Technological confirmation that low doses of medium chain fatty acids can arrest alcoholic fermentation to produce sweet wines in milder conditions

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Abstract. The usual technology for sweet white wine production requires the cessation of the alcoholic fermentation before its completion by lowering the temperature in the fermentation tank under 8-10 °C, racking the wine off the lees and adding high doses of sulphur dioxide, usually 150 mg/L or more. This process is energy-consuming and can end up introducing more sulphur dioxide in the wine than in the case of dry wine production. An alternative method for stopping the alcoholic fermentation and producing wines with natural residual sugar is the treatment of the fermenting must with medium chain fatty acids (MCFAs), immediately after a racking off the lees. In this industrial trial 10 mg/l MCFAs were used, in the form of octanoic acid, decanoic acid or 1:1 mixture, respectively, in combination with a low dose of SO_2 (60 mg/L). The treatments were performed at the normal temperature used in white wines for a controlled alcoholic fermentation (15 ºC), without decreasing the temperature any further. A control wine variant was also produced by applying the classical technology with temperature reduction at 8 ºC and addition of 150 mg/L SO_2. All the variants were prepared in triplicate, at industrial scale, in 1000 L stainless steel tanks. The must, obtained from Tămâioasă românească grapes with an initial sugar content of 261 g/L, was inoculated with ERSA 1376 yeast. The results showed that all the treatments applied when the concentration of alcohol reached about 11.0% v/v were able to stop the fermentative process in 50 hours and produce wines with about 55-57 g/L residual sugar and a final alcohol concentration of about 11.7% v/v. The microbiological analyses carried out on solid DRBCA medium to detect the viable yeast, expressed as colony forming units (CFU/mL), showed some differences between the classical technology for sweet wine production and the alternative treatments with MCFAs. At the moment of fermentation interruption, the active yeasts population in wine was on average of $1.98 \pm 0.23 \times 10^7$ CFU/mL, in all tanks. After the fermentation stopped, no viable yeasts were detected in the limpid wines above the lees, irrespective of the treatment. In the wine sediments of MCFAs-treated variants a few viable yeasts/mL were still detected, while in the lees of control wines no viable yeast was present. Anyway, racking the wines with MCFAs-stopped fermentation from the lees will prevent any possibility of refermentation. The main advantages of these alternative treatments with MCFAs are that they do not require a reduction of temperature from 15 ºC to 8 ºC and, especially, that they necessitate a much lower dose of SO_2 (in this case 60 mg/L instead of 150 mg/L).

1 Introduction

Medium-chain saturated fatty acids (MCFAs), C_6-C_{12}, are inhibitors for yeasts and lactic acid bacteria [1,2]. In wines and other fermented beverages, they are produced by yeasts depending on many variables such as: aeration regime, yeast strain, temperature, pH, yeast assimilable nitrogen or other less known factors [3]. The accumulation of MCFAs in the fermentation media is triggered by anoxic conditions and the lack of unsaturated fatty acids and of ergosterol in the must. Certain concentrations of these compounds could lead to stuck alcoholic fermentation (AF) or can make impossible the initiation of malolactic fermentation (MLF) in red wines [1,2].

These natural inhibitors caught the attention of many researchers for the production of wines with residual

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sugars and low doses of SO_2 [4-6]. The results of these studies vary, as in certain circumstances the outcomes were positive, while in others were not convincing. The aliphatic carbon chain length is an important aspect, as the longer the chain, the higher the acid hydrophobicity and inhibitory properties, due to a better solubility in the phospholipids of yeast membranes, which leads to the loss of their biological function [7]. Thus, the decanoic acid is considered more noxious than octanoic acid.

The main winemaking parameters influencing their inhibitory properties are ethanol concentration, pH, temperature and yeast strain [8,9]. Accumulation of MCFAs during AF led to an augmented yeast membrane permeability and combined with winemaking conditions, such as low pH and/or increased ethanol-induced proton influx, can lead to cytosol pH decrease, thus promoting the loss of yeast viability and subsequently the cell death [7]. Generally, yeasts need to maintain their cytosol pH between 6.0 and 6.5 to produce ATP through glycolysis, but lowering the internal pH below 6.0 not only stops ATP production, but also promotes conditions for enzymic autolysis, leading to cell degradation [10,11].

The use of MCFAs for the production of wines with residual sugars is still under evaluation as an alternative method to stop AF and in the same time limit the doses of SO_2 required for the classical method and reduce electric energy consumption necessary for cooling [4,6,9].

