# Prefermentation maceration in red winemaking: risk control and alternatives

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## ABSTRACT

Low-temperature prefermentation maceration is undeniably useful for producing fruity wines. However, this technique involves a certain number of microbiological risks. There must be adequate cooling capacity to maintain a sufficiently low temperature, as there is a real risk that indigenous flora will grow, leading to poor development of selected yeasts, sluggish fermentation kinetics, or even organoleptic spoilage. It is highly recommended to inoculate with yeast, at least partially, when the must is put into tank for prefermentation maceration, to ensure that the inoculated yeast will be the dominant strain. If the winery is not equipped with an adequate refrigeration system, the use of selective enzyme preparations makes it possible to produce fruitier wines at a reasonable cost.

**Key words: low-temperature prefermentation maceration, enzyme treatment, partial yeast inoculation, *Brettanomyces*.**

Low-temperature prefermentation maceration or cold soaking (CS) in red winemaking consists of allowing the must to macerate with the skins for a variable length of time before alcoholic fermentation (AF), generally at a temperature below 15°C. The extraction of compounds from the skin in this aqueous medium is different from the process that takes place in hydro-alcoholic medium during and after AF, as alcohol acts as a solvent and is capable of extracting tannin from seeds, protected by a lipid cuticle (1). As anthocyanins and skin tannins are relatively soluble, they are extracted during maceration in an aqueous medium (2).

In addition to phenolic compounds, CS extracts varietal aromas and aroma precursors, as well as other molecules, such as polysaccharides. From an empirical standpoint, this technique was considered to produce fruitier red wines. It has become highly successful amongst growing numbers of winemakers and has been investigated in recent research. Pineau et al. (3) demonstrated that macerating red grape skins from Bordeaux grape varieties in a model medium, with no fermentation activity, was responsible for the development of a fruity character.

This maceration prior to AF is certainly not risk-free and requires compliance with a certain number of elementary rules.

Even if precautions are taken to avoid the microbiological risks described in this article, the grapes must be healthy and at optimal ripeness. Indeed, maceration accentuates extraction of the compounds responsible for moldy, earthy off-odors (4), as well as laccase release. CS also exacerbates the herbaceous character of under-ripe grapes.

Finally, CS cannot be envisaged if the winery does not have the necessary equipment for rapid refrigeration (under 24 hours), capable of reaching and maintaining a temperature below 10°C. This key point is, unfortunately, frequently ignored by winemakers, who take ill-advised risks that could result in serious spoilage of their wine. It should be noted that, even in properly sulfured must, there is huge pressure from indigenous microflora at this stage. The diversity of microbial floral on grapes is now well-known (5). Yeasts and bacteria are present in quantities of 103 - 106 CFU/grape, with the various populations in differing proportions, according to the condition of the grapes. *Brettanomyces* spoilage yeasts have also been detected on grapes (5) and may take advantage of prefermentation maceration to acclimatize to the medium, as illustrated by the results in table 3. This point is examined in detail below.

When the winery does not have the necessary equipment or the winemaker wishes to shorten CS without loosing its benefits, pectinase-based enzyme preparations represent a very useful alternative. Indeed, progress in biotechnology and more detailed knowledge of the location of grape molecules have led to the development of enzyme preparations that act as real selective extraction tools. Exogenous enzymes on grapes mainly contain activities found naturally in grapes and may be added to the harvest to accelerate extraction by selective hydrolysis of the pectins. In particular, some of them increase color intensity (6) by promoting polymerization of the anthocyanins (6). However, commercial enzyme preparations are not all equally effective. Each enzyme acts on specific substrates and some preparations are not purified to eliminate undesirable activity (e.g. beta-glucosidase activity, which destabilizes anthocyanins by forming aglycones, or cinnamyl-esterase activity, responsible for the formation of volatile phenol off-odors).

This article initially describes the microbiological risks associated with CS, then presents the results of comparative tests using prefermentation maceration and enzyme preparations on an industrial scale.

## WHAT MICROBIOLOGICAL RISKS ARE ASSOCIATED WITH COLD SOAKING?

The major risk in cold soaking (CS) is microbiological. Premature growth of indigenous flora may hamper the development of selected yeasts inoculated later in the process. The data presented in table 1 illustrate this risk perfectly: an ADY/indigenous population ratio of 100 is required to guarantee successful inoculation.

