The effects of freezing and freeze-drying of *Oenococcus oeni* upon induction of malolactic fermentation in red wine

Sergi Maicas[1,2,]*, Isabel Pardo[1] & Sergi Ferrer[1]

1 Departament de Microbiologia i Ecologia, Facultat de Biologia, Universitat de València, Burjassot, Spain
2 Present address: Departament de Biotecnologia, Institut d'Agroquímica i Tecnologia d'Aliments, CSIC, Paterna, Spain

(Received 12 July 1999; Accepted in revised form 21 September 1999)

Summary The use of *Oenococcus oeni* starter cultures for the induction of malolactic fermentation (MLF) in wine permits control over the timing of the process and the quality of the wine. Successful inoculation of bacterial starter cultures into wine depends on the selection of suitable strains and on the preparation and conservation of those cultures. Medium for *Leuconostoc oenos* (MLO) is the best medium for easy and rapid growth of *O. oeni* cultures under laboratory controlled conditions for isolation and identification. However, this study showed that *O. oeni* cells inoculated in MLO failed to induce MLF in wine while cells grown in Medium of Preculture (MP) or wine, stored at −20 °C or freeze-dried retained the ability to induce MLF when inoculated in wine. Our results suggest that the use of freeze-dried cultures of *O. oeni* previously grown in MP is the best choice for industrial application.

Keywords Lactic acid bacteria, *Leuconostoc oenos*, malic acid, starter culture.

Introduction

The production of wine with low levels of malic acid is considered a prerequisite for the commercialization of this beverage. Traditionally, the way to reduce the quantities of this acid has been the spontaneous growth of lactic acid bacteria naturally present in wine, which develop the malolactic fermentation (MLF) i.e. the bacterial conversion of L-malic into L-lactic acid and CO_2 (Kunkee, 1967; 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 (Gao & Fleet, 1994; Henick-Kling, 1995; Maicas *et al*., 1999c). Several studies have shown that *Leuconostoc oenos*, recently reclassified as *Oenococcus oeni* (Dicks *et al*., 1995), is adapted to high ethanol concentrations and low pH values in wine and is responsible for MLF (Davis *et al*., 1985). In recent years, starter cultures technologies, involving the inoculation of *O. oeni* into wine, have been developed for inducing MLF (Edwards *et al*., 1991; Nielsen *et al*., 1996). However, failures usually occur because of the lack of adaptation of cultures to wine or because of cellular damage during storage. As the loss of viability is very high when the cells are directly inoculated into wine, starter cultures need one or more steps of reactivation and adaptation to wine before use in order to enhance the survival of the bacteria (Naouri *et al*., 1989; Nielsen *et al*., 1996).

Our aim is to enhance physiological adaptation of living cells by the incubation in preculture media such as Medium for *Leuconostoc oenos* (MLO), Medium of Preculture (MP) or wine before wine inoculation. The conditions of a pro-

*Correspondent: Fax: +34 96 3636301;
email: sergi.maicas@iata.csic.es

longed storage by freezing or by freeze-drying, on the survival rates and viability of *O. oeni* cells in the preculture media, were also investigated.

Materials and methods

Strains and culture conditions

Oenococcus oeni strains were collected from Requena (Eastern Spain), isolated and identified as described by Pardo & Zúñiga (1992). Cells were cultured in Medium for *Leuconostoc oenos* (MLO) (Caspritz & Radler, 1983) or in Medium of Preculture (MP) (Maicas *et al*., 1999b) at 28 °C without stirring.

Conservation and determination of stability of malolactic activity of cells during storage

Suspensions of *O. oeni* strains were stored at −20 °C in glycerol (40% v/v) or freeze-dried in 0.067 M glutamic acid and maintained at 4 °C. After one year, the vials were opened and percentages of surviving cells in the vials were determined by spreading onto the MLO plates. Then, cultures were diluted to reach 10^5 cfu mL^{-1}, inoculated in Monastrell red wine (pH 3.5) containing 11.1 % (v/v) ethanol and in g L^{-1}: malic acid, 3.5; glucose, 0.06 and fructose, 0.15, and incubated 7 weeks at 20 °C. The remaining malic acid quantities were quantified weekly as described by Maicas *et al*. (1999a). Wines were also inoculated with a culture grown in wine, to provide a control.

Results

Influence of the culture medium on the production of *Oenococcus oeni* biomass

The composition of the culture medium influences the adaptation of the malolactic bacteria to the stress conditions in wine (Hayman & Monk, 1982). The most favourable growth conditions for nutritionally fastidious microorganisms such as *O. oeni* occurs when a strain is inoculated in a synthetic medium (MLO) including nutritional compounds, e.g. yeast extract, tween-80 or tomato juice (Beelman *et al*., 1977; Hayman & Monk, 1982) (Fig. 1). The presence of sufficient nutrients and an appropriate pH permits developments of high levels of biomass, approx 10^9 cfu mL^{-1} in 2–3 days (Maicas *et al*., 1999a). A recently described complex medium to preculture *O. oeni* (MP) has been shown to give better adaptation of bacteria to wine conditions (Maicas *et al*., 1999b). In the present work we produced 10^{10} cfu mL^{-1} in 2–3 days, which is a ten-fold increase in the biomass production compared with MLO, the optimum medium to grow *O. oeni* strains up to date (Fig. 1). However, red wine was not suitable for direct inoculation with *O. oeni* strains since many cells died. Cells should be grown in MLO or MP before inoculation in wine in order to avoid loss

