The inhibition of *Saccharomyces cerevisiae* population during alcoholic fermentation of grape must by octanoic, decanoic and dodecanoic acid mixture

Mojmír Baroň, Michal Kumšta, Kamil Prokeš, Lenka Tomášková, and Magdaléna Tomková

Department of Viticulture and Enology, Faculty of Horticulture, Mendel University in Brno, Valticka 337, CZ-691 44 Lednice, Czech Republic

Abstract. The inhibition of alcoholic fermentation by octanoic, decanoic and dodecanoic acid mixture was investigated. Middle chain fatty acids (MCFA) mixture contained 10 grams of C_8:C_{10}:C_{12} in a ratio of 2:7:1 was dissolved in 100 ml of 70% ethanol, and in such form, it was subsequently applied into the fermenting must samples. A flow cytometry test showed that the 10 mg/L dose of MCFA mixture had a toxic effect on *Saccharomyces cerevisiae* (45.9% of viable cells) compared with the control variant (74.35%). In combination with 60 mg/L of SO_2, it had a higher efficiency (3.1% of viable cells) than using a dose of SO_2 alone (13.9%). Direct counting of yeast cells confirmed a higher concentration of dead cells with a higher concentration of MCFA. A dose of 10 mg/L of MCFA mixture caused the highest percentage of dead yeast after 24 hours (about 60%) compared to the control variant without MCFA dosage (about 24%). The results of residues showed that there is no significant quantitative difference between the treated and untreated musts because of fixing of MCFA inside the yeast cells. This method can effectively reduce the cost of production technology for wines with residual sugar and, in general, reduce the dosage of SO_2.

1. Introduction

Alcoholic fermentation is one of the most important processes in wine technology (1). Under appropriate conditions (2), there is an increase in yeast mass and fermentation of the must to a state of exhaustion of the sugar media. In recent years, the popularity of wines with residual sugar has increased, and thus it has become necessary to inhibit and stop fermentation (3).

The issue of early termination of alcoholic fermentation is rather complex. Cooling and filtration lead to an increase in costs and are laborious and generally unavailable, especially for small-scale producers. The individual application of SO_2 is not always fully reliable, and in higher concentrations leads to a reduction in future quality.

Some higher fatty acids (MCFA), particularly long chain (C_{16} and C_{18}), activate alcoholic fermentation. On the other hand, other MCFA with shorter chains, especially hexanoic C_6, octanoic $C_{8,}$ and decanoic C_10 acids, have fungicidal properties (4; 5; 6). The study of the endogenous MCFA inhibitory effect on alcoholic fermentation and malolactic fermentation were carried out (7). MCFA are produced by yeast during alcoholic fermentation and may contribute to the difficult completion of its general process. In other words, their increased concentration often accompanies problems with the process of alcoholic fermentation (8; 9; 10). The addition of MCFA has an antifungal effect and supplements SO_2. For example, to stop the fermentation of sweet wines, 150 mg/L SO_2 with 9 mg/L of MCFA added has the same efficacy as 250 mg/L of SO_2. MCFA should be added 24 hours before sulphating. Under these conditions, SO_2 causes the majority of the (if not complete) inactivation (11). It was found that the doses sufficient to inhibit varies with the type of yeast tested. For example, *Kluyveromyces marxianus* is less sensitive than *Saccharomyces cerevisiae* (12).

A study comparing the composition of MCFA and their esters in wine treated and untreated with MCFA showed that concentrations remained within the normal range of 2.6 to 12.4 mg/L (MCFA) and 0.2 to 0.81 mg/L (ethylesters) without increase of the concentration of any volatiles (12).

These features, combined with the wholesomeness and increasing popularity of wines with residual sugar, led to the idea that MCFA could serve as a substitute, or rather a complementary method, for the use of SO_2 in wine technology. In 2016, a communication was presented regarding the use of saturated higher fatty acids (MCFA) in oenology in commission II "Oenology" in OIV, and the Czech Republic applied to allow this as new oenological method.

2. Material and methods

2.1. Experimental design

To demonstrate the efficacy of MCFA on yeast inhibition, two inhibition tests were performed. The MCFA solution was applied into the fermenting must of a '*Welschriesling*' variety.

Table 1. Design of experiment.