The fact that the addition of MCFAs for AF cessation reduces the production of acetaldehyde compared to the chilling process and reduces the diacetyl content compared to cross-flow filtration, both important SO_2-binding compounds in wine, can contribute to the reduction of required SO_2 [12].

Keeping normal temperatures of the fermentation media and not cooling the media, generally enhance the inhibitory effect of MCFAs, especially during the mid or the end of AF, due to an increased fluidity of cell membranes caused by accumulated ethanol [13]. A laboratory-controlled experiment on synthetic growth medium also demonstrated that higher temperatures increase inhibitory property of MCFAs [9].

The stopping of AF with MCFAs requires also a certain amount of ethanol present in the medium. The ethanol concentration and low pH enhance the inhibitory effect of MCFAs [8,9]. Otherwise, yeasts and bacteria can tolerate higher concentrations of MCFAs in the absence or low ethanol content [14].

The addition of 10 mg/l MCFAs as individual dose or as mixture dose was found to be enough to stop the AF to obtain sweet wines and at this maximal concentration the sensorial properties of wines are not affected or are slightly modulated depending on the conditions during AF, such as the use of different yeast strains [4,6]. The yeast strains respond differently to inhibitors and are able to adapt and to trigger several mechanisms for detoxification in the presence of inhibitors which can influence sensorial profiles of wines [15-17]. One of the detoxification mechanisms that is considered to potentially impact the sensory properties of wines is the formation of fatty acid ethyl esters [4,6,18-21]. However, the sensory effect is not major and it will be studied during the evolution of these wines obtained by stopping fermentation with various MCFAs.

The aim of the present study is to demonstrate and confirm at an industrial scale that the addition of low doses of MCFA and lower doses of SO_2 represents an alternative technology for sweet wine production, able to arrest AF without the need to chill the wine or to add high doses of SO_2, as is the case in the classical technology.

2 Material and methods

2.1 Winemaking

The grapes used in this study were of Tămâioasă românească variety, clone 36Pt, an aromatic variety with a predominant terpenic profile. The grapes were grown in Pietroasa wine centre, Romania, and harvested manually at technological maturity, on September 16th, 2022.

The grapes were processed at an industrial level using a Puleo Vega 10 crusher-destemmer. The crushed grapes were treated with a dose of 60 mg/kg $K_2S_2O_5$ and transported with a helical rotor pump through a heat exchanger and then left for 24 hours for a pre-fermentative maceration at controlled temperature in a stainless-steel tank. The temperature of crushed grapes was lowered to 10-12 °C and the mash treated with two enzymes: one pectolytic enzyme Speed UP Aroma – (Sodinal), in a dose of 2 g/q and another pectolytic enzyme with a strong beta 1,3-glucanase activity, Vinotaste Pro (Lamothe Abiet), in a dose of 5 g/q. To improve the extraction of aroma precursors, a recirculation regime of 2 minutes every 2 hours was set. The macerated crushed grapes were transferred into a pneumatic press (Bucher Vaslin Xplus 80 IT) with which free run and press musts were extracted. The free run must was obtained through draining and soft pressing with several cycles up to a maximum of 0.6 Bar. The must resulted from pressing at over 0.6 Bar was excluded from the experiment because they carry a much higher load of polyphenols, affecting the quality of the wines. The resulted free-run must was then treated with pectolytic enzyme for clarification in a dose of 2 g/hl Enozim Lux (Agrovin) and with 10 g/hl PVPP (LaFood). Two hours after the enzyme treatment, a dose of 20 g/hl Na-bentonite Microcol Alpha (Laffort) was added in order to inactivate enzymatic activities. After two days of settling, the clear must was transferred to a 200-hl stainless-steel tank. At this stage, the parameters of limpid grape juice were: sugars = 261 g/l; titratable acidity expressed as tartaric acid = 4.6 g/l; pH = 3.55; NH_4^+ = 20 mg/l; α-amino acids, expressed as eq. isoleucine = 130 mg/l. To adjust the total acidity, the dose of 2.5 g/l tartaric acid was used, thus increasing the titratable acidity to 7.0 g/l and lowering the pH to 3.03. The resulted must was inoculated with a suspension of 30 g/hl of selected *Saccharomyces cerevisiae* yeast, strain ERSA 1376 iFruit (Enologica Vason) with 30 g/hl of organic nutrient ActiveManno White (LaFood), rich in protective agents for yeast (sterols, unsaturated fatty acids, glutathione, α-amino acids, etc.). This yeast strain was used because on our previous small-scale experiments performed between 2018 and 2021 it was the one which maintained the most

