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# Kurzbericht

# Applications of lysozyme in enology - Technical report

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## Origin and activity of lysozyme

The enzyme lysozyme is a natural product found in many animal secretions such as egg whites or human tears. It has been used for years as a biopreservative in the processing and storage of hard cheese (Proctor and Cunningham, 1988). For enological means lysozyme is extracted, purified and freeze-dried. The enzyme activity (muramidase) degrades the cell wall of gram positive bacteria, such as lactic bacteria, but not acetic bacteria which are protected by an external membrane (Pilatte et al., 2000). The action of lysozyme is immediate, and after a few hours, the lysozyme is inactive in white wines or eliminated by flocculation with tannins in red wines (Green and Daeschel, 1994).
The advantage of lysozyme is that its activity gets stronger when the pH-value increases, e.g. in the conditions where lactic bacteria are more likely to spoil must or wine. This is in contrast to $SO_2$, so that there is a good synergy between these two products. It is important to notice that lysozyme has no effect on yeasts like Brettanomyces and does not prevent oxidation, so it cannot replace the application of $SO_2$ completely.
Lysozyme has been tested in wines since the early nineties in Italy (Amati et al., 1992; Amati et al., 1996) and France (Gerbaux et al., 1997) under OIV and EU authorization. Different applications have been tested successfully (Gerbaux et al., 1999; Gerland et al., 1999); each is very specific and therefore requires technical knowledge and expertise to be efficient.
The use of lysozyme in must and wines is allowed by EU legislation since the end of October 2001 with a maximum level of 500 mg/l.

In the following the main applications in enology will be explained.

## Postponing of malolactic fermentation (MLF)

There are cases in red wine vinification where the malolactic fermentation (MLF) occurs early; then problems associated with malolactic fermentation (e.g. high volatile acidity, off-flavour) may occur, and the maceration will have to be stopped, even if it is too early for the intended quality of wine.
This problem is common in the case of carbonic maceration. Addition of 10 g lysozyme per hectolitre of must (calculated on the final liquid volume) just after tank filling causes a retardation of MLF and solves the above mentioned problems (figure 1).
With destemmed (not crushed) berries (semi-carbonic maceration), two different settings can be distinguished:
- hot climate with high lactic bacteria populations (105 to 106 lactic bacteria per milliliter): the lysozyme has to be used at the same time as the $SO_2$ addition (e.g. south of France, Languedoc-Roussillon)
- moderate climate with normal lactic bacteria populations (103 to 104 lactic bacteria per milliliter): if the grapes are not contaminated by Botrytis, lysozyme can replace the $SO_2$ addition to must with the same good delay of MLF.

In traditional vinification with destemming and crushing the best results are obtained by addition of 20 g lysozyme per hectolitre during alcoholic fermentation (AF) at a relative density near 1.020.

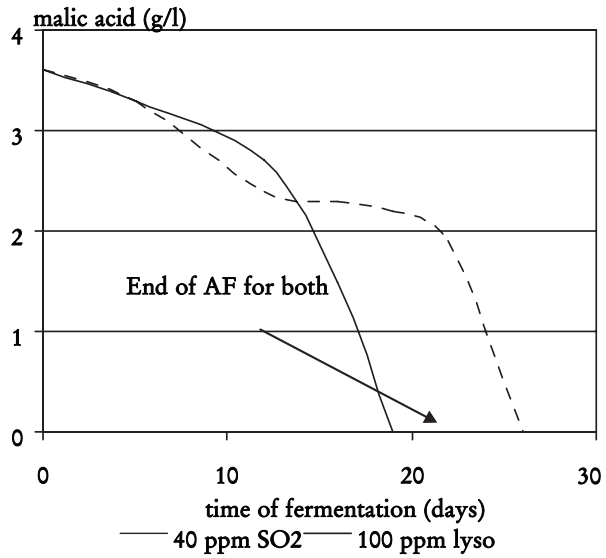


Figure 1: Comparison of the MLF retardation of $SO_2$ and lysozyme with carbonic maceration (GERBAUX et al., 1997)

## Inhibition of malolactic fermentation (MLF) in white or rosé wines

Lysozyme was originally tested as a substitute for $SO_2$; in this case it showed good results with respect to microbiology: compared to $SO_2$ the inhibition of MLF was as or even more efficient with lysozyme (figure 2).

