



**HAL**  
open science

# Rapid assessment of [*Oenococcus oeni*] activity by measuring intracellular pH and membrane potential by flow cytometry, and its application to the more effective control of malolactic fermentation

Marielle Bouix, Sarrah Ghorbal

## ► To cite this version:

Marielle Bouix, Sarrah Ghorbal. Rapid assessment of [*Oenococcus oeni*] activity by measuring intracellular pH and membrane potential by flow cytometry, and its application to the more effective control of malolactic fermentation. *International Journal of Food Microbiology*, 2015, 193, pp.139-146. 10.1016/j.ijfoodmicro.2014.10.019 . hal-01195510

**HAL Id: hal-01195510**

**<https://hal.science/hal-01195510>**

Submitted on 28 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



# Rapid assessment of *Oenococcus oeni* activity by measuring intracellular pH and membrane potential by flow cytometry, and its application to the more effective control of malolactic fermentation <sup>☆</sup>



M. Bouix <sup>a,\*</sup>, S. Ghorbal <sup>a</sup>

<sup>a</sup> AgroParisTech, INRA, UMR 782 Génie et Microbiologie des Procédés Alimentaires, Grignon, France

## ARTICLE INFO

### Article history:

Received 31 August 2014

Received in revised form 3 October 2014

Accepted 17 October 2014

Available online 22 October 2014

### Keywords:

*Oenococcus oeni*

Physiology

Activity

Flow cytometry

Malolactic fermentation

## ABSTRACT

The aim of this study is to highlight the changes in the physiological cellular state of *Oenococcus oeni* during malolactic fermentation (MLF), and to use its cellular parameters to improve existing knowledge of *O. oeni* behaviour and to more effectively control the performance of the bacteria during MLF in wine.

To do this, measurements of intracellular pH, transmembrane potential and vitality were performed using flow cytometry with different fluorescent probes: CFDA-SE and CDCF, DiBAC and CFDA, respectively. The kinetics of the cellular changes in these parameters were determined during MLF in FT80 synthetic medium and in white wine, as were the kinetics of malic acid consumption.

pH<sub>in</sub> measurement throughout the entire growth shows that the pH was equal to the pH of the culture medium during the early stage, increased to pH 6 in the exponential phase, and then decreased to equilibrate with the pH of the medium in the late stationary phase. Membrane potential increased in early MLF and then decreased. The decrease in pH<sub>in</sub> and membrane potential occurred when all of the malic acid was consumed. Finally, we showed that the higher the ΔpH (pH<sub>in</sub> – pH<sub>ex</sub>) in *O. oeni* cells was, the shorter the lag phase of the MLF was. To better manage the initiation of MLF in wines, the physiological state of *O. oeni* cells must be taken into account. These results allow us to understand the sometimes random initiation of MLF in wines inoculated with *O. oeni* and to suggest ways to improve this control.

© 2014 Elsevier B.V. All rights reserved.

## 1. Introduction

Malolactic fermentation (MLF) takes place after alcoholic fermentation and consists of the decarboxylation of malic acid into lactic acid and CO<sub>2</sub>. As a consequence, the organoleptic quality and the microbiological stability of wine are improved. MLF is mainly driven by the lactic acid bacterium *Oenococcus oeni*, which is able to decarboxylate malic acid under unfavourable conditions such as low pH (Drici-Cachon et al., 1996), high ethanol concentration and the presence of other compounds from yeast metabolism (Lonvaud-Funel, 1999). In practice, it is difficult to control MLF in wines because the cell physiology of *O. oeni* is poorly known. One criterion of growth physiology is the culturability measured using the plate count technique. However, in the case of *O. oeni*, the plate count technique requires a very long incubation time of about seven to ten days, which is not compatible with a real-time control of MLF. Moreover, viable and culturable cells can be weakly active or very active. This technique is not sufficient to assess the malolactic activity and effectiveness of the bacteria. Activity can be measured by kinetic parameters such as lag phase, specific growth

rate, specific substrate degradation rate or end product formation rate, but the assessment of all of these parameters also requires a long time (Rault et al., 2008).

Bacteria have developed several mechanisms that allow them to survive and grow in an acidic environment. For example, some lactic acid bacteria maintain the transmembrane pH gradient constant by decreasing their intracellular pH (pH<sub>in</sub>) when extracellular pH (pH<sub>ex</sub>) decreases (Siegmund et al., 2000). The mechanisms by which lactic acid bacteria regulate their pH<sub>in</sub> are based on the structure and the function of the plasma membrane (Garbay and Lonvaud-Funel, 1994). In *O. oeni*, the conversion of L-malic acid to L-lactic acid is not energetic, and the production of ATP is obtained by a chemiosmotic mechanism that involves the two components of the proton motive force, ΔpH and Δψ, which is sufficient to drive ATP synthesis by membrane ATPase (Cox and Henick-Kling, 1989, 1995; Olsen et al., 1991; Salema et al., 1994). In *O. oeni*, the ATPase complex is of the (F<sub>1</sub>F<sub>0</sub>)H<sup>+</sup>-ATPase type (Salema et al., 1996b) and can function reversibly when coupled with a proton pump via the synthesis of ATP. In wine (acidic medium), this ATPase activity is very important to the survival of *O. oeni*. The role of ATPase in relation to acid tolerance has been demonstrated with ATPase-deficient mutants (Tourdot-Maréchal et al., 1999). Since all the ATPase-deficient mutants lack malolactic activity, a link has been suggested between ATPase and malolactic activity in *O. oeni* (Galland

<sup>☆</sup> This work is dedicated to the memory of Monique Charpentier.

\* Corresponding author. Tel.: +33 130814534; fax: +33 130815597.

E-mail address: [marielle.bouix@agroparistech.fr](mailto:marielle.bouix@agroparistech.fr) (M. Bouix).

et al., 2003), and, moreover, it has been observed that ATPase activity was maximal in the exponential growth phase of *O. oeni* (Carrete et al., 2002). It has been demonstrated (Salema et al., 1996b) that MLF generates both a transmembrane pH gradient and an electrical potential gradient  $\Delta\Psi$  in *O. oeni*, and that the latter controls the rate of fermentation. It has also been shown that malate influx leads to the increase of the negative charge in the cell, subsequently improving membrane polarisation (Loubiere et al., 1992; Salema et al., 1994, 1996a) and decreasing the  $\Delta\Psi$  at the end of MLF when malate is exhausted. Cox and Henick-Kling (1995) observed a rise in  $\text{pH}_{\text{in}}$  due to the consumption of protons by the malolactic enzyme, and some authors hypothesized that the lactic acid and carbon dioxide were co-extruded in symport with protons (Poolman et al., 1991; Salema et al., 1994). The generated  $\Delta\text{pH}$  was the major component of the proton motive force responsible for the ATP generation observed (Cox and Henick-Kling, 1995). Metabolism is thus closely linked to the cellular parameters  $\Delta\text{pH}$  and  $\Delta\Psi$ , but most of the studies were performed on cells in the stationary phase or in vesicles (Salema et al., 1994), or on cells in the middle of the exponential phase (Augagneur et al., 2007), and always on only one point of the growth curve. Consequently, there is a lack of information about the kinetics of these phenomena. We think that the kinetics of changes in the  $\text{pH}_{\text{in}}$  and the membrane potential  $\Delta\Psi$  during MLF could make it possible to improve existing knowledge about *O. oeni* behaviour and to better control the performance of the bacteria in MLF.

