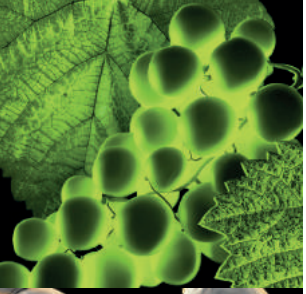

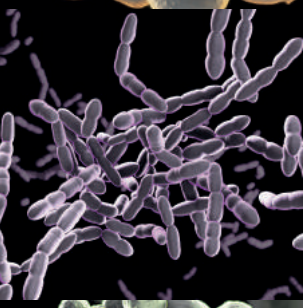

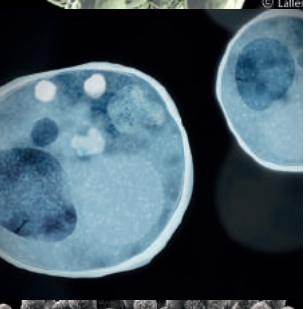
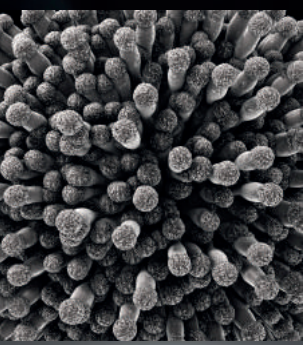


# THE PRODUCTION OF SELECTED WINE BACTERIA FOR OPTIMAL WINEMAKING: THE MBR® PROCESS

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A successful malolactic fermentation (MLF) in wine is easier and faster with an active and efficient population of selected wine bacteria. Winemakers use selected wine bacteria not only to secure MLF, but to regulate the process time, to avoid the growth of spoilage microorganisms, and also as a tool to modulate the flavours and texture of wine, as well to reduce the use of energy in the winery. One of the most efficient tools for MLF is the use of Lallemand's MBR® (*Membrane Be Ready*) selected wine bacteria recognized and appreciated by winemakers for its reliability and for its direct inoculation in wine. This article will review how the MBR® process efficiently prepares selected wine bacteria for malolactic fermentation.

## 1. The challenges in production of selected wine bacteria

For more than 30 years Lallemand has been producing active freeze-dried bacteria. A unique expertise was acquired on the importance of the production process on the vitality and malolactic activity after direct inoculation of the selected wine bacteria into wine.

Because of the extremely challenging wine matrix, the production of selected wine LAB is highly specific and not comparable to the production of other LAB for dairy or probiotic applications. If we produce an *Oenococcus oeni* like we produce a LAB for dairy for example, even with typical optimal growth conditions, we will obtain a large amount of biomass but with low MLF activity and low survival rate when introduced into wine because, as seen in Table 1, the wine conditions are very different from the optimal conditions of LAB growth.

| | **Wine conditions** | **Optimum conditions for *O. œni* growth** |
|---|---|---|
| **pH** | 2.8 – 4.0 | 4.5 – 5.5 |
| **Ethanol (% v/v)** | 10 – 17 | 0 |
| **Total $SO_2$ (mg/L)** | 0 – 100 | 0 |
| **Temperature (°C)** | 14 – 30 | 30 |
| **Polyphenolic compounds** | Present | Absent |

**Table 1: Comparison of wine/must conditions and optimum parameters for *O. œni* growth.**

Understanding the unique conditions found in wine, we have designed our specific MBR™ production process to obtain cells adapted to wine conditions and to ensure good survival and high malolactic activity expression upon their direct inoculation into wine. Each of our MBR® wine bacteria is produced to obtain their optimal physiological state with regard to vitality and metabolic activity (MLF activity and aroma metabolism).

## 2. The basis of the MBR® process: Understanding *O. œni* stress resistance

To develop the resistance mechanisms of the cells, certain stresses have to be applied during the production process, to guarantee a good survival and viability of the LAB upon direct inoculation in wine. These resistance functions are related to stress mechanisms, as the bacteria cell develops strategies to overcome the stress consequences enabling them to survive in wine conditions.

