# The LACTOENOS 350 PreAc® a new tool to manage malolactic fermentation in white wines

*Vincent RENOUF*

*LAFFORT - BP17 - 33072 BORDEAUX - vincent.renouf@laffort.com*

*LAFFORT has continued the advance in pre-acclimated bacterial development with **LACTOENOS 350 PreAc®**, which follows on from the highly successful **450 PreAc®** strain. **LACTOENOS 350 PreAc®** PreAc exhibits high acid stress tolerance, allowing it to perform malolactic fermentation in wines with a pH of 2.9, high alcohol tolerance (16 %), and good thermal tolerance (15 °C), and so is particularly well-suited for malolactic fermentation in sparkling wine base. With minimal diacetyl and acetic acid production, **LACTOENOS 350 PreAc®** is ideally suited for malolactic fermentations where wine neutrality is required, and is also suitable for late co-inoculation at the end of alcoholic fermentation. A genetic analysis of **LACTOENOS 350 PreAc®** has allowed marker genes to be identified for enhanced performance, which will be used to direct research into future bacterial strain isolation.*

Malolactic fermentation (MLF), resulting from the action of lactic bacteria, mainly *Oenococcus oeni* species, is an essential stage in the winemaking process. The vast majority of wines made from red grape must worldwide undergo MLF. Besides the malolactic conversion itself and the **decrease of the wine acidity**, lactic bacteria had organoleptics impact. In particular, Bloem and de Revel (2007) demonstrated the contribution of lactic bacteria to revealing oaky aromas. From a microbiological standpoint, good management of MLF offers the advantage of **preventing spoilage caused by other microorganisms**, as continuing fermentation activity after AF ensures that the ecosystem is constantly occupied and offers no opportunity for spoilage microorganisms to develop (***Brettanomyces***, etc.) (Renouf and Murat 2008). Furthermore, sulfiting the wine after MLF affords additional protection. Finally, promoting the early onset of MLF by **using malolactic starter reduces the energy costs associated with MLF**: the faster MLF is completed, the lower the heating costs (Renouf et al. 2008).

Nevertheless, MLF is less widely implemented in white wines. Its use depends on the grape variety and vineyard region, as well as the winemaker's preference. **In the case of great Chardonnay, MLF is essential to control acidity**, as these wines frequently have very low pH and high acidity. Full MLF results in a considerable decrease in the total acidity of a dry white wine: approximately 0.4 g of $H_2SO_4$ per gram L-malic acid is consumed, accompanied by a significant increase in pH (5-10% of the initial pH). Finally, MLF makes a significant contribution to the aromatic complexity of Chardonnay wines. : «*A Chardonnay wine that has not undergone MLF cannot be considered a great Chardonnay. Not only does MLF not attenuate varietal aroma, but it also develops or stabilizes certain nuances, revealing, to a certain extent, the wines aromatic fullness*» (Ribéreau-Gayon et al., 1998).

In other vineyard areas, MLF in white wine may be useful for different reasons. In the Charente vineyards, for example, it is beneficial for wines to be distilled into Cognac, as, during MLF, l**actic bacteria convert part of the ethanal** produced by yeasts back into ethanol. In the example presented in table I, the ethanal content was halved thanks to MLF.

MLF is not generally applied to white wines made from other grape varieties, to maintain a certain acidity and freshness. However, it should be noted that consumer expectations change and current market trends prefer white wines with softness and body on the palate. MLF (total or partial, applied to the entire production or a few batches to be included in the final blend) may have a positive impact without any significant detrimental impact on varietal and fermentation aromas (Table I).

