Using directed breeding to improve a reference strain unsuited to modern winemaking conditions

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INTRODUCTION

Temperature is one of the key parameters in winemaking, due to its impact on fermentation kinetics and the chemical quality of the resulting wine. (***Ribéreau-Gayon et al., 2000a***). Fermentation temperatures range from 13 to 35 °C. White must is usually fermented at low temperatures (<20°C) to obtain wines with a more complex aromatic profile. (***Torija et al., 2003; Masneuf-Pomaède et al., 2006; Molina et al., 2007***). On the contrary, red must is generally fermented at higher temperatures (24-30°C), to optimize color extraction from the anthocyanins in grape skins. (***Ribéreau-Gayon et al., 2000a***). The impact of temperature on the fermentation of must has been examined in detail in the literature. In particular, temperature affects the growth rate of yeast (***Watson, 1987***) and its CO_2 production rate (***Bely et al., 1990a; Bely et al., 1990b; Bely et al., 1990.; Sablayrolles & Barre, 1993a; Sablayrolles & Barre, 1993b***).

Furthermore, temperature affects cell viability at the end of fermentation (***Watson, 1987; Sablayrolles & Barre, 1993a***) by decreasing proton pump activity in the plasma membrane, (***Alexandre et al., 1993; Piper, 1995***) as well as changing the fatty acid (***Torija et al., 2003; Coleman et al., 2007; Beltran et al., 2008***) and sterol composition of the cell membrane. (***Larue et al., 1980; Beltran et al., 2008***).

Temperature also interacts strongly with other fermentation parameters, such as pH (***Ough, 1966a***), sugar content (***Ough, 1966b***), ethanol content (***Ough, 1966a; D'Amore & Stewart, 1987***), and nitrogen availability (***Sablayrolles & Barre, 1993a; Sablayrolles & Barre, 1993b***), accentuating the inhibiting effect of these physicochemical parameters on cell activity. Due to these interactions, an excessively high temperature may cause fermentation to slow down or even stop, especially when other factors reach critical values (i.e. low pH, high ethanol content, nitrogen deficiency, etc.) (***Piper, 1995; Alexandre & Charpentier, 1998; Bisson, 1999; Coleman et al., 2007***).

Although temperature is a carefully-controlled parameter in modern wineries, overheating is relatively common during the fermentation of red must, when the cap reaches significantly higher temperatures than the juice (32°C - 37°C) (***Guymon & Crowell, 1977***). This has a negative impact on yeast viability and may lead to stuck fermentations (***Ribéreau-Gayon et al., 2000b; Coleman et al., 2007***).

To avoid these incidents due to «overheating», it is essential to choose a yeast strain suited to high-temperature fermentation conditions. The genetic diversity of *Saccharomyces cerevisiae* strains in terms of tolerance to high temperatures in winemaking has previously been described (***Rainieri et al., 1998***). As Rainieri observed (1998), this research clearly demonstrated that heat-tolerance resulted from the interaction of several genes, making it extremely complicated to obtain heat-tolerant strains that also have other desirable traits for winemaking (which are also polygenic) (i.e. ethanol tolerance, low volatile acidity production, appropriate aromatic contribution, etc.). *Breeding* techniques, more specifically a series of backcrosses, may be used to overcome this difficulty. This approach, known as *directed breeding,* frequently used in agricultural engineering, consists of repeated backcrosses to obtain a specific trait that gives an individual host an identified advantage without using genetic modification techniques. It took two years' research to adapt this new approach for enology.

This research involved a *directed breeding* program, based on phenotype observations, to develop specific improvements in a commercial yeast strain, ZYMAFLORE F10®, making it more heat-tolerant and reliably able to complete fermentation. After 4 stages of backcrossing, the progeny population shared approximately 93% of the genome of the initial commercial strain. One heat-tolerant clone was selected from this population and compared with the commercial strain under difficult fermentation conditions, to assess the technological enhancement of the initial strain. Finally, this new strain was compared with the original strain and other strains in fermentation tests, to assess the organoleptic qualities of the resulting wines.

MATERIALS AND METHODS

Developing the H4 4th-generation population by backcrossing

The first-generation hybrid (H1) was produced by crossing spores from industrial strains F10® and B, via micromanipulation. The next-generation hybrids, H2, H3, and H4, were obtained by crossing spores from the parent F10® with spores from the H1, H2, and H3 clones, respectively, selected by phenotype. In the end, the fourth generation had over 93% of its genome in common with the initial F10® strain (figure 1).