Stress can be defined as any physical, chemical, or nutritional change that affects the survival and growth of a microorganism. The various stresses will induce physiological and molecular changes that will allow cells to adapt to new environmental conditions. Many studies have investigated the resistance of *O. œni* to wine stressors to understand the physiological reactions of this bacterium under these conditions (Guzzo *et al*., 1994, Guzzo *et al.,* 2000, Tourdot-Maréchal *et al.,* 2000, Da Silveira *et al.,* 2003, Beltramo *et al.,* 2004).

Several mechanisms have been identified describing the adaptation capacity of *O. œni* species:

1- In wine acidic conditions, the maintenance of the intracellular pH is essential to bacteria survival (Figure 1, point A).

Both activation of ATP-ase pump activities and MLF pathway contribute to maintain internal cellular pH, by consuming and/or excreting intracellular protons.

2- The membrane is the first target of the various stresses (Figure 1, point C). The barrier role of the membrane is affected by acid, heat or ethanol stresses. To adapt to these stress conditions, lipidic membrane composition and membrane fluidity are modulated.

3- One of the most important mechanisms in adaptation and stress resistance is the synthesis of new proteins such as the Heat Shock Proteins (HSP) (Figure 1, point B). These proteins permit to repair or degrade intracellular proteins.

It has been demonstrated within the species *O. œni* the induction of various stress genes under stress conditions (Guzzo *et al.,* 1994, Garbay et Lonvaud-Funel, 1996, Guzzo *et al.,* 1997)

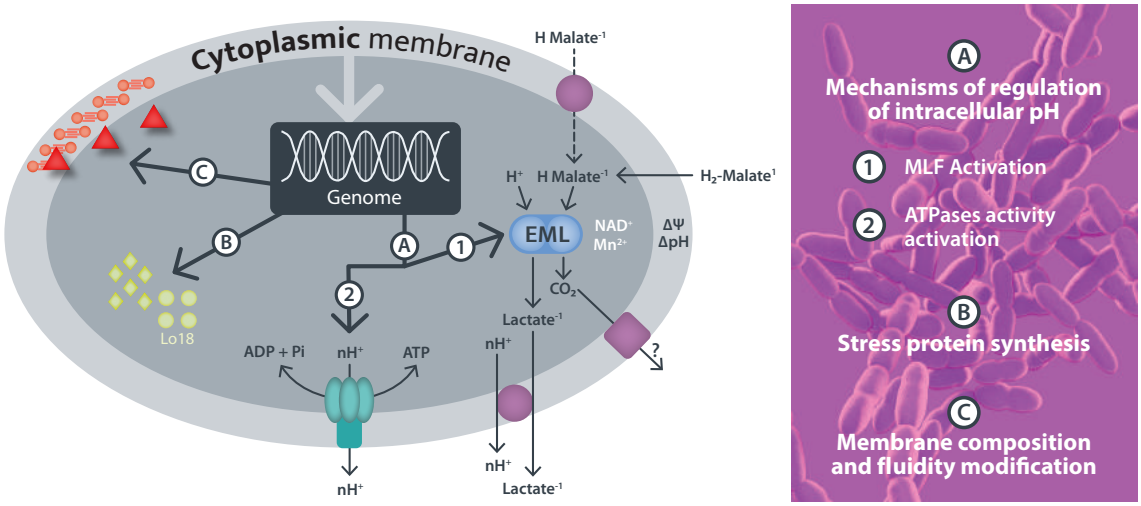


**Figure 1. Main mechanisms of resistance to wine conditions within the species *O. œni*. (Adapted from Desroche et al 2004)**

## 3. The MBR® process of production

Thus, one of the key steps during the production is the stress adaptation. As described above, the bacteria cells gradually adapt to the stressful wine conditions through different mechanisms. This step occurs during production in the main fermenter (Figure 2).

