Low SO_2 winemaking

Bio-protection for microbial control pre-fermentation

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INTRODUCTION

Reducing the additions of SO_2 before fermentation and during the maturation process with natural solutions is of interest to many winemakers. The maximum allowable level of total SO_2 in wine in Australia is 250ppm for any wine under 35g/L of sugar and 300ppm for all other wines (Australian and New Zealand Food Standards 4.5.1). Importantly, consumer preferences are leaning towards minimal intervention or low SO_2 wines if not preservative free, whilst demanding a quality product. The objective of this article is to discuss the use of bio-protection strategies pre-fermentation as a tool for winemakers to increase microbial protection thus reducing the amount of SO_2 required.

MICROBIAL POPULATIONS PRE-FERMENTATION

The pre-fermentation stage has the most microbial variation in the winemaking process. Many *Acetobacter* spp., non-*Saccharomyces* and *Saccharomyces* species of yeast, lactic acid bacteria, mould and fungi may be present (Table 1). The method of harvesting, state of sanitation of both equipment and grapes, temperature and, critically, length of time before processing will affect the presence of these populations. These factors are even more important when fermentation is left to commence naturally (spontaneous fermentation) as indigenous microflora have more time to proliferate.

Typically, species such as *Hanseniaspora, Kloeckera, Pichia, Candida,* and *Metschnikowia genera* dominate the first stage of uninoculated fermentations. Species such as *Torulaspora delbrueckii, Kluyveromyces* spp., *Zygosaccharomyces bailli, Schizosaccharomyces pombe* and *Issatchenkia* spp., may also be present (Table 1). These species start the fermentation and proliferate up 10^6-10^7 cells/mL until factors such as alcohol, nutrient limitation and competition cause a decline in population. Whilst some of these non-*Saccharomyces* species give desirable attributes to a wine, some are more likely to produce undesirable sulfur compounds and/or volatile acidity (Albertin *et al*. 2014). Typically, half way through the fermentation, *Saccharomyces cerevisiae* takes over the fermentation (Di Mario *et al*. 2007, Fernandez *et al*. 1999, Fleet *et al.* 1984, Hiero *et al*. 2006, Zott *et al.* 2008, Comitini *et al*. 2017, Zott *et al*. 2008).

BIO-PROTECTION – WHAT IS IT?

Bio-protection is a chemical-free way of protecting a wine from spoilage, in this case by using desirable microorganisms to occupy the space and deterring other microorganisms from proliferating. Bio-protection strategies may be used in the pre-fermentation stage to increase microbial protection, particularly relevant when SO_2 is used

Table 1. Effect of specific microorganisms on wine quality * ; ** and * : increasing detection in must (Albertin *et al*. 2014).**

Process stage	Microorganism	Organoleptic impact	Pre-fermentative influencing factors
Grapes	*A. pullulans*	NA	Not relevant as they do not grow in must
	Cryptococcus spp		
	Rhodotorula		
	Debaromyces hansenii		
Must (to 6 Baume)	*Hanseniaspora uvarum ****	VA increase Fast growing species	-Competition from other yeast (EGIDE®) -Low temperatures (10°C) will limit growth of this species - low NTU will limit growth of this species
	Candida spp *Candida zemplinina*	Low sugar/ethanol yield Negative organoleptic impact	-Lower temperatures and high NTU favour the growth of this species
	*T. delbrueckii**	-Reduced VA production -Thiol liberation -Increase in fruit -Increase in mouthfeel	-Low temperatures will limit growth of this species -the presence of SO2 will limit growth of this species
	*Metschnikowia spp***	-Terpene liberation	- Very cold tolerant – ability to grow at extremely low temperatures - Slow growing
	*Pichia kluyverii**	-Thiol liberation	- the presence of SO2 will limit growth of this species
	*K. thermotolerans**	Lactic acid production	
Alcoholic fermentation (6 Baume to 0)	*Saccharomyces cerevisiae*	Strain dependant	-Ability to tolerate SO2 and proliferate -Not impacted by turbidity, temperature, nor yeast addition