Fermentation

The fermentation tests used synthetic grape must (***Marullo et al., 2006***), with a slightly-modified composition to mimic the fermentation conditions of red must: (i) two sugar concentrations were tested (S+ and S-), containing 260 and 230g/L, respectively. (ii) Anaerobic growth factors were added to the medium to offset the anaerobic conditions of the fermentations, at two different concentrations (A+ and A-), depending on the dilution of the stock solution.

The medium was inoculated at 10^6 cell/mL.

CO_2 release was measured throughout fermentation in 1.2L fermenters, kept closed to maintain anaerobic conditions, with constant agitation. The apparatus was weighed every 20 minutes. The CO_2 (dCO_2/dt) production rate was calculated, as well as certain parameters, particularly kinetic factors, such as the latency phase (h), maximum fermentation rate or Vmax (g/L/h), and total fermentation time (h).

The progeny clones from each crossing stage were tested in small volumes (300mL), with agitation, under anaerobic and S+A- conditions, at a temperature of 28°C.

RESULTS

Impact of temperature on fermentation completion for nine commercial strains.

To assess the impact of temperature on red wine fermentation, we measured the capacity of nine commercial strains (A - I; strain G was ZYMAFLORE F10®) commonly used in red winemaking, to ferment a synthetic medium at 24 and 28°C isotherm. The S+A+ medium used contained high concentrations of sugars and anaerobic growth factors. At 24°C, all the commercial strains except one (strain I) completed the fermentation (table 1). In the same medium, when the temperature was raised to 28°C, five strains (A, E, F, G and I) stopped fermenting prematurely, leaving over 9g/L residual sugar.

Apart from strain I, which had failed to complete the fermentation at 24°C, the stuck fermentation was directly attributable to the increase in temperature. These observations were used to define the phenotypes of temperature-resistant (B, C, D and H = '***TR***') and -sensitive strains (A, E, F, and G = '***TS***'). Figure 2 shows the fermentation performance of strains B and G (F10®) at 24 and 28°C.

The impact of temperature on kinetic parameters was also assessed: maximum CO_2 production rate (Vmax), duration of the latency phase, and total fermentation time (table 1). These data were extremely interesting as they suggested that over ***50% of industrial winemaking strains were likely to stop fermenting in a must at 28°C*** (isotherm). Most of the strains used for red wine were, therefore, heat-sensitive, so excessively high temperatures should be avoided, especially during the cell division phase and at the end of AF. This corroborates the empirical experience of winemakers, who take care to maintain a reasonable temperature during the fermentation of red must (***Ribéreau-Gayon et al., 2000b***).

Backcross stages to bring a 'TR' trait in a 'TS' strain

The aim was to enhance the performance of a heat-sensitive commercial strain '***TS***' (strain G, ZYMAFLORE F10®), while conserving its useful fermentation and organoleptic characteristics. This optimization was achieved by adding the 'TR' heat-resistant characteristic from strain B via a series of crossings (figure 1). Strain B was chosen among the '***TR***' strains for its excellent fermentation capacities.

The '***TR***' phenotype of the hybrids was checked after each crossing stage by fermentation at 28°C. To evaluate phenotype differences between the various strains, we also used much tougher fermentation conditions, dividing the amount of anaerobic growth factors added by five (medium S+A-). In this type of medium, the B and F10® strains exhibited the same '***TR***/***TS***' phenotype as the corresponding commercial strains. At each backcrossing stage, the fermentation capacity of over 10 progeny was assessed to select the best strain, which was then retained for future crossings with F10®. In each generation, observations confirmed that the phenotype of the hybrid had been enhanced as compared to the initial F10® strain.

In this way, the heat-resistant characteristic from strain B was conserved and the fermentation performance of F10® was clearly improved in its progeny.

Comparison of the impact of temperature and CO_2 rate under different conditions in the medium, using two strains that were genetically very similar.