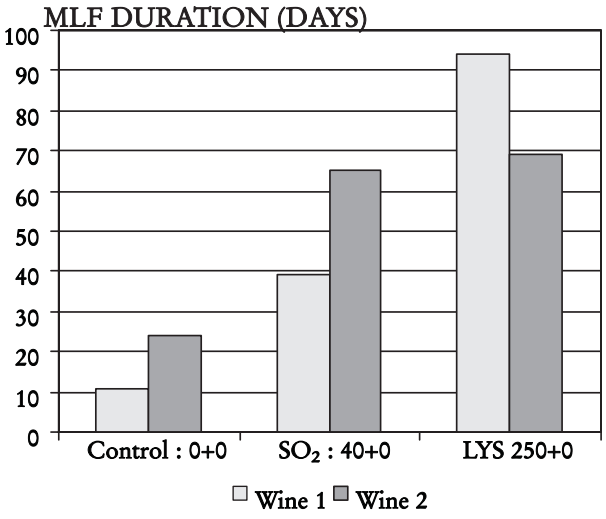


Figure 2: Comparison of the MLF inhibiting effects of lysozyme and $SO_2$ with wine from 'Gewürztraminer' grapes (GERBAUX et al., 1997).

Because of problems with oxidation lysozyme is now used in combination with $SO_2$, which, however, can be applied at lower rates than when used alone.
For example, in the vinification of rosé wines in the south of France, an addition to the must of 20 g lysozyme per hectolitre showed very good results in 1999 and 2000: in the untreated control variant MLF started before the end of the alcoholic fermentation, whereas no MLF occured with the lysozyme-treated variant.
But it has to be mentioned that the inhibition of MLF is the most delicate application of lysozyme and is not effective in all cases. Lysozyme can not prevent MLF under all conditions, but is only a tool to decrease the bacteria level, and has to be used in combination with $SO_2$ and good hygienic conditions.

## Remedy against sluggish fermentation

One of the reasons for sluggish alcoholic fermentation (AF) is the multiplication of lactic bacteria before the end of the AF, often combined with an increase of volatile acidity. In such cases the bacteria consume the sugars after the malic acid and the quality of the resulting wines is often poor. The common treatment is a sulphitation (4 to 6 g/hl), this, however, is problematical since $SO_2$ not only affects the bacteria but also the yeasts, and the AF will not be completed without insemination of a new yeast culture.
The application of lysozyme is advantageous, because lysozyme specifically affects the bacteria and after the fast death of bacteria due to the lysozyme treatment the yeasts can finish the AF normally (figure 3).
The increase of volatile acidity is stopped (figure 4 and figure 5). The dose of lysozyme used is 25 to 40 g/hl; if the development of bacteria is detected in time (before reaching an amount of 106 bacteria per ml), the treatment can postpone the MLF (enough to allow the yeasts to finish the AF). If the MLF has started already, it is better to wait until the end of MLF and to treat just after the end of malic degradation (GERBAUX et al., 1999; PUJOL, 1999).

## Microbiological stabilization during aging

The utilization of lysozyme instead of $SO_2$ for stabilization after MLF is advantageous because it permits a longer time for tannin and anthocyanin complexation. This reaction is inhibited by $SO_2$ but not by lysozyme, while the effect on the elimination of lactic bacteria is quite similar (figure 6).

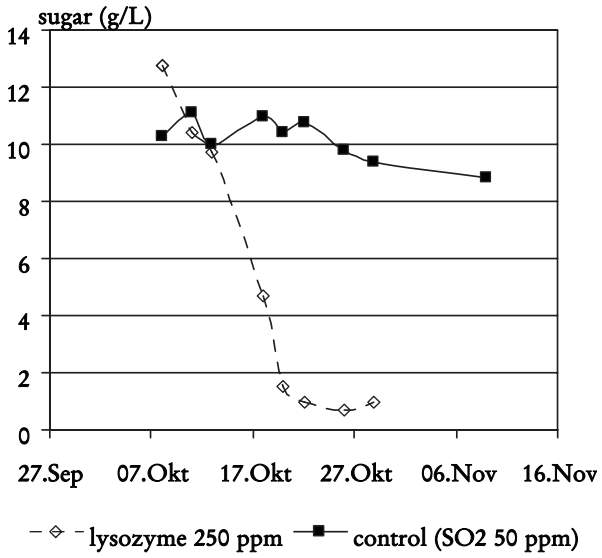


Figure 3: Effect of lysozyme and $SO_2$ on sluggish fermentations in wines from the Côte-du-Rhône