The proliferation of indigenous flora is clearly correlated with maceration temperature (table 2). While temperatures above 10°C are more favorable for extracting and stabilizing color, CS must be carried out at low temperatures, (i.e. below 8-10°C), to avoid the development of indigenous flora present in the must and prevent spontaneous fermentation.

Not only do indigenous species deplete the nutrients in the medium, they are also perfectly suited to the conditions in the must. As a result, when the ADY are inoculated, they have to compete to occupy the same ecological niche. Indeed, not only do the grape microorganisms that have survived the osmotic shock of crushing rapidly consume the available nitrogen, as well as the growth and survival factors available in the must (sterols, vitamins, minerals, etc.), but they also enhance their capacity to adapt to the medium during maceration. For example, figure 1 shows the population growth of total yeasts and *non-Saccharomyces* yeasts during 7 days' CS at 10°C (5). *Non-Saccharomyces* yeasts were in the majority during maceration and remained at high levels during fermentation, despite the development of the *Saccharomyces* yeasts. As these yeasts are perfectly adapted to the medium, their growth metabolism resumes as soon as the *Saccharomyces* start to decline. V. Renouf showed that, among *non-Saccharomyces* yeasts, *Brettanomyces bruxellensis* was in the minority when the must is put into tank but represented a majority after fermentation, as it was extremely resistant to conditions in the medium. Apparently, therefore, the adaptation period for yeasts in the must and *Brettanomyces bruxellensis* at the beginning of maceration enhanced their capacity to take over from *Saccharomyces* at the end of fermentation. In addition to adapting more easily, these microorganisms may also cause off-odors, due to the production of volatile acidity by bacteria or volatile phenols by *Brettanomyces bruxellensis*. Phytotoxins or fermentation inhibitors (8) may also be produced if CS is not properly controlled.

Sulfuring the grapes is an excellent method for controlling populations of indigenous flora in the must. However, it is not advisable to sulfur the must excessively (depending on the condition of the grapes), as this may lead to difficulties later in fermentation, indirectly facilitating the development of *Brettanomyces bruxellensis*.

Indeed, excessive sulfuring, resulting in a wine with a higher total $SO_2$ content, delays the growth of lactic bacteria and the beginning of MLF, leading to a long lag phase, giving ***Brettanomyces bruxellensis***, which is particularly resistant to $SO_2$, an opportunity to develop.

In view of all these factors, CS is clearly a risky technique. As previously mentioned, temperature is one of the keys to success, as is the time when yeast is added. For this reason, several protocols for inoculating with yeast were studied. One hypothesis was that early inoculation of selected microorganisms into the medium would enable them to colonize the microbial ecosystem, thus inhibiting the development of the indigenous flora responsible for spoilage. Yeast inoculation before CS and in several stages was compared with adding yeast after CS.

## CONTROLLING MICROBIOLOGICAL RISKS: IMPORTANCE OF INOCULATION TIME IN ENSURING PROPER GROWTH OF ADY

### *Comparison of seeding before / after CS*

One batch of Merlot (Bordeaux, 2006) was divided evenly among 3 vats. The characteristics of the must were as follows: potential alcohol 14 % v/v., Total Acidity 3 g/L $H_2SO_4$, pH 3.5, Free $SO_2$ 30 mg/L, Total $SO_2$ 62 mg/L, Malic Acid 2 g/L, assimilable nitrogen when must put into tank 65 mg/L. Note that these were particularly difficult conditions with a high potential alcohol and severe nitrogen deficiency. The temperature in all 3 tanks was maintained at 14°C (57°F) for 4 days. Yeast was added to tanks A and B after CS but to tank C before CS. All 3 samples were inoculated with the ZYMAFLORE® RB2 yeast (20 g/hL, 200 ppm) rehydrated with DYNASTART® yeast rehydration nutrient (30 g/hL, 300 ppm). DIAMMONIUM PHOSPHATE (DAP) was added to each tank in two stages: ½ at the end of CS and second ½ after a third of AF, totaling 70 g/hL (i.e. 140 mg/L assimilable nitrogen for the yeasts).