*Table I: Example of the impact of total or partial MLF on the analytical characteristics of white wines.*

| | | Charente wines | | Bordeaux wines | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ugni-blanc | | Sauvignon A | | Sauvignon B | | Sémillon C | |
| | | Control without FML | Total MLF | Control without FML | Total MLF | Control without FML | Partial MLF | Control without FML | Partial MLF |
| L-malic acid (g/L) | | 4,4 | 0,16 | 3,78 | 0,22 | 2,42 | 1,24 | 2,2 | 0,58 |
| Ethanal (mg/L) | | 94 | 46 | | | | | | |
| Volatile acidity (g/L $H_2SO_4$) | | 0,34 | 0,42 | 0,18 ±0,00 | 0,22 ±0,06 | 0,25 ±0,00 | 0,28 ±0,01 | 0,15 ±0,02 | 0,24 ±0,03 |
| Total acidity (g/L $H_2SO_4$) | | 6,22 | 4,54 | 6,68 ±0,12 | 5,02 ±0,04 | 5,64 ±0,02 | 5,18 ±0,00 | 5,15 ±0,12 | 4,27 ±0,03 |
| pH | | 3,04 | 324 | 2,95 ±0,03 | 3,12 ±0,04 | 3,10 ±0,02 | 3,22 ±0,06 | 3,10 ±0,00 | 3,17 ±0,01 |
| Varietal aromas | 4-mercaptopentanone | | | 4,4 ±0,2 | 3,2 ±0,4 | 2,8 ± 0,8 | 1,8 ±0,3 | 2,0 ±0,0 | 2,8 ±0,2 |
| Volatile thiols (ng/L) | 3-mercaptohexan-1-ol | | | 66 ±3 | 68 ±6 | 78 ±14 | 55 ±4 | 280 ±12 | 250 ±4 |
| | 3MH acetate | | | 14 ±4 | 12 ±3 | 12 ±3 | 9 ±1 | 24 ±2 | 31 ±5 |
| Fermentative aromas (mg/L) | Phényl-2-ethanol | | | 72 ±8 | 98 ±4 | 126 ±6 | 132 ±12 | 93 ±2 | 100 ±7 |
| | Phenylethanol acetate | | | 0,8 ±0,0 | 0,5 ±0,1 | 0,8 ±0,0 | 0,7 ±0,0 | 0,6 ±0,0 | 0,5 ±0,0 |
| | Isoamyl acetate | | | 4,0 ±0,2 | 3,5 ±0,0 | 4,4 ±0,0 | 4,0 ±0,1 | 5,0 ±0,0 | 4,7 ±0,4 |

However, unfortunately, **low pH and/ or high acidity are the two main obstacles to bacterial developmen**t and malolactic activity in wine.

Malolactic activity is one consequence of the action of the malolactic enzyme located in the bacteria's cytosol and an L-malic acid transporter in the cell walls. The malolactic enzyme, identified in the 1970s by Lonvaud's team in Bordeaux, is actually a complex of two identical protein subunits. This activity requires a pH in the vicinity of 5.75 (Figure 1). This pH is not only essential for enzyme activity, but also for maintaining a favorable electrochemical gradient for L-malic acid to be brought into the cell by the malate permease.

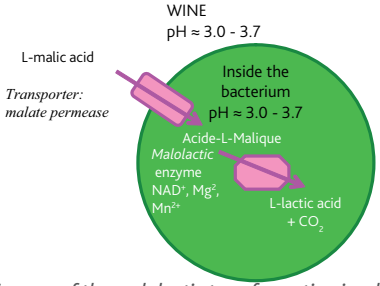



*Figure 1: Diagram of the malolactic transformation in a bacterium and the differences in pH between the extracellular medium (wine) and the inside of the cell (the bacterium's cytosol).*

LAFFORT
*l'œnologie par nature*

Thus, the malolactic reaction takes place inside the cell at a pH much higher than that in wine. The bacterium must therefore buffer its intracellular pH, despite the relatively high acidity of its environment. In an acid medium like wine, the lower the pH, the more effort *O. oeni* cells devote to controlling pH. Total acidity, i.e. the concentrations of citric, tartaric, succinic, and even malic acid, is also essential. Acids from the wine penetrate the cell in undissociated form, then dissociate inside it. The proton release causes a decrease in intracellular pH, thus reducing not only malolactic activity but also the electrochemical gradient necessary for L-malic acid entry. The cell is, therefore, required to make extra effort to export protons in wine with high total acidity.