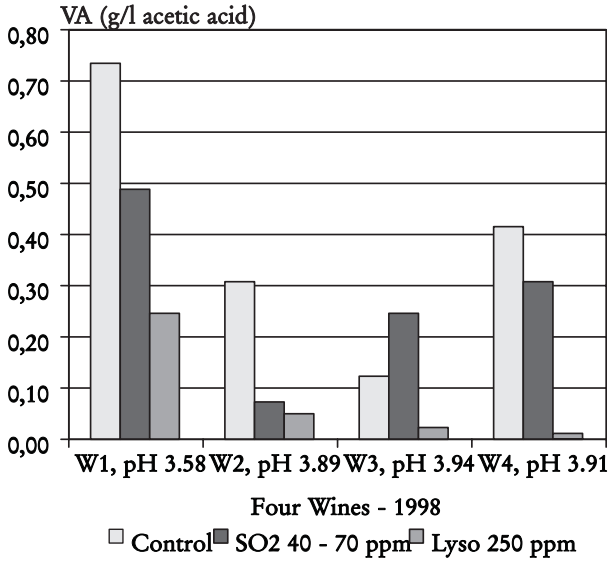


Figure 4: Effect of lysozyme and $SO_2$ on the formation of volatile acidity in sluggish fermentations at laboratory scale (wines from Côte-du-Rhône)

The wines with lysozyme addition have a higher colour intensity (figure 7) and preserve a more fruity aromatic profile. This is very important with some grape varieties, like 'Pinot Noir', in cool regions, where it is difficult to obtain good and stable red colour.

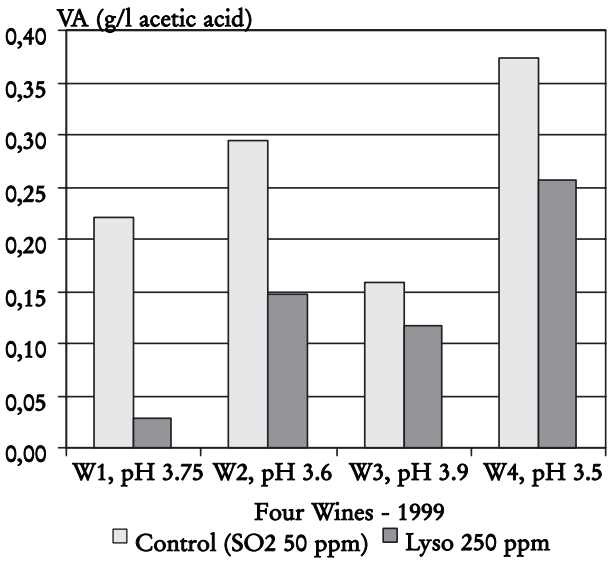


Figure 5: Effect of lysozyme and $SO_2$ on the formation of volatile acidity in sluggish fermentations at winery scale (wines from Côte-du-Rhône)

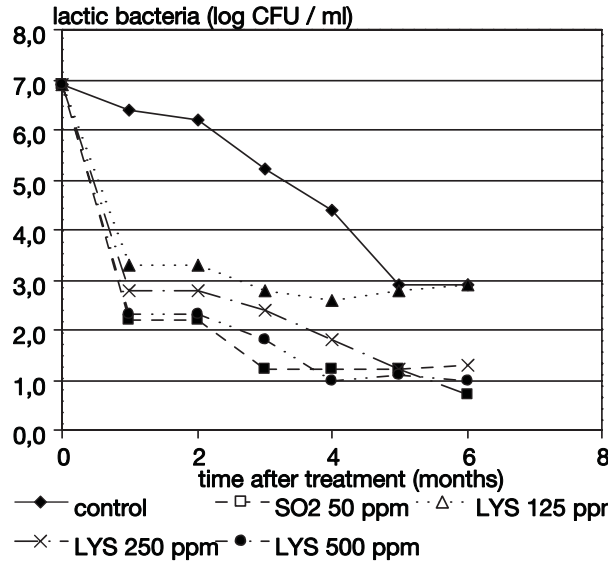


Figure 6: Lactic bacteria population after MLF in a 'Pinot Noir' wine from Burgundy (GERBAUX, 1997)

The results obtained by ITV France in an experimental cellar (GERBAUX, 1997) are confirmed in big wineries on the aspect of colour, but in 1999, it was difficult to diminish the lactic bacteria population, both with $SO_2$ or lysozyme.

