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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 ☆

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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_{in} 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_{in} and membrane potential occurred when all of the malic acid was consumed. Finally, we showed that the higher the ΔpH ($pH_{in} - pH_{ex}$) 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.

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1. Introduction

Malolactic fermentation (MLF) takes place after alcoholic fermentation and consists of the decarboxylation of malic acid into lactic acid and CO_2. 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_{in}) when extracellular pH (pH_{ex}) decreases (Siegumfeldt et al., 2000). The mechanisms by which lactic acid bacteria regulate their pH_{in} 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 $\Delta\psi$, 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 $(F_1F_0)H^+$-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

☆ This work is dedicated to the memory of Monique Charpentier.

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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 pH_{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 ΔpH 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 ΔpH 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 pH_{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 pH_{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 pH_{in}. Bis-(1,3-bibutylbarbituric 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 pH_{in} are 5,6 carboxyfluorescein diacetate succimidyl ester (cFDA-SE) for the 5 to 7 range of pH_{in} (Breeuwer et al., 1996), and 5–6 carboxy 2′7′-dichlorofluorescein (CDCF) for the lower pH_{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 pH_{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 °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, NaH_2PO_4, $2H_20$: 0.5 g, $MgCl_2$: 0.05 g, $MnSO_4$,H_20: 0.01 g, KCl: 0.15 g, $CaCl_2$: 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 °C, 20 min, and incubated at 30 °C. Some cultures were performed in white wine (grape variety: Pinot Meunier, 11% ethanol, SO_2: 40 mg/L, pH 3.2) (provided by Moet et Chandon), supplemented with L-malic acid (final concentration: 5 $g.L^{-1}$), filtered on a 0.45-μM membrane to eliminate other bacteria, and incubated at 20 °C after inoculation with MC1. Plate count was performed in FT80 agar (FT80 broth, 15 $g.L^{-1}$ 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 $cells.mL^{-1}$ (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 °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 succimidyl ester (cFDA SE), bis-(1,3-dibutylbarbituric acid) ($DiBAC_4(3)$), 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 μL of Syto9 and 1.5 μL 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 °C in the presence of 10 μL of Chemchrome V8, 10 μL of CDCF (188.9 mM in acetone) or 5 μL 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

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