Whereas sterol deficiency and high temperature were the limiting factors for the F10® strain, the 4th-generation strain, known as **FX10®**, showed enhanced resistance to these factors under all the fermentation conditions tested. A synergy was also observed between sterol deficiency and high temperature. Furthermore, the duration of the latency phase and Vmax were statistically identical pour both strains, while the CO_2 production rate was identical during the first 30% of the fermentation. This indicates that the first part of the fermentation was similar and not too fast for these two strains, facilitating even extraction from the grapes in the aqueous phase and the beginning of the hydro-alcoholic phase. Another observation was that, in the second stage of fermentation, the activity of F10® slowed down sharply, leading to prematurely stuck fermentation (figure 3), especially under conditions combining ***a low sterol content and high temperatures***. On the contrary, its progeny **FX10®** maintained a high fermentation rate (high CO_2 production rate). This result clearly showed that, although fermentation kinetics were similar during the early stages, the directed breeding technique gave F10® greater resistance, not only to high temperatures, but also to sterol deficiency.

CONCLUSION ON *DIRECTED BREEDING*

The *directed breeding* technique consists of crossing two strains, selecting the precise characteristic(s) to be retained from each one. This technological breakthrough, applied to winemaking for the first time by **SARCO** laboratories and the general enology laboratory at the Faculté d'Œnologie de Bordeaux, opens up a number of interesting perspectives. It is important to note that neither *directed breeding*, nor simple *breeding*, involve genetic modification techniques. In fact, genetically modified organisms are produced by adding a trait from another species, or a trait from an individual in a species by means of an artificial technique (e.g. using a plasmid to introduce a trait rapidly). Both *directed breeding* and *breeding* are based on the naturally-occurring sexual reproduction of yeasts, conducted in a controlled manner in a laboratory. Finally, *directed breeding* is a true innovation as compared to simple *breeding*, which merely consists of crossing two strains and observing the combination of traits from both parents in the resulting hybrid. However, due to genetic complexity, some optimum traits are not reproduced, which makes this technique somewhat unreliable. On the contrary, *directed breeding* makes it possible to give a specific trait to an existing strain without modifying its other desirable qualities.

The most important application of *directed breeding* is to adapt commercial yeast strains to new winemaking conditions or techniques. This was the aim with ZYMAFLORE F10®. It was crossed with a heat-resistant parent with excellent fermentation capacity, then the 'best' strain from each generation was backcrossed with the initial F10® strain, to obtain a strain with over 93% of its genome, thus retaining all its organoleptic characteristics.

This new strain, **ZYMAFLORE FX10®**, was tested under real winemaking conditions for large-volume fermentation in several countries, to confirm the two key points: that it had better fermentation capacities than ZYMAFLORE F10® and retained all the main characteristics of the parent strain, particularly in terms of mouthfeel. These aspects are discussed in the following paragraphs.

Comparison of the fermentation capacities of ZYMAFLORE FX10® and ZYMAFLORE F10

The test consisted of fermenting Merlot (2007) grapes from the Bordeaux area in 200hL tanks. Two parallel tests were run, using identical fermentation parameters (skin contact, extraction, temperature, aeration, nutrients, etc.). The only difference was the yeast strain: the first tank was inculated with **ZYMAFLORE FX10®** and the second with ZYMAFLORE F10®. Enzymes, 3g/100kg (30ppm) Lafase HE Grand Cru®, were added to the grapes and the must was inoculated with 20g/hL (200pmm) yeast the day after tanking. The temperature was maintained at 26°C throughout alcoholic fermentation (AF). Development of the yeast strains inoculated in both tanks was confirmed by genetic analysis (PCR), half-way through fermentation.

A one-day difference was noted in the latency phase for F10® yeast (possibly for adapting to the difficult conditions), but, interestingly, both graphs had the same slope, i.e. the same fermentation rate during the first stage (Figure 4). However, the kinetics at the end of fermentation were markedly different: fermentation with **FX10®** ended very cleanly, whereas it slowed down considerably with F10®, once the density dropped below 997.

The wines were analyzed at the end of AF, after 10 days post-fermentation skin contact (table 2). There were no significant differences between the analysis results for the two wines.

This confirmed the enhanced performance of **FX10®** compared to F10® under slightly limiting conditions (13% v/v alcohol). Under slightly tougher conditions, the fermentation with F10® would probably have stopped prematurely.

Comparison of the organoleptic characteristics using ZYMAFLORE FX10® and ZYMAFLORE F10®

ZYMAFLORE F10® yeast is known for its specific organoleptic characteristics: wines fermented with this strain are elegant, with good structure, and silky tannins (low reactivity), and retain all their typical varietal aromas. **ZYMAFLORE FX10®** yeast was designed to exhibit the same aptitude for making wines suitable for ageing.