Flow cytometry (FCM) is a rapid method for cell-by-cell analysis. It can be applied in combination with several fluorescent probes. It is used to count viable bacteria (Jepras et al., 1995) and yeasts (Bouix et al., 1999). Esterase substrates such as fluorescein diacetate (FDA) and carboxyfluorescein diacetate (cFDA) are widely used for viability assessment (Breeuwer and Abee, 2000b; Bunthof et al., 2001; Ben Amor et al., 2002). However, due to the change in  $\text{pH}_{\text{in}}$  during growth, we have previously shown that it is preferable to use BacLight™ staining rather than cFDA staining to enumerate *O. oeni* (Bouix and Ghorbal, 2013).

cFDA is a non-fluorescent precursor that is taken up by the cell and converted by non-specific esterase to a fluorescent compound, cF. cF can be actively extruded from the cells (Breeuwer et al., 1994), and this extrusion can be used as a tool to assess the vitality of yeasts (Bouix and Leveau, 2001), *Lactobacillus* (Rault et al., 2008) and *O. oeni* (Da Silveira and Abee, 2009). FCM is also used to characterise cellular physiological parameters such as membrane potential and  $\text{pH}_{\text{in}}$ . Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)) is the most commonly used dye for bacteria among the membrane potential-sensitive probes. Oxonols are anionic probes that enter depolarised cells and bind to lipid-rich compounds, resulting in green fluorescence (Mason et al., 1995). The more the cells are depolarised, the brighter the green fluorescence will be (Gabier et al., 2005). The main dyes used to assess  $\text{pH}_{\text{in}}$  are 5,6 carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) for the 5 to 7 range of  $\text{pH}_{\text{in}}$  (Breeuwer et al., 1996), and 5–6 carboxy 2',7'-dichlorofluorescein (CDCF) for the lower  $\text{pH}_{\text{in}}$  range (Nedergaard et al., 1990). By using different probes and FCM, it is possible to assess the viability, the vitality, the membrane potential and the  $\text{pH}_{\text{in}}$  of microbial cells in a short time.

The aim of this study was, first, to highlight the change in the components of the proton motive force, energetic state and vitality of *O. oeni* cells during MLF, using FCM. We then determined the best physiological cellular state for inoculation of bacteria in wine in order to better control MLF.

## 2. Materials and methods

### 2.1. Strain and cultures

The MC1 strain of *O. oeni* was isolated from wine by Moet et Chandon, and was stored at  $-80\text{ }^{\circ}\text{C}$ . Unlike most of the *O. oeni* strains, MC1 does not metabolise citric acid. All the cultures were performed in a flask, in FT80 synthetic medium (glucose: 5 g, fructose: 5 g,

L-malic acid: 5 g, yeast extract: 5 g, beef peptone: 2.5 g, tryptone: 2.5 g,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ : 0.5 g,  $\text{MgCl}_2$ : 0.05 g,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ : 0.01 g, KCl: 0.15 g,  $\text{CaCl}_2$ : 0.13 g, Tween 80: 1 mL, qsp water 1 L) (Cavin et al., 1989), adjusted to pH 3.2 or pH 5 before sterilisation at  $120\text{ }^{\circ}\text{C}$ , 20 min, and incubated at  $30\text{ }^{\circ}\text{C}$ . Some cultures were performed in white wine (grape variety: Pinot Meunier, 11% ethanol,  $\text{SO}_2$ : 40 mg/L, pH 3.2) (provided by Moet et Chandon), supplemented with L-malic acid (final concentration:  $5\text{ g}\cdot\text{L}^{-1}$ ), filtered on a  $0.45\text{-}\mu\text{m}$  membrane to eliminate other bacteria, and incubated at  $20\text{ }^{\circ}\text{C}$  after inoculation with MC1. Plate count was performed in FT80 agar (FT80 broth,  $15\text{ g}\cdot\text{L}^{-1}$  agar). All the pre-cultures were performed in the FT80 broth at pH 5 for 96 h. The culture in synthetic medium and in wine was inoculated at a concentration corresponding to approximately  $10^6\text{ cells}\cdot\text{mL}^{-1}$  (optical density of 0.025 at 600 nm).

Bacterial growth was monitored using two methods. The enumeration of culturable cells was performed by plating appropriate serial dilutions on FT80 agar. The count was obtained as the number of colony-forming units after incubation for 15 days at  $30\text{ }^{\circ}\text{C}$ . The enumeration of viable and dead cells was performed by flow cytometry after fluorescence staining with the Live-Dead BacLight™ kit, as described below. All the measurements were performed once a day during MLF. Each data point was the mean of three independent cultures.

### 2.2. Probes

Chemchrome V8 containing carboxyfluorescein diacetate (cFDA) (AES-Chemunex, Ivry sur Seine, France), 5,6 carboxy 2',7' dichlorofluorescein diacetate (CDCF), 5,6 carboxyfluorescein diacetate succinimidyl ester (cFDA SE), bis-(1,3-dibutylbarbituric acid) (DiBAC<sub>4</sub>(3)), and the Live-Dead BacLight™ Viability kit were obtained from Molecular Probes (Life Technologies, St. Aubin, France).

### 2.3. Live-Dead BacLight™ staining

One millilitre of culture containing approximately  $10^6$  cells was centrifuged in an MSE Micro Centaur SANYO centrifuge (13,000 g, 1 min) and the pellet was suspended in 1 mL of McIlvaine's buffer at pH 7.3 (citric acid: 0.1 M; disodium hydrogenophosphate: 0.2 M), and incubated for 15 min at room temperature in the dark in the presence of 1.5  $\mu\text{L}$  of Syto9 and 1.5  $\mu\text{L}$  of PI (solutions ready to use in the kit) (Bouix and Ghorbal, 2013).

### 2.4. CFDA, CDCF and CFDA SE staining

One millilitre of culture containing approximately  $10^6$  cells was centrifuged in an MSE Micro Centaur SANYO centrifuge (13,000 g, 1 min). The pellet was resuspended in 1 mL of McIlvaine's buffer at pH 7.3, or in phosphate buffer (orthophosphoric acid: 0.1 M; disodium hydrogenophosphate: 0.2 M), and incubated for 10 min at  $40\text{ }^{\circ}\text{C}$  in the presence of 10  $\mu\text{L}$  of Chemchrome V8, 10  $\mu\text{L}$  of CDCF (188.9 mM in acetone) or 5  $\mu\text{L}$  of cFDA SE (8.96 mM in acetone).