**Malolactic starters are the best tools for managing malolactic fermentation**. They trigger MLF, following inoculation and an adaptation phase, thus not only saving time but also ensuring more **reliable fermentation and controlling microbial spoilage**. At present, most of the malolactic starters available for winemaking are strains of *O. oeni*, isolated from the red wines in which they are mainly used. They are selected on good tolerance for high ethanol content and low temperatures, as well as the absence of undesirable metabolic traits (e.g. the genes involved in producing biogenic amines, etc.). From a physiological standpoint, stress caused by ethanol and temperature has a common target: the cell membrane. These factors affect its fluidity and, consequently, enzyme activity. Among these enzymes, ATPase pumps play a key role in the mechanism for resisting acid stress, due to their involvement in proton transport. Consequently, if a strain's homeostasis enables it to regulate its membrane characteristics to resist high ethanol content or low temperatures, it should also be able to adapt to low pH. Thus, the use of a starter selected for red wines to trigger MLF in more acidic white wines requires an adaptation phase, so that the cells gradually become acclimatized to an increasingly acid environment. The method consists of mixing the started into a series of vat samples where the pH is modified by adding the appropriate quantity of potassium bicarbonate (Figure 2). The aim is to put the bacteria into an increasingly acidic environment, so that the cells synthesize the enzymes involved in stress resistance, to optimize their survival rate following inoculation into the wine.

*Inoculation protocol with gradual adaptation of the malolactic starter required for 50 hL wine at pH=3.0.*

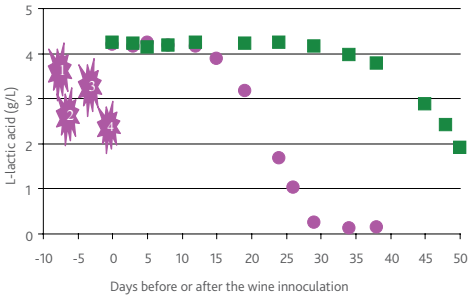
**Day D1:**
⇨Mix 2.5 L non-chlorinated, non-sulfited water with 2.5 L wine, deacidified to pH= 4.0 (potassium bicarbonate).
⇨Inoculate with bacteria (total dose for 50 hL)
**Day D2**
⇨Add 5 L wine deacidified to pH= 3.5 (potassium bicarbonate).
**Day D4**
⇨Use the 10 L preparation to inoculate 5 hL wine
**Following days:**
⇨Monitor the degradation of L-malic acid in the wine. When the L-malic acid content reaches two-thirds of its initial value, the preparation is ready to inoculate the 50 hL vat.

Maintain a constant temperature above 18°C

Use clean containers and avoid contact with air.

N.B: If other parameters in the wine, besides pH and acidity, are unfavorable: ACV >13 %, total $SO_2$ > 30 mg/L, add the equivalent of 20 g/hL of **MALOSTART®**.

Example: Sauvignon Blanc
initial pH = 2.98
ACV= 13.2 %
total $SO_2$= 12 mg/L
L-malic acid = 4.2 g/L
Total acidity = 6.68 g/L $H_2SO_4$
Temperature 22°C




● Inoculation with strain A, using the inoculation protocol including gradual adaptation to pH. 1: 1st stage (D-8 before final inoculation), 2: 2nd stage (D-7 before final inoculation), 3: 3rd stage (D-5 before inoculation), 4: Final inoculation + 20 g/hL of *MALOSTART®*

■ *Inoculation with strain A according to the standard protocol (+ 20 g/hL of MALOSTART®).*

*Figure 2 :Example of protocol for inoculation with gradual adaptation to pH and comparison with direct inoculation into an «acidic» dry white wine.*

With acclimatization phases in deacidified media, the latency phase was halved and MLF was completed 32 days after inoculation, whereas only 50 % of the L-malic acid had been degraded 50 days after direct inoculation. **This shorter completion time minimizes the risk of spoilage that occurs when MLF is too sluggish**. At the end of MLF, following inoculation with acclimatized starter, the volatile acidity and diacetyl concentrations were only 0.14 g/L $H_2SO_4$ and 0.2 g/L, respectively, whereas concentrations following direct inoculation were as high as 0.25 g/L $H_2SO_4$ volatile acidity and 0.4 g/L diacetyl. By measuring the levels of glutathione (Figure 3) in two wines, thirty days after the end of the MLF, we find that the wine, in which the **MLF was the fastest, has a wealth of glutathione more than 2 times bigger than the other modality.**

