

Malolactic fermentation– A review of recent research on timing of inoculation and possible yeast-bacteria combinations

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SUMMARY: Despite a considerable research effort, the malolactic fermentation (MLF) process remains an imperfectly controlled process and at times MLF can be difficult to get started. One possible explanation for this difficulty is that the wine may be lacking essential nutrient factors for the lactic acid bacteria. Another possible argument is that inhibitory substances are accumulated in wine. Sulfur dioxide and alcohol concentrations and pH have been included among the most significant parameters influencing the growth of malolactic bacteria in wine. King and Beelman suggested that the growth of *Oenococcus oeni* during alcoholic fermentation might be retarded by the production of toxic compounds by yeasts other than ethanol and sulfur dioxide. Moreover, the malolactic fermentability of wines produced from the same must differ according the yeast strain used in alcoholic fermentation. Previous studies have shown the inhibition of malolactic starter cultures by active growing yeasts due to the production of high levels of SO₂ during the early stage of alcoholic fermentation and the effect of acetic acid formed by *Kloeckera* yeasts and some lactobacilli on growth of *Saccharomyces*. The goal of our recent investigations is to better understand these interactions, to quantify the nutrient demand by yeast and the nutrient requirements of wine lactic acid bacteria, and to determine whether there are other synergistic interactions between *S. cerevisiae* and *O. oeni* in wine. A better understanding of these aspects of wine microorganism physiology will allow us to better match combinations of yeast and bacteria starter cultures with grape varieties, and select the timing of yeast and bacteria inoculations.

Interactions between yeast and bacteria

Wine is not only a product made out of grapes and modified by technical means. Wine is a product of a complex microbial fermentation of the grape juice involving the sequential development of various species of yeasts and lactic acid bacteria. In wine fermentation, the perhaps earliest use of biotechnology, the final quality of the wine can be modified in two major ways, (1) by the quality of the grapes and juice preparation, and (2) by the selection of most suitable yeasts and bacteria and control of fermentation condition. The task of the winemaker is to ensure that the desired fermentative yeasts and malolactic bacteria strains predominate in the juice and wine and carry out the fermentations. Today, most major producers of wine inoculate the juice with selected strains of yeast and bacteria. Very often, starter culture failures are due to improper preparation and inoculation procedures. In some cases, starter culture failure may be due to antagonistic interactions between yeast and bacteria (Table 1).

Competition for nutrients	
Production of inhibitors	
<i>Yeast - bacteria</i>	<i>Bacteria - yeast</i>
<ul style="list-style-type: none"> • Alcohol • SO₂ • Medium chain fatty acids (octanoic and decanoic acid) 	<ul style="list-style-type: none"> • Acetic acid • Bacteriocin

Table 1 - Yeast - bacteria interactions

Effect of yeast growth and SO₂ on bacterial survival

One possible argument is that inhibitory substances are accumulated in wine. Sulfur dioxide and alcohol concentrations and pH have been included among the most significant parameter influencing the growth of malolactic bacteria in wine. Already in 1994 Henick-Kling *et al.* demonstrated the inhibition of malolactic starter cultures by active growing yeasts due to the production of high levels of SO₂ during the early stage of alcoholic fermentation. Growth and malolactic activity of *Oenococcus oeni* starter cultures inoculated together with the yeast was evaluated in 1991 Chardonnay juice containing SO₂ and in Chardonnay juice free of SO₂ (Table 2).

A Chardonnay juice	B Chardonnay juice
<ul style="list-style-type: none"> • 23° Brix • pH 3.3 • Malic acid 3.30 g/l • SO₂: 17/35 mg/l (free/total) (50 mg/l added at pressing) 	<ul style="list-style-type: none"> • 20° Brix • pH 3.3 • Malic acid 6.00 g/l • SO₂: non added

Table 2 - Content of free and total SO₂

The viability of bacterial cultures rapidly declined to between 10² and 10³ CFU/ml when inoculated together with the yeast in a sulfited juice. Bacterial cultures only recovered after completion of alcoholic fermentation. Malolactic fermentation (MLF) was completed between 32 and 55 days, except by culture OSU in wine fermented with K1 and EC1118 (Table 3). Bacteria cultures inoculated into the sulfited juice without added yeast died at the same rate as in the juice with added yeast. Thus, they couldn't find an effect of the yeast but of the SO₂ added to the juice. The bacteria did not affect fermentation rate of the yeast.