the classical sensory profile of Tămâioasă românească wines in Pietroasa wine centre [4]. This inoculum provided 2,78 x 10^6 of active yeast cells/ml in the must. The added nutrient increased the α-amino acids, expressed as eq. isoleucine from 130 to 152 mg/l. During the AF, an organic nutrient Nutristart ORG (Laffort) was added in a dose of 15 g/hl at about 48 hours from the inoculation and another dose of 15 g/hl at about 72 hours from inoculation.

The moment of AF cessation was determined following the fermentation diagram with the evolution of sugar, density, alcohol and temperature and was established so that to obtain wines with of 12% v/v alcohol and over 45 g/l reducing sugars.

The AF cessation was carried out after 400 hours (over 16 days) of fermentation through racking off the lees, treatment with 60 g/hl Na-bentonite Microcol Alpha (Laffort) combined with fermentation inhibitors detailed in Table 1. The sulphur dioxide doses were applied using a solution of 10% m/v $K_2S_2O_5$, while MCFAs were used as solutions of 10% m/v (octanoic or decanoic acids) in 70% v/v ethanol. The industrial-control batches were produced in 3000-liter stainless steel tanks with controlled temperature, in which it was possible to reduce the temperature to 8 °C, as the classical method requires. The alternative experimental batches using MCFA inhibitors were produced in 1000-liter floating lid stainless steel tanks without temperature control, for energy saving during the cessation process. All the experimental variants were done at industrial scale in triplicates as detailed in the Table 1.

Table 1. Experimental variants done in triplicates.

Experimental variants	Octanoic acid, mg/l	Decanoic acid, mg/l	SO_2, mg/l	Temp., °C
Control	0	0	150	8
Oc10	10	0	60	15
De10	0	10	60	15
OcDe5	5	5	60	15

2.2 Analyses and process control

To avoid the risks associated with fermentations that are too slow or arrested before the established moment of AF cessation, the number of yeasts in the fermenting must was determined and the yeast viability verified to be over 90% [22], threshold below which some fermentation issues can occur.

This evaluation was performed at 96 hours from inoculation by microscopy, using sample dilutions of fermenting must, a Thoma counting chamber and vital methylene blue dye [22]. The counting was done in 5 squares of the Thoma chamber, one in each corner of the grid and one in the central part. All viable cells (the unstained ones) were counted, and then, separately, the non-viable cells (the stained cells) were also counted. The viability was calculated as percentage, by dividing the viable cells to total cell number and multiplying by 100.

To count the viable cell number after the cessation of AF the technique of cultivation on selective medium was used. The culture medium used for the CFU technique is DRBCA (Dichloran Rose Bengale Chloramphenicol Agar) (Scharlau, Spain). To isolate the yeasts, 31.6 g of powdered medium were dissolved in 1 litre of distilled water and sterilized by autoclaving at 115 °C for 15 minutes. The medium was supplemented with chloramphenicol to inhibit the growth of bacteria. Representative samples were taken from the stainless-steel tanks and analysed after decimal dilutions made in aseptic conditions. The inoculation on the solid medium in Petri dishes was done using the lawn microbiological technique, applying 0.1 ml of the undiluted and diluted samples, then spread using a Drigalski spatula. The Petri dishes thus seeded were placed in the thermostat with the lid down at 25 ºC, from 3 to 5 days. Counting of the developed colonies for each dilution was done by naked eye, as the number of colonies were between 15 and 300. The results were expressed as an average of the number of colonies for each dilution. $CFU = m \cdot c \cdot 10$, where: m - the arithmetic mean of the colonies counted on 3 plates seeded with the same dilution; c - the inverse of the dilution from which the seeding was done; 10 - coefficient of reporting the result per 1 ml.

During the entire period of the AF, the process control was ensured by monitoring the temperature and the refractive index with an Abbe refractometer, which allows the estimation of the parameters of residual sugar, density and alcohol concentration of wines through several equations (modernized refractometric methodology used by the team from the Unit of Viticulture and Oenology, USAMV) [23].