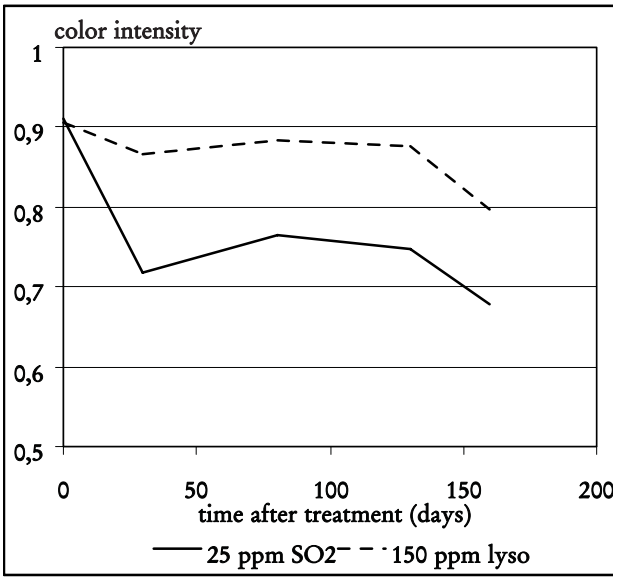


Figure 7: Colour changes after MLF in a 'Pinot Noir' wine from Burgundy (GERBAUX, 1997)

It is important to notice that lysozyme has no effect on acetic bacteria nor on yeasts, so in this application, wines must be surveyed regarding their content with those two types of microorganisms. Lysozyme treatment has to be used in combination with $SO_2$ if a contamination is suspected especially with Brettanomyces.

## Specific applications for sparkling wine production

To avoid MLF lysozyme can be used at three stages of the process of sparkling wine production.

1. In must or wine during fermentation: After four years of trials a process was adapted in Champagne which provided good inhibition of the MLF with a reduced application amount of 3 to 4 g/hl (30 to 40 mg/l) $SO_2$ (CHABOCHE, 1999). But the usually very low pH-values (2.9 to 3.1) in sparkling wine delimit the conditions for lysozyme. Thus, the mortality effect on the bacteria is only from 1 to 2 log, instead of 3 to 6 at higher pH-values. From figure 8 you can see that a weak dose of lysozyme (5 g/hl) is not enough to block the multiplication of bacteria, while the complementary dose of lysozyme permits to block it. The efficiency also depends on the bacteria population level, so the effect of lysozyme was lower in 1999, when the bacteria population was much higher than in normal years (105 to 106). Furthermore it was observed that dividing of the entire dose into smaller frequent additions was more effective against the bacteria multiplication, because the lysozyme works quickly, but at low pH-values it gets inactivated very soon.
   Decreasing temperature after fermentation is an important factor to prevent MLF, e.g. in wines transferred from cellar to laboratory (about 20 °C) MLF started immediately.

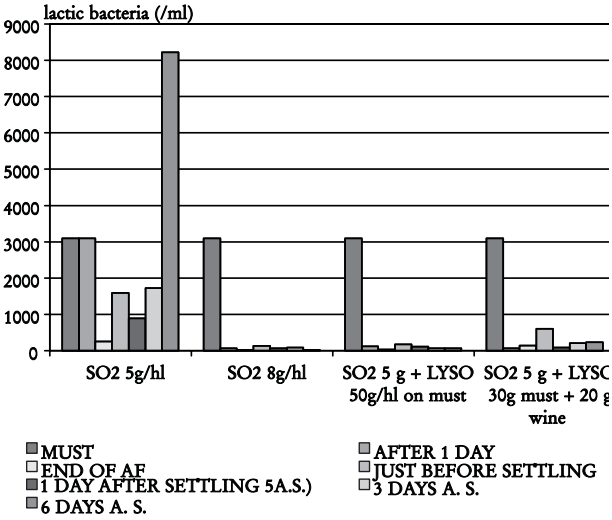


Figure 8: Comparison of the effects of traditional and new process (= lysozyme + lower dosis of $SO_2$) in Champagne

2. At tirage (bottling for second fermentation): MARCHAL et al. (2000) showed that the addition of 10 g/hl (100 mg/l) lysozyme to a Luxembourg Cremant wine at bottling stage had a good influence on the reduction of viable lactic bacteria (figure 9). Furthermore they found that lysozyme additions from 100 to 400 mg/l avoid MLF in the bottle (figure 10).
   Similar effects were found with Cava wines, it was also possible to reduce the dose to 50 g/hl because it is better from health and technological aspects to have lower residual lysozyme concentrations.
3. In the yeast culture (pied de cuve): The yeast inoculum is certainly the most dangerous source of contamination of the wine, because the media of multiplication of the yeast contains sugar and nutritive elements. A frequent lysozyme addition of 500 mg/l can prevent bacteria multiplication.
   Of course, all the usual hygienic precautions have to be followed to avoid MLF.