### 2.5. Viability measurement

The BacLight™ double-stained sample was centrifuged (13,000 g, 1 min) and the pellet was suspended in McIlvaine's buffer at pH 3.2 before FCM analysis.

### 2.6. Vitality measurement

Based on the energy-dependent extrusion of cF from the cell after conversion of cFDA (Breeuwer et al., 1994), we developed a vitality descriptor on the same principle as for yeast (Bouix and Leveau, 2001) or *Lactobacillus* (Rault et al., 2008). Two millilitres of the ChemChrome-stained sample were centrifuged (13,000 g, 90 s), and the pellet was resuspended in 2 mL of McIlvaine's buffer at pH 3.2. One millilitre was

immediately analysed by FCM to measure the fluorescence intensity  $FI_0$ . One millilitre was incubated for 15 min at 40 °C, centrifuged, and the pellet was resuspended in Mcllvaine's buffer (pH 3.2) before FCM analysis. The fluorescence intensity  $FI_{15}$  was measured. The excretion percentage was calculated as follows:  $\Delta FI_{15} = 100(FI_0 - FI_{15}) / FI_0$ . The more the cells were active, the greater the excretion and the descriptor  $\Delta FI_{15}$  were.

### 2.7. Intracellular pH ( $pH_{in}$ ) measurement

To measure the  $pH_{in}$  and establish the calibration curve, two probes are required to cover the range of pH. CDCF was used for pH values ranging from 3 to 4.5 (Nedergaard et al., 1990), and cFDA SE was used for pH values ranging from 4 to 7 (Breeuwer et al., 1996; Breeuwer and Abee, 2000a).

Cells containing CDCF or CFDA SE were centrifuged (13,000 g, 90 s), and the pellet was resuspended in 3 mL of Mcllvaine's buffer (or phosphate buffer) at the pH value equal to the pH of the culture (5 or 3.2) before FCM analysis. The fluorescence intensity of the cells was directly linked to the  $pH_{in}$  in the range of the calibration curves. Calibration curves were determined for each cell sample. After staining, cells were centrifuged (13,000 g, 90 s) and resuspended in Mcllvaine's buffer with pH values ranging from 3 to 7. Five microlitres of nigericin (0.2 mM in ethanol) and 5  $\mu$ L of valinomycin (0.2 mM in ethanol) were added to equilibrate the  $pH_{in}$  and the pH medium ( $pH_{ex}$ ) before FCM analysis.

### 2.8. Membrane potential evaluation

One millilitre of culture was centrifuged (13,000 g, 1 min) and the pellet was resuspended in 1 mL of Mcllvaine's buffer, pH 7.3, and incubated for 20 min at 40 °C in the presence of 5  $\mu$ L of DiBAC (3) (0.5 mg.mL<sup>-1</sup> in DMSO). Samples stained with DiBAC<sub>4</sub> (3) were analysed by FCM. The result indicated the depolarised cell number and the depolarisation level: the higher the fluorescence intensity was, the more the cells were depolarised.

### 2.9. Flow cytometry analysis

FCM analysis was performed with a CyFlow SL cytometer (PARTEC, Sainte Geneviève des Bois, France). The cytometer was equipped with a solid blue laser emitting at 488 nm, and four band-pass filters: a forward-angle light scatter (FSC) combined with a diode collector, a side-angle light scatter (SSC) and two fluorescence signals collected with photomultiplier tubes, a 530-nm band-pass filter (515–545) to collect green fluorescence (FL1 channel), and a 630-nm long pass filter to collect the red fluorescence (FL2 channel). The FCM analysis was performed using logarithmic gains and specific detector settings adjusted on a sample of unstained cells in order to eliminate cellular autofluorescence. Gating on FSC/SSC was used to discriminate bacteria from the background.

Data were collected and analysed with FlowMax software (PARTEC, Sainte Geneviève des Bois, France). The subpopulations were identified using dot plots. Gates were defined in the dot plots of FSC, SSC, green fluorescence and red fluorescence, thus allowing the software to separate the different events. Data were analysed using statistical tables that indicate numbers and percentages of stained cells determined by each detector, as well as the fluorescence intensity of each fluorescent signal.

### 2.10. Microscopic analysis

Labelled fluorescent cells were analysed with an Axiostar + epifluorescence microscope (CARL ZEISS, Le Pecq, France) equipped with a 50-Watt mercury lamp. A JAI M70 camera (Imazys, Suresnes, France) linked to ITEX software (Imazys, Suresnes, France) was used to take microphotographs.

### 2.11. Malic acid concentration

The kinetics of malic acid consumption were monitored using the enzymatic method (Enzytec, Biopharm, Saint Dizier au Mont d'Or, France). One millilitre of the culture was centrifuged and the supernatant was used to determine the residual quantity of malic acid once daily.

### 2.12. Energy charge

The energy charge was calculated as the ratio as follows:  $EC = (ATP + 1/2 ADP)/(ATP + ADP + AMP)$ .

For the measurement of nucleotide acids, ADP and AMP were converted to ATP according to (Gorman et al., 2003).

For the extraction of nucleotides, 800  $\mu$ L of DMSO and 100  $\mu$ L of 10 mM EDTA were added to 100  $\mu$ L of cell suspension precisely adjusted to 10<sup>6</sup> cells.mL<sup>-1</sup>, mixed for 1 min and then centrifuged (13,000 g, 5 min). Nine hundred microlitres of supernatant were added to 4 mL of 20 mM Tris buffer and stored at -20 °C before analysis. The ATP concentration was measured with an ATP meter (MicroLumat LB 96PEG&G Berthold, Germany).

### 2.13. Evaluation of activity of *O. oeni* culture

The aim of this experiment was to establish the link between the physiological cellular state of *O. oeni* cells ( $pH_{in}$ ) and the activity of the cells measured by the lag phase of the MLF. In order to evaluate the role of the physiological cellular state of *O. Oeni* on the initiation of MLF, a culture of *O. oeni* in FT80 medium, pH 3.2, was performed, and  $pH_{in}$  and malic acid concentration were measured at different times. At each of these times, 10<sup>8</sup> *O. oeni* cells were taken from this culture and inoculated in 100 mL of fresh FT80 medium, pH 3.2, and incubated at 30 °C. Malic acid was analysed in these sub-cultures once a day throughout MLF. The MLF lag time was calculated as the time where the initial malic acid concentration remained constant. The duration of MLF was the sum of the MLF lag time and the malic acid consumption time (from 5 g/L to 0). The same experiment was carried out on white wine: samples were taken during MLF in wine, analysed (malic acid concentration and  $pH_{in}$ ), and 1 mL (adjusted to 10<sup>8</sup> cells) was transferred to 100 mL of fresh white wine (malic acid concentration adjusted to 5 g/L), incubated at 20 °C. MLF was monitored by measuring the malic acid concentration once daily. The MLF lag time and the malic acid consumption time were calculated as described above. The shorter the MLF lag time was, higher the activity of *O. oeni* cells was.

