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Quantitative Approach to Determining the Contribution of Viable-but-Nonculturable Subpopulations to Malolactic Fermentation Processes[▽]

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Different sizes of viable-but-nonculturable cell subpopulations of a lactic acid bacterium strain were induced by adding increasing amounts of SO_2. The experimental data obtained here were fitted to a segregated kinetic model developed previously. This procedure allowed us to determine in quantitative terms the contribution of this physiological state to malolactic fermentation.

The persistence of stressed, damaged, or viable-but-nonculturable (VBNC) cells during microbial fermentation underlines the requirement of alternative methods for detecting and characterizing these emergent states not otherwise detectable by traditional culture-based methods (13). Flow cytometry (FC) has evolved as an outstanding tool in bioprocesses due to its usefulness in cell physiology monitoring (5, 12). The persistence of nonculturable cells during microbial fermentation has been attributed to changes in water activity, acidity, redox potential, nutrient availability, and starvation (14, 17, 18, 24, 25) or to the use of preserved starter cultures (20). Additionally, the quantification of catalytic activity is critical to bioprocess optimization, as it measures the individual contributions of different cell subpopulations to the global process (2, 13). Despite the loss of culturability under standard conditions, it is strongly suspected that VBNC cells remain alive, maintain the transport system and biosynthesis, and are able to metabolize substrates (16, 26). Gene expression has also been demonstrated previously (9, 23). However, although the physiology, biochemistry, and genetics of the VBNC state have been studied over the years, its functionality and biological implication are still issues under intense debate (1, 21, 22).

In this work, cider malolactic fermentation (MLF) was selected as a model system to clarify the role played by VBNC cells in bioprocesses. MLF was carried out under different SO_2 concentrations (0, 30, and 60 ppm total) for inducing VBNC states. The fermentation medium was sterile apple must or "green" cider (obtained just after alcoholic fermentation and containing 5.6% [vol/vol] ethanol), obtained as previously described (11). Sodium bisulfite was used for SO_2 treatments. MLF was carried out in duplicate at 22°C statically in 250-ml bottles. An indigenous strain of *Lactobacillus hilgardii* was inoculated at an optical density at 600 nm of ~0.1 to start MLF. Flasks were shaken just before sampling in order to homogenize the biomass content. Samples were taken aseptically at time intervals until malic acid was consumed (≤0.5 g liter^{-1}), and cells were collected and processed for further analysis as described previously (20). Supernatants were filtered (0.45 μm pore size) and frozen (−20°C) until chemical analysis. The amount of malic acid was determined by high-pressure liquid chromatography (Alliance 2690; Waters) with a photodiode array detector (Waters 996), as reported previously (19).

Evolution of bacterial subpopulations during MLF. Viable cells (measured as CFU ml^{-1}) were monitored by a plate counting method on MRS (in triplicate), as reported previously (20). Total cell counts were determined by DRAQ5 single staining, and metabolically active and dead cells were monitored by a dual-staining (Chem Chrome 6 [CV6] and propidium iodide [PI]) FC protocol carried out using a Cytomics FC 500 instrument (Beckman Coulter), as described previously (20). This dual-staining protocol was based on the detection of membrane integrity (PI) and intracellular esterase activity (CV6) as the metabolic probe. The evolution of total, viable, VBNC, and dead cells during MLF is shown in Fig. 1. A subpopulation of VBNC cells (calculated as the difference between metabolically active and viable cells) was found in both controls (Fig. 1a and d), as was detected previously under stress conditions during cider MLF (11). The use of sodium bisulfite caused a drop in cell viability even to the extent of a total absence (Fig. 1b, c, e, and f). Malic acid was consumed in all assays, showing the metabolic activity associated with the VBNC state, as previously observed (26). The addition of increasing SO_2 amounts did not really correspond to an increase in cell death rate but accelerated the transition to VBNC states. The effect was more drastic with green cider fermentation, due to a synergistic effect of SO_2 and other inhibitors, such as ethanol. Actually, SO_2 has been reported to lead wine yeast and bacteria to adopt VBNC states rather than to undergo cell death (6, 7, 15).