Physico-chemical parameters of the resulted wines were determined using OIV methods [24]: alcoholic strength – distillation and pycnometer technique (OIV-MA-AS312-01A); reducing sugars – Luff-Schoorl method (OIV-MA-AS311-01A); density - hydrometry (OIV-MA-AS2-01); total acidity - potentiometric titration (OIV-MA-AS313-01); volatile acidity – steam distillation (OIV-MA-AS313-02); free and total SO2 - aeration-oxidation method (OIV-MA-AS323-04A); pH (OIV-MA-AS313-15); total dry extract and sugar-free extract – Tabarié method (OIV-MA-AS2-03B).

3 Results and Discussions

The risk associated with slow fermentative process due to high sugar concentration was evaluated in the 200-hl batch, from which must samples were extracted at 96 h form inoculation.

The total (T), viable (V) and non-viable (N) yeasts in the fermenting base must was evaluated by direct counting on Thoma chamber and results are included in Table 2. As the viability proportion (V%) of yeast was over 90% and the viable yeast population reached 1.07 x 10^8 cells/ml, it meant that there were no risks associated with slow/arrested AF.

In order to obtain the desired parameters of sweet wines, particularly the concentrations of alcohol and residual sugars, the kinetic of AF was followed through a refractometric method to estimate the level of these parameters and to determine the optimal moment for AF cessation (Fig. 1).

Table 2. Evaluation through microscopy of the total, viable and non-viable yeasts in the fermenting base must (96 hours from inoculation).

V, cells/ml	N, cells/ml	T, cells/ml	V%
1.07 x 10^8	6.00 x 10^6	1.13 x 10^8	**94.69**

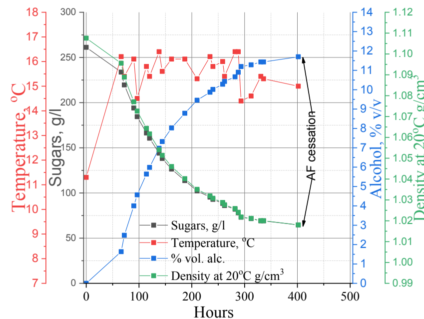

Figure 1. Alcoholic fermentation kinetics of base must from which the experimental variants are derived (200 hl batch).

The chosen moment for AF cessation was set after 400 hours of fermentation, when the alcohol concentration reached 11.5% v/v and residual sugar was estimated to be slightly above 60 g/l. From the moment of applying the treatments for stopping the AF the evolution of residual sugar (Fig. 2) and of the alcohol (Fig. 3) was followed.

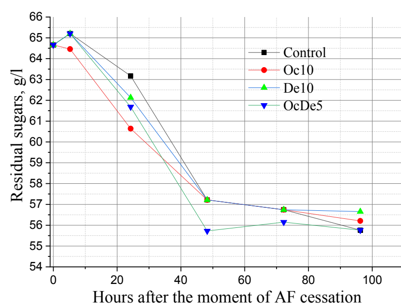

Figure 2. Evolution of residual sugar in the experimental industrial variants from the moment of applying the cessation treatments (values are average of repetitions).

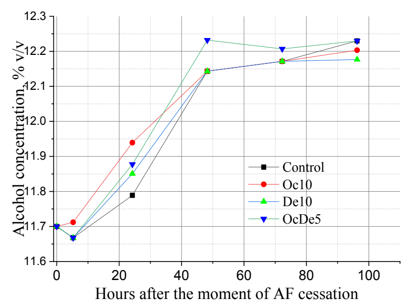

Figure 3. Evolution of alcohol concentration in the experimental industrial variants from the moment of applying the cessation treatments (values are average of repetitions).

In order to evaluate the efficacy of MCFAs compared with classical method used for AF cessation, viable yeast populations were determined in all variants and repetitions by the CFU technique on DRBCA medium.

To have a reference (Table 3), viable cells were also determined with the same technique prior to AF cessation, in the tanks containing only inoculated must, just before selected yeast inoculation (T0) and 400 hours after inoculation, which is the moment when the AF cessation was started (T1).

Table 3. Viable yeast population using CFU technique on DRBCA medium supplemented with chloramphenicol.