## 3. Results

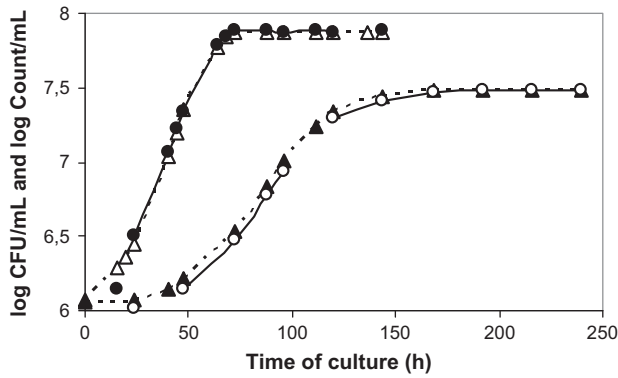
### 3.1. Growth curve, viability after BacLight staining and culturability assessment

Growth curves of *O. oeni* established by plate counts and FCM after BacLight™ staining are shown in Fig. 1. At pH 3.2, the duration of the growth was longer than at pH 5: the lag phase was 24 h, the exponential phase began as of 50 h, and the stationary phase after 168 h of culture. The final cell concentration was  $2.28 \times 10^7$  cells.mL<sup>-1</sup>. At pH 5, the lag phase was shorter (<24 h) and the stationary phase began as of 72 h. The final cell concentration was  $6.3 \times 10^7$  cells.mL<sup>-1</sup>. The FCM counts were well correlated with the plate counts throughout the culture for the two pH conditions. The R<sup>2</sup> correlation was 0.99 for the two culture conditions.

### 3.2. Transmembrane potential change during MLF

With the DiBAC probe, the fluorescence intensity increases with the depolarisation level of the cells. FCM was used to evaluate the change in the percentage of depolarised cells compared to total cells and the





**Fig. 1.** *O. oeni* counts in FT80 broth at pH 5 ( $\Delta$ ): plate count; ( $\bullet$ ): FCM; pH 3.2 ( $\blacktriangle$ ) plate count, ( $\circ$ ): FCM. The maximum standard deviations for these measurements were 0.073 for plate counts and 0.045 for FCM counts. The error tool bars were removed to make the graph clearer.

depolarisation level (fluorescence intensity of cells) (Fig. 2a). In FT80 medium at pH 3.2, the transmembrane potential of *O. oeni* cells was weak at the beginning and at the end of MLF, with 95% and 98% of depolarised cells and a high depolarisation level (FI = 50, arbitrary unit). During the exponential growth phase, the transmembrane potential of *O. oeni* cells increased since we observed only 7.5% of weakly depolarised cells at 168 h (strong decrease of fluorescent intensity level; FI = 18, arbitrary unit). Microscopic observations of *O. oeni* cells

confirm the bright fluorescence of cells at the beginning of growth and at the end of the stationary growth phase and a weak fluorescence of cells in the exponential phase (Fig. 2b).

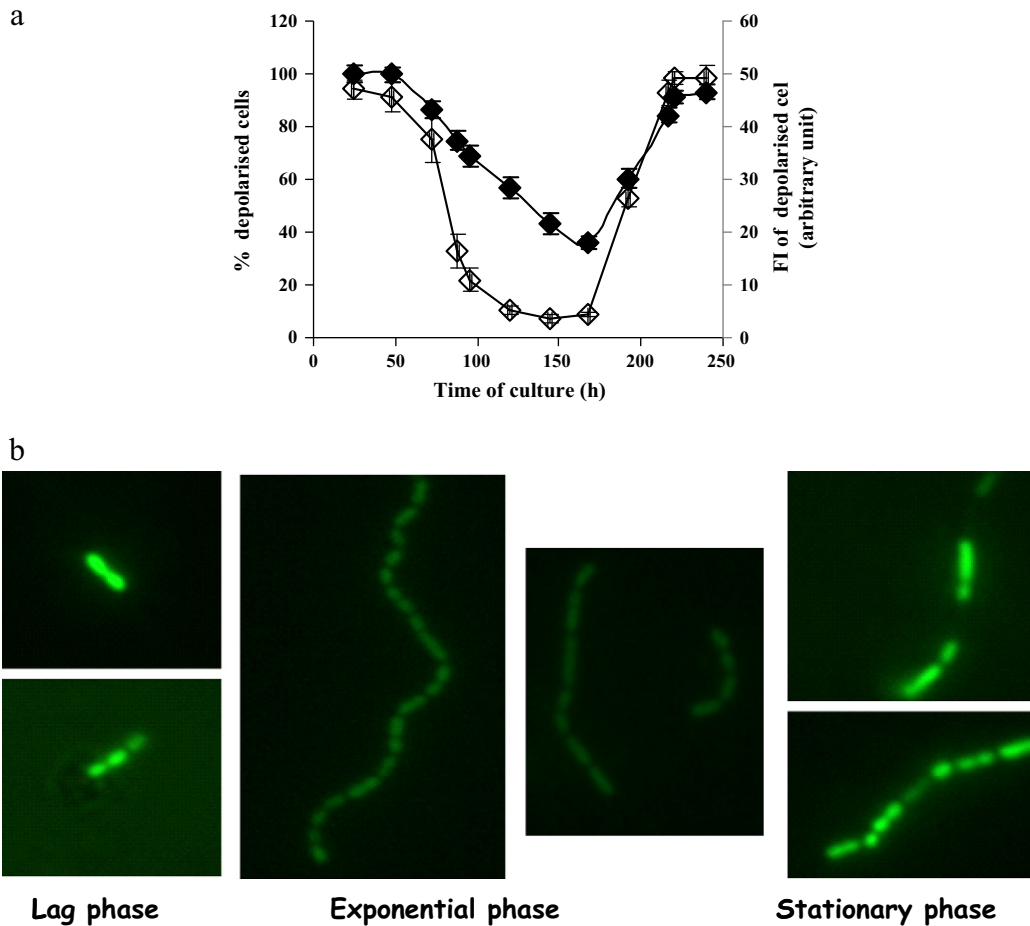
### 3.3. $pH_{in}$ change during MLF

The calibration curve is carried out at each measurement. The choice of one of the two probes is based on the value of each previous measurement. At the beginning of MLF in FT80 medium at pH 3.2, the  $pH_{in}$  of the cells was very low, increased during cell growth to a maximum of  $pH_{in}$  6.1 at 168 h, which corresponded to the entry into the stationary phase, and then decreased to pH 3.26 at the end of the stationary phase. Surprisingly, the  $pH_{in}$  at the beginning and at the end of MLF seemed to be close to the medium pH. Measuring the change in  $pH_{in}$  during MLF in FT80 medium at pH 5 confirmed that the *O. oeni* cell  $pH_{in}$  was equilibrated with the medium pH at the beginning and at the end of MLF, regardless of the pH (Fig. 3).