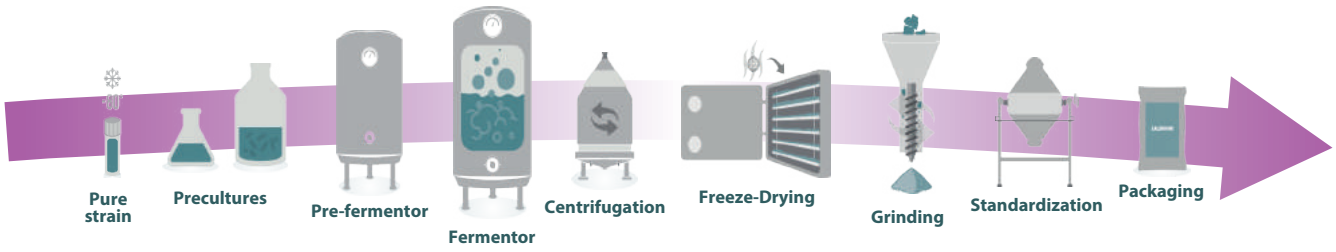


**Figure 2. Different steps of production of selected wine bacteria in freeze-dried form (Source: Lallemand Oenology – process of production for direct inoculation freeze-dried bacteria MBR®).**

Figures 3 and 4 show the same LAB strain produced under optimal growth conditions versus with our MBR® process and the impact how it performs under wine conditions. As expected, the MBR® process leads to better survival and viability as seen in Figure 3 and activity with fast malic acid degradation (Figure 4).

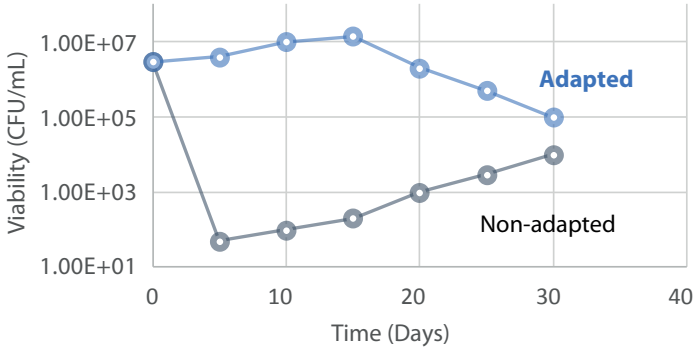


**Figure 3. Survival rate (viability) after inoculation into wine of the same strain produced in optimal conditions (non-adapted) and with our MBR® process (adapted).**

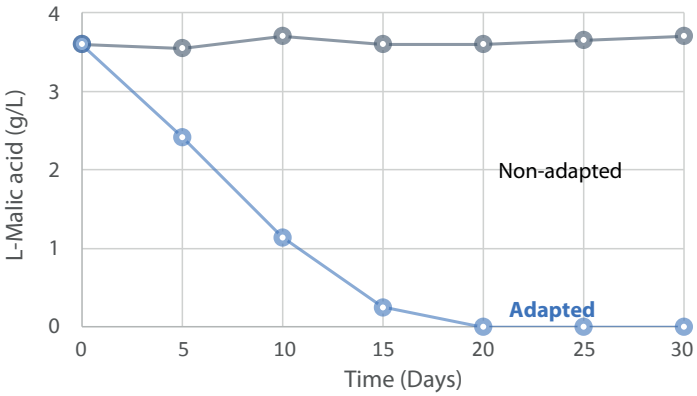


**Figure 4. Kinetics of malic acid degradation of the same strain produced in optimal conditions (non-adapted) and with our MBR® process (adapted)**

However, not all LAB strains are able to withstand this process of production. Some strains cannot be produced because of weak growth rate, low or no malolactic enzyme expression, or an inability to survive the challenging process of production and lyophilization. Due to their huge diversity and uniqueness, each strain of *Oenococcus oeni* is different and reacts differently to the production process. For each strain, we need to develop and optimize a specific recipe and a specific MBR® process protocol , to ensure their performance in wine.

We also know that the production process is a key factor that impacts the performance of the LAB. It was shown that the inoculation rate is not the main criteria to ensure a good start and performance of MLF. For example, in Figure 5, when inoculated, the MBR® bacteria population grows from $10^6$ cells/mL, upward, without and drop in population at inoculation. When compared to the a regular commercial preparation of Bacteria A for example, you can see a drop in population from $10^7$ cells/mL to $10^6$ cells/mL right at the onset of inoculation, showing the extreme sensitivity of this type of bacteria preparation compared to the MBR® bacteria.

