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A mathematical model of the link between growth and L-malic acid consumption for five strains of *Oenococcus oeni*

N. Fahimi · C. Brandam · P. Taillandier

Abstract In winemaking, after the alcoholic fermentation of red wines and some white wines, L-malic acid must be converted into L-lactic acid to reduce the acidity. This malolactic fermentation (MLF) is usually carried out by the lactic acid bacteria *Oenococcus oeni*. Depending on the level of process control, selected *O. oeni* is inoculated or the natural microbiota of the cellar is used. This study considers the link between growth and MLF for five strains of *O. oeni* species. The kinetics of growth and L-malic acid consumption were followed in modified MRS medium (20 °C, pH 3.5, and 10 % ethanol) in anaerobic conditions. A large variability was found among the strains for both their growth and their consumption of L-malic acid. There was no direct link between biomass productivities and consumption of L-malic acid among strains but there was a link of proportionality between the specific growth of a strain and its specific consumption of L-malic acid. Experiments with and without malic acid clearly demonstrated that malic acid consumption improved the growth of strains. This link was quantified by a mathematical model comparing the intrinsic malic acid consumption capacity of the strains.

Keywords *Oenococcus oeni* · Growth rate · Malo-lactic fermentation · Model · Strain variability

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Introduction

Oenococcus oeni is the species of lactic acid bacteria (LAB) most frequently associated with malo-lactic fermentation (MLF) in winemaking (González-Arenzana et al. 2012; Kunkee 1967, 1974; Lerm et al. 2010; Rankine 1977; Ruiz et al. 2009; Saguir et al. 2009). Abundant knowledge about *Saccharomyces cerevisiae* has been accumulated over a long period but knowledge of *O. oeni* is much less developed. However, MLF is a critical step in winemaking as this fermentation, consisting of the enzymatic decarboxylation of L-malic acid into L-lactic acid, is required during the making of most red wines and some white and sparkling wines. In addition to its deacidification effect, MLF increases microbiological stability and enhances wine flavours and aromas (Alberto et al. 2001; Armada et al. 2010; Reguant et al. 2000; Ugliano et al. 2003). Therefore, achieving successful MLF is a key factor for the quality and the cost of wine.

MLF performed after alcoholic fermentation (AF) by *S. cerevisiae* can be difficult to manage because the physicochemical conditions of wine, such as high concentration of ethanol (Ingram and Butke 1984; King and Beelman 1986; Rosa and Sa-Correia 1992), low pH (Henick-Kling 1989), low temperature Asmundson and Kelly 1990; Maicas et al. 2000), nutrient depletion (Remize et al. 2006), presence of fatty acids (Guerrini et al. 2002a, b); Guilloux-Benatier et al. 1998) and sulfur dioxide (SO_2) addition (Romano and Suzzi 1993)may be inadequate for *O. oeni* activity.

Inadequate biological conditions may also cause the failure of MLF by release of some common inhibitory metabolites from yeasts, such as SO_2 (Carreté et al. 2002; Henick-Kling and Park 1994; Osborne et al. 2006), specific inhibitory metabolites produced by some strains of *S.*

cerevisiae (Nehme et al. 2010; Taillandier et al. 2002) and probably inhibitory metabolites produced by indigenous strains of LAB (Knoll et al. 2008).

Several studies have demonstrated that choosing a strain well adapted to wine conditions is of primary importance in controlling MLF (Nehme et al. 2008). It is only in the last decade that the literature has presented data about the genome of *O. oeni*, describing its wide genotype diversity (Borneman et al. 2010; Guerrini et al. 2002a, b; Lechiancole et al. 2006; Olguin 2010; Rivas et al. 2004). For industrial uses, it is indispensable to investigate the development and activity of the *O. oeni* species to compare its phenotypic characteristics. In this study, the physiological variability of five selected strains of *O. oeni* isolated from different media (red wines, sparkling wines and ciders) was evaluated. Although the majority of studies reported in the literature have used an air atmosphere, in the present work, the experiments were carried out in a nitrogen atmosphere (anaerobic conditions) to be close to the conditions of winemaking where, after dissolved oxygen consumption by the yeast during alcoholic fermentation, there is no dissolved oxygen in the must since there is no aeration and no agitation in the process.

Growth and L-malic acid consumption kinetics of the strains were followed and compared. In order to better understand why strains react differently under wine-making conditions, the link between specific growth and specific L-malic acid consumption was evaluated and quantified using a kinetic model.

Materials and methods

Strains and storage conditions

Five strains of *O. oeni* named A, B, C, D, and E were tested in this work. These strains belong to the DIVOENI ANR collection at the Faculty of Oenology, Bordeaux, France (no. ANR-07 BDIV 011-01) and came from different sources: reference strain A is commonly referred ATCC BAA 1163 in the literature, B and C were indigenous strains isolated from Champagne and Normandy cider, D and E were commercial oenological strains. The strains were kept frozen at −20 °C in MRS broth (Biokar diagnostic, Beauvais, France) containing 20 % glycerol (v/v).