In order to compare the characteristics of these two strains, a test was carried out under non-limiting conditions, so that ZYMAFLORE F10® could ferment completely and express its full potential. Cabernet Sauvignon grapes (2007), from a high-quality plot in the Bordeaux area, were fermented in 200L tanks. The must analysis results were as follows: Sugars 198g/L, potential Alcohol 11.4%v/v., TA 2.80g/L H_2SO_4, pH 3.60, malic acid 2.80g/L, available nitrogen 63mg/L. The nitrogen deficiency was corrected by adding **THIAZOTE®** twice, once when the yeast was added and again when fermentation was one-third completed, and the must was chaptalized with sugar during fermentation. The must was separated into three samples, each inoculated with 20g/hL of a different yeast strain: the first with ZYMAFLORE F10®, the second with **ZYMAFLORE FX10®,** and the third with ZYMAFLORE F15®. The other fermentation parameters were identical. Development of the yeast strains inoculated in all three tanks (finger printing) was confirmed by genetic analysis (PCR), half-way through fermentation.

The AF kinetics (figure 5) were extremely similar in all three samples; the fermentation rate (slope) was similar throughout the first stage of fermentation. The sample with **ZYMAFLORE FX10®** completed fermentation quickly and cleanly.

All the samples were inoculated with the same strain of pre-acclimated bacteria, **LACTOENOS 450 PREAC®**, and had the same lag phase before malolactic fermentation (MLF) started (no negative impact on bacterial development). Table 3 shows the post-AF analysis results for the wines.

A triangular tasting was organized using wines made with F10® and **FX10®**. No difference was perceived between the two wines (statistically significant at 0.1%). This corroborated the results of the other industry-scale tests with these two strains, carried out during the 2007 vintage in several regions and wineries in France, Italy, Spain, California, Chile, and South Africa.

A tasting was also organized to describe the wines made with **FX10®** and F15®, particularly any differences between wines made using these two red winemaking strains in the ZYMAFLORE® range. The results are presented in figure 6. Both wines received good marks and were well-balanced, but their profiles were quite distinctive: The wine made with FX10® had good structure, with silky tannins, and all the character of its terroir. The wine made with F15® also had good structure, but it was round and fruitier.

The **FX10®** wine certainly corresponded to a *Grand Cru* wine with nice ageing potential, while the F15® wine was more expressive.

Comparison of the fermentation and organoleptic qualities of ZYMAFLORE FX10® and a control under more difficult conditions

Once the similarity of wines made with ZYMAFLORE F10® and **ZYMAFLORE FX10®** had been confirmed by tasting, it was important to test the new strain under more difficult conditions. The test was set up using Cabernet Sauvignon (2007) from a vineyard plot in the southeast of France intended for high-quality wine. Both samples received the same treatment in terms of yeast nutrients, extraction management, temperature control, etc. The only difference was the yeast strain: the control was fermented with the strain usually used by the winery for wine with good aging potential. Development of the yeast strains inoculated in both tanks was confirmed by genetic analysis (PCR), half-way through fermentation.

The alcoholic and malolactic fermentation kinetics were very similar for both samples and the wines were analyzed at the end of MLF (table 4). There was a significant difference in volatile acidity, with 37% less in the **ZYMAFLORE FX10®** wine than the control, which had a mean alcohol content of 14.7%v/v. This is particularly important when a wine is to be barrel-aged for 12 - 18 months. Furthermore, the **FX10®** wine had a deeper color and more massive structure (HCl index) at that stage.

Comparative tasting revealed that these two wines each had distinctive personalities. The wine fermented with **FX10®** yeast was elegant and fruity on the nose, with noticeable tannins that were, however, very soft and silky compared to those of the wine fermented by the control yeast. The control also had a fruity aroma, but it seemed «rougher», less soft and supple on the palate than the **FX10®** wine. This assessment confirmed that the organoleptic characteristics revealed in the **FX10®** wine were certainly inherited from F10®.

CONCLUSION

The directed breeding technique enhanced the fermentation performance of ZYMAFLORE F10® yeast, known as the ideal strain for fine wines intended for long ageing. Its progeny, **ZYMAFLORE FX10®**, was shown to offer perfectly safe fermentation while maintaining all its organoleptic properties, producing elegant wines with good structure.

Directed breeding is a technological breakthrough, which opens up a number of avenues for research, especially as fundamental research has now made it possible to locate certain winemaking traits more precisely on the genome. These tools, together with selection and characterization, will make it possible to develop higher-performance strains that correspond more closely to winemakers' needs.