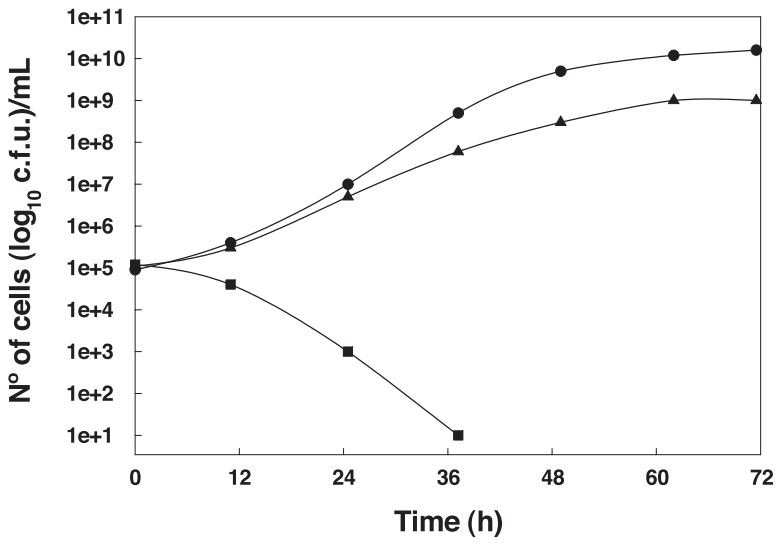

Figure 1 Effect of different culture media on *Oenococcus oeni* MA4 survival. Cells were inoculated in MLO (▲), MP (●) or wine (■). Cells were incubated at 28 °C (MLO and MP) or 20 °C (wine). The data presented are mean values from three separated experiments.

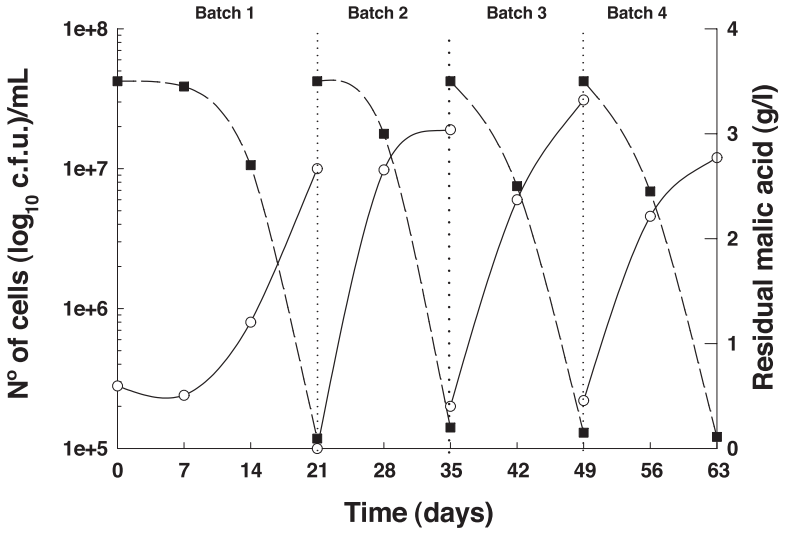

Figure 2 Time course of four successive malolactic fermentations in wine with *Oenococcus oeni* MA4. ○, cfu ml^{-1}; ■, residual malic acid. Cells used in batch 1 were precultured in MP. Cells grown in a previous batch were diluted to give a concentration of about 1×10^5 cfu mL^{-1} in fresh wine (batches 2–4). The data presented are mean values from three separated experiments.

of viability of the organisms. Successive reinoculations in wine were done without any loss of viability although the nutritional shortage in wine permits development of quantities of biomass of only about 10^7 cfu mL^{-1} and requires at least 2–3 weeks for each batch (Fig. 2).

Storage of cultures: survival and maintenance of malolactic activity

The rates of survival of *O. oeni* cells in MLO or in wine were higher than those recorded in MP, after the storage for one year at –20 °C (Table 1). Nevertheless, when the living cells were inoculated in fresh wine (about 10^5 cfu mL^{-1}) only cells previously grown and stored in MP or wine were able to perform the MLF in 3–4 weeks. These results were similar to those obtained by direct inoculation to wine of cells growing in wine (Table 2), providing an acceptable method for storage of cultures. Freeze-dried suspensions of *O. oeni* cells were also stored for one year to check the survival rate and the retention of their ability to degrade the malic acid. The cells showed a similar performance to that was previously described for storage at 20 °C (Table 2). When cells were grown in MP, less than 10% of the initial cells survived (with the only exception of the *O. oeni* strain MA4). Stability was improved for some strains grown in MLO or wine (Table 1). However, mirroring the results for storage at −20 °C, only cells that have gained adaptation by inoculation in MP or wine retained the ability to induce MLF when inoculated in wine (Table 2). *O. oeni* strain VV5 was able to degrade L-malic acid, but it took almost two months. As part of this project to optimize the conditions of storage for the retention of malo-