Culture conditions

Reactivation

One hundred µl of the frozen strains A, B, C, D, and E were reactivated for 65 h in 10 ml of MRS broth supplemented with L-malic acid (4 g L^{-1}) at 28 °C, pH 5.2, without agitation in Erlenmeyer flasks.

Preculture

The modified MRS, noted MRS_m, (MRS broth + 4 g $L^{-1}$of L-malic acid + 2 g $L^{-1}$of D-fructose) was used with the pH adjusted to 4.8 by means of an 85 % orthophosphoric acid solution. After autoclaving, 5 % (v/v) of ethanol was added and then the medium was inoculated at 1 % (v/v.) using reactivated cultures. The precultures were incubated at 28 °C in Erlenmeyer flasks without agitation.

Malolactic fermentation (MLF) conditions

Two types of fermentation were performed:

- Fermentation in a 4-L bioreactor: For these cultures, the pH of MRS_m was adjusted to 3.5 and 10 % (v/v) of ethanol was added. The incubation was carried out at 20 °C. The volume of the preculture added for inoculation was adjusted so as to start the MLF with 2×10^6 CFU/mL. Strains were grown under anaerobic conditions: in 0.45 bar pressure of nitrogen atmosphere in the headspace of a 4-L bioreactor and with 100 rpm stirring. Three culture replicates were used for each strain.
- Fermentation in 250-ml Erlenmeyer flasks: in these cultures, conditions were the same as above except for a slight difference in the atmospheric conditions: a small quantity of nitrogen was injected continuously into the medium instead of having a head-space pressure of 0.45 bar of nitrogen gas, and the bacterial culture was in contact with atmospheric oxygen once a day when the flask is opened for sampling.

Analytical methods

Growth

Bacterial growth was followed by measuring the optical density (OD) in a spectrophotometer (Hitachi U-2000) at 620 nm using a quartz cuvette with a 1-cm light path. Biomass was also determined by colony counts on MRS agar plates. The MRS agar was completed with 4 g L^{-1} L-malic acid and 5 g L^{-1} agar. Its pH was adjusted to 5.7 using a 10 M NaOH solution. A specific correlation between OD and number of colonies was determined for each bacterium and used to inoculate fermentations at 2×10^6 CFU mL^{-1}.

L-malic acid concentration

L-malic acid concentration was determined using an enzymatic assay (Roche Boehringer Mannheim/R-Biopharm, Darmstadt, Germany, kit no. 10,139,068,035) and the results were expressed in g L^{-1}.

Determination of kinetic parameters

Growth and L-malic acid consumption kinetics were smoothed by a cubic spline function using a Microsoft Excel™ macro. The smoothed kinetics were then used to calculate the kinetic parameters of the fermentations:

- Specific growth rate μ:

$$\mu = \frac{1}{X} \times \frac{dX}{dt} \qquad (h^{-1})$$

- Specific L-malic consumption rate ν:

$$v = \frac{1}{X} \times \frac{d[mal]}{dt} \qquad [(\text{g L}^{-1}\ \text{h}^{-1}\ (\text{OD}_{620}\ \text{unit})^{-1}]$$

with [*mal*] the L-malic acid concentration in g L^{-1}, X the biomass concentration in OD units and t the time.

Several parameters were defined so that strains and fermentations could be compared:

- The lag phase: several definitions are employed in the literature (Swinnen et al. 2004) but the most widespread is the time found by extrapolating the tangent of the exponential part of the growth curve back to the inoculum level. In this study, fermentations were stopped when malic acid had been totally consumed, which was often before the exponential part of the growth curve was completed. The lag phase was identified with the specific growth rate curve here, by extrapolating the tangent at the beginning of growth rate acceleration and the horizontal tangent at the initial point.
- The MLF duration was the total time taken for the L-malic acid concentration to fall to zero.
- The growth phase duration was the difference between the MLF duration and the lag phase duration.
- ΔOD_{620} was determined as the difference between the maximum OD and the initial OD.
- Productivity in the growth phase (Pg) was defined as ΔOD_{620} divided by the growth phase duration.
- Overall productivity (P) was defined as ΔOD_{620} divided by the MLF duration. These two productivities, P and Pg, were expressed in units of $OD_{620}\ h^{-1}$.

Results and discussion

Anaerobic growth

Figure 1a shows the growth profiles of all strains in anaerobiosis in the bioreactor. The values presented are the average of three independent replicas for each strain, with standard deviations.

Without reaching the stationary phase, the bacteria showed different behaviour but a common general profile was observed: a lag phase followed by an increase of OD_{620}. The duration of the lag phases (determined from specific growth rate curves in Fig. 2) varied between 76 h for strain D and 156 h for strain E. Strains A, B, and C showed similar growth kinetics and the best development, with the highest variations in OD (Table 1). However, strains D and E presented the highest maximum specific growth rate. The initial OD of strains D and E decreased after inoculation, certainly because they were more sensitive than the other strains to the culture medium variations (higher ethanol content and lower pH compared to the inoculum preparation). Concerning the overall productivity (P), the best strains were A, C and B, followed by E and D. The same classification was obtained when we considered productivity during the growth phase (Pg), i.e. productivity after the lag phase.