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at much lower rates. Research by the Laffort group has led to the launch of Egide®, a strain-specific combination of two non-*Saccharomyces* yeast species *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* which have been demonstrated to occupy the microbial space in the pre-fermentation stage. Both species are commonly found among indigenous microflora and known for their favourable impact/no defect (Table 1). At a strain level, both strains of *T. delbrueckii* and *M. pulcherrima* have a positive organoleptic impact on wine and have also been found to be extremely cryotolerant (Figure 2). *M. pulcherrima* also has beneficial characteristics in that it has very low fermentative capacity (meaning that it won't dominate the fermentation). Both species have extremely good implantation capacity and do not have a requirement for rehydration (Figure 1).

SO_2 additions should, by way of their anti-microbial action, reduce the populations of all microbes. However, studies have shown that at low levels, certain strains are more affected than others (Albertin *et al*. 2014). Conversely, *Hanseniaspora uvarum,* which can produce high levels of volatile acidity in wine, was found to increase in population with low levels of SO_2 (Table 2). A standard SO_2 addition made at the crusher can affect the indigenous populations present in must. Trials conducted on white must (Figure 2a) showed the addition of 50ppm of SO_2 does not allow non-*Saccharomyces* populations to increase. This addition of SO_2 is effective in inhibiting native populations of non-*Saccharomyces* (no more non-*Saccharomyces* were counted in white wines and a reduction in red wines for which the initial population was much larger – data not shown). In red must where the populations of indigenous microflora are higher due to skin contact, without SO_2 (Figure 2b) non-*Saccharomyces* populations increase. With the addition of Egide and 20ppm of SO_2, the population was higher but comprised selected microorganisms that are known to be non-impacting or positively impact the quality of wine (Figure 2a and 2b). The non-*Saccharomyces* population is replaced by a selected and non-fermentative population. In Fig 2a, a 20ppm addition (SO_2-) was not enough to prevent the development of indigenous non-*Saccharomyces* after cold settling whilst only *T. delbrueckii* and *M. pulcherrima* were detected (hence no indigenous non-*Saccharomyces*) in the 20ppm assay where *T. delbrueckii* and *M. pulcherrima* were added. It is speculated that the SO_2 added allows for the growth of species that can tolerate some levels of SO_2, and takes away the competition by inhibiting those species most sensitive to SO_2.