Determining the efficacy of MCFA in fermenting must	Flow cytometry Flow cytometry
	Direct counting in Bürker chamber
Determination of MCFA residues in treated wines	GC chromatography

2.1.1. MCFA solution

The MCFA solution contained C_8:C_{10}:C_{12} in a ratio of 2:7:1. From a total of 10 g of mixture, there was 2 g of C_8, 7g of C_10 and 1 g of C_12. This mixture was dissolved in 100 mL of 70% ethanol. In such a form, different doses of MCFA mixture were subsequently applied into samples of grape must. For GC analysis, the finished wine after the first racking was used.

2.2. Biological material

'Welschriesling' must: Glucose + fructose content 200 g/L, pH 3,0, titratable acids 11.3 g/L, and the must was treated with 20 mg/L SO_2. Mixture of MCFA was added after seven days of fermentation, glucose + fructose content in this time was 50 g/L.

2.3. Flow cytometry test

The first inhibition test was performed using a flow cytometry.

2.3.1. Determination of live and dead cell concentration

The cell concentration in the samples was measured with no sample preparation by direct analysis of the sample must using a flow cytometry.

2.3.2. Determination of yeast viability

Determination of yeast viability using flow cytometry was performed after the sample preparation. 1 mL of each sample was collected in a micro test-tube, centrifuged and washed 2x with demineralized water. 200 μL of washed cell suspension was added to fluorescein diacetate (FDA) and propidium iodide (PI) at a concentration of 20 μg/mL and 10 μg/mL, respectively. The samples were incubated at room temperature, in the dark, for ten minutes. The experiment was carried out twice; further samples from the first attempt were measured again one hour later. One drop of prepared sample was used for direct microscopic observation using ultraviolet radiation.

2.4. Direct counting in a Bürker chamber

The second inhibition test was performed by direct counting in a Bürker chamber. The fermenting must contain 50 g/L of glucose + fructose was homogenized by stirring. Then it was put into bottles to the volume 0.75 L, to which was added a different dose of MCFA mixture according to Table 2. Each variant was repeated, in the second iteration. The counting was performed 24 and 168 hours after application of the MCFA.

Table 2. Samples with added concentration of MCFA mixture.

	Dose of MCFA mixture [mg/L]
Sample 1	0
Sample 2	4
Sample 3	5
Sample 4	6
Sample 5	7
Sample 6	8
Sample 7	9
Sample 8	10
Sample 9	20
Sample 10	40

2.4.1. Counting in a Bürker chamber

The sample was coloured by methylene blue solution to better distinguish the dead and live yeast. On a clean glass of the counting chamber, a sample was pipetted and treated with methylene blue for easy differentiation of live and dead cells. Under the microscope (Olympus CX 31) at 40x magnification, each of the fields of the Bürker counting chamber was monitored. 72 fields were evaluated at 6 columns to 12 squares, 1 square having an area of 1/25 mm^2. The dead yeasts are distinguished from the live ones by the intensity of their colour. The dead cells are coloured dark blue as a result of the disruption of their protective phospholipid layer, thus absorbing a bigger quantity of the pigment. On the contrary, the live cells are coloured blue. Samples were properly diluted so that the number of microorganisms ranged from 10 to 40 cells per square.

2.5. Test of MCFA residues

The purpose of the experiment was testing residues of MCFA in treated wines after application into the fermenting must.

2.5.1. Experimental design

- Control sample (only 100 mg/L SO_2)
- Variant with 10 mg/L of MCFA - mixture C_8, C_10 and C_12 (2:7:1).

Each variant had a volume of 350 litres. Subsequently, the finished wine after the first racking (approximately 30 days after must inoculation) was measured with GC-MS (Gas Chromatography/Mass Spectrometry).

The concentration of individual volatile compounds in wine was determined according to the unpublished method of extraction with methyl-t-butylether. 20 mL of wine was pipetted into a 25-mL volumetric flask together with 50 μL of 2-nonanol solution in ethanol; this compound was used as an internal standard (in concentration of 400 mg/L) and 5 mL of a saturated $(NH_4)_2SO_4$ solution. The flask content was thoroughly stirred, and thereafter a volume of 0.75 mL of the extraction solvent (MTBE with added 1% of cyclohexane) was added. After another thorough stirring and separation of individual phases, the upper organic layer (supernatant) was placed into a micro test-tube together with the produced emulsion and centrifuged; the clear organic phase was dried up with anhydrous magnesium sulphate. Extract samples (adjusted in this way) were thereafter used for the GC-MS analysis.