L-malic acid degradation under anaerobiosis conditions

Figure 1b shows the L-malic acid consumption profiles of each strain A, B, C, D, and E under anaerobic conditions. Results are again average values of three replicas with standard deviation.

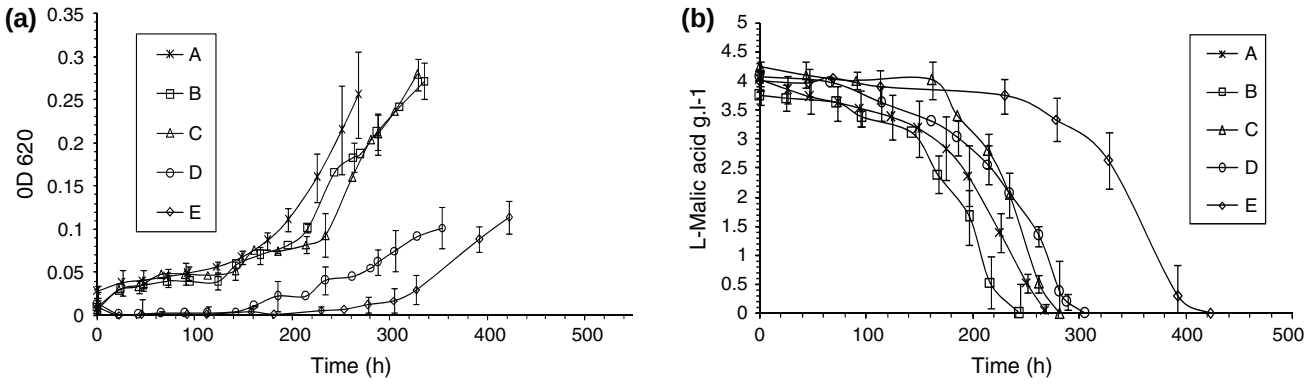

Fig. 1 Growth and L-malic acid consumption of A, B, C, D, and E *O. oeni* strains in fermentation medium MRS_m in anaerobic conditions for the cultures carried out in the bioreactor: **a** growth and **b** L-malic acid consumption