In the juice without added SO₂ *Oenococcus oeni* cultures maintained their viability (Figure 2). Malolactic fermentation was completed in 9 to 20 days (Table 3).

Bacteria	Yeast			
	EC1118	K1	71B	Wädenswil
A				
• OSU	> 50	> 50	40	40
• X-3	55	55	40	40
• Inobacter	55	55	32	32
B				
• OSU	18	13	9	9
• X-3	20	18	13	13
• Inobacter	18	13	18	9

Table 3 - Time to complete (days after inoculation) malolactic fermentation by *Oenococcus oeni* cultures OSU, X-3 and Inobacter in wines fermented with wine yeast cultures EC1118, K1, 71B and Wädenswil.

A: Concurrent yeast-bacteria fermentation in Chardonnay juice 17/35 mg/l SO₂ (free/total), pH 3.3, TA 6.5 g/l.

B: Concurrent yeast-bacteria fermentation in non-sulfited Chardonnay juice pH 3.3, TA 12.0 g/l.

The four yeast strains differed strongly in the amount of SO₂ produced during alcoholic fermentation. The relative amount produced was consistent for each yeast in the sulfited and the non-sulfited juice (table 4). Yeast K1 produced in both juices more SO₂ than EC1118, and both produced more than 71B or Wädenswil. Yeast strain K1 with the largest production of SO₂ was also the most inhibitory to MLF, when bacteria were inoculated after completion of alcoholic fermentation. Yeasts 71B and Wädenswil, producing only small amounts of SO₂, were the most favorable to MLF and bacterial viability.

Yeast	in non-sulfited juice		in sulfited juice (17/35 mg/l)	
	free (mg/l)	total (mg/l)	free (mg/l)	total (mg/l)
• K1	7	27	7	42
• EC1118	9	27	8	30
• 71 B	9	8	15	17
• Wädenswil	4	3	2	13

Table 4 - SO₂ production by four wine yeast strains

In this study, clearly the SO₂ added at pressing and produced by the yeast during alcoholic fermentation was the dominant factor determining the success of the inoculation with bacterial starter cultures." They couldn't find other interactions such as competition for nutrients or the production of antibiotic substances by the yeast or the bacterial culture being inhibitory.

Recent studies of Thomas Henick-Kling and Kathy Arnink showed a big difference between yeast strains not only with regard to SO₂ production but also with regard amino nitrogen consumption (Table 5).

		average			
		1° amino N	free SO ₂	total SO ₂	adjusted
		mg/L	mg/L	mg/L	pH
	juice	119.38			3.50
wine	D47	67.03	5.3	9.9	3.32
	W27	79.87	5.0	7.0	3.30
	Yeast 1	76.17	5.1	15.4	3.32
	W15	63.62	4.9	8.5	3.29
	BM45	57.46	5.1	55.9	3.29
	EC1118	70.08	5.6	15.3	3.29
	Yeast 2	55.39	5.8	12.7	3.30
	W46	52.92	5.6	12.5	3.29
	AMH	85.19	5.4	9.0	3.30
	CY3079	68.88	4.6	12.3	3.30

Table 5 - SO₂ production and amino nitrogen changes in a Chardonnay after alcoholic fermentation with different yeast strains

Table 6 shows the results of laboratory scale fermentations in this Chardonnay. In the wines fermented with the yeast exhibiting high amino nitrogen demand, but also produced higher levels of SO₂ induction of MLF was difficult or impossible. Whereas yeasts with lower nitrogen demand seem to support MLF. But beside influence of the yeast we could also observe difference in the sensibility of the MLF starter cultures used for induction of MLF. Direct inoculation starter strains, which had been pre-adapted on production proofed to be less sensitive (strain 54, strain 98, strain 101, strain Bio).