Thus, in FT80 medium, pH 3.2, there was a change in  $pH_{in}$  of almost three pH units during the growth of *O. oeni*, which corresponded to a  $\Delta pH$  ( $pH_{in} - pH_{ex}$ ) ranging from zero at the beginning of the culture to 2.8 at the entry into the stationary growth phase. The  $pH_{ex}$  increased slightly, from 3.2 to 3.3 at the end of the exponential growth phase.

### 3.4. Vitality and energy charge changes during MLF

In FT80 medium, pH 3.2, the vitality measured by the percentage of cF extrusion from cells (descriptor  $\Delta FI_{15}$ ) by FCM was 28% at the



**Fig. 2.** Change in *O. oeni* cell depolarisation during MLF in FT80, pH 3.2. a: Percentage of depolarised cells ( $\diamond$ ) and depolarisation level (FI) ( $\blacklozenge$ ) after DiBAC staining. The percentage of depolarised cells decreased up to 168 h (maximum polarisation). b: Photograph of *O. oeni* cells during MLF after DiBAC staining. The fluorescence intensity of cells after DiBAC staining was lower in the exponential phase than in the lag phase or at the end of the stationary phase.

beginning of the growth, increased to 82% at 168 h, and decreased to 30% at the end of the stationary growth phase. The maximum value of the energy charge also occurred at 168 h (Fig. 4). These measurements were performed without the addition of energetic substrate, corresponding to the intrinsic values of vitality and energy charge in the cells during MLF.

### 3.5. Malic acid consumption and cellular parameters of *O. oeni* changes during MLF

In Fig. 5a, we have superposed the consumption of malic acid and  $pH_{in}$  change during the growth of *O. oeni* in FT80 medium, pH 3.2. The malic acid concentration reached zero at 168 h and the  $pH_{in}$  increased from 3.2 to 6.1 during malic acid consumption. When the malic acid concentration tended to zero, the  $pH_{in}$  rapidly decreased. All these results were validated in wine and the data are shown in Fig. 5b: in wine, due to the necessity to adapt to ethanol concentration and to the temperature (20 °C instead of 30 °C in synthetic medium), the MLF lag phase was more time-consuming. However, like in synthetic medium, the polarisation level of cells,  $pH_{in}$  and vitality were weak during the lag phase of the MLF, increased during growth with the consumption of malic acid to reach a maximum when malic acid was exhausted, and immediately decreased to their initial values. The maximum  $pH_{in}$  value was the same in wine and in synthetic medium for an equal malic acid concentration (5 g/L).

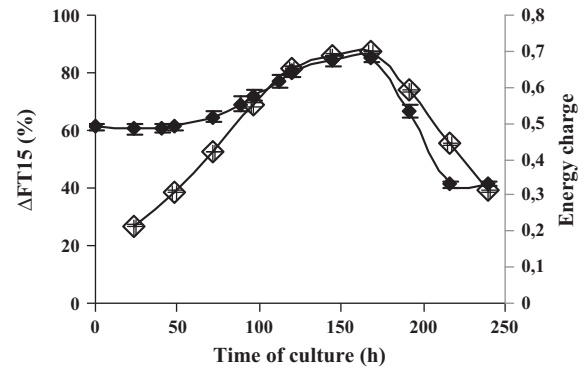


Fig. 4. Vitality (◊) and energy charge (•) during MLF in FT80 medium, pH 3.2. The two values increased up to 168 h of culture and then decreased.

### 3.6. Relationship between maximum $pH_{in}$ , malic acid concentration and activity of *O. oeni* cells

FT80 medium pH 3.2, or aliquot of wine was inoculated with *O. oeni* cells from different stages of a pre-culture and then in various physiological states (various  $pH_{in}$ , polarisation levels and residual acid malic concentrations named “inoculum values” in Fig. 6). Regardless of the medium, synthetic or wine, the higher the  $pH_{in}$  and polarisation level (lower fluorescence intensity) of inoculated cells were, the shorter the lag phase of the MLF was (Fig. 6). On the contrary, the malic acid consumption time was the same, regardless of the physiological state of *O. oeni* cells before inoculation.

## 4. Discussion

### 4.1. Vitality descriptor

In this study, we propose a descriptor  $\Delta FI_{15}$  to measure the vitality of cells. The principle of this measurement is based on the energy-dependent cF extrusion ability (Breeuwer et al., 1994). The cells were loaded with cF and the fluorescence intensity ( $FI_0$ ) was measured. Then, during a second incubation, the cF was excreted even more rapidly when the cells had a high energy charge. The more active the cells were, the greater the extrusion was and, therefore, the greater the difference in the fluorescence of cells before and after incubation over the same time period was. This descriptor has been used for yeast (Bouix and Leveau, 2001) and *Lactobacillus* (Rault et al., 2008). cF extrusion was also used to assess the activity of *O. oeni* in several stress conditions with ethanol (Da Silveira and Abee, 2009). These latter authors added an energy source to the buffer and their measurements were performed at pH 5.5. In our descriptor, we compensated for the variations of  $pH_{in}$  during MLF on the measurement of fluorescence of the cF (pH-dependent) by taking the difference of fluorescence intensities ( $FI_0 - FI_{15}$ ) divided by the initial fluorescence intensity ( $FI_0$ ) into account. Otherwise, we did not use an energy source in our experiment to measure the extrusion of cF from the cells at each point in time in MLF conditions, and measurements were performed in pH 3.2 buffer (wine pH).

### 4.2. Cell parameter changes during MLF

The work of Augagneur et al. (2007) showed that the presence of citrate in buffer impacts the  $pH_{in}$  value by increasing the  $\Delta pH$ . In preliminary experiments, we used two buffers (phosphate and citrate) for staining cells for  $pH_{in}$  measurements with the two probes, CDCF and CFDA SE. No significant difference was observed between the two buffers and a good correlation ( $R^2 = 0.996$ ) was obtained between the two  $pH_{in}$  measurement series with the two buffers (data not