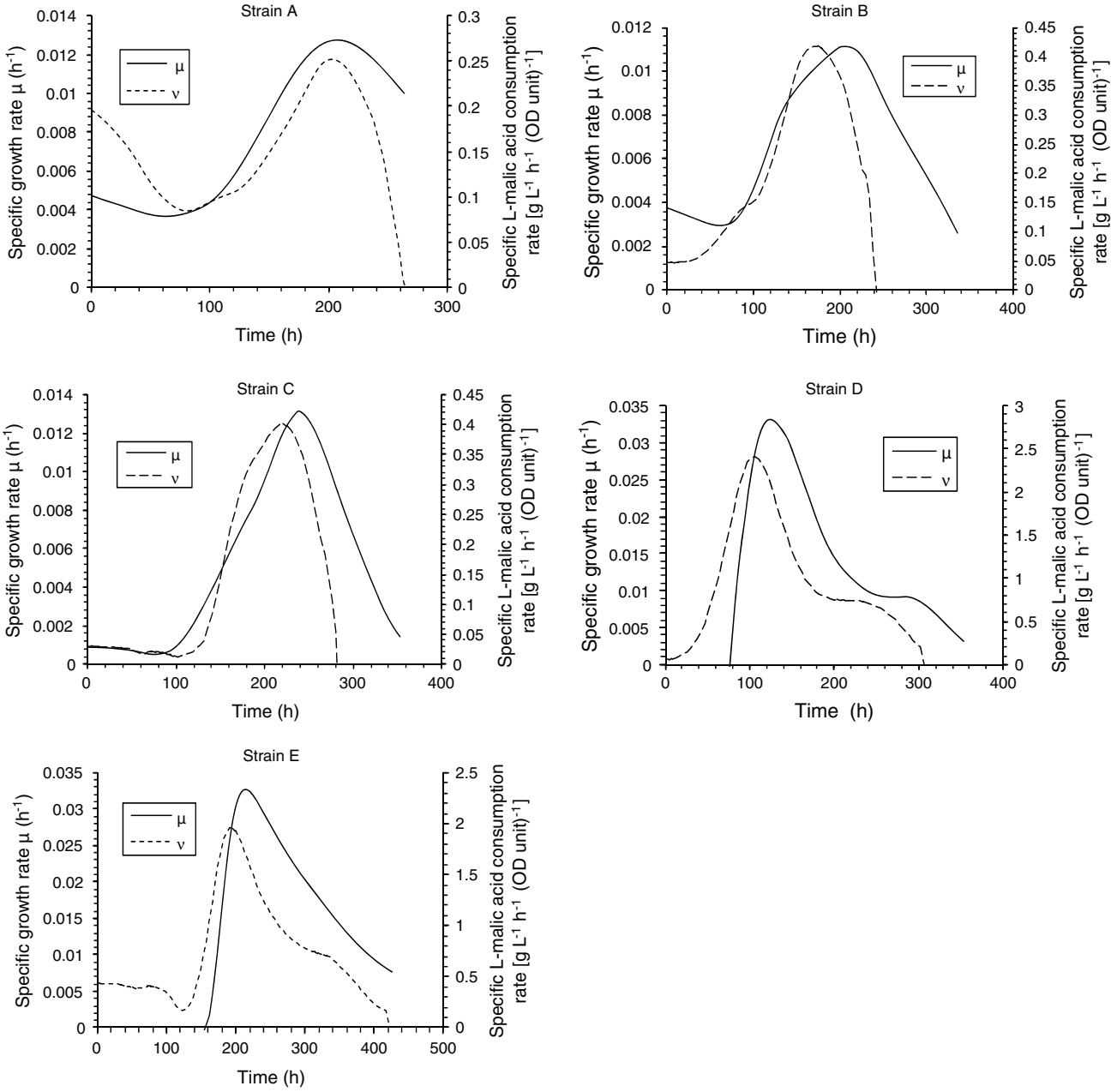

Fig. 2 Specific growth rate and specific L-malic acid consumption rate of *O. oeni* A, B, C, D, and E strains for the cultures carried out in the bioreactor in anaerobic conditions

The L-malic acid consumption was very slow at the beginning and then accelerated. All strains consumed all the L-malic acid present in the medium, i.e. 4 g L^{-1}. Strain B was the fastest consumer, finishing MLF after 242 h, and the slowest was strain E, which needed 422 h to complete the same process. Strains A, B, C and D had similar overall rates of L-malic acid consumption, between 1.33 and 1.55×10^{-2} g L^{-1} h^{-1}, whereas strain E consumed only 0.95×10^{-2} g L^{-1} h^{-1} (Table 2). Strain A was the fastest growing and it finished MLF after strain B. Despite the fact that strains B and C had very similar growth profiles (Fig. 1a), strain C finished MLF 39 h later than strain B. Strains D and E required less biomass than the others to perform MLF (Fig. 1a, b). When growth and consumption of L-malic acid, and both biomass productivities were compared with the overall rates of L-malic acid consumption for the five strains, no proportionality was found. It was thus concluded that, from one strain to another, there was no direct link between the growth, the biomass reached, and the MLF duration, as has indeed been shown

Table 1 Growth parameters of *O. oeni* A, B, C, D, and E strains in MRS_m in bioreactor under anaerobic conditions

Strain	A	B	C	D	E
Duration of lag phase (h)	110	90	120	76	156
Duration of the growth phase during MLF (h)	157	152	161	229	266
ΔOD_{620} during MLF	0.23	0.156	0.192	0.061	0.104
Productivity in growth phase (Pg) (OD_{620} unit h^{-1}) $\times 10^4$	14.65	10.26	11.92	2.66	3.90
Overall productivity (P) (OD_{620} unit h^{-1}) $\times 10^4$	8.61	6.44	6.83	2.00	2.48
μ_{max} Maximum specific growth rate reached (h^{-1}) $\times 10^2$	1.2	1.1	1.3	3.3	3.3

in previous works. For example among the 16 strains of *O. oeni* studied, many were found to perform malolactic fermentation without growing in the wine (Arnink and Henick-Kling 2005). It has also been demonstrated that although the growth, and the malolactic activity, of three isolates of *O. oeni* (UNQOe 31, UNQOe 71, UNQOe 73) from Argentinean wine were affected by a high ethanol concentration but not at the same extend, these two activities were linked (Bravo-Ferrada et al. 2011).

So, regarding the growth and malolactic fermentation efficiency of the five strains cultivated under nitrogen atmosphere, a large diversity was observed. A physiological explanation proposed by several authors (Guzzo et al. 2000, 2002; Kroll and Booth 1983) is that this diversity can be linked to their greater or lesser ability to maintain an intracellular pH compatible with the functioning of metabolic pathways in presence of ethanol and in acidic conditions. The malate decarboxylation causes alkalinization of the cytoplasm, thus increasing the pH difference between inside and outside the cell (Poolman et al. 1991; Salema et al. 1994). For the access of L-malic acid into the cytoplasm, cellular homeostasis of the bacterium is required (Guzzo et al. 2002; Kroll and Booth 1983). Hence, strains A, B, C, D, and E probably have different tolerance to a variation of the pH gradient induced by transfer of L-malate into the cytoplasm.

Specific growth rates and specific L-malic acid consumption rates

Nevertheless, in order to test the link between growth and malolactic fermentation for an individual strain, the specific activities of the two phenomena were calculated and compared. Figure 2 shows that, for all strains, specific L-malic acid consumption rate and specific growth rate (ν and μ) had the same profile versus time.