Table 1 Survival rate (%) for *Oenococcus oeni* strains, grown in different culture media, after one year storage at 4 °C (freezed-dried cultures) or at −20 °C. The data presented are mean values from three separated experiments

	Storage conditions					
	−20 °C			Freeze-drying		
***O. oeni* strain**	**MLO**	**MP**	**Wine**	**MLO**	**MP**	**Wine**
MA4	78.9	20.0	87.5	57.9	62.5	29.1
TE3	95.2	26.9	93.3	27.4	5.2	20.8
VV5	92.3	10.5	73.3	23.3	5.8	26.0
BM3	72.0	13.3	88.9	7.7	2.1	8.5
TV3	82.0	10.4	80.0	3.3	1.5	8.3

Table 2 Time required to detect MLF in wine (weeks) with different *Oenococcus oeni* strains after storage (a direct inoculation control was also included). The data presented are mean values from three separate experiments

	Storage conditions								
	Direct inoculation			−20 °C			Freeze-drying		
O. oeni strain	MLO	MP	Wine	MLO	MP	Wine	MLO	MP	Wine
MA4	a	3	3	a	3	3	a	3	3
TE3	a	3	3	a	4	3	a	3	3
VV5	5	3	3	a	3	3	7	3	3
BM3	a	3	3	a	3	4	a	3	3
TV3	a	4	3	a	4	3	a	3	3

a After 7 weeks, malic acid degradation in wine was not detected.

lactic ability, we compared the survival rates for the same *O. oeni* strain grown in a culture medium and stored by freezing or freeze-drying. Results showed higher retention of malolactic activity for storage of cells at −20 °C for one year than for freeze-drying, in agreement with results reported by Henick-Kling (1991). However, malolactic activity and time required to detect MLF were very similar irrespective of cell storage conditions.

Discussion

MLO is the best medium for easy and rapid growth of *Oenococcus oeni* cultures under laboratory controlled conditions for isolation and identification. However, cells lose their natural resistance to the adverse environmental conditions in wine and fail as starter to induce MLF (Henick-Kling, 1995). The growth of the starter bacteria in an appropriate preculture medium such as MP (Maicas *et al*., 1999b) reduces the preparation time of starter cultures, prepares the cells to survive after storage and allows growth in wine. Moreover, cells efficiently perform MLF in wine. Storage at −20 °C is an effective procedure too, but it takes 2–3 months to establish growth in wine. However, this technique also requires growth of cells in adaptation media prior to inoculation to ensure an adequate survival rate. Despite loss of viable cells when stored at −20 °C, MP is a better choice compared with rates recorded in wine, since adapted surviving cells are able to perform MLF immediately after being inoculated in wine. This also involves a reduction of production costs and of contamination problems often linked to starter cultures. The results obtained in freeze-drying experiments were similar to those described for storage at 20 °C. The use of freeze-dried cultures is easy and inexpensive in industry.

Acknowledgments

This work has been partially supported by grants from the Comisión Interministerial de Ciencia y Tecnología (ALI93–0246) and by a grant from the M.E.C. (Spanish Government) to S.M. The authors are grateful to À. Natividad and P. González-Cabo for technical assistance.

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.

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.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D. (1995). Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. *International Journal of Systematic Bacteriology*, **45**, 395–397.

Edwards, C.G., Jensen, K.A., Spayd, S.E. & Seymour, B.J. (1991). Isolation and characterization of native strains of *Leuconostoc oenos* from Washington state wines. *American Journal of Enology and Viticulture*, **42**, 219–226.

Gao, C. & Fleet, G.H. (1994). The degradation of malic by high density cell suspensions of *Leuconostoc oenos*. *Journal of Applied Bacteriology*, **76**, 632–637.

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. *Proceedings of the Focus Eastern US Chardonnay Symposium* (edited by T. Henick-Kling). Pp. 112–117. Front Royal: University of Davis. Virginia: Virginia Polytechnic Institute and State University.

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.

Maicas, S., González-Cabo, P., Ferrer, S. & Pardo, I. (1999a). 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. (1999b). Continuous malolactic fermentation in red wine using free *Oenococcus oeni*. *World Journal of Microbiology and Biotechnology*, in press.

Maicas, S., Gil, J.V., Pardo, I. & Ferrer, S. (1999c). Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Research International*, in press.

Naouri, P., Chagnaud, P., Aranaud, A., Galzy, P. & Mathieu, J. (1989). Optimization of the conditions for preparing bacterial cultures for malolactic fermentation. *Journal of Biotechnology*, **10**, 135–150.

Nielsen, J.C., Prahl, C. & Lonvaud-Funel, A. (1996). Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *American Journal of Enology and Viticulture*, **47**, 42–48.

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.

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.