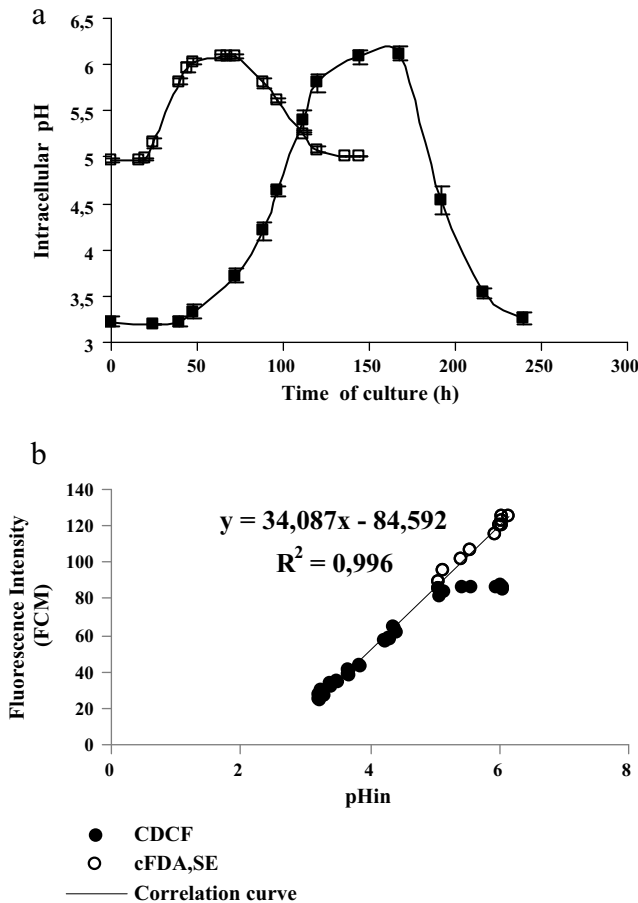


Fig. 3. a: Change in  $pH_{in}$  during MLF in FT80 medium, pH 3.2 (■), and FT80 medium, pH 5 (□). At the beginning and at the end of MLF,  $pH_{in}$  is equal to  $pH_{ex}$ .  $pH_{in}$  increased to 6.1 during growth. b: Calibration curve with CDCF and CFDA SE.

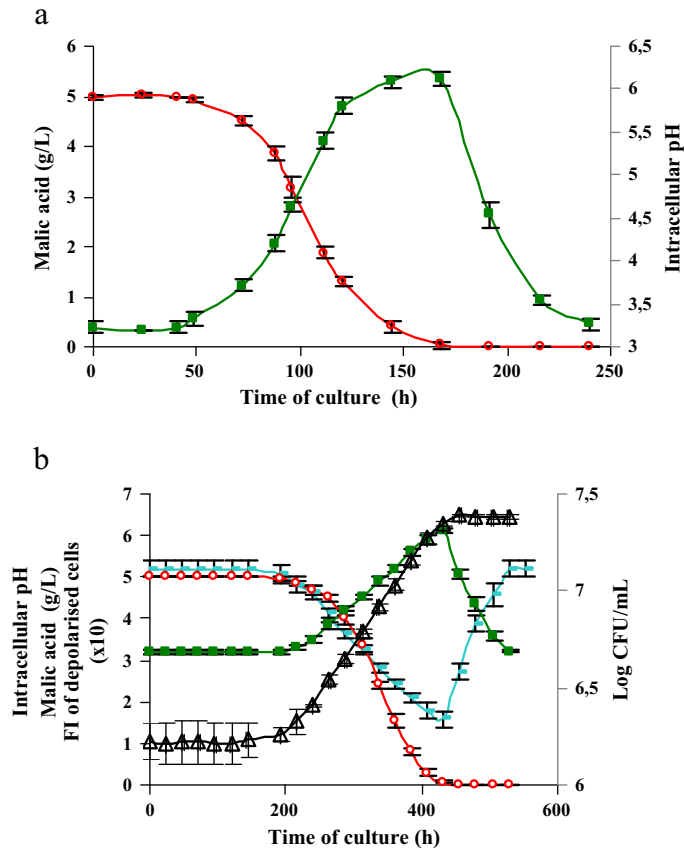


Fig. 5. Malate consumption and cellular parameters of *O. oeni* changes during MLF in FT80 medium, pH 3.2 (a) and in wine (b). a: FT80 medium, pH 3.2, malic acid (O),  $pH_{in}$  (■). The maximum  $pH_{in}$  increased until malic acid reached 0. b: White wine, malic acid (O),  $pH_{in}$  (■), FI depolarised cells (—) and plate count ( $\Delta$ ).

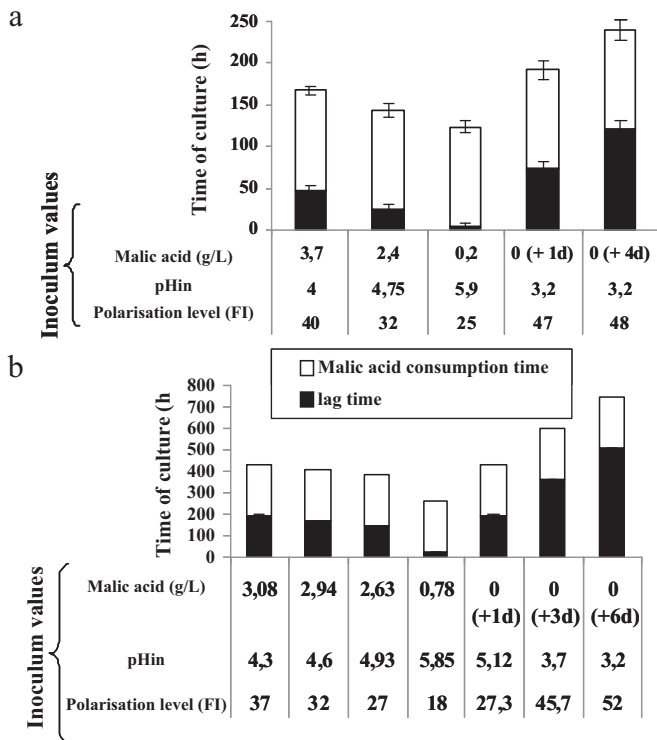
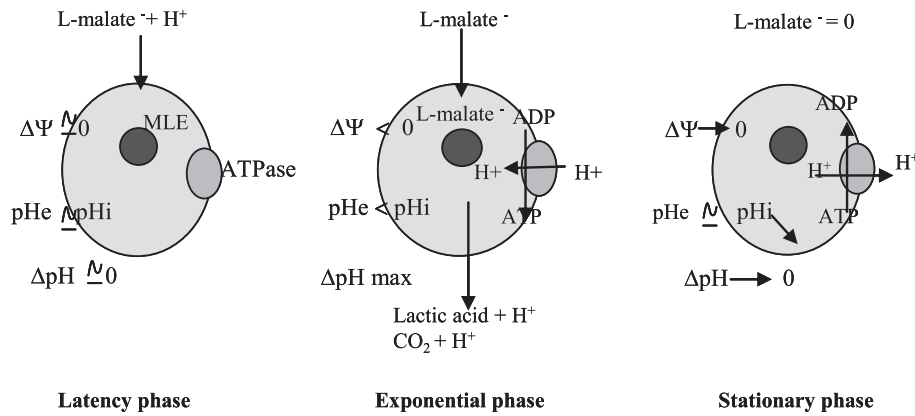


Fig. 6. Cellular parameters of *O. oeni* and lag time of MLF in FT80, pH 3.2 (a) and in white wine (b). The higher the  $pH_{in}$  and polarisation level (lower fluorescence intensity) of inoculated cells were, the shorter the lag time of the MLF was, but the malic acid consumption time remained constant.

shown). The difference with the results of Augagneur et al. (2007) can be explained by the fact that our MC1 strain is citrate-negative.