The yeast population and assimilable nitrogen content were measured before and after CS (table 4). Although the must in tanks A and B, where yeast was added after CS, was properly sulfured, there was a sharp increase in the population of indigenous flora (x 2.102 to x 7.102 UFC), which was also responsible for significant consumption of the assimilable nitrogen in the must (figure 2). As a result, when the yeast was added after CS, very little nitrogen was available for the ADY. Furthermore, the ADY had to compete with large quantities of indigenous flora that were already acclimatized to the medium. In tank C, inoculated with yeast before CS, the yeast (ADY) population increased by a factor of 10 during maceration, thus achieving the desired effect of colonizing the medium with the selected inoculated strain, without triggering AF. By the end of CS, a large amount of the available nitrogen in this tank had been consumed by the ADY, so post-maceration addition of DAP was advisable to start fermentation.

Yeast development, assessed by genetic analysis at mid-fermentation, was positive for tank C and negative for tanks A and B (table 5). In these two tanks, one or more indigenous strains were responsible for AF. The inoculated yeast strain did not develop, in spite of the addition of 20 g/hL yeast, so medium was colonized by one or more indigenous strains, with unknown capacity to achieve a complete AF.

Inoculation at 20 g/hL (200 ppm) generally adds 2.106 cell/mL (depending on the quality of the ADY), which, in this test, represented a population only 5 - 10 times larger than the indigenous population in tanks A and B when yeast was added (after CS). The probability that ADY would develop properly, with a population under 10 times larger than that of indigenous flora, was very low (table 1). As a result, in this test, the indigenous population after maceration was so large, active, and perfectly adapted to the medium that adding 10 times as much ADY was insufficient to ensure that the inoculum could develop. The indigenous flora prevented the selected yeast from developing and colonizing the medium. As expected, in these two tanks, yeast activity stopped before all the sugars were consumed (table 6) and a starter was required to ferment the remaining sugars. The indigenous strain or strains responsible for AF were not, therefore, resistant enough to complete fermentation. Fortunately, the wine in tanks A and B did not have high volatile acidity or any of the off-odors frequently caused by this type of competition. However, the difficulty in completing fermentation in these two tanks delayed the start of MLF.

### *Comparison of adding yeast before / after cs or in several stages*

For wineries that do not have proper cooling equipment but still wish to use prefermentation maceration, the addition of yeast must be adapted to minimize the microbiological risks. One way of doing this is to add yeast in two stages, before and after cold soaking. To illustrate this technique, we present the results obtained during a test fermentation of a batch of Cabernet Sauvignon (Médoc, 2007). A batch of must was divided into 3 samples:

- Tank 1: yeast added before CS, no cooling.
- Tanks 2 and 3: maintained at 15°C (59°F) for 3 days (cooled by adding dry ice):
  - tank 2 was inoculated in two stages with the same yeast strain: 5 g/hL (50ppm) before and 15 g/hL (150ppm) after CS
  - tank 3 was inoculated with 20 g/hL yeast after CS.

The yeast strain used in all cases was ZYMAFLORE FX10®.

In tank 2, adding 5 g/hL yeast (i.e. approximately 5.105 cell/mL) before CS resulted in gradual colonization of the ecological niche by the selected strain without triggering fermentation. The yeast population at the end of CS was still below the threshold for triggering AF (2.106 cell/mL) (see table 7). The second addition of yeast, before the temperature in tank A started to rise, caused fermentation to start rapidly (figure 3). The importance of adding the yeast as soon as possible after CS should be emphasized: the temperature should not be left increasing before inoculating.

In the sample inoculated before CS, the yeast population increased by a factor of 1,000 during the 3 days' maceration and AF started the day after inoculation (table 7, figure 3).

When yeast was added after CS, the indigenous flora proliferated during maceration (table 7). This tank experienced a long lag phase before fermentation started and a slowdown towards the end of AF (figure 3). The inoculated yeast failed to develop. This was due to competition between the selected and indigenous yeasts, as previously described.

In order to guarantee the successful growth of ADY, it is also essential to acclimatize the yeasts. While it is important to add yeast as soon as possible, it is also true that ADY must be gradually acclimatized, especially when it is inoculated into cold must. It is, therefore, vital to reduce the temperature of the yeast inoculum by several additions of must, so that the temperature difference between the yeast preparation and the must in the tank does not exceed 10°C.