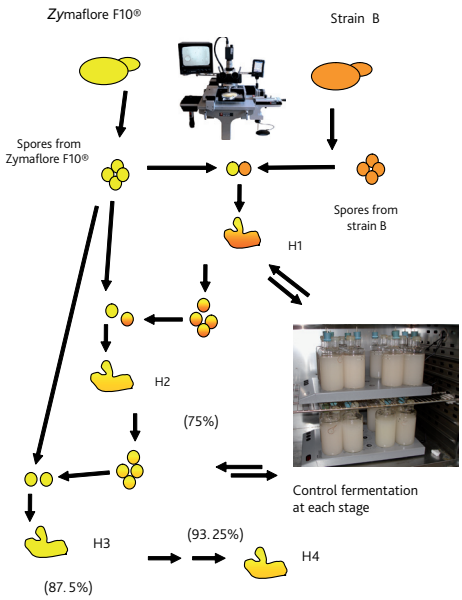

Figure 1: Principle of directed breeding used to produce Zymaflore FX10®.

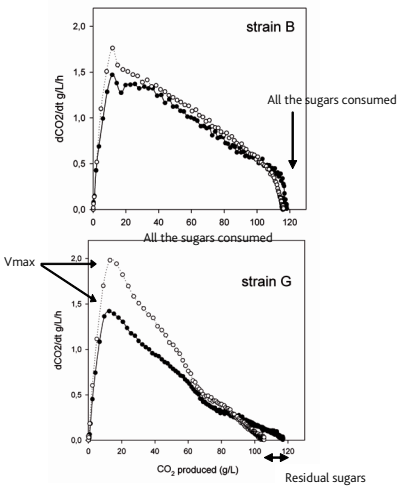

Figure 2: Impact of temperature on CO_2 production (sugar consumption) for heat-resistant strain B and heat-sensitive strain G. Black dots: fermentation at 28°C, white dots: 24°C.

	S+A+ medium at 24°C				S+A+ medium at 28°C			
		Red.		Latency		Red.		Latency
	AF time	sugars	Vmax	phase	AF time	sugars	Vmax	phase
Strains	(h)	($g.L^{-1}$)	($g.L^{-1}.h^{-1}$)	(h)	(h)	($g.L^{-1}$)	($g.L^{-1}.h^{-1}$)	(h)
A	249	1.0	1.52	15	**SF**	9.2	1.73	10.3
B	158	1.0	1.46	17.5	175	1.0	1.76	11.3
C	287	0.6	1.53	12.7	332	1.8	1.56	12.7
D	307	0.4	1.38	13.3	410	0.8	1.49	14.3
E	378	0.9	1.22	13.0	**SF**	12.9	1.64	12.3
F	417	1.8	1.43	13.3	**SF**	32.8	1.99	11.7
G	343	0.7	1.38	44.7	**SF**	32.8	1.92	35.7
h	264	0.4	1.42	13.0	264	1.2	1.55	13.3
I	**SF**	8.6	1.23	18.7	**SF**	48.0	1.41	13.7

SF = Stuck Fermentation

Table 1: Impact of temperature on nine commercial red winemaking strains. These data represent the means of two independent experiments. Strain G is Zymaflore F10®.

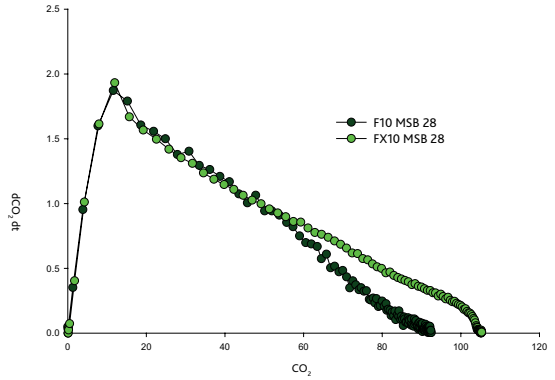

Figure 3: Fermentation kinetics of Zymaflore F10® and ZYMAFLORE FX10® at 28°C in S-A- medium.
ZYMAFLORE FX10® was resistant to high temperatures and sterol deficiency.