Firstly, during the lag phase, the specific consumption rate of L-malic acid and the specific growth rate were low. The strains needed to adapt their metabolisms to the difference between inoculum and culture conditions.

For strains D and E, death of some of the cells was expressed by a negative specific growth rate at the beginning of the culture. Secondly, net increases in both specific rates, ν and μ, were observed and the maximum rates μ_{max} (Table 1) and ν_{max} (Table 2) were obtained at the beginning of the growth acceleration if we observe the times in which we obtain those values on Fig. 1. Finally, the specific L-malic acid consumption rate, ν, slowed to zero because the L-malic acid was exhausted. The specific growth rate, μ, was also observed to decrease for the five strains but its value was greater than zero at the end of the experimentation.

Values of the specific activities ν and μ differed considerably from one strain to another as can be seen from the μ_{max} and ν_{max} in Tables 1 and 2, and from Fig. 2.

Figure 2 also shows that, for four of the strains, the specific growth rate, μ, started to slow down just after the specific L-malic acid consumption rate ν started to decrease, with a shift in time (δt) between the decline of ν and the decline of μ ($t\mu_{max} - t\nu_{max}$) for strains B, C, D and E (Table 2). This shift was absent in the case of strain A, and its duration was similar for strains C, D, and E, and was about twice as long in the case of strain B. If we analyse the physiological assumptions in the literature, it has been shown that the malolactic reaction provides energy by the translocation of molecules of malate (in its monoanionic form) and lactate. The shift observed between the decline of the specific activities ν and μ could be related to the accumulation of L-malic acid and/or of energy produced by consumption of the L-malic acid system. Between strains, differences in the time shift could be explained by the amount of L-malic acid and/or energy each strain is able to store.

Effect of L-malic acid on growth

To evaluate the effect of consumption of L-malic acid on the growth of *O. oeni*, cultures were performed in the absence of L-malic acid and compared to cultures conducted in the same fermentation medium but with 2 g L^{-1} and 4 g L^{-1} of L-malic acid. These specific cultures were made only for strains D and E, in Erlenmeyer flasks under the atmosphere conditions described in the Materials and methods section.

Figure 3 shows the specific growth rates and the specific L-malic acid consumption rates of the strains D and E in the presence of 4 g L^{-1} of L-malic acid and in the absence of this acid in the culture medium. For both strains, we

Table 2 L-malic acid consumption parameters and kinetic model parameters of *O. oeni* strains A, B, C, D, and E in MRS_m in bioreactor under anaerobic conditions

Strain	A	B	C	D	E
MLF duration (h)	267	242	281	305	422
Overall rate of L-malic acid consumption (g L^{-1} h^{-1}) × 10^2	1.51	1.55	1.48	1.33	0.95
ν_{max} Maximum specific consumption rate of L-malic acid (g/L/h/OD_{620} unit)	0.24	0.42	0.4	2.4	1.95
δt* (h)	0	38	17	17	21
Ki	35.8	54.5	45.7	70.8	62.2
Kmal (g L^{-1})	1.1	1.23	1.14	0.47	0.8

* $\delta t = t\mu_{max} - t\nu_{max}$

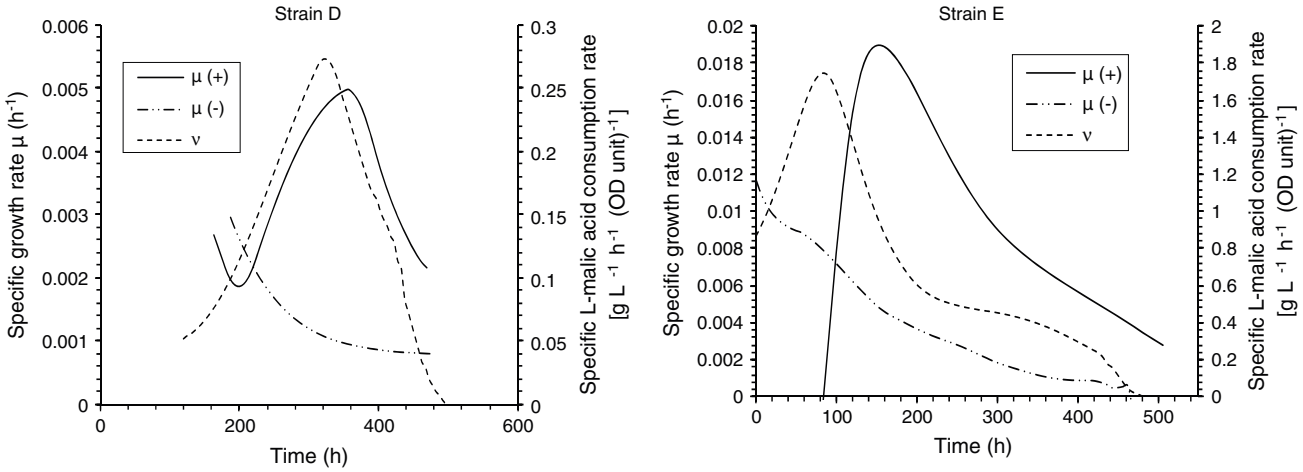

Fig. 3 Specific growth rates (μ) of strains D and E for cultures carried out in Erlenmeyer flasks in presence (+) and in absence (−) of L-malic acid in the fermentation medium MRS_m. Evolution of their specific L-malic acid consumption rate