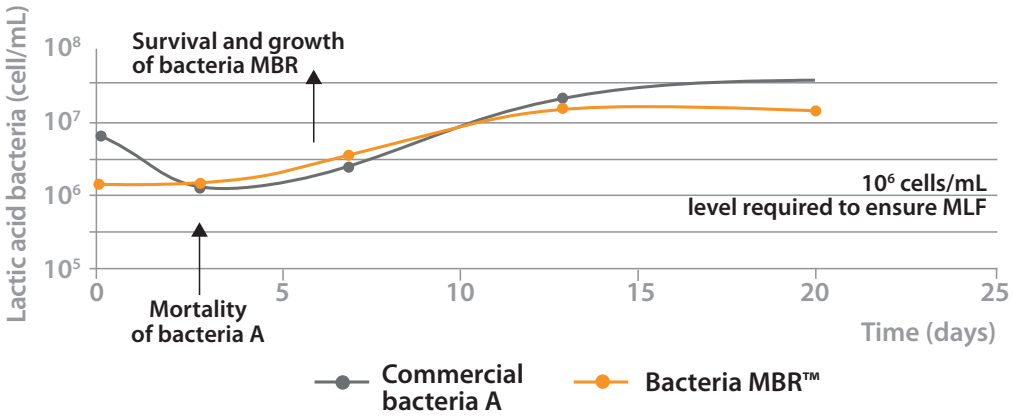


**Figure 5: Survival (cells/mL) right after inoculation into wine using 2 different commercial selected wine bacteria (regular versus MBR®)**

This translates into a more efficient MLF as seen in Figure 6, where the duration of MLF was significantly shorter with MBR® bacteria compared to the other commercial preparation. The process of production of bacteria cells in product A is less effective to adapt to the wine conditions compared to our MBR® bacteria. Comparing bacteria population is not an appropriate measure for efficiency for MLF when the production processes are different. In all cases, MBR® bacteria, because of their adapted state, always outperform other preparations of bacteria.

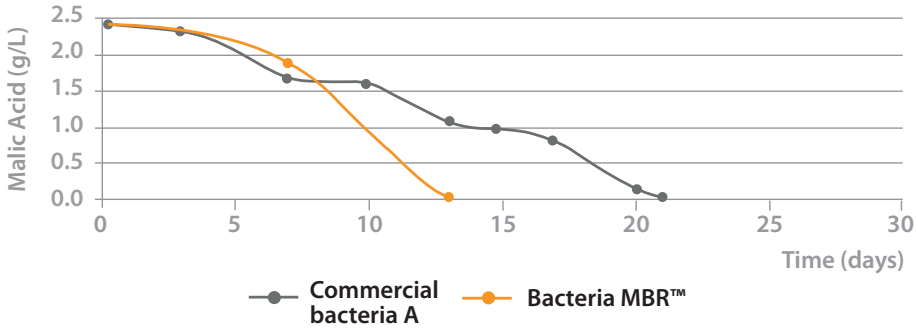


**Figure 6. Kinetics of malic acid degradation using 2 different commercial selected wine bacteria (regular versus MBR®)**

## 4. New insights - MBR® process

### Liquid form underperforms compared to MBR® form

A recent study investigated the differences of performance of the same strain produced in liquid or MBR® form, in wines with limiting conditions: low pH or high tannins concentrations (Matsumoto *et al.* 2022).

The study compared the performance of five different LAB strains, either inoculated into wine in liquid form (cultured in grape juice medium and harvested at a later stage of exponential phase, and called "liquid bacteria"), or produced under freeze-dried form with Lallemand MBR® process (direct inoculation into wine after a short step of rehydration).

As seen in Figure 7 , the bacteria produced in liquid form were not able to grow in the wine. Seven days after inoculation, percentage of survival was below 100%. Adding tannins into this wine, made the medium even more difficult for the survival and the growth of bacteria in liquid form. Whereas, the MBR® bacteria could survive and grow in all conditions (even with 2.5 g/L addition of inhibitory tannins). The bacteria in liquid form had very low survival rate (<10%) in these extreme conditions.