Determination of malolactic activities of viable and VBNC subpopulations. In order to quantify the malolactic activity of VBNC cells, experimental data for cell subpopulations and malate uptake were fitted (MicroMath Scientist version 2.0) to a segregated kinetic model developed previously (20). For kinetic modeling, the cell concentrations of bacterial subpopulations were expressed in g liter^{-1}, using corresponding cali-

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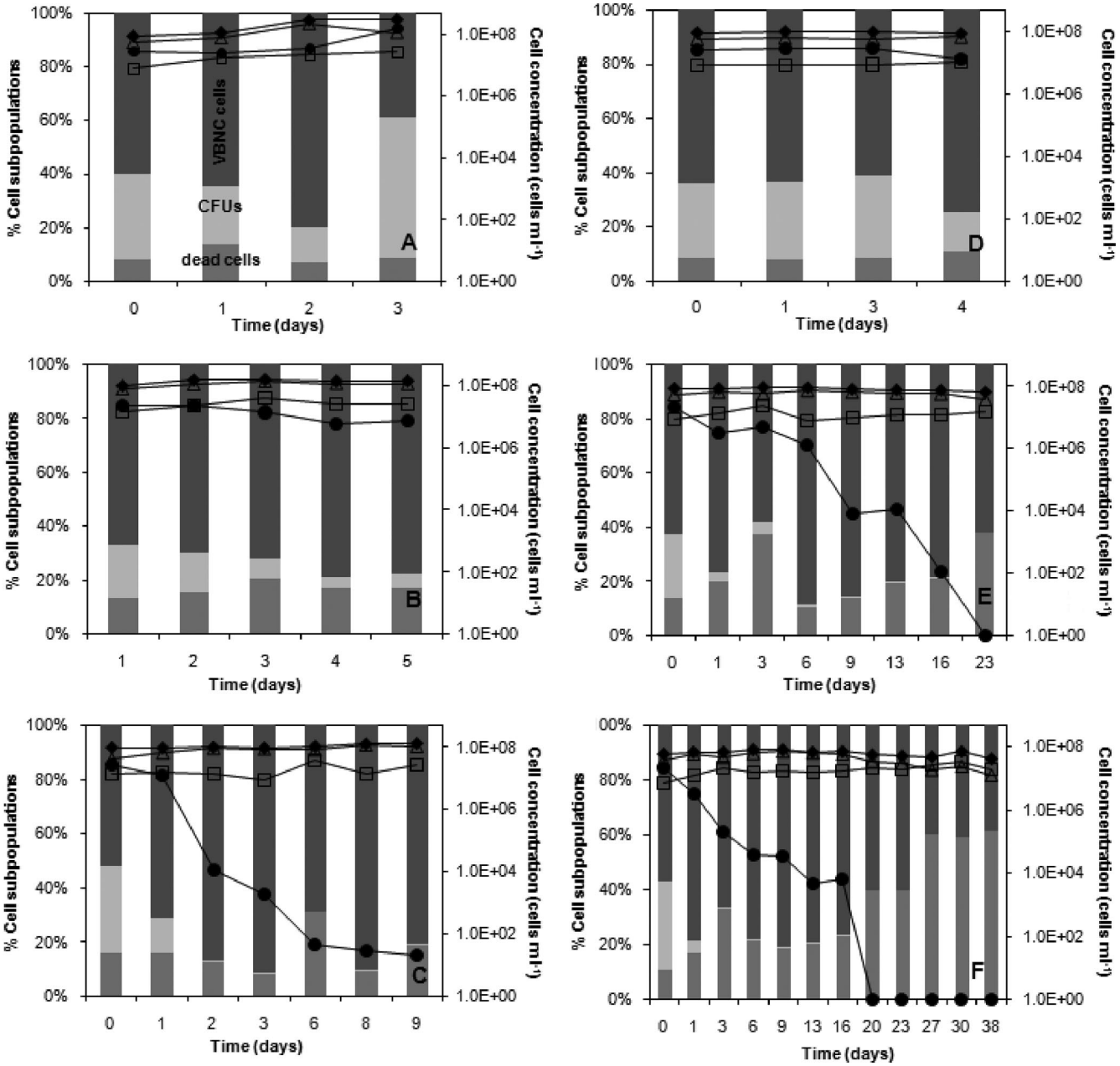
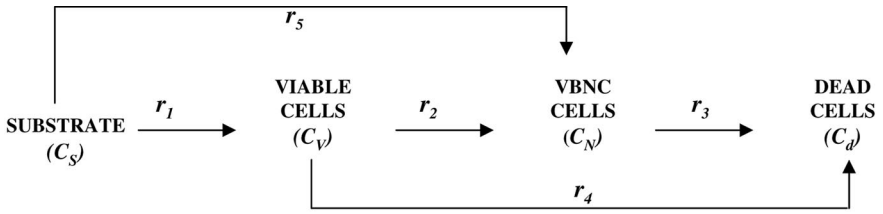