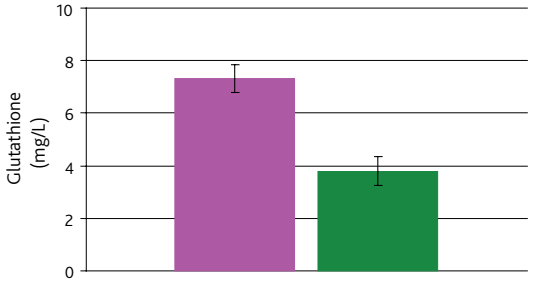



*Figure 3: Level of glutathione in the two wines of the figure 3. ■ is the wine inoculated with the adapted bacteria and ■ is the direct inoculation.*

But, the acclimatization phase increases the winemaker's workload. While this acclimatization process is easily implemented under laboratory conditions, where it is easy to keep containers and equipment clean, control temperature, and carry out frequent analyses, etc., this is much more difficult in a full-scale winery.

In order to solve these problems and facilitate the winemaker's task, recent research has resulted in **the selection of *O. oeni* strains with enhanced tolerance of low pH**. The traditional selection protocols were «reversed» to select strains tolerant of low pH. Rather than isolating strains that completed MLF rapidly, then examining their behavior under high-stress conditions, the first stage was to isolate *O. oeni* strains from white wines with low pH (< 3.1) and high total acidity (> 6 g/L of $H_2SO_4$), then use physiological and genetic testing in the laboratory to confirm their tolerance to acidity. Only strains exhibiting suitable characteristics at that stage were tested for their fermentation capabilities, resistance to high ACV, low temperatures, spoilage metabolisms, etc.

A collection of a hundred strains isolated in this way were assessed to identify those most resistant to low pH. Simple tests, monitoring MLF in dry white wines at different pH, confirmed the capacity of certain strains to resist pH stress. These strains were compared with malolactic starters (Figure 4) intended for red wines (A - D).

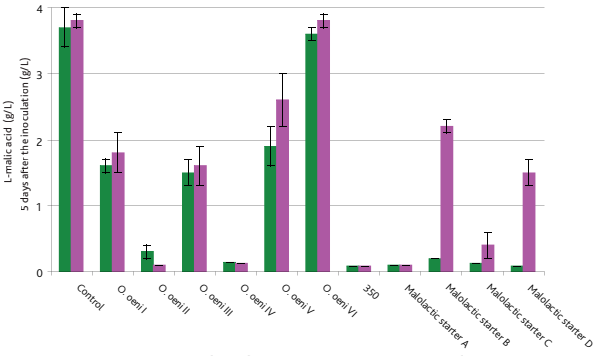



*Figure 4: MLF test in sample white wines at pH 3.4 and 3.0, comparing experimental strains (O. oeni I, II, III, IV, V, VI, and 350) with commercial malolactic starters (Malolactic starters A, B, C, and D) (temperature = 18°C initial L-malic acid = 3.8 g/L)*

All the malolactic starters designed for red wines completed MLF perfectly at pH 3.4 but only one of them was also successful at pH 3.0. The difference in malolactic starter activity levels at the different pH levels was very striking. There was much less variation among the *O. oeni* strains derived from acidic wines. Those that completed MLF at pH 3.4 were also successful at pH 3.0. **It is the case of the strain 350. The strain 350 was isolated from an acidic (pH=2,8) and alcoholic (ACV=15,2 % vol.) wine where MLF was spontaneously performed beside low temperature (15°C). Other similar tests showed that the strain 350 is the most effective to resist and to perform MLF at low pH among the collection of more one hundred *O. oeni*.**

The results observed were obviously due to genetic differences, so identifying the genes has made it possible to include genetic testing to optimize the selection process for new strains. **These innovative tools to characterize the intrinsic abilities of *O. oeni* strains were for the first time used during the study of the strain 350.** For example, a gene encoding for an ATPase pump involved in cadmium transport was revealed to be implied in the resistance of acid stress of *O. oeni* cells. Among the strains in figure 4, it is interesting to note that those with the best aptitude for winemaking were also the only two (*O. oeni* II and 350) to possess this gene (Figure 5).