found the logical correlation between the two specific rates (ν and μ) that had already been observed in bioreactor experiments. However, the values of the specific activities reached in the bioreactor were higher than those reached in Erlenmeyer flasks, especially for strain D (Figs. 2, 3). This difference was probably due to the different atmosphere conditions.

For cultures in Erlenmeyer flasks without L-malic acid, the growth rates (μ) obtained remained very low compared to those obtained with the L-malic acid, and they decreased over time. In addition, biomasses reached by strain D in the presence of 2 g L^{-1} (data not shown) and 4 g L^{-1} of L-malic acid were respectively 1.5 and 1.9 times higher than the biomass obtained without L-malic acid in the culture medium. Also, for strain E, the biomass was 1.3 and 2.3 times higher with 2 (data not shown) and 4 g L^{-1} of L-malic acid than that reached without L-malic acid. In the work of Saguir and Manca de Nadra (1997), where the cultures were carried out in MRS medium supplemented with 15 % of tomato juice, the growth of the *Leuconostoc oenos* strain isolated from Argentinean wine was greater with 2.5 g L^{-1} of malic acid in the medium than without the acid. However, the gain in biomass in the first case was only multiplied by 1.2. In this case, the growing conditions were not stressful (pH 4.8 and 30 °C) compared to the conditions of our cultures (pH 3.5 and 20 °C). Moreover, the addition of tomato juice in the MRS medium may have brought a certain amount of citric and malic acids, which consequently promoted growth in the medium not supplemented with pure L-malic acid. In this case, the difference between the biomasses reached for cultures with and without pure L-malic acid would be low. Hence, the gain in biomass in this work (Saguir and Manca de Nadra 1997) cannot be compared to the gains we obtained. Other works have shown that L-malate enhances the growth yield of *O. oeni* ATCC BAA-1163 when its growth is compared in presence and in absence of L-malate but only at low pH (3.2) and in the absence of ethanol; the μ_{max} reached in the exponential phases was slightly higher in the presence of L-malate (0.042 h^{-1}) than without malate (0.038 h^{-1}) (Augagneur et al. 2007).

In conclusion, a clear effect of L-malic acid on the specific growth rates and on biomasses reached was observed in the cultures of strains D and E. These results