However, our results were in agreement with those of Augagneur et al. (2007) who observed a  $\Delta pH$  equal to 2.6 ( $pH_{in} = 5.8$ ) for a culture with a malic acid concentration of 37 mM, i.e., the same  $\Delta pH$  as in our study with the same concentration of malate. On the contrary, the  $\Delta pH$  was only equal to 1 ( $pH_{in} = 4.2$ ) for the culture without malic acid; the  $\Delta pH$  was thus greater when the culture under acidic conditions was performed in the presence of malic acid. These measurements were performed at only one point in time on the culture while in our work, we established the kinetic change of  $pH_{in}$  in the entire fermentation.

It is now well accepted that MLF is a proton motive force generating process (Cox and Henick-Kling, 1995; Loubiere et al., 1992; Salema et al., 1996b). Nevertheless, most of the previous studies were performed on cells harvested at the end of the exponential phase. Due to the simplicity and lower time consumption of FCM associated with fluorescent probes, we simultaneously measured  $pH_{in}$  and  $\Delta\Psi$  throughout MLF, both in synthetic medium and in wine, and determined their changes. Moreover, with the vitality descriptor  $\Delta FI_{15}$ , we confirmed ATP synthesis, as suggested by (Galland et al., 2003). The kinetics of physiologic cellular changes of *O. oeni* are illustrated in Fig. 7: at the beginning of the growth,  $\Delta\Psi$  is low and  $pH_{in}$  is equal to  $pH_{ex}$  ( $\Delta pH = 0$ ). The vitality is weak. During the growth of *O. oeni*,  $\Delta\Psi$  increases (negative charge inside the cell) when malate enters the cell (Loubiere et al., 1992). According to Salema et al. (1996b), the malolactic enzyme clives malic acid, and lactic acid and  $CO_2$  are co-excreted with protons, which causes the  $pH_{in}$  to increase to 6–6.1 ( $\Delta pH = 2.8$ –2.9). This  $\Delta pH$  allows the entry of protons and the increase of the proton motive force with the synthesis of ATP by the ATP synthase (Cox and Henick-Kling, 1989, 1995). When malic acid is totally exhausted,  $pH_{in}$ ,  $\Delta\Psi$  and vitality decrease. These results were in accordance with the



**Fig. 7.** Changes in force proton motive components and functioning of ATPase during MLF. At the beginning of the growth,  $\Delta\Psi$  was low and  $pH_{in}$  was equal to  $pH_{ex}$  ( $\Delta pH$  0). During the growth of *O. oeni*,  $\Delta\Psi$  increases when malate enters the cells, the malolactic enzyme clives malic acid, and lactic acid and CO<sub>2</sub> are co-excreted with protons, which causes the  $pH_{in}$  to increase. This  $\Delta pH$  allows the entry of protons and the increase of the proton motive force with the synthesis of ATP by ATP synthase. When malic acid is totally exhausted,  $pH_{in}$  and  $\Delta\Psi$  decrease.

literature cited above. However, in the case of FCM, we specified the course of the cellular phenomenon during MLF from the kinetic point of view.

No values have been reported in literature for  $pH_{in}$  in cells not undergoing growth. Our measurements indicated that the  $pH_{in}$  value was equal to the pH of the medium ( $pH_{in} = pH_{ex}$ ). This can be explained by the metabolism of the bacteria as described above. Nevertheless, we can wonder about the survivability of the bacterium under such harsh conditions.

#### 4.3. Relationship between cellular parameters and activity of *O. oeni*

On the basis of these kinetics, we confirmed our hypothesis that the higher the vitality, the  $pH_{in}$  and the polarisation of inoculated cells are, the shorter the lag time of the MLF will be. In our experiments, in FT80 medium, the lag time varied from 4 h to 120 h, depending on whether the  $pH_{in}$  of inoculated *O. oeni* cells was equal to 5.9 or 3.2. In white wine, with the same range of  $pH_{in}$  of inoculated cells, the lag time varied from 24 h to 504 h (21 days), respectively. The lag phase was longer in wine than in synthetic medium, probably due to the presence of ethanol and other inhibitory compounds. However, the lag phase in wine was not longer when pre-cultured was performed in FT80 than in wine, i.e., 24 h from FT80 to wine or from wine to wine (data not shown), which suggests that the physiological state of cells was more important in reducing the lag phase than adaptation to ethanol. It is interesting to point out that the lag time rapidly increases in fresh medium after the malic acid concentration in pre-culture reaches zero. In practice, when inoculating wines from a pre-culture, the simple measurement of malic acid concentration could be used to control the physiological state of inoculation cells. Validation in red wine is in progress.

Use of freeze-dried starter cultures in MLF, either with direct inoculation or with re-hydrated cells, still provides random results. We think MLF is sometimes sluggish because there is no criterion to control the physiological state of cells at the time of inoculation. Given our results, we think it would be interesting to integrate these physiological cellular state parameters into the production of active functional freeze-dried starters or into the re-hydration phase to improve the onset of MLF.

## 5. Conclusion

FCM was quickly able to assess the  $pH_{in}$  and membrane potential of *O. Oeni* cells during MLF. The results obtained in synthetic medium at two pH values and in white wine showed that the  $pH_{in}$  underwent

considerable changes during MLF. These variations are undoubtedly linked to the complex energy metabolism of *O. oeni*.

The simple measurement of the cellular parameters ( $pH_{in}$ ,  $\Delta\Psi$  and  $\Delta FI_{15}$ ) makes it possible to better understand the cellular mechanisms of *O. oeni*. Together with malic acid concentration, it provides us with simple tools to evaluate cellular activity before inoculating bacteria in wine and to more effectively control malolactic fermentation.

## Acknowledgements

We thank Moët et Chandon for providing us with their *O. oeni* strain and wine.