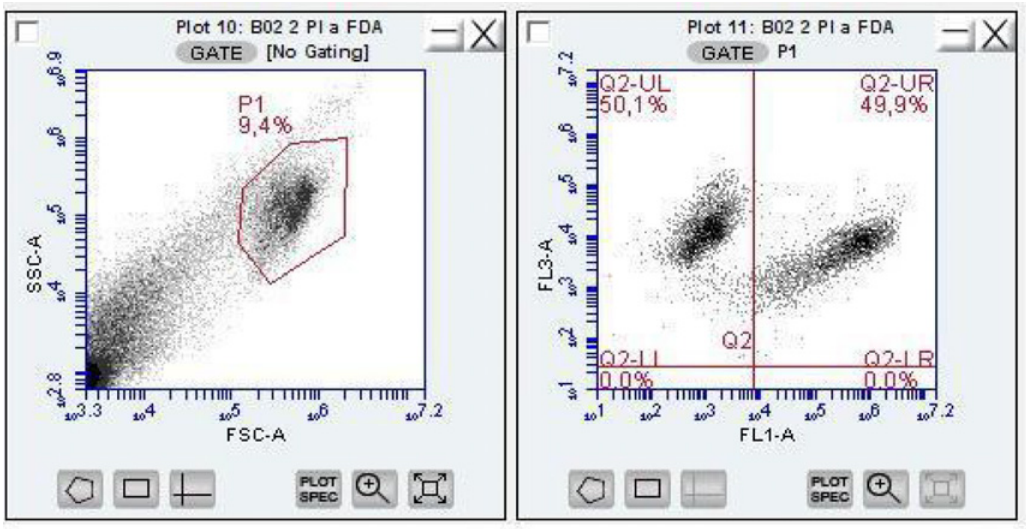

Figure 1. Dot-plot diagrams showing the separation. 1a – left: of yeast from bacteria and impurities. 1b – right: by the FDA and PI labelled yeas.

Table 3. Cell concentration in the experimental must samples.

	MCFA [mg/L]/ 28 hours before test	SO_2 [mg/L]/ 4 hours before test	The number of cells in 1 mL sample
Sample 1 (C)	0	0	8.3×10^6
Sample 2	10	0	9.4×10^6
Sample 3	10	60	8.0×10^6
Sample 4	0	60	11.9×10^6

Instruments: Shimadzu GC-17A, Autosampler: AOC – 5000, Detector: QP-5050A, Software: GCsolution. Separation conditions: column: DB-WAX 30 m × 0.25 mm; 0.25 μm stationary phase (polyethylene glycol). Voltage of the detector 1.5 kV. Individual compounds were identified on the basis of MS spectrum and retention time.

3. Results

3.1. Flow cytometry

Table 3 shows that the lowest cell (both, live or dead) concentration 8×10^6 is in sample 3 treated with 10 mg/L mixture of MCFA 28 hours before the measurement, and 60 mg/L of SO_2 4 hours before the measurement.

In Fig. 1a (below), the total amount of yeast separated by the flow cytometer from bacteria and impurities is marked in red. The value of 9.4% represents the area of yeast. The dot plot diagram in Fig. 1b shows the value of live (49.9%) and dead (50.1%) yeast.

The yeasts were separated from bacterial cells and debris by flow cytometry (see Fig. 1a). Then, based on FSC and SSC parameters, cells labelled FDA were evaluated as "live" while cells labelled PI were evaluated as "dead" (see Fig. 1b). The number of live and dead cells was monitored using flow cytometry.

Submitted samples contained a large number of bacteria and particles of a non-cellular nature, affecting cytometric analysis and the measured data. However, with FSC and SSC parameters, it was possible to locate the yeast population so it could be analysed separately. Bacteria from the impurities could not be sufficiently separated in the submitted samples. For samples 3 and 4, a significant change in the representation of "viable" cells was noted after transfer into the marking culture solution.

Figure 2 shows that the dose of MCFA has an inhibitory effect on *Saccharomyces cerevisiae* compared with the control. In combining SO_2, it has a higher efficiency than using a dose of SO_2 itself. The highest concentration of viable yeast content was in the control (variant C). By contrast, the lowest concentration was in variant 3, where MCFA and SO_2 were added.

3.2. Direct counting in a Bürker chamber

The counting was performed 24 and 168 hours after different doses of application of the MCFA mixture.

Figure 4 shows a higher concentration of dead cells with a higher concentration of MCFA. In the case of variant 8 (concentration 10 mg/L MCFA), the percentage of dead yeast was highest – about 60% (compared to variant 1 – about 24%). From Fig. 5, it is obvious that after more time (168 hours) the number of dead cells were higher than in the variants after 24 hours. The highest concentration of dead cells was measured in variant 10 (40 mg/L of MCFA mixture) – about 94%.