	05	3	34	36	54	55	59	74	75	76	77	98	10	Bi
AMH	+	-	+	-	+	-	+	-	-	-	+	+	+	+
BM45	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CY3079	-	-	-	-	-	-	-	-	-	-	-	-	/	-
D47	-	/	-	/	+	/	-	-	-	/	+	+	+	/
EC1118	+	-	+	+	+	+	+	-	-	-	+	+	+	+
Yeast1	+	-	+	+	+	+	+	-	-	-	+	+	+	+
Yeast2	-	-	+	-	+	-	+	-	-	-	+	+	+	+
W15	+	-	+	-	+	+	+	-	-	-	+	+	-	+
W27	-	-	+	+	+	+	-	-	-	-	+	+	+	+
W46	+	-	+	-	+	+	+	-	-	-	+	+	+	+

+ = MLF complete - = no MLF / = some replicates finished

Figure 6: MLF Success in Laboratory-Scale Chardonnay

Results of laboratory fermentations had been compared to a plating procedure, which has been tested as a simple method for predicting possible yeast bacteria pairings. This method was inspired by the use of antibiograms. It is based on the susceptibility of bacteria to a specific substance. This substance, applied via filter paper, will diffuse radially into the agar and influence the growth of the bacteria on the plate. A zone of growth inhibition, i.e. “a clear zone” around the disk, indicates toxic substances or toxic concentrations. The diameter of the zone is proportional to the susceptibility or resistance. In this study the filter paper was impregnated with wine. If yeast chosen for AF produces harmful or beneficial substances during AF, a zone of growth or inhibition or a dense zone of enhanced growth would indicate it. Also this method would be quick and easy it has its limitations, since for some strains growth is not a good indication that bacteria are converting malic acid to lactic acid. As mentioned earlier some bacteria perform malolactic fermentation with no growth in a wine. A good example will be the commercial strain Enoferm ALPHA. Thus, plating method will not predict all successful yeast-bacteria pairs.

Comparing our data from the plating results and completion of MLF in real wine in the laboratory-scale fermentations seem to confirm this. Plating results predicted a positive effect from yeast strains CY3079 and D47 on growth of the bacteria strains shown in the table, but no MLF occurred in the Chardonnay wines fermented by these yeasts in the laboratory. These yeast strains might use up some. But the plating method is still useful in separating different effects yeast might have on ML bacteria. Some effects are purely nutritional.

To gain more knowledge about the influence, not only of the yeast-bacteria pairing, but also on the influence of the wine matrix, a number of yeast/bacteria pairings had been tested in Finger Lakes wineries in 2001. The barrels of Chardonnay that quickly completed MLF consisted of the following pairings of yeast and bacteria. Two barrels of each pairing were used.

W27 yeast with EQ101, EQ98, and EQ32 strains of bacteria.

CY3079 yeast with EQ101, EQ98, and EQ32 strains of bacteria

D47 yeast with EQ101, EQ54, and L41 strains of bacteria

CY3079 and D47 with the 105 strains of bacteria, in tanks

The other (red) varieties in this study completed MLF.

Results in industry scale winemaking indicate Chardonnay is a particularly difficult grape variety for successful MLF.

2002 the 10 most used Lallemand wine yeast strains had been tested with 5 Lallemand starter cultures for direct inoculation on laboratory and industrial scale.

Further studies are necessary to better understand the factors that influence these bacteria in various wine conditions. Additional studies, making additions to wine or synthetic medium to determine which nutrients in some wines are limiting to bacteria, will be performed. We also need to determine what wine compounds have possible stimulatory or inhibitory effects on *Oenococcus*. Yeast or bacteria in wine might produce these compounds.

Based on the preliminary results from these studies we proposed a yeast-bacteria table and a nutrient management for bacteria and yeast to achieve best results by using the right yeast-bacteria couple and/or the right nutrient addition.