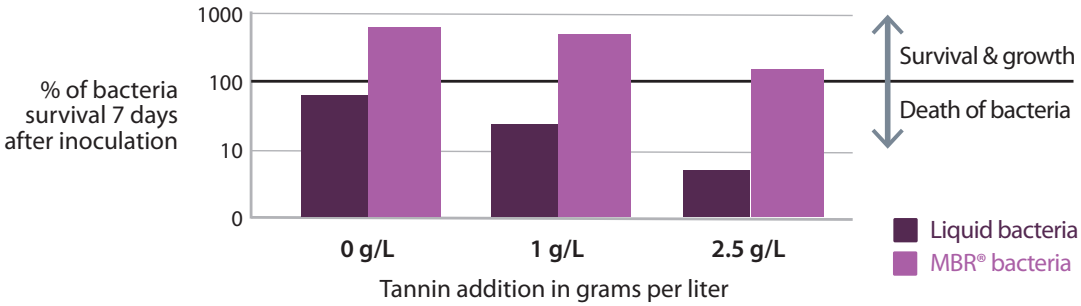


**Figure 7. Survival of bacteria, 7 days after inoculation into a wine with the addition or not of inhibiting tannins (average of results obtained with 5 bacteria strains used in liquid form or MBR® form) – adapted from M. Breniaux, 2017.**

These results highlighted the importance of the bacteria production process to ensure a fast initiation and fully achieved MLF, especially under difficult conditions.

## Performance of MBR® bacteria with proteomics

Matsumoto et al. (2022) further compared the physiological state of the bacteria LAB6 in the two forms (liquid versus MBR® ) using a proteomic approach. Proteomics is the large-scale study of the proteome, which is the entire set of proteins produced or modified by an organism or system. Proteins are vital parts of living organisms, with many functions.

To compare the proteome of MBR® cells to liquid culture cells, they used 2 strategies:

- untargeted proteomics: almost 300 proteins were identified
- targeted proteomics of 18 proteins

The analysis of proteomic results revealed that protein responses in MBR® bacteria were very specific in terms of stress regulation and could explain the resistance induced by the process. It showed that:

- An up-regulation of the general stress response. For instance, some chaperone proteins can protect cells in harsh conditions such as the Lo18 chaperone protein; its importance was confirmed in this study. Lo18 is a molecular chaperon that prevents protein aggregation and contributes to the stabilisation of the cell membrane in response to ethanol, acid and heat stress.
- An up-regulation of the stringent response. The stringent response is usually linked to nutritional deprivation. However, in some other bacteria, this stringent response could help to resistother stress (acidity, osmotic stress…)
- A down regulation of non-essential metabolic activities (for instance the down regulation of process in lipid metabolism and cell envelope biogenesis). One of the hypotheses is that during the production process of the MBR® cells, the lipid composition of the cell membrane is already modified to obtain a very fluid and stable bacteria membrane (something that was investigated while developing the MBR® process). As the adaptation has already been completed during preconditioning of the MBR® cells, there is no need for the cells to up-regulate these lipid metabolisms.

This work gives new evidence of the interest of the MBR® process to optimize strain performance and confirms or provides new clues to explain how this process of production makes cells resistant to harsh wine conditions.

## Conclusion

If the selection and characterization of a selected wine bacteria are essential, the performance of this strain is also very dependent on how it will be produced. The process of production is crucial to activate resistance mechanisms of bacteria cells to withstand the wine conditions.

MBR™ production process highly improves the ability of the selected wine lactic acid bacteria strain to survive, grow and quickly perform MLF in wine. The efficiency of a MBR® wine bacteria strain is higher than same strain produced in liquid form.

Recent studies confirm the MBR® industrial production process induces an upregulation of resistance mechanisms. The presence of major components of the stress response facilitates protein homeostasis and physiological changes that further ensure the integrity of cells. These new findings explain the excellence of our MBR® process and how the production process can impact the efficiency and robustness of selected wine bacteria.

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