3.3. Determination of MCFA residues in treated wines

To determine MCFA residues after application into the fermenting must to stop alcoholic fermentation, the finished wine after the first racking was measured with GC-MS (Gas Chromatography/Mass Spectrometry).

The results of the GC-MS show that there is no significant quantitative difference in treated and untreated wines. The difference is between C_8 is 0.383 mg/L, C_10 0.226 mg/L and C_12 0.0019 mg/L in treated and untreated wines. These small differences are caused by absorption or assimilation by the yeast. Only a small part is esterified and remains as a residue in the wine.

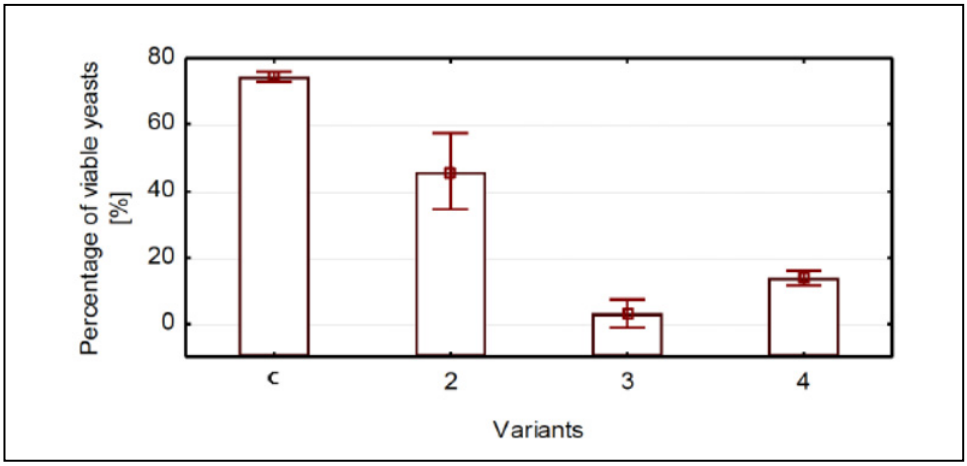

Figure 2. Percentage of live yeast in the tested must.

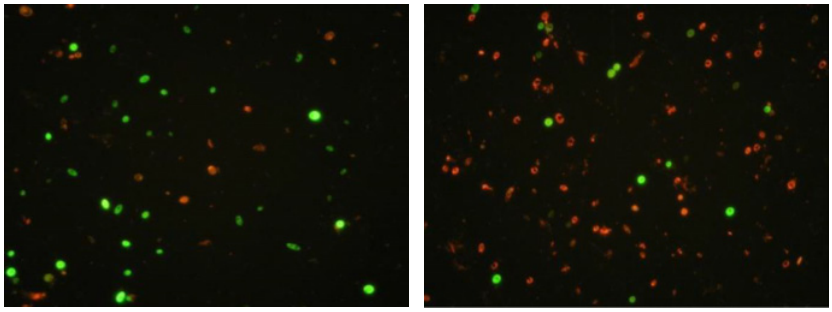

Figure 3. Microscopic view of the lifetime of protoplasts using fluorescein diacetate, propidium iodide and ultraviolet radiation. "Live" yeasts are marked green, and "dead" yeasts are marked red. 3 a) control sample, 3 b) variant 2.

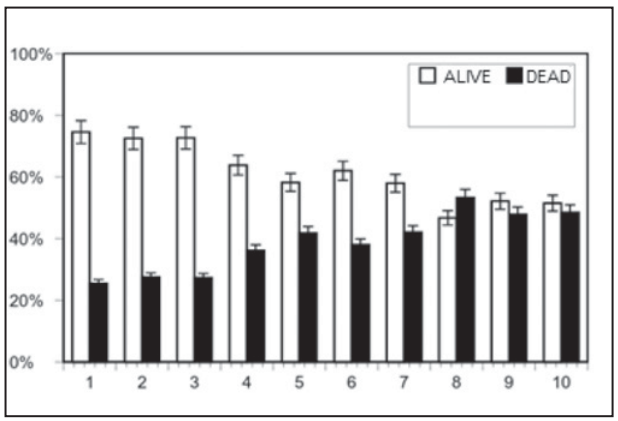

Figure 4. Percentages of live and dead yeast cells after 24 hours of addition of HFA mixture.