TIMING OF INNOCULATION

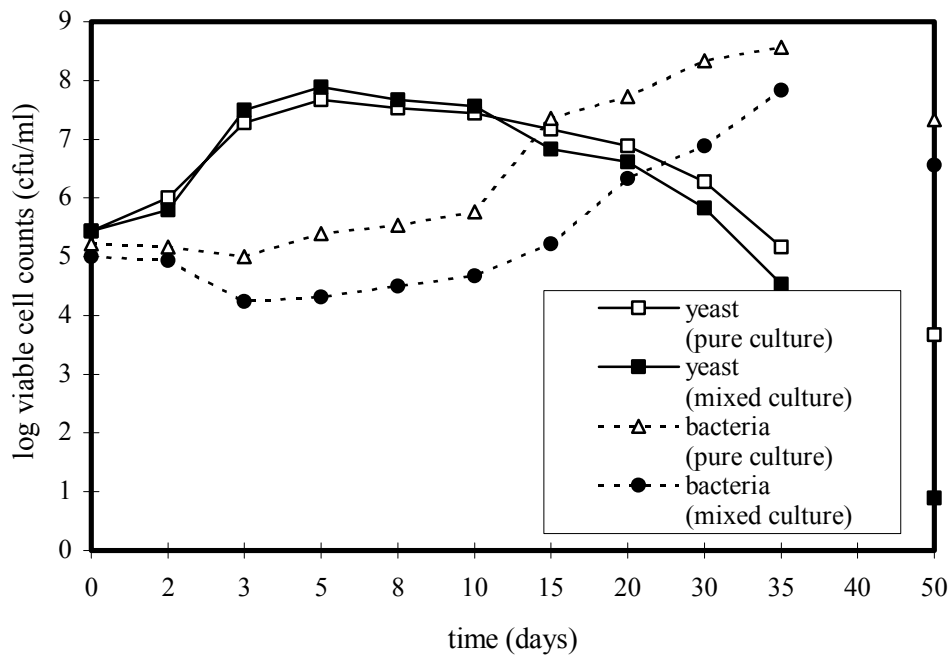
There is also still a lack of understanding on the influence of timing and level of inoculation on the flavor contribution of malolactic fermentation.

Beelman and Kunkee showed that MLF in presence of fermentable sugars does not necessarily lead to the production of excessive amounts of acetic acid by the bacteria, as long as yeast fermentation starts promptly and goes to completion. However, there might be a danger that strong bacterial growth can inhibit yeast growth, leading to stuck alcoholic fermentation and subsequent bacterial spoilage of the wine with excessive acetate and other off-flavors (Lafon-Lafourcade).

We achieved positive results by inducing simultaneous malolactic and alcoholic fermentation. Over several vintages (since 1996), we investigated interactions between wine yeast and malolactic starter bacteria with special regard to the malate and citrate metabolism and the formation of acetate and diacetyl. Under low pH conditions no acetic acid was formed out of sugars.

Even under high pH conditions in a 2000 Riesling wine, early inoculation did not lead to an excessive production of acetic acid, but helped to suppress wild bacteria growth and benefit juice hygiene.

Already studies of King and Beelman (1986) suggested that the growth of *Oenococcus oeni* (former *Leuconostoc oenos*) PSU-1 might be retarded in the first days of alcoholic fermentation by the production of toxic compounds by yeasts other than ethanol and sulfur dioxide. Comparing bacterial growth curves in pure and mixed culture with yeast they found that the presence of rapidly growing yeast was definitely antagonistic to bacterial development. Figure 1 shows typical kinetics of yeast and bacteria survival and growth in a model wine system.



Figure

1 - Growth of wine yeast and bacteria in pure and mixed culture in grape juice/wine system at 20°Brix (from King and Beelman, 1986)

They supposed the inhibition of bacterial growth being due to the presence of yeast metabolites and or the removal of substances important to bacterial nutrition by the yeast. Data in figure 1 show the transition from lag to logarithmic phase of bacterial growth in mixed culture being coincident with the death phase of yeast growth cycle, which may result from essential bacterial nutrients returned to the system as a result of yeast autolysis. Comparing yeast growth curves in pure and mixed cultures, they could demonstrate yeast growth through the stationary phase being unaffected by the presence of bacteria. However an accelerated death rate of the yeast in mixed culture was observed when rapid bacterial growth occurred - We could confirm these findings in our experiments. During simultaneous yeast and malolactic fermentation initial and active growth of the yeast was not affected. But towards the end of alcoholic fermentation, depending on the yeast strain, we could observe a slightly faster decline of the yeast population.