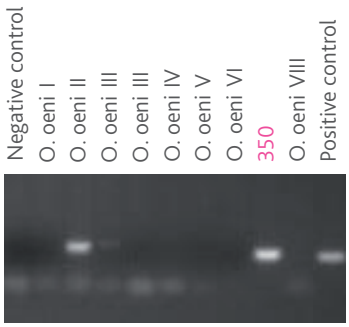



*Figure 5: Photograph of the gel electrophoresis results of the PCR test for the genetic marker encoding the ATPase pump cadmium transporter gene (Renouf et al. 2007).*

Another gene encoding for a membrane ATPase (clpL gene) involved in acid stress resistance, was present in all strains tested, although with some variations in the nucleotide sequence. In fact, there are two possible sequences: H and B. As is the case in humans, where genes involved in eye color are present in all individuals in varying sequences (genetic polymorphism), resulting in different eye colors, all the *O. oeni* strains had the clpL gene, but in two different sequences, which probably accounts for the physiological differences. Very recently, Renouf et al. 2009 reported that a collection of several hundred strains of *O. oeni* could be divided into two groups, according to physiological and genetic criteria. Analysis of the clpL gene sequence revealed that the strains in the first group all had sequence H, whereas the other half had sequence B. The strains in the first group adapted better to low pH, while those in the second group were better-suited to higher pH (Table II). In our works of selection, **the strain 350 has the sequence H that confirms its remarkable genetic predisposition to resist at low pH.**

*Table II: Genetic differences and adaptation among a selection of strains isolated from white wines, corresponding to the two groups defined by Renouf et al. (2009).*

| | | Group 1 of *O. oeni* strains * | Group 2 of *O. oeni* strains |
|---|---|---|---|
| Frequency of polymorphic sites in the target sequence of the clpL gene | | sequence H : 100% | sequence H : 50%<br>sequence B : 50% |
| Implantation rate** | pH = 3,25 | 0,68 | 0,12 |
| | pH = 3,5 | 2,2 | 0,98 |
| | pH = 3,25 | 4,8 | 6,4 |

* The strain 350 belongs to the group H which is group of the more resistant strains to acid stress.

**ratio of the population in a wine on d+2 to the population at the time of inoculation ACV=12.4 % (% Vol.), Malic Ac.= 1.8 g/L

Then, the **strain 350** has been associated with **the PreAc production process which exclusively developed by LAFFORT** to ensure a first acclimatizing stage of the bacterium during their production. After, at the winery, a simple and rapid final acclimatization in presence of nutrients (**ENERGIZER®**) optimises **the efficiency of the LACTOENOS 350 PreAc® inoculum.**

Study 6-A illustrates the effectiveness of the **LACTOENOS 350 PreAc®** in real winemaking situation (pH=3.12 and ACV= 13.4 %). **LACTOENOS 350 PreAc®** completed MLF 25 after inoculation, whereas the starter used as a control had hardly started MLF 30 days after inoculation (and only after 20 g/hL **MALOSTART®** activator was added).

Test 6-b illustrates the use of the **LACTOENOS 350 PreAc®** in late co-inoculation, i.e. before the end of AF, at 0°Brix. Early inoculation facilitated adaptation of the starter to the medium. As one could expect, considering the failure of the starter inoculated after AF, MLF would probably have been even more problematic if the bacteria had been added before AF. This reflected the effect of alcohol stress, PAC = 14.4 % (% Vol.), in addition to low pH. Note that, in wine white, besides facilitating adaptation of the starter to the alcohol content, starting MLF by co-inoculation has the advantage that the medium is less oxidative, thus minimizing the development of buttery or stale odors and ensuring that the wine retains its aromatic potential.

