomitantly with L-malic acid consumption (Table 3).

Fermentations at pH 3.5 with 3.5 g l^{-1} of malic acid

The ability of high densities of *O. oeni* cells to perform MLF in wine was enhanced when a comfortable pH 3.5 was provided and only 3.5 g l^{-1} of malic acid were to be degraded. After 3 weeks, malic acid was almost completely degraded by using any of the strains used in this work at about 1×10^8 c.f.u. ml^{-1} with the only exception being *O. oeni* T46. The use of such densities of cells resulted in satisfactory levels of metabolism after 1 week providing a satisfactory method for wine. We also recorded an efficient metabolism of malic acid at lower levels, i.e. 1×10^6–1×10^7 c.f.u. ml^{-1} by using M42, N172 and G6 strains (Figure 1d). Roughly 50% of the 3.5 g l^{-1} of malic acid available at the beginning of the experiment were consumed in 3 weeks.

Evolution of other metabolites during the malolactic fermentation

Ethanol, sugars and organic acids usually present in wine were also periodically quantified in the 60 batch fermentations developed in this study. No significant differences have been found in any one of the wines analyzed, including the non-inoculated controls (data not shown). As an example, the concentrations of fumaric, citric and acetic acids were respectively 0.002, 0.060 and 1.400 g l^{-1}, respectively, before and after MLF. This is a remarkable fact given that in a traditional fermentation, i.e. by inoculating only about 10^5 c.f.u. ml^{-1} and after an incubation period of 1–3 months, fumaric and citric acids were metabolized (Maicas 1998). As expected, lactic acid concentration increased as a consequence of the L-malic metabolism. The production of non-volatile compounds, which has also been reported for traditional vinifications with *O. oeni* (Maicas *et al.* 1999a) was not significantly detected under the current non-proliferating conditions (data not shown).

Discussion

High-density cell suspensions of several *O. oeni* strains are able to rapidly degrade L-malic acid present in wine. Inocula lower than 1×10^7 c.f.u. ml^{-1} require a previous phase of cellular growth usually involving weeks or months and not always successfully accomplished. Under non-proliferating conditions, the minimal inoculation concentrations to achieve optimal degradations of malic are about 1×10^7 c.f.u. ml^{-1} at pH 3.5 and 1×10^8 c.f.u. ml^{-1} at pH 3.1. Wine conditions are not determined by the winemakers but by the must production and by primary stages of wine alcoholic fermentation. Therefore, the ability of various malolactic strains degrade L-malic acid arises as a crucial aspect in winemaking (Beelman *et al.* 1977; Caillet & Vayssier, 1984; Edwards *et al.* 1991), particularly when non-standard wines are to be deacidified. Culturing of *O. oeni* under controlled conditions in synthetic media offers the possibility of inoculation with sufficiently high numbers of cells to induce MLF in wine (Maicas *et al.* 1999b). This is specially desired when a sure and rapid consumption of L-malic acid is required or when an intractable wine needs to be deacidified. The use of high densities of cells permits a substantial deacidification of wine (Tables 1–4) while these same strains usually failed to induce MLF when inoculated at low densities (Pardo & Zúñiga 1992). Basically, the rate of bacterial growth and malolactic fermentation increased as the wine pH