Similar results were obtained in a 2006 test when yeast was added to Syrah must from southeast France in several stages (5 days' CS at 12°C). As this experiment was successful and the winery wanted to continue using CS, this two-stage technique for adding yeast has been adopted by the winemaker.

Even when the microbiological risks are under control, cold soaking is, in many ways, an expensive technique. First of all, it necessitates adequate refrigeration equipment. Furthermore, as the total maceration period is longer, the winery must either have enough tank space, or plenty of time... The use of enzyme preparations was studied, with the aim of minimizing these constraints and reducing the time required for CS, while retaining all the benefits.

## USING SPECIFIC ENZYME PREPARATIONS AS AN ALTERNATIVE TO COLD SOAKING

The aim of adding prefermentation enzymes in red winemaking is to accelerate selective extraction. This technique was compared to CS.

For example, the results of a test carried out at a Pessac Léognan "Grand Cru" (2006) are presented below. A batch of good-quality Cabernet Sauvignon was divided evenly between two tanks. In the tank ENZ was added enzyme (4g/100kg LAFASE HE GRAND CRU®) and yeast (20 g/hL ZYMAFLORE F15®) when the must was put into tank and the temperature was maintained to allow AF to start quickly. Tank CS was maintained at 7°C for 5 days, then yeast was added to the cold must after CS (same yeast strain and inoculating rate). The other fermentation parameters were identical: temperature of AF, frequency and intensity of pumping over, post-fermentation maceration period, etc. After running off, both samples were stored separately and analyzed.

Table 8 shows the results in terms of free-run + press wine production and phenolic content after post-MLF sulfuring. The total wine yield (free-run and press) was improved by 31% by using the LAFASE HE GRAND CRU® enzyme preparation.

Standard analyses of the wines did not reveal any differences between the two samples (results not shown).

In terms of phenolics, the color of the enzyme-treated sample was 18% more intense than that of the CS sample. Similarly, the enzyme-treated sample had a higher tannin content (+ 22.5% compared to the CS sample).

The tannins were also more polymerized (HCl index) than those in the CS sample. In view of the significant improvement of the total polyphenol index (OD 280) of the enzyme-treated sample, a more detailed analysis of the phenolic content of the wines was carried out by HPLC (table 8). The wine in the enzyme-treated sample contained higher concentrations of polymerized phenolic compounds (tannins that remain more stable over time and protect the wine from oxidation), caftaric and caffeic acids (caftaric acid hydrolyzes to form caffeic acid, which plays a role in protecting wine color, by co-pigmentation with anthocyanins), and quercetins (yellow pigment from black grapes). Adding prefermentation enzymes, therefore, resulted in producing a larger quantity of a wine that generally has a more intense color, which remains more stable over time, and higher concentrations of more-polymerized tannins. Interestingly, in view of these results, this *Château* decided to use the same enzyme preparation on a systematic basis.

A similar test was carried out the same year using Merlot from the Entre-Deux-Mers and a combination yeast/enzyme of ZYMAFLORE RX60® / LAFASE FRUIT®. The results obtained were comparable to those shown above. After three months' ageing, a tasting was organized with 19 enologists. In a triangular test, 58% of the tasters found a difference between the two samples (results significant above 5%). Descriptive analysis of the wines revealed statistically significant differences in terms of fruitiness, with a preference for the enzyme-treated sample. There were no significant differences between the two wines for the following criteria: olfactory intensity, softness, fruitiness on the palate, and length. The differences were still perceptible when the two batches had been bottle-aged for 18 months.

The results of these two tests demonstrated that the use of specific enzyme preparations (LAFASE HE GRAND CRU® and LAFASE FRUIT®) produced wines with comparable, or even better, analytical and tasting results to those obtained with low-temperature prefermentation maceration. This confirmed the results obtained during several seasons of comparative tests of this type, in several countries.

Furthermore, comparing the cost of these two techniques (table 9), it was noted that using 4g/100kg maceration enzyme cost approximately 6 times less than refrigerating the grapes for CS (excluding the cost of maintaining low temperatures during maceration).