Time of analysis	CFU/ml	Log_{10} (CFU)
T0 (0 h) – base clarified must, before selected yeast inoculation	3.50 ± 0.50 x 10^4	4.54 ± 0.06
T1 (400 h) - must in fermentation, just before AF cessation	1.98 ± 0.23 x 10^7	7.29 ± 0.05

This T1 is considered a critical point for the cessation process, showing the total population of viable yeast cells which have to be inhibited to arrest the AF when MCFAs are added in experimental samples. As shown in Table 3, the yeast cells still viable at T1, 400 h after inoculation with selected yeasts, were about 1.98 ± 0.23 x 10^7 CFU/ml. This was selected as the starting point of AF cessation, because the yeast population was already declining and it also met the prerequisite for the optimum required ethanol concentration.

The procedure for stopping the AF involved racking off the wine from the gross lees and splitting the wine in smaller tanks, where variants and repetitions were obtained, by adding the fermentation inhibitors described in Table 1. A subsequent treatment with 60 g/hl Na-bentonite was also done in order to aid the clarification and to remove most of the heat unstable proteins. Five days after stopping the fermentation viable yeast populations were determined, both in the limpid wine and in the wine sediment in each tank. Interestingly, all the limpid wine samples were free of viable yeasts five days after AF cessation, irrespective of the type of treatment used for attaining the result (Table 4).

Table 4. Viable yeast population using CFU technique on DRBCA media supplemented with chloramphenicol (5 days after AF cessation).

Samples	Limpid wine		Wine sediment (fine lees)	
	UFC/ml	Log10UFC	UFC/ml	Log10UFC
Control	n.d.	n.d.	n.d.	n.d.
Oc10	n.d.	n.d.	7.67±4.70	0.77±0.34
De10	n.d.	n.d.	6.33±1.89	0.78±0.12
OcDe5	n.d.	n.d.	6.00±0.82	0.77±0.06

However, in the sediment collected from the bottom of the tanks in which fermentation was stopped by using MCFAs and low doses of SO_2, some viable cells were still present, which was not the case of the control samples, were fermentation was stopped with higher doses of SO_2 and cold temperature (Table 4). The viable yeasts found in the sweet wines obtained by using 10 mg/l MCFAs, irrespective of the type or combination of the acid used, varied between 5 and 12 yeasts/ml of sediment. Therefore, a prompt subsequent racking is necessary for these wines, to prevent any possibility of refermentation.

These results confirm that 10 mg/l MCFA can replace the classical approach for AF cessation in sweet wine production, even if several days after treatment the sediments may still contain a few viable yeasts. These viable cells are not a major issue, because the fine lees formed during wine clarification are usually removed shortly after.

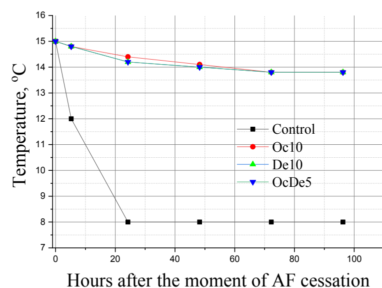

Figure 4. Evolution of temperature in the stainless-steel tanks with the experimental industrial batches from the moment of applying AF cessation treatments.

Using MCFAs with lower doses of SO_2 (60 mg/l) instead of low temperature with high doses of SO_2 (150 mg/l) allows reducing the total amount of SO_2 in the final wines. Another important advantage of MCFA treatments is the energy savings, since there is no need to cool the tanks anymore to lower the temperature from 15 ºC to 8 ºC and to maintain it this low for 5 days (Fig. 4).

To stabilize the wines, a second treatment with 60 g/hl Na-bentonite and a second racking off from the lees were applied, for a complete removal of haze-forming protein. For tartaric acid, the wines were cold stabilized without additional costs, through exposure to natural temperatures for two months during winter, as the experimental tanks are placed on a platform outside. After stabilization, the resulted wine samples were filtered through Hobrafilt S10N depth filter sheets with a porosity up to 0.8 µm. After other four months, the wines were ready for bottling, when they were again filtered through Hobrafilt ST5N depth filter sheets with a reduced porosity of up to 0.3 µm. At bottling, 20 mg/LSO_2 was added and nitrogen was used as a protection against oxidation during filling.

The bottled wines were submitted to the usual physico-chemical analyses and the results are presented in Table 5. These results confirm that with 10 mg/l MCFAs treatments sweet wines with similar parameters with the control wines can be successfully produced. As it can be seen in Table 5, with the exception of free and total SO_2, all the determined parameters for wines obtained by MCFAs treatments are not statistically different from the control samples, obtained with the classical method. The free and total SO_2 were, as intended, lower in the wines produced with MCFAs treatments, with about 30 and 80 mg/L, respectively.