Risk of volatile acidity production

However some authors showed, that strong bacterial growth can inhibit the yeast growth, leading subsequently to high amounts of volatile acidity.⁴ Figure 2 shows 3 different phases of bacterial growth and metabolic metabolism in wine.

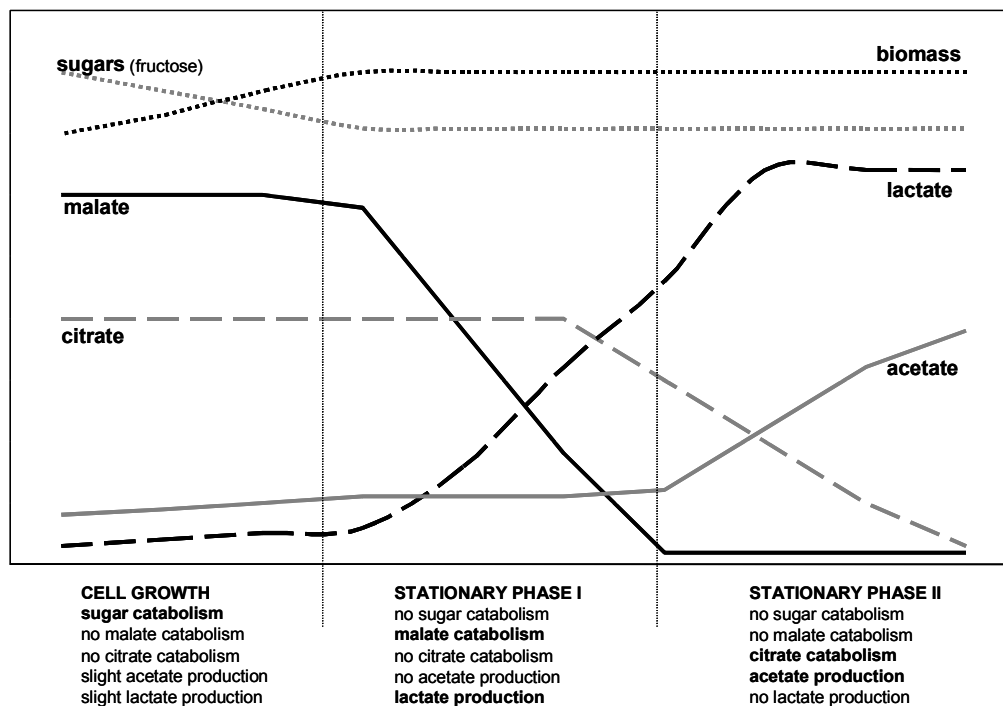


Figure 2: Metabolism of sugars and organic acids during MLF in wine

Radler reported small sugar consumption (0,2 – 2 g) during bacterial growth. Thus, during growth (phase I) small amounts of acetic acid and D-lactic acid can be produced (Figure 2). reaching cell counts higher than 5×10^6 cfu/ml malic acid degradation starts (Phase II). No acetic acid is produced during degradation of malic acid. Phase III is characterized by the degradation of citric acid and sugars accompanied by an increase in acetic acid. However, only when degradation of organic acids (malic acid, followed by citric acid and other organic acids, such as fumaric acid³) is complete, malolactic bacteria will start to degrade sugars resulting in an important increase in volatile acidity. Our experiments confirmed that no acetic acid was produced during growth and active MLF. Only when malic acid was degraded half way and bacteria started to utilize citric acid, an increase in acetic acid was observed. No difference in the final acetic acid concentration was found between the trials with simultaneous inoculation and inoculation post alcohol fermentation. The direct relationship between citric acid degradation and a measurable increase of acetic acid content was demonstrated in the experiment (data not shown).