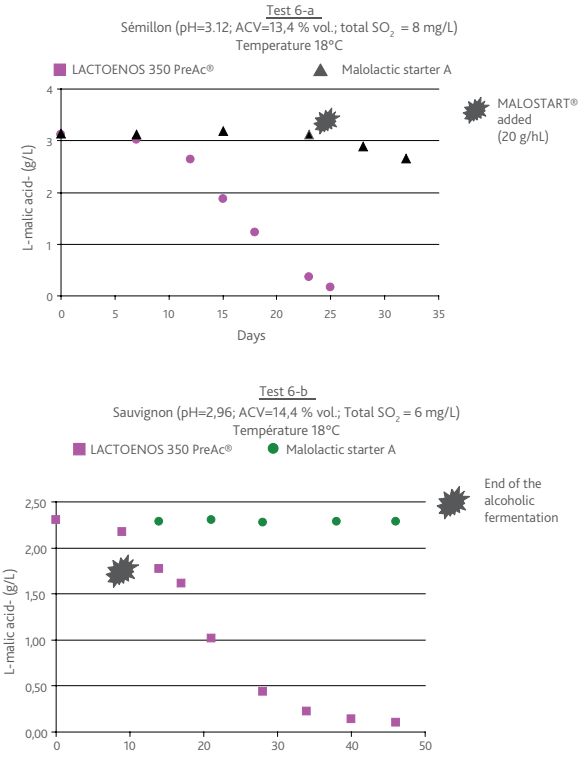



*Figure 6: Results of trials for the* **LACTOENOS 350 PreAc®**

In white winemaking, MLF is an effective tool for controlling acidity and promoting the desired sensations of richness and volume on the palate. However, the malolactic starters currently marketed for red wines have great difficulty dealing with the frequently low pH of white wine. Successful MLF requires a multi-stage adaptation process to accustom the bacteria to increasingly low pH, so that the cells gradually cope with the final acidity of the wine. These highly-effective protocols are essential in cases where the parameters, particularly alcohol content, are not conducive to rapid completion of MLF. But these protocols could be relatively constraining for the winemakers. Therefore we have focused our research on the selection of *O. oeni* strains intrinsically resistant to acid stress in white wines to provide a simpler method.

Thanks to recent progress in knowledge of bacterial genetics and physiology, effective tools for characterizing strains are now available. **We have controlled the presence of certain genes implied in stress resistance**. In combination with these innovative tools and standard laboratory testing techniques, the bacteria **LACTOENOS 350 PreAc® was developed**. Its used in real conditions at cellar during winemaking from the vintage 2008 gave entirely satisfactory results. **The LACTOENOS 350 PreAc® provides a really efficient tool to perform the MLF in whites wines even in the more acidic.**

## BIBLIOGRAPHIE


Bloen, A., de Revel, G., 2007. Fermentation malolactique en barriques, le rôle des bactéries lactiques dans la libération de l'arôme boisé. Rev. Oenol. 123, 25-27.

Renouf, V., Murat, M.L., 2008. L'utilisation de levains malolactiques pour une meilleure maîtrise du risque Brettanomyces. Rev. Œnol. 126, 11-15.

Renouf, V., Barona de la Fuente, F., Murat, M.L., 2008 Ce que coûtent réellement les levains malolactiques. Rev. Fr. Œnol. 232, cahier technique.

Renouf, V., Delaherche, A., Claisse, O., Lonvaud-Funel, A., 2007. Correlation between indigenous Oenococcus oeni strain resistance and the presence of genetic marker. J. Ind. Microbiol. Biotechnol. 35, 27-33.

Renouf, V., Cadet Vayssieres, L., Claisse, O., Lonvaud-Funel, A., 2009. Genetic and phenotypic evidence for two groups of Oenococcus oeni strains and their prevalence during winemaking. Appl. Biotech. Microbiol. 10.1007/s00253-008-1843-1

Ribéreau-Gayon P., Dubourdieu D., Donèche B., Londaud A., 1998. Traité d'oenologie, Tome I, Microbiologie du vin, Vinifications. Ed Dunod. 541.