FIG. 1. Evolutions of different *L. hilgardii* subpopulations during MLF in apple must (A), apple must plus 30 ppm SO_2 (B), apple must plus 60 ppm SO_2 (C), green cider (D), green cider plus 30 ppm SO_2 (E), and green cider plus 60 ppm SO_2 (F). Subpopulations of VBNC cells, viable cells (CFU), and dead cells are plotted as percentages of the total cell counts on the principal *y* axis. Total (◆), VBNC (△), viable (CFU) (●), and dead (□) cells are expressed as cells ml^{-1} on the secondary *y* axis. Note the different values along the *x* axis in the different graphs.

TABLE 1. Kinetic parameters[a] determined during MLF in apple must and green cider in the absence and presence of SO_2

Fermentation medium	$1/\beta'$ (g $liter^{-1}$)	$1/\beta''$ (g $liter^{-1}$)	k_1 (h^{-1})	k_2 (h^{-1})	k_3 (h^{-1})	k_4 (h^{-1})	k_5 (h^{-1})
Apple must	0.14	0.18	0.034	0.001	5.0×10^{-4}	1.0×10^{-4}	0.053
Apple must plus 30 ppm SO_2		0.12		0.017	5.8×10^{-4}	1.0×10^{-4}	0.021
Apple must plus 60 ppm SO_2		0.10		0.048	6.5×10^{-4}	1.5×10^{-4}	0.014
Green cider		0.050		0.002	5.5×10^{-4}	1.5×10^{-4}	1.5×10^{-4}
Green cider plus 30 ppm SO_2		0.048		0.031	6.0×10^{-4}	1.8×10^{-4}	1.1×10^{-4}
Green cider plus 60 ppm SO_2				0.088	1.0×10^{-3}	3.0×10^{-4}	

[a] β' and β'', the inverses of the maximum concentrations of viable and VBNC subpopulations, respectively; k_1 and k_5, growth kinetic constants of viable and VBNC subpopulations, respectively; k_2, transformation constant from the viable state to the VBNC state; k_3 and k_4, death kinetic constants of viable and VBNC subpopulations, respectively.



Equations

$$r_1 = k_1 C_V (1 - \beta' C_V) \quad (1)$$

$$r_2 = k_2 C_V \quad (2)$$

$$r_3 = k_3 C_N \quad (3)$$

$$r_4 = k_4 C_V \quad (4)$$

$$r_5 = k_5 C_N (1 - \beta'' C_N) \quad (5)$$

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\left(\frac{dC_x}{dt}\right) + m_s C_x \quad (6)$$

Nomenclature

Cell concentration (gl^{-1}) = $C_X = C_V + C_N$

$Y_{X/S}$ = *apparent yield coefficient (g cell* g^{-1} *substrate)*

m_S = *cell maintenance coefficient (g substrate* h^{-1} g^{-1} *cell)*

$\beta' = 1/C_{V\,max}$

$\beta'' = 1/C_{N\,max}$

r_1 *and* r_5, *growth net rates of viable and VBNC cells*

r_2, *transformation rate from viable to VBNC state*

r_3 *and* r_4, *death net rates of viable and VBNC cells*

FIG. 2. Scheme and equations of the segregated kinetic model (adapted from reference 20).