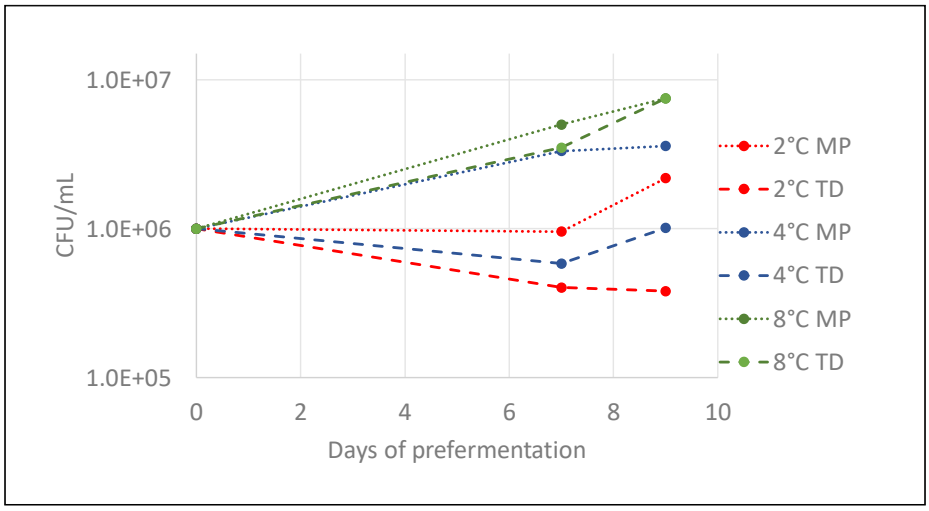

Figure 1. Must inoculation with Zymaflore Egide 5g/hL. Populations monitoring over time according to the temperature. In between 2 and 4°C: ***T. delbrueckii*** **populations fall. 4°C: after initial decline,** ***T. delbrueckii*** **is able to grow after 7 days. 8°C: Similar evolution of** ***T. delbruekii*** **and** ***M. pulcherrima*** **populations.**

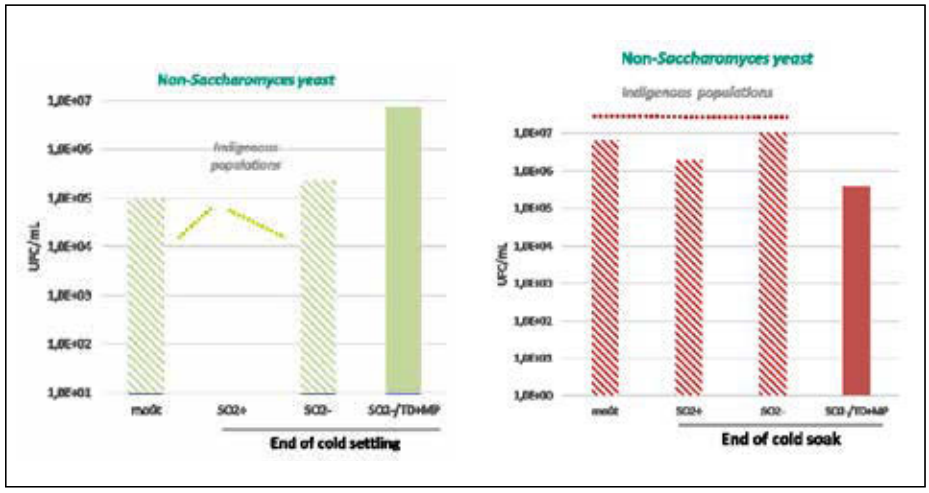

Figure 2a. Levels of non-*Saccharomyces* yeast present in the must, with 50ppm of SO_2 added (SO_2+), with 20 ppm of SO_2 added (SO_2-) and a combination of ***T. delbrueckii and M. pulcherrima*** **with 20ppm of SO_2 (SO_2-/TDMP) in Semillon must, 2017 Bordeaux; Figure 2b. Levels of non-*Saccharomyces* yeast present in the must, with 40ppm of SO_2 added (SO_2+), without SO_2 added (SO_2-) and a combination of** *T. delbrueckii* and *M. pulcherrima* **without SO_2 (SO_2-/^{TDMP}) in Merlot must, 2016 Bordeaux.**

Table 2. Influence of SO_2 addition to the must on the different yeast species during the pre-fermentation phase (Albertin *et al.* 2014) (-) initial must sulfited with 50ppm SO_2 and (+) initial must was sulfited with 50ppm SO_2 and then had a second addition of 25ppm SO_2 after two days.

	SO_2	
	-	+
S. cerevisiae	↘	↗
C. zemplinina	→	→
H. uvarum	↗	↘
T. delbrueckii	↘	↗

Table 3. Applications and functions of Egide in the winemaking process flow.