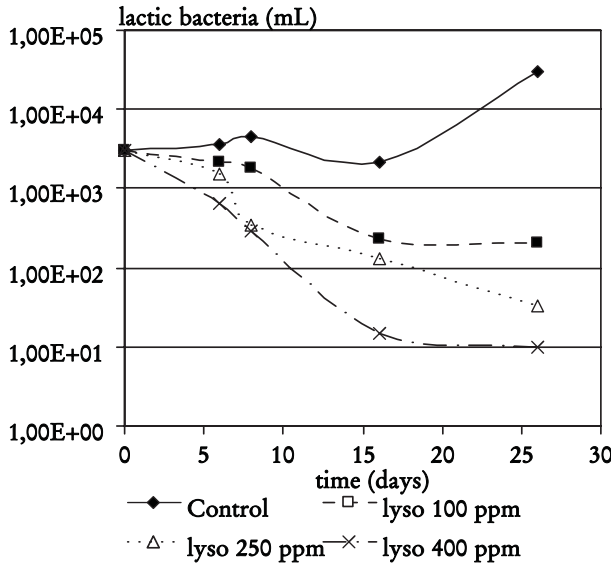


Figure 9: Effect of lysozyme on the number of malic acid bacteria in a Cremant bottle during second fermentation

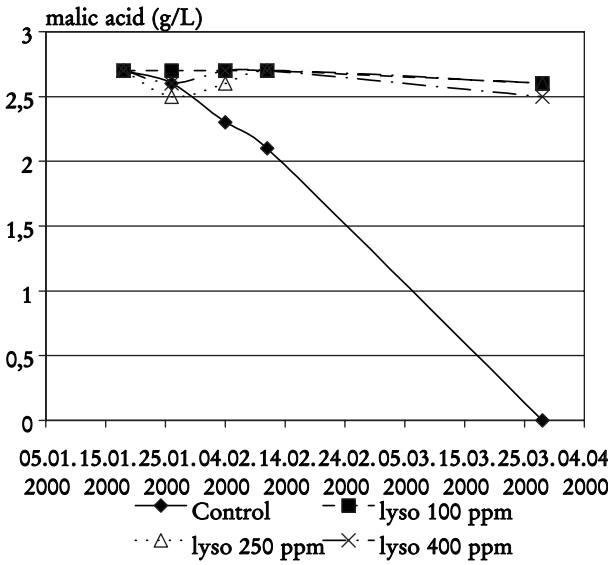


Figure 10: Effect of lysozyme on the viable lactic bacteria in a Cremant bottle during second fermentation

## Secondary effects

A few days after lysozyme treatment in red wines, lysozyme gets inactivated because of their reaction with tannins. With very light red wines small losses of colour can appear. With normally coloured red wines there are no secondary effects. But with white wines 50 to 80 % of the added lysozyme remains present in the wine (CHABOCHE, 1999; MARCHAL et al., 2000). This residual lysozyme does not have any effect on organoleptic properties of the wine, nor on its alimentary safety because lysozyme is a natural protein.

Possible enological effects of lysozyme:

1. Strong reaction with protein stability tests such as heating test, bentotest or TCA reaction. According to test results these wines should then be treated with very high bentonite doses for stabilization. Fortunately in practice, however, no haze formation is observed in these wines under severe conditions, for example when stored for one week at 40 °C, then for one week at 4 °C and finally one week at 40 °C. So it is recommended to make the bentonite fining with respect to the protein test value of the same wine without lysozyme.
2. Strong reaction after addition of metatartaric acid with intensive haze formation, like in protein-rich wines, but in this case it is not reversible. So this type of protective should not be used with lysozyme treated white wines.
3. Protective effect on effervescent capacity of sparkling wine. Lysozyme has no direct positive effect on effervescence, but reduces the negative effect of bentonite treatment (MARCHAL et al., 2002). It seems that lysozyme present in wine is very reactive with bentonite, more than the natural proteins of wine, which are known to have a positive effect on foam properties of wine. This should be due to the very high iso-electric point of lysozyme. At common pH-values of wine, lysozyme has a very high surface electric charge, that explains bentonite reactivity.
4. Probably there is a protective effect of lysozyme in white wine against tartaric precipitation. This effect has to be studied in the future.

With rosé wines the effects of lysozyme treatment can be described as being „between reds and whites“, depending on the tannin level of the wine.
To avoid all these possible difficulties a procedure can be applied which removes the lysozyme residues by fining or reduce the levels by blending the treated wine with a big quantity of another wine.

## Conclusion

Many years of experiments have shown that lysozyme is a good tool to control lactic bacteria multiplication

and to manage the start of the MLF. It can replace $SO_2$ in some cases where $SO_2$ is not very efficient, but lysozyme is generally used together with $SO_2$, resulting in wines with lower final $SO_2$ levels.

As lysozyme is a protein that partially remains in the treated white wine (only in whites, not in reds), there are some secondary effects to know for correct application.

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