bration curves determined previously (20). This model includes different cell physiological states and levels of malate consumption by metabolically active cells (Fig. 2). Enzymatic assays carried out as reported previously (10), using CV6-negative, PI-positive whole cells (referred to as dead cells in this work), showed a lack of malolactic activity, as expected. Thus, the total biomass involved in malate conversion (C_X) was considered to be formed by VBNC (C_N) and viable (C_V) subpopulations. Since malate metabolism is not directly linked to cell growth or energy obtainment (4), the initial equation for substrate uptake (equation 6 in Fig. 2) is simplified as follows: $-(dS/dT) = m_V C_V + m_N C_N$, where m_V and m_N are cell maintenance coefficients of viable and VBNC subpopulations, respectively. These parameters can be considered a measure of the individual contribution of each subpopulation to the global process. This equation was finally expressed as follows: $-(dS/dT) = q_V C_V + q_N C_N$, where q_V and q_N are specific uptake rates of viable and VBNC subpopulations, respectively.

The experimental data and predicted values of different cell subpopulations during MLF are shown in Fig. 3. The values of the kinetic parameters are given in Table 1. The values obtained for k_1 and k_5 (growth kinetic constants) indicated that VBNC cells divided in apple must at a rate higher than that of viable cells ($k_5 > k_1$). The values of death constants, k_3 and k_4, showed slightly higher cell death rates as SO_2 content increased, with $k_3 > k_4$ in all cases (higher VBNC death net rate).

Finally, malic uptake experimental data were also fitted to the equation $-(dS/dT) = q_V C_V + q_N C_N$, and parameters were calculated in each case, assuming a constant value during fermentation (Fig. 3; Table 2). In all cases, the q_V/q_N ratio was close to 2.0 during fermentation experiments. It was stated previously that the catalytic capacity of damaged cells is generally lower than that of viable cells (3). Entrance into the VBNC state can be accompanied by a reduction in substrate transport and metabolic activity levels in order to minimize cellular energetic requirements (16), as has been observed with nonculturable lactococci in response to sugar starvation (8).

TABLE 2. Specific uptake rates of viable and VBNC subpopulations during MLF in apple must and green cider at concentrations of 0, 30, and 60 ppm SO_2

Fermentation medium	q_V (g malate h^{-1} g^{-1} CFU)	q_N (g malate h^{-1} g^{-1} VBNC cells)	q_V/q_N	R^2
Apple must	1.10	0.54	2.04	0.97
Apple must plus 30 ppm SO_2	0.79	0.36	2.19	0.98
Apple must plus 60 ppm SO_2	0.48	0.17	2.82	0.99
Green cider	0.77	0.35	2.20	0.98
Green cider plus 30 ppm SO_2	0.13	0.07	1.94	0.99
Green cider plus 60 ppm SO_2	0.11	0.05	2.14	0.97