Function	Action	Benefits
Juice and grape transportation	• Limit the growth of undesirable microorganism, prevent uncontrolled fermentation during transport whilst using less SO_2	Protection against uncontrolled fermentation
Cold settling (white and rose wines)	• Limit the growth of undesirable microorganisms whilst using less SO_2 (or not) • Hold off alcoholic fermentation by *S. cerevisiae*	Lower refrigeration costs (Ability to settle at a higher temperature – Refer to Table 4 for guidlines
Stabulation	• Limit the growth of undesirable microorganisms whilst using less SO_2 (or not) • Hold off alcoholic fermentation by *S. cerevisiae*	Lower refrigeration costs (Ability to conduct stabulation at a higher temperature – Refer to Table 4 for guidlines)
Cold soak	• Limit the growth of undesirable microorganisms • Greater extraction at higher temperature whilst using less SO_2 • Hold off alcoholic fermentation by *S. cerevisiae*	Lower total SO2 levels Has been demonstrated to enhance the implantation of S. cerevisiae upon inoculation
Wild fermentations	• Limit the growth of undesirable microorganisms	Lower VA

WHEN CAN I APPLY EGIDE AS A BIO-PROTECTION STRATEGY?

Whilst the applications are many, Egide has two main functions:

- to colonise the medium (and thus represent the majority of detected microflora)
- to limit the development of other microorganisms during the pre-fermentation phase

The practical applications are outlined in Table 3.

Juice and grape transportation are key given the low doses and lack of requirement for rehydration (Figure 3). Analysis of the survival rates of both microorganisms when rehydrated vs no rehydration showed similar levels of survival after several hours. In Australia we transport a large amount of both whole juice and grapes right across the country. Being able to add this form of bio-protection without rehydration makes it simple for any vineyard crew.

In instances where cold settling is undertaken, bio-protection strategies may enable higher temperatures, thus increasing the activity of settling enzymes and minimising refrigeration costs. Stabulation, the process of keeping whole juice circulating over juice lees at -2°C to 2°C for one to three weeks to increase mouthfeel and aroma precursors, may be conducted at a higher temperature with bio-protection (see Table 4 for guidelines), thereby minimising requirements for refrigeration. In reds, cold soak on skins to increase maceration in certain varietals may be conducted at higher temperatures, increasing the rate of extraction and minimising refrigeration costs.

The use of bio-protection in wild fermentations is not an obvious application. But with bio-protection, there is some control over undesirable species such as *H. uvarum* and *Acetobacter* spp., thus supporting spontaneous fermentation with lower volatile acidity. By adding Egide with a small amount of SO_2, the entire microbial space is occupied by desirable species. In a spontaneous fermentation it has also been demonstrated that the levels of *Acetobacter* spp. are not present or at a lower level (Table 5, see page 26). The inoculation of Egide and a strain of *S. cerevisiae* was required to achieve the same low levels of ethyl acetate as a must inoculated with *S. cerevisiae* with SO_2. The spontaneous fermentation with Egide demonstrated similar levels of ethyl acetate to the must without any preliminary SO_2 addition.

Table 4. Trials conducted on rosé must whereby 2 and 5g/L of Egide were inoculated to determine the optimal dose rates without risking fermentation of Egide.

Egide 2.5 g/hL				
	3 days	7 days	9 days	11 days
2°C	Risk: weak	Risk: weak	Risk: moderate	Risk: high
4°C	Risk: weak	Risk: weak	Risk: moderate	Risk: high
8°C	Risk: weak	Risk: weak	Risk: high	Risk: high

Egide 5 g/hL				
	3 days	7 days	9 days	11 days
2°C	Risk: weak	Risk: weak	Risk: moderate	Risk: high
4°C	Risk: weak	Risk: moderate	Risk: moderate	Risk: high
8°C	Risk: weak	Risk: high	Risk: high	Risk: high

A WORD ON SO_2

SO_2 has both an antimicrobial and anti-oxidative effect in its free form (Jackowetz and de Orduña 2012, Carreté *et al*. 2002). It can be added at the grape stage with the intention of reducing the microbial load due to its anti-microbial effect. It is also able to inhibit the activity of oxidising enzymes from grapes, preventing excessive browning (Main and Morris 1991). Oxidising enzymes from *Botrytis cinerea* and other rot species may require higher levels of SO_2 to inhibit their enzymatic activity. The levels of undesirable microorganisms such as *Acetobacter* spp may be higher in rot-affected fruit (Barbe *et al.* 2001, Mills *et al.* 2002).