This is a significant reduction of SO_2 for sweet wines produced at an industrial scale. The experiment also demonstrated at a large-scale the possibility of stopping fermentation to produce sweet wines without cooling the wine at 8 °C.

However, even though after AF cessation the colour (OD_{420nm}) of wines produced with MCFAs and lower doses of SO_2 was not significantly affected as compared to control wines (Table 5), during the subsequent evolution the experimental wines with MCFAs are expected to be a bit more sensitive to oxidation. Depending on the level of oxygen exposure during stabilization and filtration treatments, low-sulphite wines may require additional doses of SO_2 to ensure their protection against chemical oxidation on longer periods of storage.

Further work is planned in order to evaluate the colour evolution as well as the sensory and volatile profile changes for the wines produced with the use of MCFAs and low doses of SO_2 at industrial-scale. Our previous results on small-scale winemaking experiments using doses of MCFAs between 10 and 30 mg/L revealed that at doses of 10 mg/L MCFAs the yeast strain ERSA 1376 ensured a sensorial quality similar with the one of the control samples, not inducing significant changes of the sensory profile [4]. However, this fact needs to be assessed also at industrial scale and in evolution. To ensure a minimum impact on the sensory profile, for the wines produced with MCFA treatments, the use of low-ester producing yeasts would be recommended.

Table 5. Physico-chemical analyses of the industrial scale experimental wines obtained by stopping the fermentation by different treatments.

Physico-chemical parameter	**Treatment**			
	Control	**Oc10**	**De10**	**OcDe5**
Alcoholic strength, % v/v	12.2±0.1^a	12.3±0.1^a	12.3±0.2^a	12.3±0.1^a
Reducing sugars, g/l	49.6±1.0^a	49.4±0.4^a	50.2±2.6^a	51.0±1.7^a
Density (20°C), g/l	1013.4±0.5^a	1013.7±0.3^a	1014.3±1.1^a	1014.4±0.7^a
TA, g/l tartaric acid	7.1±0.1^a	7.1±0.1^a	6.9±0.1^a	7.0±0.1^a
VA, g/l acetic acid	0.22±0.01^a	0.23±0.01^a	0.23±0.02^a	0.22±0.01^a
Free SO_2, mg/l	**56.7±2.1^a**	**27.9±1.6^b**	**25.7±0.9^b**	**25.8±1.5^b**
Total SO_2, mg/l	**169.0±4.6^a**	**84.0±2.3^b**	**84.7±2.7^b**	**82.2±2.5^b**
OD_{420nm}	0.013±0.001^a	0.014±0.002^a	0.014±0.002^a	0.013±±0.002^a
pH	3.03±0.01^a	3.06±0.03^a	3.04±0.03^a	3.05±0.02^a
Total dry extract, g/l	79.0±1.0^a	79.9±0.7^a	81.3±2.3^a	81.4±1.7^a
Sugar-free extract, g/l	28.3±0.1^a	29.0±0.5^a	28.8±0.7^a	28.7±0.2^a

Different letters on each row indicate a statistically significant difference between the averages for the assessed varieties at a probability level of 95% (α = 0.05) determined by one-way ANOVA and Tukey test ($p < 0.05$).

4 Conclusions

The industrial-scale experiment conducted with Tămâioasă românească, a high sugar accumulating muscat-type variety, highlighted some benefits of using MCFAs to stop the alcoholic fermentation and produce sweet wines. The parameters of the wines produced with MCFA treatments showed non-significant differences irrespective of the type of acid used, in our case octanoic and decanoic acid, applied independently or in combinations.

The microbiological analyses revealed the efficiency of MCFAs inhibitors at low doses in combination with a reduced dose of SO_2 and without the need of cooling the tanks as it is the case in the classical process of AF cessation.

The most important aspects for successful implementation of MCFAs for sweet wine production should focus on the optimal selection for the moment of AF cessation and on correct and timely separation of the sweet wines from the lees after MCFA treatments produced their effects.

Considering the previous knowledge of the factors affecting MCFA inhibitory properties and our results, for an optimal AF cessation the fermenting must should contain at least 11% v/v alcohol, to ensure synergism. Also, the pH correction of the high sugar musts should be seriously considered when the starting pH is high, as the MCFAs work better at low pHs, which increase they inhibitory effect.

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