Depending on availability of oxygen and redox potential, citric acid can be used as an electron acceptor, which will result in acetic acid production, or citric acid can be degraded to diacetyl. Reduction of diacetyl to acetoin and butandiol is also depending on the redox conditions in the wine. Diacetyl is considered as a major flavor compound, which at concentrations between 0.02 and 2 mg/l can give the wine a distinct buttery aroma. The taste-threshold for acetoin and butandiol is much higher than the concentrations at which they are typically found in wine. Thus, they do not contribute to the wine flavor. Further studies are necessary to get more knowledge on precise data on oxygen level and redox potential and their influence on final diacetyl and acetic acid concentrations. MLF in presence of lees always results in lower diacetyl levels, since the reductive power of the yeast supports the reduction of diacetyl to the less flavor active compounds.

Advantages for co-inoculation for example in a white wine will smaller levels of these lactic and buttery flavors and a more fruit driven wine style.

In collaboration with Massey University in New Zealand one yeast strain (CY-3079) and two malolactic strains (EQ54 and ALPHA) were combined in different vinifications. For each yeast/bacterium-combination, malolactic bacteria were inoculated either together with the yeast (simultaneous AF/MLF) or after conclusion of alcoholic fermentation (sequential AF/MLF).

Chardonnay grapes from a commercial vineyard in the Fernhill area, Hawke's Bay, NZ, were used. The grapes were machine harvested at 7.30 am (low portion stalks). 620 kg of fruit were pressed at 11.30 am with 1.3 bar in a bag press to yield 400 L. No SO₂ was added and the must was cold settled at 4°C for 24h. DAP was added to give 300 mg/L. The must had 20.7 Brix, pH 3.28 and 10 g/L TA (as tartaric acid). Vinifications were carried out in triplicate in 25 L carboys. Microorganisms were inoculated according to the manufacturer's recommendations. After conclusion of both alcoholic and malolactic fermentation, the musts were fined with bentonite, cold settled, racked off, adjusted to 36 mg/L free SO₂ and bottled.

Only small differences were found between malolactic strains for any treatment (either simultaneous or sequential AF/MLF). However, considerable differences were found with regard to time for malic acid degradation between treatments with simultaneous and sequential AF/MLF (Table 7):

	Simultaneous AF/MLF	Sequential AF/MLF
EQ54	26 days	74 days (residue)
ALPHA	19.5 days	68 days

Table 7: Time until end of malic acid degradation during vinification of Chardonnay must with simultaneous and sequential AF/MLF.

From an initial concentration of 5 g/L, malic acid was degraded in the same time than sugar was converted to alcohol during simultaneous AF/MLF (approx. 3 weeks). If MLB were inoculated after AF, MLF was difficult and took considerably longer. For the sequential treatment with EQ54, a residue of around 100 mg/L remained (Table 1).

Lactic acid formation was proportional to the degradation of malic acid and final concentrations were similar in all treatments.

Sugar metabolism

Regarding the degradation of glucose and fructose, no considerable difference was visible between simultaneous and sequential AF/MLF during the first two weeks. However, 20 days after complete simultaneous AF/MLF, both glucose and fructose were not detectable in the co-inoculation situation, whereas a residue of combined glucose and fructose of approx. 700 mg/L remained in sequential treatments with both strain ALPHA and EQ54. Since glucose and fructose can serve as carbon and energy sources to many microorganisms, it is suggested that the complete absence of free glucose and fructose will increase microbiological stability.

Organic acid metabolism

Degradation of citrate was different in treatments with simultaneous or sequential AF/MLF. In simultaneous treatments citrate was degraded faster and slightly more acetate produced (Figure 3)

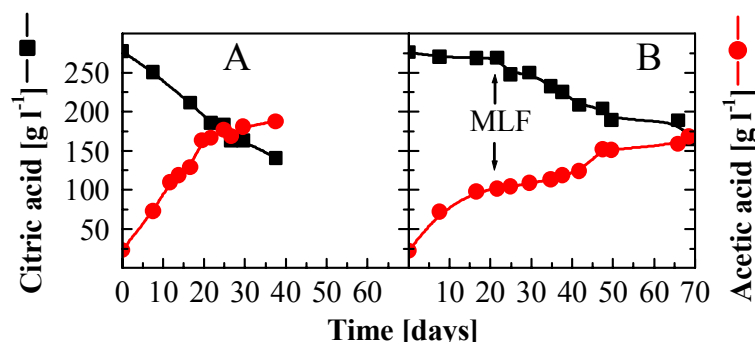


Figure 3: Citrate and acetate concentrations during vinification of Chardonnay must with simultaneous and sequential AF/MLF (ML strain ALPHA).
A, simultaneous AF/MLF; B, sequential AF/MLF.