SO_2 can also be found in bound form — this form does not have an antimicrobial or antioxidative function. During alcoholic fermentation SO_2 is completely bound by acetaldehyde, an intermediate of alcoholic fermentation (Jackowetz and de Orduña 2012). Many other compounds are able to bind to SO_2 affecting the levels of free SO_2.

Table 5. Semillon 2017 assay, Wine Experimental Cellar Bordeaux. RS – Residual Sugar, NTU – measurement of turbidity; YAN – yeast assimilable nitrogen; AB – Acetobacter spp; LAB - Lactic acid bacteria.

	SO_2+ control	No SO_2	With the addition of 50ppm TD/MP	With the addition of 50ppm TD/MP
		Control	(no SO_2)	Spontaneous AF (no SO_2)
SO2 at harvest	4 g/hL	-	-	-
EGIDE®	-	-	3 g/hL	3 g/hL
12°C / 24h, then settling				
Inoculated with S. cerevisiae	20 g/hL	20 g/hL	20 g/hL	-
RSg/L	174	174	174	174
NTU	226	221	217	220
YAN mg N/L	151	148	150	149
SO2T mg/L	26	5	5	4
AAB CFU/mL	<1,8E+5	<1,8E+5	Not detected	Not detected
LAB CFU/mL	1,20E+03	9,80E+02	6,41E+02	8,60E+02
Ethyl acetate et the end of AF (mg/L):	42	65	40	65

ALCOHOLIC FERMENTATION AND EFFECTS OF EGIDE POST FERMENTATION

The species of *T. delbrueckii* and *M. pulcherrima* are inoculated at extremely low rates (20ppm as opposed to 200-300 ppm for a standard wine yeast for fermentation), and have extremely low fermentative capabilities (they are very slow to grow). In order to start fermenting, these strains would both need to be able to grow and proliferate up to approximately 1e7 cells/mL before starting to ferment.

Table 4 provides recommended rates of Egide depending on time and temperature trials. Once inoculated, *Saccharomyces cerevisiae* would outcompete both *T. delbrueckii* and *M. pulcherrima* which has faster fermentation kinetics and much greater tolerance to alcohol. By taking over the majority of the non-*Saccharomyces* species in the must, hence leaving less space to undesirable non-*Saccharomyces* spp. such as VA-producing *Hanseniaspora* species, the finished wines trialled to date have resulted in lower levels of ethyl acetate (Table 5).

Lactic acid bacteria are not affected therefore MLF is not affected (Table 5). It is highly recommended to minimise any lag between alcoholic fermentation and malolactic fermentation as spoilage microorganisms such as *Brettanomyces bruxellensis* and *Acetobacter* spp. can proliferate here in the absence of SO_2. Chitosan-based options may be used to control microflora post-fermentation to minimise the use of SO_2. For example, Bacticontrol® may be used to inhibit lactic acid bacteria while Oenobrett can be used to inhibit further growth of *Brettanomyces* spp. A reduction in the proliferation of *Acetobacter* spp. may be assisted by the addition of Microcontrol®. Low levels of SO_2 and the use of oxygen scavengers such as tannins at the beginning of AF whilst incorporating good winemaking practices have the ability to limit the ingress of oxygen thus preventing oxidation.

CONCLUSIONS

Selected strains of *T. delbrueckii* and *M. pulcherrima* are able to minimise the amount of SO_2 required by replacing the population of unknown non-*Saccharomyces* species prior to alcoholic fermentation with known non-fermentative species which have a positive organoleptic impact. This can lead to a reduction in undesirable yeast species and lower ethyl acetateas as well as a lower total SO_2 level. Anywhere there is a gap between picking and fermentation Egide may be used successfully.

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