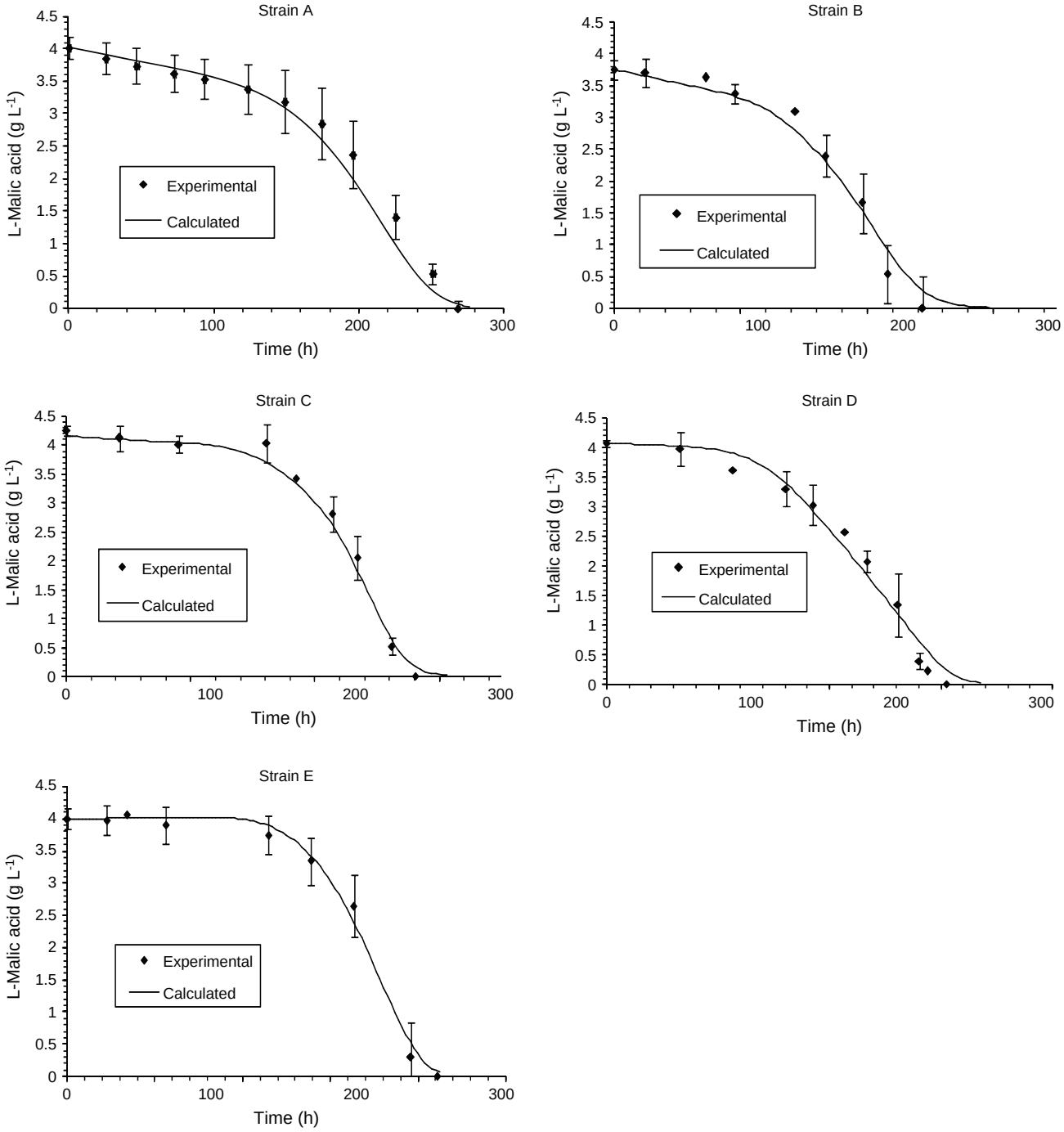

Fig. 4 Experimental and calculated L- malic acid concentrations during the cultures of *O. oeni* strains A, B, C, D, and E in anaerobic conditions in the bioreactor

regarding the beneficial effect on the growth of *O. oeni* need to be clarified for all of the five strains studied.

Modelling

The specific L-malic acid consumption rate (ν) seemed to be proportional to the specific growth rate μ (Figs. 2, 3). Moreover, at the end of the consumption of L-malic acid, specific consumption ν decreased, surely caused by the L-malic acid limitation. To quantify the link observed, a mathematical model was developed. The following equation is proposed:

$$v = ki \times \mu \times \frac{[mal]}{[kmal] + [mal]},$$

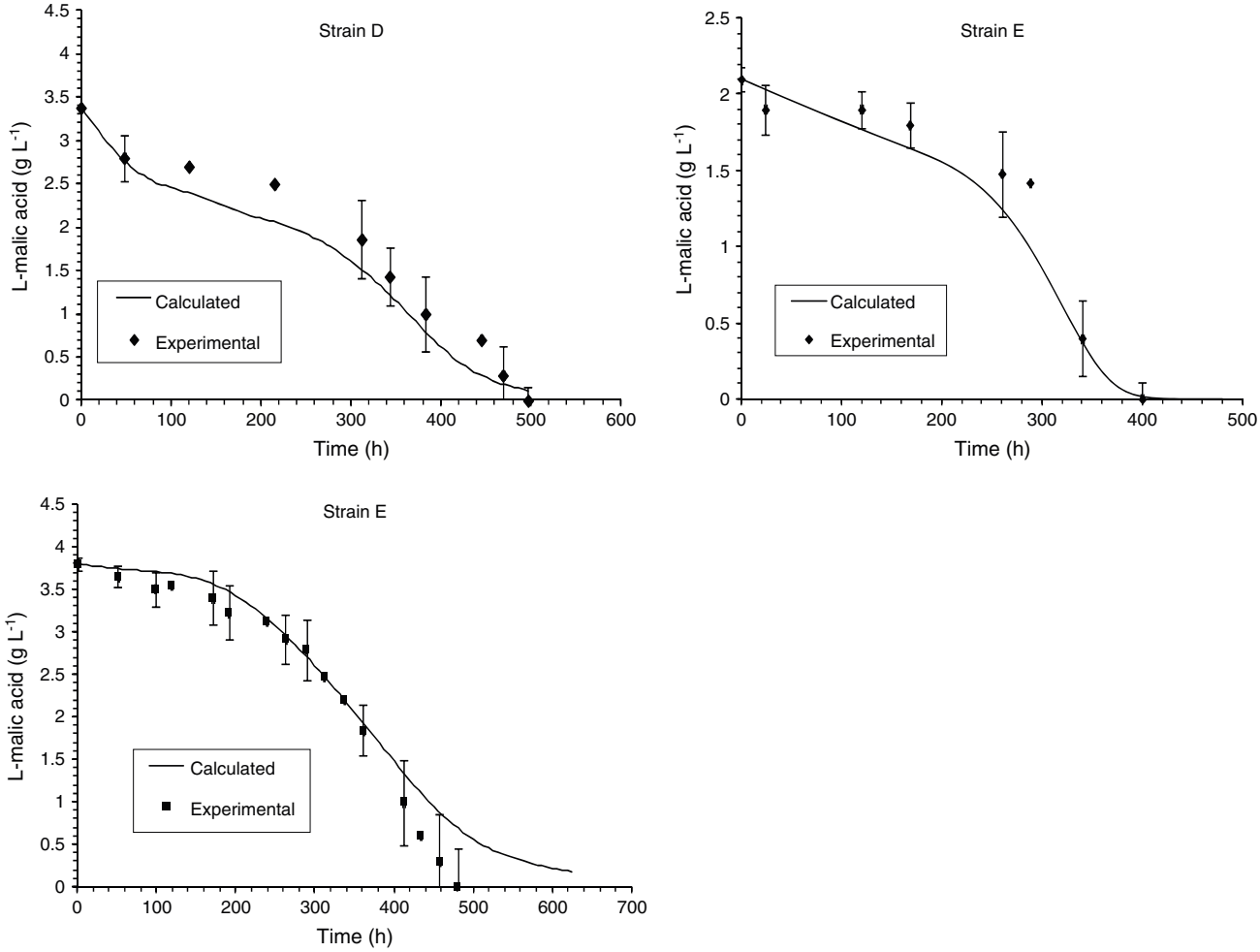

Fig. 5 Experimental and calculated L-malic acid concentration during the cultures of *O. oeni* strains D (malic acid concentration of 4 g L^{-1}) and E (malic acid concentration of 2 and 4 g L^{-1}) in Erlenmeyer flasks

- *v*: specific L-malic acid consumption rate
- μ: specific growth rate
- [*mal*]: L-malic acid concentration

ki is a parameter representing the coefficient of proportionality between ν and μ; it informs us about the intrinsic capacity of the cells of a strain to consume L-malic acid. *kmal* is a substrate limitation parameter (expressed in g L^{-1}), a low value of *kmal* means that the bacteria are able to grow with a low concentration of L-malic acid in the medium, conversely a high value of *kmal* means the bacteria require a high minimum threshold of L-malic acid concentration to ensure their growth via the malate metabolism. These two parameters (*ki* and *kmal*) were determined by minimizing the sum of the squared deviations between experimental and calculated values of specific L-malic acid consumption rate. Values found for each strain, A, B, C, D, and E, of *O. oeni* are shown in Table 2.

Knowing the initial concentration of L-malic acid in the culture medium and after determination of *ki* and *kmal*, the modelled specific L-malic acid consumption rate (ν_m) was used to calculate the concentration of L-malic acid corresponding to the experimental biomass (x_{exp}) measured during bacterial growth.

$$v_m = \frac{1}{x_{exp}} \times \frac{\Delta[mal]}{\Delta t}$$

It was thus possible to deduce the L-malic acid concentration for different time intervals during the MLF and compare it to experimental values (Fig. 4). The profiles of calculated L-malic acid concentration for each strain were similar to the experimental profiles obtained previously. The shift observed experimentally between specific L-malic acid consumption rate and specific growth rate was not taken into account in the proposed model, but it did not affect the result

for calculated L-malic acid concentration. Thus, the proposed model seems to be suitable for predicting the concentration of L-malic acid consumed by *O. oeni* bacteria from the values of biomass concentration versus time.

The parameter ki represents the intrinsic capacity of a strain to consume L-malic acid, independently of its growth. Strain A has the lowest value of the constant *ki*, 35.8, followed by strain C. The *ki* of strain B is 1.5 times higher than that of strain A. It can be observed that strains E and D have the highest *ki*, respectively 1.98 and 1.74 times the *ki* of strain A. This explains the good consumption of L-malic acid by strains D and E despite their slow growth. Strain D has the lowest *kmal*, followed by strain E and then strains A, B and C. Strains D and E have the highest capacities to use L-malic acid (high *ki*) and they can grow in the medium with a low concentration of L-malic acid (low *kmal*).

In order to validate the proposed equation independently, the L-malic acid consumption was calculated in experiments in Erlenmeyer flasks for each of the strains D and E using the model defined previously in the bioreactor. Although the specific activities reached in the bioreactor were different from those obtained in Erlenmeyer flasks due to oxygen supply during sampling, the good fit between the modelled data and the experimental results (Fig. 5) confirms that the model equation seems to be suitable for calculating the L-malic acid used by the strains. These results are very encouraging. Nevertheless, this model cannot be used to predict L-malic acid consumption in cases where there is no growth. In this case, the model predicts no MLF, whereas it has been shown that many strains perform MLF without growing in the wine (*35*).

In conclusion, this model can quantify the link between the 2 activities, growth and L-malic acid consumption. It is useful since it brings out parameters characterizing the strains (*ki* and *kmal*) and comparing their phenotype activities of MLF and growth. For a strain that has been characterized, it can be used to predict the consumption of L-malic acid knowing its initial concentration. It would also be very useful in the industry to only follow the growth of the bacteria and deduct its MLF state thanks to the proposed model.

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