However, the differences in final acetic acid concentrations were small and statistically only significant for ML strain EQ54 (Table 8).

	Simultaneous AF/MLF	Sequential AF/MLF
EQ54	0.195	0.147
ALPHA	0.187	0.168

Table 8: Final acetate concentrations (g/L) in Chardonnay wine produced by simultaneous or sequential AF/MLF with ML strains ALPHA and EQ54.

Sensory evaluation

Several tests were carried out to evaluate sensory differences between the treatments. According to the sensory evaluation, small differences were detectable between wines due to the stage of inoculation (more fruitiness for the co-inoculation situation). but little or no differences were attributable to the ML strain.

The results in the New Zealand Chardonnay show that inducing simultaneous AF/MLF can give a considerable time advantage in the production of wines. Regarding the possibility of SO₂ addition the wines earlier and the absence of free fructose or glucose in wines from simultaneous AF/MLF, it is suggested that wines made by this technique will be microbiologically more stable.

Overall, no arguments were found against using the technique of inducing AF and MLF simultaneously in low pH white musts.

Conclusion

If early inoculation is still considered being too risky the application of Lysozyme should be considered. Under optimum conditions for bacterial growth such as high pH, highly mature or moldy grapes or temperatures around 22°C, an early addition of lysozyme (500 mg/L) will reduce undesired lactic acid bacteria and help to ensure that the alcoholic fermentation is completed. MLF can be induced later, towards the end of alcoholic fermentation (Figure 4).

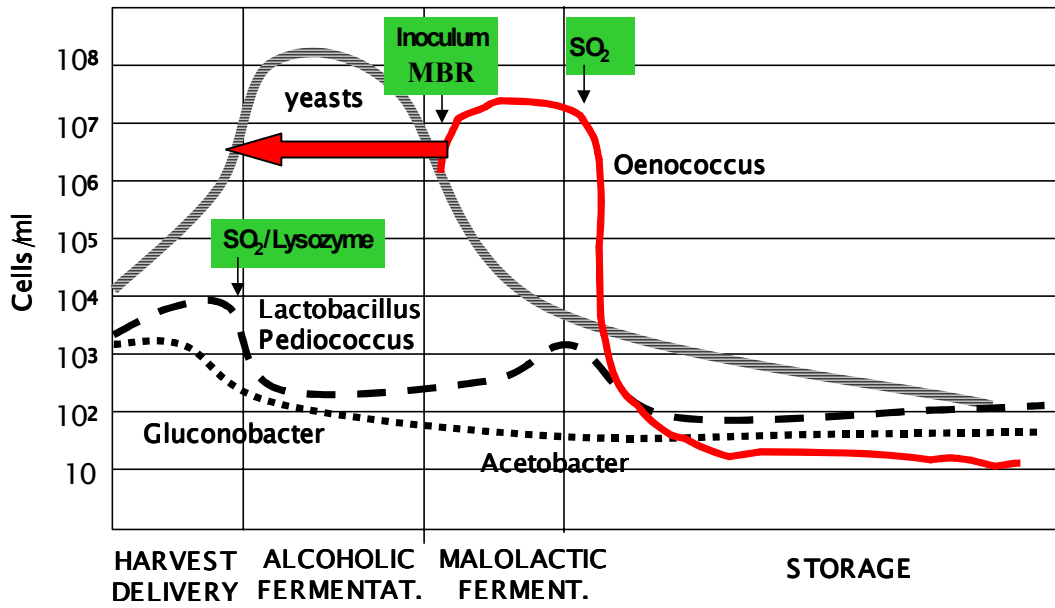


Figure 4: Controlled malolactic fermentation