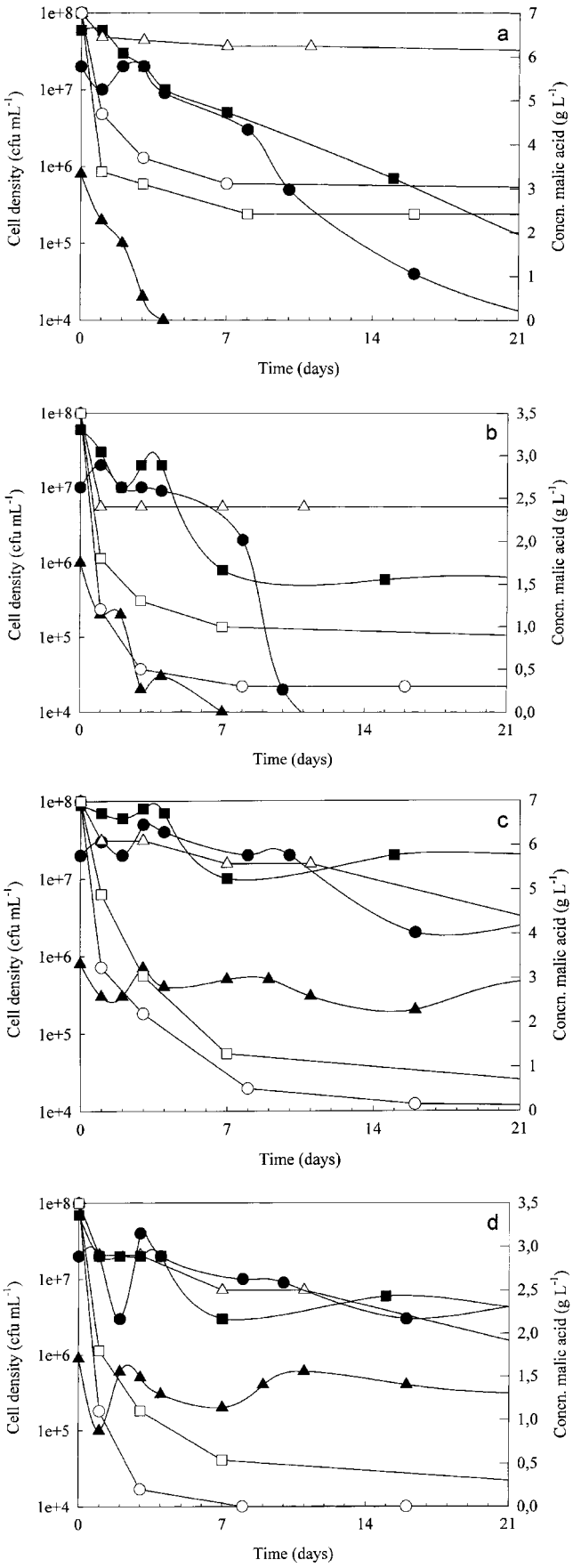

Figure 1. Time course of malolactic fermentation in wine by *Oenococcus oeni* G6 inoculated at different cell densities. Initial wine conditions were: (a) pH 3.1 and malic acid 7.0 g l^{-1}; (b) pH 3.1 and malic acid 3.5 g l^{-1}; (c) pH 3.5 and malic acid 7.0 g l^{-1}; (d) pH 3.5 and malic acid 3.5 g l^{-1}. Other vinification conditions are described in Materials and Methods. Viable cells: ▲, 1×10^6 c.f.u. ml^{-1}; ●, 1×10^7 c.f.u. ml^{-1}; ■, 1×10^8 c.f.u. ml^{-1}; Malic acid concentration at batches inoculated with: △, 1×10^6 c.f.u. ml^{-1}; ○, 1×10^7 c.f.u. ml^{-1}; □, 1×10^8 c.f.u. ml^{-1}.

was increased from 3.1 to 3.5. Therefore, pH has a selective effect upon the strains that arise in wine, which remain in a viable but non-proliferating state for long periods and conduct the MLF. Moreover, it appears to be that at low pH values, bacterial development is difficult and as cell growth is necessary to efficiently achieve malic acid degradation under proliferating conditions, no detectable MLF is produced. The negative impact of pH on the growth rate of *O. oeni* in wine was previously established (Bousbouras & Kunkee 1971; Liu & Gallander 1983). We report here that when *O. oeni* is inoculated at high suspensions about 1×10^7–1×10^8 c.f.u. ml^{-1}, the inhibition of MLF by low pH is diminished, as bacterial development is not essentially necessary to develop the deacidification.