## References

- Augagneur, Y., Ritt, J.-F., Linares, D., Remize, F., Tourdot-Marechal, R., Garmyn, D., Guzzo, J., 2007. Dual effect of organic acids as a function of external pH in *Oenococcus oeni*. *Arch. Microbiol.* 188, 147–157.
- Ben Amor, K., Breeuwer, P., Verbaarschot, P., Rombouts, F.M., Akkermans, A.D.L., de Vos, W.M., Abee, T., 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Appl. Environ. Microbiol.* 68 (11), 5209–5216.
- Bouix, M., Ghorbal, S., 2013. Rapid enumeration of *Oenococcus oeni* during malolactic fermentation by flow cytometry. *J. Appl. Microbiol.* 114, 1075–1081.
- Bouix, M., Leveau, J.-Y., 2001. Rapid assessment of yeast viability and yeast vitality during alcoholic fermentation. *J. Inst. Brew.* 107, 217–225.
- Bouix, M., Grabowski, A., Charpentier, M., Leveau, J.-Y., Duteurtre, B., 1999. Evaluation rapide de la contamination des moûts par cytométrie en flux. *J. Int. Sci. Vigne Vin* 33, 25–32.
- Breeuwer, P., Abee, T., 2000a. Assessment of the intracellular pH of immobilized and continuously perfused yeast cells employing fluorescence ratio imaging analysis. *J. Microbiol. Methods* 39, 253–264.
- Breeuwer, P., Abee, T., 2000b. Assessment of viability of microorganisms employing fluorescence techniques. *Int. J. Food Microbiol.* 55, 193–200.
- Breeuwer, P., Drocourt, J.-L., Rombouts, F.M., Abee, T., 1994. Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow-cytometry. *Appl. Environ. Microbiol.* 60, 1467–1472.
- Breeuwer, P., Drocourt, J.-L., Rombouts, F.M., Abee, T., 1996. A novel method for continuous determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe 5 (and 6-) carboxyfluorescein succinimidyl ester. *Appl. Environ. Microbiol.* 62, 178–183.
- Bunthof, C.J., Bloemen, K., Breeuwer, P., Rombouts, F.M., Abee, T., 2001. Flow cytometric assessment of viability of lactic acid bacteria. *Appl. Environ. Microbiol.* 67, 2326–2335.
- Carrete, R., Vidal, M., Bordons, A., Constanti, M., 2002. Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. *FEMS Microbiol. Lett.* 211, 155–159.
- Cavin, J., Prevost, H., Lin, J., Divies, C., 1989. Medium for screening *Leuconostoc oenos* strains defective in malolactic fermentation. *Appl. Environ. Microbiol.* 55, 751–753.
- Cox, D.J., Henick-Kling, T., 1989. Chemiosmotic energy from malolactic fermentation. *J. Bacteriol.* 171, 5750–5752.
- Cox, D.J., Henick-Kling, T., 1995. Protonmotive force and ATP generation during malolactic fermentation. *Am. J. Enol. Vitic.* 46, 319–323.



- Da Silveira, M.G., Abee, T., 2009. Activity of ethanol-stressed *Oenococcus oeni* cells: a flow cytometric approach. *J. Appl. Microbiol.* 106, 1690–1696.
- Drici-Cachon, Z., Guzzo, J., Cavin, J.-F., Diviès, C., 1996. Acid tolerance in *Leuconostoc oenos*. Isolation and characterization of an acid-resistant mutant. *Appl. Microbiol. Biotechnol.* 44, 785–789.
- Gabier, A.C., Reitz, J., Gourdon, P., Leveau, J.-Y., Bouix, M., 2005. Intracellular physiological events of yeast *Rhodotorula glutinis* during storage at +4 °C. *Int. J. Food Microbiol.* 105, 97–109.
- Galland, D., Tourdot-Maréchal, R., Abraham, M., Chu, K.C., Guzzo, J., 2003. Absence of malolactic activity is a characteristic of H<sup>+</sup>-ATPase-deficient mutants of the lactic acid bacterium *Oenococcus oeni*. *Appl. Environ. Microbiol.* 69, 1973–1979.
- Garbay, S., Lonvaud-Funel, A., 1994. Characterization of membrane-bound ATPase activity of *Leuconostoc oenos*: growth conditions. *Appl. Microbiol. Biotechnol.* 41, 597–602.
- Gorman, M.W., Marbie, D.R., Ogomoto, K., feigi, E.O., 2003. Measurement of adenine nucleotides in plasma. *Luminescence* 18, 173–181.
- Jepras, R.I., Carter, J., Pearson, S.C., Paul, F.E., Wilkinson, M.J., 1995. Development of a robust flow cytometry assay for determining numbers of viable bacteria. *Appl. Environ. Microbiol.* 61, 2696–2701.
- Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Anton. Leeuw.* 76, 317–331.
- Loubiere, P., Salou, P., Leroy, M.-J., Lindley, N.D., Pareilleux, A., 1992. Electrogenic malate uptake and improved growth energetics of the malolactic bacterium *Leuconostoc oenos* grown on glucose-malate mixtures. *J. Bacteriol.* 174, 5302–5308.
- Mason, D.J., López-Amorós, R., Allman, R., Stark, J.M., Lloyd, D., 1995. The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. *J. Appl. Bacteriol.* 78, 309–315.
- Nedergaard, M., Desai, S., Pulsinelli, W., 1990. Dicarboxy-dichlorofluorescein: a new fluorescent probe for measuring acidic intracellular pH. *Anal. Biochem.* 187, 109–114.
- Olsen, E.B., Russel, J.B., Henick-Kling, T., 1991. Electrogenic L-malate transport by *Lactobacillus plantarum*: a basis for energy derivation from malolactic fermentation. *J. Bacteriol.* 173, 6199–6206.
- Poolman, B., Molenaar, D., Smid, E., Ubbink, T., Abee, T., Renault, P.P., Konings, W.N., 1991. Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. *J. Bacteriol.* 173, 6030–6037.
- Rault, A., Bouix, M., Beal, C., 2008. Dynamic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 physiological characteristics during fermentation. *Appl. Microbiol. Biotechnol.* 81, 559–570.
- Salema, M., Poolman, B., Lolkema, J.S., Loureiro-Dias, M.C., Konings, W.N., 1994. Uniport of monoanionic L-malate in membrane vesicles from *Leuconostoc oenos*. *Eur. J. Biochem.* 225, 289–295.
- Salema, M., Capucho, I., Poolman, B., San Romão, M.V., Loureiro-Dias, M.C., 1996a. In vitro reassembly of the malolactic fermentation pathway of *Leuconostoc oenos* (*Oenococcus oeni*). *J. Bacteriol.* 178, 5537–5539.
- Salema, M., Lolkema, J.S., San Romão, M.V., Loureiro-Dias, M.C., 1996b. The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. *J. Bacteriol.* 178, 3127–3132.
- Siegumfeldt, H., Rechinger, K.B., Jakobsen, M., 2000. Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Appl. Environ. Microbiol.* 66, 2330–2335.
- Tourdot-Maréchal, R., Fortier, L.C., Guzzo, J., Lee, B., Diviès, C., 1999. Acid sensitivity of neomycin-resistant mutants of *Oenococcus oeni*: a relationship between reduction of ATPase activity and lack of malolactic activity. *FEMS Microbiol. Lett.* 178, 319–326.