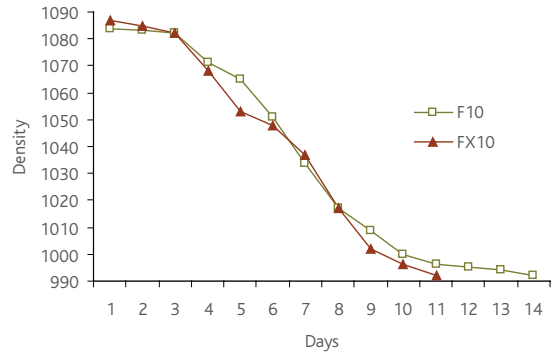

Figure 4: Comparison of the alcoholic fermentation kinetics of the F10® and FX10® yeast strains (first trial). Strains finger printing confirmed by PCR.

Wine	*F10®*	*FX10®*
Alcohol (% v/v) (+/- 0.19)	13	13
Reducing sugars (g/L) (+/- 0.44)	1	1
Total acidity (g/L H_2SO_4) (+/- 0.15)	4.66	5.11
Malic acid (g/L)	4.19	4.35
Volatile acidity (g/L H_2SO_4) (+/- 0.04)	0.25	0.27
pH (+/- 0.05)	3.71	3.63
Free SO_2 (mg/L) (+/- 3.8)	0	0
Total SO_2 (mg/L) (+/- 12.5)	42	43

Table 2: Standard analyses of the wines after AF (first trial).

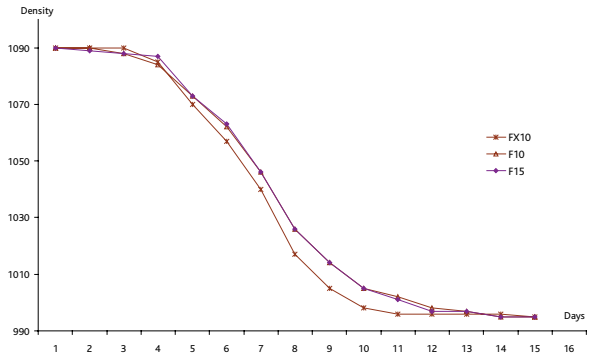

Figure 5: Kinetics of alcoholic fermentation (second trial). Strains finger printing confirmed by PCR.

Wine	*FX10®*	*F10®*	*F15®*
Alcohol (% v/v) (+/- 0.19)	12.30	12.20	12.20
Reducing sugars (g/L) (+/- 0.44)	1.1	1.7	1.3
Volatile acidity (g/L H2SO4) (+/- 0.04)	0.13	0.14	0.14
Modified Color Intensity	7.29	7.53	7.33
OD 280	59	61	60
Tannins (g/L)	3.3	3.5	3.4
Gelatin index	49	48	58
HCl index	15.9	16.5	16.4

Table 3: Standard analyses of the wines after AF (standard analyses) and MLF (phenolic compounds) (second trial).

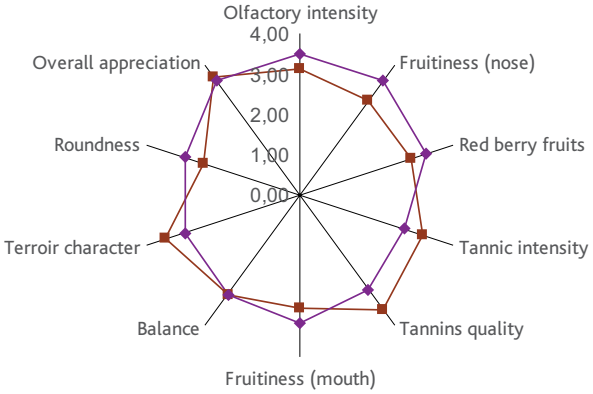

Figure 6: Comparative descriptive tasting of wines made with FX10® and F15® (second trial).

Wine	*FX10®*	*Control*
Alcohol (% v/v) (+/- 0.19)	14.60	14.85
Reducing sugars (g/L) (+/- 0.44)	1.7	1.9
Total acidity (g/L H_2SO_4) (+/- 0.15)	3.41	3.45
Volatile acidity (g/L H_2SO_4) (+/- 0.04)	0.33	0.52
pH (+/-0.05)	3.92	3.88
Modified Color Intensity	14.69	13.21
OD 420	5.430	4.860
OD 520	7.360	6.730
OD 620	1.900	1.620
Tannins (g/L)	3.6	4.0
HCl index (%)	19.2	16.4

Table 4: Standard analyses of the finished wines after MLF (third trial).

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