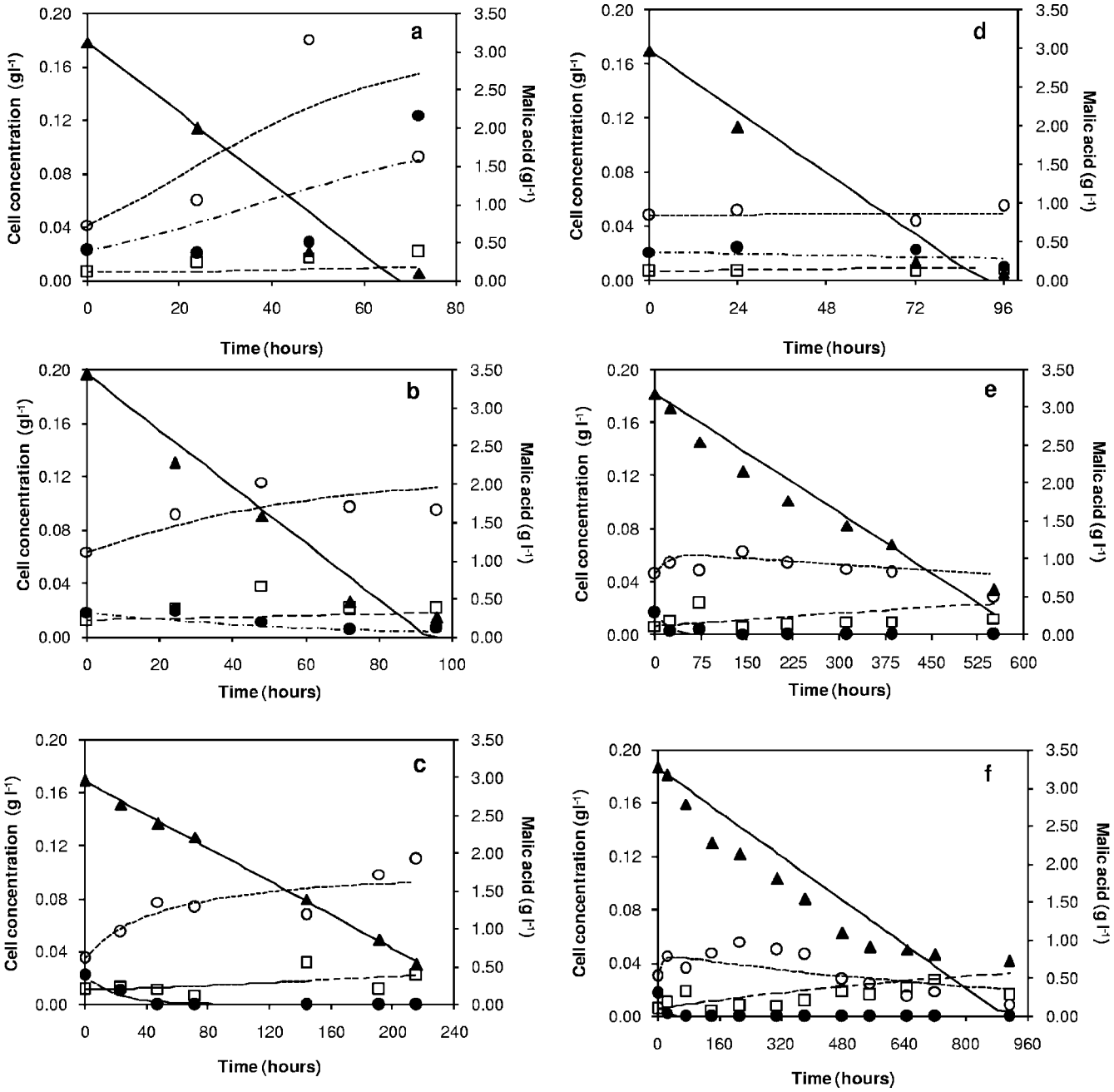


FIG. 3. Experimental data (symbols) and calculated values (lines) for *L. hilgardii* VBNC (○), viable (CFU) (●), and dead (□) cells and L-malic acid consumption (▲) during MLF in apple must (a), apple must plus 30 ppm SO_2 (b), apple must plus 60 ppm SO_2 (c), green cider (d), green cider plus 30 ppm SO_2 (e), and green cider plus 60 ppm SO_2 (f).

Conclusions. VBNC cells conducting MLF showed a state of reduced metabolic activity. The specific malate uptake rate of the VBNC subpopulation was approximately 50% of that found for the viable population in all cases tested, irrespective of medium composition and SO_2 concentration. VBNC cells became the majority of the total population in all cases (70 to 90%). These results may help to clarify, in quantitative terms, the real contribution of the VBNC subpopulation to fermentation processes. This outcome could be useful for bioprocess control and optimization on an industrial scale.

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