## CONCLUSION

Although low-temperature prefermentation maceration (cold soaking) produces fruitier wines, this technique involves a number of microbiological risks: poor development of the selected strain, off-odors due to competition between native and selected populations, sluggish or even stuck fermentations etc. The *sine qua non* condition for successful CS is proper control of maceration temperatures. Furthermore, to ensure development of the selected yeast strain, it must dominate the indigenous flora and minimize its growth from the beginning of maceration. For this reason, it is strongly advised to add at least some of the yeast before maceration, especially if the winery does not have sufficiently powerful refrigeration systems. The partial or total addition of yeast when the must is put into tank also minimizes the adaptation and development of spoilage microorganisms, such as ***Brettanomyces***.

The use of selective enzyme preparations is an excellent alternative, which reduces maceration time and facilitates the extraction of useful compounds in the aqueous phase, at a very affordable cost, potentially even lower than that of low-temperature prefermentation maceration. Pre-fermentation enzyme-treatment produces wines which retain a fruitier character, even after several months' ageing. Research continues to achieve a more detailed understanding of the phenomena involved in extracting compounds from the skin during this maceration phase. Particular importance will be placed on identifying the various phenolic fractions in the grapes.

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| ADY / indigenous population ratio | 0.1 | 1 | 10 | 100 |
|---|---|---|---|---|
| develop in the must | 0.1 - 1% | 15% | 95% | 100% |

**Table 1: Development of ADY in must: ADY / indigenous population ratio (7).**

| Temperature (°C) | 15 | 20 | 25 | 30 |
|---|---|---|---|---|
| Generation time (h) | 8 | 4 | 3 | 2 |

**Table 2: Saccharomyces cerevisiae proliferation time according to temperature (7).**

| | |
|---|---|
| D0 (tanking) | < 10 |
| D4 (after CS) | $2.2.10^2$ |

**Table 3: Cabernet Sauvignon Cold Soaking 8°C / 4 days - Brettanomyces yeast count before and after maceration. (Results in CFU/mL, counted after culture on enrichment medium)**

| | Tank A *Yeast added after CS* | Tank B *Yeast added after CS* | Tank C *Yeast added before CS* |
|---|---|---|---|
| Before CS | $1.6.10^3$ | $1.2.10^3$ | $1.5.10^3$ (before yeast added) |
| At the end of CS | $3.9.10^5$ (before yeast added) | $8.0.10^5$ (before yeast added) | $1.5.10^5$ |

**Table 4: Cold Soaking Merlot 14°C / 3 days: Yeast population before and after CS. (Results in CFU/mL, yeast population counted on specific agar medium).**

| | Tank A *Yeast added after CS* | Tank B *Yeast added after CS* | Tank C *Yeast added before CS* |
|---|---|---|---|
| Monitoring yeast development *(PCR and electrophoresis)* | Negative: medium not colonized by the inoculated strain | | Positive: medium colonized by the inoculated strain |

**Table 5: Cold Soaking Merlot 14°C / 3 days: Results of the mid-fermentation yeast development tests.**

| | Tank A *Yeast added after CS* | Tank B *Yeast added after CS* | Tank C *Yeast added before CS* |
|---|---|---|---|
| Alcohol (% v/v) | 13.80 | 14.05 | 13.95 |
| Reducing sugars (g/L) | **4.4** | **3.9** | 1.6 |
| Volatile acidity (g/L $H_2SO_4$) | 0.36 | 0.40 | 0.39 |

**Table 6: Cold Soaking Merlot 14°C / 3 days: Analytical profiles of the wines after AF.**

| | Tank 1<br>*Yeast added before CS* | Tank 2<br>*Yeast added in 2 stages* | Tank 3<br>*Yeast added after CS* |
|---|---|---|---|
| **D 0: before CS** | $9.4.10^4$<br>(before yeast added) | $3.1.10^4$<br>(before 1st yeast added) | $1.5.10^4$ |
| **D 3: after CS** | $1.2.10^7$<br>(fermentation started) | $1.5.10^6$<br>(before 2nd yeast added) | $3.1.10^5$<br>(before yeast added) |