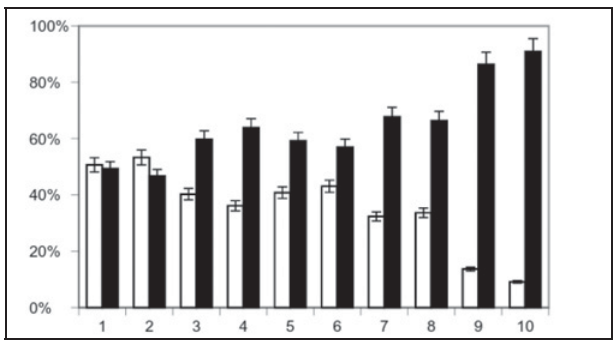

Figure 5. Percentages of live and dead yeast cells after 168 hours of addition of MCFA mixture.

4. Discussion

The results of flow cytometry (Chapter 4.1) show the highest inhibitory effect with the MCFA and SO_2 combination compared to the control variant and must treated with MCFA or SO_2 individually (Fig. 2). These results were achieved at a comparable concentration of yeast cells (dead or live) in a medium and confirm the results of Rib'erau-Gayon., et al., (2006) by providing precise quantification of live and dead cells in the defined medium (Figs. 1 and 3).

Even more accurate results in relation to the used concentration of MCFA mixture were obtained from direct cell counting. Here, 24 hours after application of a MCFA mixture (Fig. 4), there is a much higher concentration of dead cells than the control variant in the case of concentrations of 10, 20 and 40 mg/L MCFA mixture. These results confirm and quantify the results obtained by other authors (e.g., (4; 5; 6)).

Very interesting results have been obtained in the measurement of residues in treated and untreated wines (Sect. 4.3). The yeast basically detoxifies their bodies by esterification of the MCFA (7) which have penetrated into their cells, thus resulting in changes in the sensory characteristics of the wine.

According to our results (Table 4) the quantity of ethylesters MCFA is the same for untreated wine and for wine, to which was applied 10 mg/L mixture of MCFA. This measurement was done after the first racking (12; 13; 14). This shows that a sufficiently low concentration of MCFA has a sufficiently high inhibitory effect on

Table 4. Comparison of treated and untreated wine with MCFA.

T-tests; grouped: Group 1: with MCFA; Group 2: without MCFA											
MCFA	Group 1 [mg/L]	Group 2 [mg/L]	t	sv	**p**	n1	n2	SD 1	SD 2	F	P
C8	4.62	4.237	0.9051	38	**0.3703**	20	20	1.442	1.405	1.052	0.8911
C10	1.091	0.865	1.48	38	**0.0953**	20	20	0.539	0.36	2.246	0.0591
C12	0.0331	0.0350	0.2643	38	**0.7928**	20	20	0.029	0.020	2.061	0.0909
Ethylesters C8	0.498	0.505	0.132	38	**0.8952**	20	20	0.16	0.179	1.246	0.6291
Ethylesters C10	0.113	0.0953	1.16	38	**0.2523**	20	20	0.054	0.4678	1.331	0.4964

Saccharomyces cerevisiae during fermentation to achieve the stopping of fermentation. The part of the higher fatty acid is eliminated by fixation on the body of the dead yeast (13; 14).

5. Conclusions

Higher fatty acid is not currently used for inhibiting the activity of yeast during alcoholic fermentation. Previously published works demonstrate the properties of individual MCFAs, but do not offer results from wine production conditions and quantification of the effect of these substances on the yeast population after their use. The use of MCFA to stop fermentation is very promising technology. From the results obtained, it is evident that the application of MCFA to the fermentation media causes inhibition of the metabolic activity of the yeast, causing its death and stopping alcoholic fermentation (Figs. 2, 3, 4 and 5). This method can effectively reduce the cost of the production technology of wines with residual sugar and, in general, reduces the dosage of SO_2. This technology is also very simple for use in small wineries, where is no need for energy consuming cooling systems – the higher the temperature, the better the function. The great advantage of this method is its easy application, immediate action, and low cost. MCFA are harmless substances, naturally occurring in wine. Because of their very low concentration of use – less than 10 mg/L (Table 4), they have no effect on the final quality of the wine, primarily because the part of added MCFA is fixed on the yeast bodies and removed with each racking.