The use of some of the strains here shown enables the deacidification of recalcitrant wines that could not be metabolized any other way. *O. oeni* G6 has been the strain best adapted to the range of wine conditions assayed in this work and it can be used as starter culture to induce MLF in difficult red wines. However, further endeavours are to be made provided that the concept of a worldwide useful strain is not acceptable nowadays under criteria of quality and distinctiveness. A battery of strains able to perform MLF under well-controlled conditions should be supplied to winemakers in order to minimize risks of wine spoilage or fermentation stops. Moreover, the inoculation of high densities of non-proliferating cells into wine does not produce negative volatile compounds. This behaviour is commercially important because a low concentration of such secondary metabolites like as diacetyl and acetoin is a quality factor in red wine (Rankine 1977).

References

Beelman, R.B., Gavin, A. & Keen, R.M. 1977 A new strain of *Leuconostoc oenos* for induced malo-lactic fermentation in eastern wines. *American Journal of Enology and Viticulture* **28**, 159–165.

Bousbouras, G.E. & Kunkee, R.E. 1971 Effect of pH on malolactic fermentation in wine. *American Journal of Enology and Viticulture* **22**, 121–126.

Caillet, M.M. & Vayssier, Y. 1984 Utilisation des biomasses de bactéries lactiques pour le déclenchement de la fermentation malolactique. *Revue Francaise d'Oenologie* **24**, 65–69.

Caspritz, G. & Radler, F. 1983 Malolactic enzyme of *Lactobacillus plantarum*. *Journal of Biological Chemistry* **258**, 4907–4910.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H. 1985 Practical implications of malolactic fermentation: a review. *American Journal of Enology and Viticulture* **35**, 290–301.

Davis, C.R., Wibowo, D., Lee, T.H. & Fleet, G.H. 1986 Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. *Applied and Environmental Microbiology* **51**, 539–545.

Edwards, C.G., Jensen, K.A., Spayd, S.E. & Seymour, B.J. 1991 Isolation and characterisation of native strains of *Leuconostoc*

oenos from Washington State wines. *American Journal of Enology and Viticulture* **42**, 219–226.

Hayman, D.C. & Monk, P. 1982 Starter culture preparation for the induction of malolactic fermentation in wine. *Food Technology in Australia* **34**, 16–18.

Henick-Kling, T. 1991 Use of malolactic starter cultures in the winery. In *Proceedings of the Focus Eastern U.S. Chardonnay Symposium*, ed. Henick-Kling, T., pp. 112–117. Front Royal: University of Davis.

Henick-Kling, T. 1995 Control of malo-lactic fermentation in wine: energetics, flavour modification and methods of starter culture preparation. *Journal of Applied Bacteriology Supplement* **79**, 29S–37S.

Kunkee, R.E. 1967 Malo-lactic fermentation. *Advances in Applied Microbiology* **9**, 235–279.

Liu, J. & Gallander, J.F. 1983 Effect of pH and sulfur dioxide on the rate of malolactic fermentation in red table wines. *American Journal of Enology and Viticulture* **34**, 44–46.

Maicas, S. 1998 Study of different techniques to induce malolactic fermentation in wine using *Oenococcus oeni*. Ph.D. Thesis. Universitat de València, València, Spain.

Maicas, S., Gil, J.V., Pardo, I. & Ferrer, S. 1999a Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Research International* **32**, 491–496.

Maicas, S., González-Cabo, P., Ferrer, S. & Pardo, I. 1999b Production of *Oenococcus oeni* biomass to induce malolactic fermentation in wine by control of pH and substrate addition. *Biotechnology Letters* **21**, 349–353.

Maicas, S., Pardo, I. & Ferrer, S. 1999c Continuous malolactic fermentation in red wine using free *Oenococcus oeni*. *World Journal of Microbiology and Biotechnology* **15,** 737–739.

Pardo, I. & Zúñiga, M. 1992 Lactic acid bacteria in spanish red rosé and white musts and wines under cellar conditions. *Journal of Food Science* **57**, 392–405.

Rankine, B.C. 1977 Developments in malolactic fermentation of Australian red table wines. *American Journal of Enology and Viticulture* **28**, 27–33.

Wibowo, D., Eschenbruch, R., Davis, C.R. & Lee, T.H. 1985 Occurrence and growth of lactic acid bacteria in wine: a review. *American Journal of Enology and Viticulture* **36**, 302–313.