**Table 7: Cold Soaking Cabernet Sauvignon 15°C / 3 days: Yeast population in the various samples: results in cell/mL, counted by epifluorescence microscopy).**

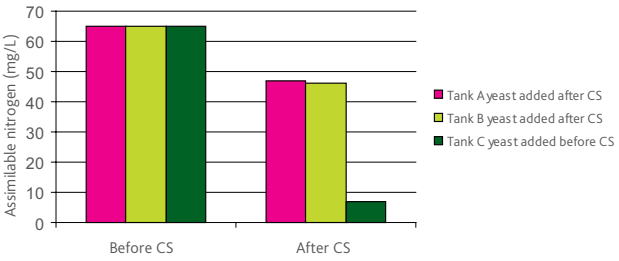


**Figure 2. CS Merlot 14°C / 3 days. Assimilable nitrogen content before and after CS**

| | CS Tank<br>*Not enzyme-treated with CS* | ENZ Tank<br>*Enzyme-treated without CS* |
|---|---|---|
| Yield of free-run + press wine | 100% (reference) | 131% |
| Modified Colouring Intensity | 12.2 | 14.4 |
| OD 280 | 78 | 82 |
| Anthocyanins (mg/l) | 743 | 726 |
| Tannins (g/l) | 4 | 4.9 |
| HCl index (%) | 29 | 35 |
| HPLC analysis of phenolic content (+/- 5%) | | |
| Gallic acid (mg/L) | 52 | 60 |
| Polymerized phenolic compounds (mg/L) | 1029 | 1329 |
| Caftaric acid (mg/L) | 9 | 22 |
| Caffeic acid (mg/L) | 3 | 4 |
| Quercetin glucoside (mg/L) | 8 | 12 |
| Quercetin aglycone (mg/L) | 15 | 22 |

**Table 8: Cold Soaking Cabernet Sauvignon 5 days -7°C / Enzyme added: LAFASE HE GRAND CRU®. Yields of free-run + press wine / Analysis of the wines' phenolic content after MLF.**

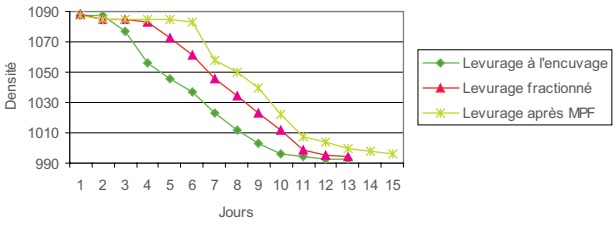


**Figure 3: Changes in fermentation kinetics in the various samples. In green: control (no CS), in pink: CS with yeast added in stages, in pale green: CS with yeast added after maceration.**

| Chilling the must by adding dry ice | Using an enzyme preparation (***LAFASE FRUIT®***) |
|---|---|
| $CO_2$ consumed per hL<br>to cool the must by 10°C: 10kg<br>Cost of $CO_2$ per kg: approximately 0.25 €<br>**Total excluding the cost of maintaining the low temperatures for several days: 2.5 € / hL finished wine** | Using 4 G/100kg specific extraction enzyme preparation: **approximately 0.4 € / hL** |

**Table 9: Comparison of the cost of using a specific enzyme preparation and chilling the must.**

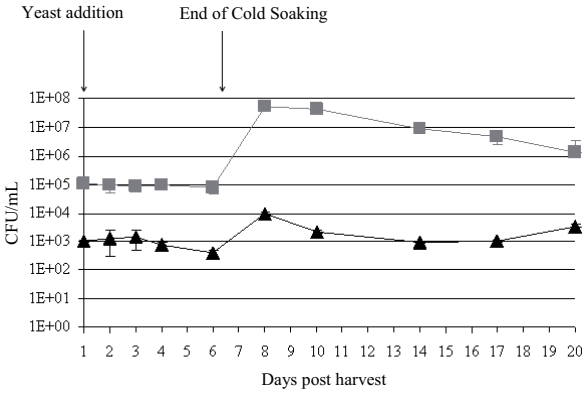


**Figure 1: Changes in the total yeast population (■) and non-Saccharomyces yeasts (▲) in a batch of 2003 Merlot during 7 days' initial low-temperature maceration (5).**


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