References

[1] Hui, Y. H., et al. Handbook of Food Products Manufacturing: Principles, Bakery, Beverages, Cereals,. New Jersey : y John Wiley & Sons, Inc., 2007. ISBN: 978-0-470-04964-8

[2] Fugelsang, K. C. and Edwards, Charles G. Wine microbiology. 2 New York : Springer, 2007. ISBN: 978-0-387-33349-6

[3] Mitchell, Richard and Hall, Colin Michael. Wine Marketing: A Practical Guide. Boston: Elsevier, 2008. ISBN: 9780750654203

[4] Callul, M., et al. HPLC analysis of fatty-acids in wine. American Journal of Enology and Viticulture. 1991, **3**, pp. 268–273

[5] Guilloux-Benatier, M., Le Fur, Y. and Feuillat, M. Influence of fatty acids on the growth of wine micoorganisms Saccharomyces cerevisiae and Oenococcus oeni. Journal of Industrial Microbiology & Biotechnology. 1998, **20**, pp. 144–149

[6] Viegas, C. A., et al. The H+-ATPase in the plasma membrane of Saccharomyes cerevisiae is activated during growth latency in octanoic acd - supplemented medium accomanying he decrease in intracellular pH and cell viability. Applied and Environmental Microbiology. 1998, **64**, pp. 779–783

[7] Bardi, L., Cocito, C. and Marzona, M. Saccharomyces cerevisiae cell faty acid composition and release during fermentation without aeration and in absence of exogenous lipids. International Journal of Food Microbiology. 1999, **47**, pp. 133–140

[8] Pina, C., et al. Ethanol tolerance of five non-Saccharomyces wine yeasts in comparison with a strain of Saccharomyces cerevisiae - influence of different culture conditions. Food Microbiology. 2004, **21**, pp. 439–447

[9] Sajbidor, J., Malik, F. and Kissantalova, H. The relationship between the viability of selected dried wine yeast and their lipid-composition and fatty-acid profile. Food Botechnology. 1992, **6**, pp. 187–196

[10] Torija, M. J., et al. Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. International Journal of Food Microbiology. 2003, **85**, pp. 127–136

[11] Rib'erau-Gayon., P., et al. *Handbook of Enology Volume 1 The Microbiology of Wine and Vinifications.* **2**. s.l.: John Wiley & Sons, 2006. ISBN 0-470-01034-7

[12] Viegas, C. A., Sacorreia, I. and Novais, J. M. Synergistic inhibition of the growth of Saccharomyces

bayanus by ethanol and octanoc or decanoic acids. Biotechnology Letters. 1985, **7**, pp. 611–614

[13] Bábiková, P., et al. *Increasing the efficiency of sulphur dioxide in wine by using of saturated higher fatty acids.* 1. s.l.: Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2012. pp. 17–22, **60**. ISSN 1211-8516

[14] Baroň, Mojmír. *Effectiveness of saturated higher fatty acids for inhibition of yeasts and malolactic activities in wine*. 1. s.l.: Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2014. pp. 23–26, **62**. ISSN 1211-8516

[15] Correia, I. S., et al. Leakage induced by ethanol, octanoic and decanoic acids in Saccharomyces cerevisiae. Yeast. 1989, **5**, pp. 123–127

[16] Lafon-Lafourcade, S., Geneix, C. and Riberau-Gayon, P. Inhibition of alcoholic fermentation of grape must by fatty-acids produced by yeast and their elimination by yeast ghosts. Applied and Enviromental Microbiology. 1984, **61**(2), pp. 1246–1249

[17] Viegas , C. A., et al. Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. Applied and Environmental Microbiology. 1989, **55**(1), pp. 21–28

[18] Viegas , C. A. and Sacorreia, I. Activation of plasma-membrane ATOase of Saccharomyces cerevisiae by octanoic-acid. Journl of General Microbiology. 1991, **137**, pp. 645–651

[19] Viegas, C. A., et al. Regulation of the expression of the H+-ATPase genes PMA1 and PMA2 during growth and effect of octanoic-acid in Saccharomyces cerevisiae. *Biochemica Et Biophysica Acta-Gene Structure and Expression*. 1994, **1217**, 1, pp. 74–80

[20] Comitini, F. and Ciani, M. The inhibitory activity of wine yeast starters on malolactic bacteria. Annals of Microbiology. 2007, **57**(1), pp. 61–66

[21] Geneix, C., Lafon-Lafourcade, S. and Ribereau-Gayon, P. The effect of fatty acids on the viability of Saccharomyces cerevisiae. *Comptes Rendus De L'Academic Des Sciences*, Série Iii-Sciences De La Vie-Life Sciences. 1983, **19**, pp. 943–947