

## How to reduce SO<sub>2</sub> additions in wine with the aid of non-conventional yeasts

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**Abstract.** Among the factors that influence a wine's profile, the contributions of microbial biodiversity are widely recognized. Besides the increased aromatic complexity through multistarter fermentations with *Saccharomyces cerevisiae* and non-*Saccharomyces* species, non-conventional yeasts display many different biochemical pathways giving further inputs. Hence, the aim of this article is to explore the opportunities of a new generation of starter cultures to optimize the effects of sulphur dioxide (SO<sub>2</sub>) and thus reduce its addition in grape must and wine, in line with consumers needs and expectations. Non-conventional yeasts naturally produce glutathione, a non-protein thiol that can limit oxidative phenomena when released during fermentation. Acetaldehyde, which firmly bind with SO<sub>2</sub> limiting its antimicrobial and antioxidant effect, is also produced by non-*Saccharomyces* strains in variable amounts and thus can be reduced by selecting low producers. *Lachancea thermotolerans* shows the very uncommon metabolism of grape sugars to lactic acid, decreasing the wine pH and favouring the most effective SO<sub>2</sub> chemical conformation. Finally, other molecules released by non-conventional yeasts present antimicrobial effect, improving the stability of wines through biocontrol and bioprotection. Those are some of the innovative solutions embracing non-conventional yeasts that are proposed to help achieving more sustainable, stable, and fresher wines, with less alcohol and less sulphites added.

### 1. Introduction

According to the most recent report by the International Organisation of Vine and Wine (OIV), in 2023 a total estimated of 237.3 million hectolitres of wine were produced. With around 85 countries in the world making wine and 200 countries consuming it, the international trade is strong, and global exports reached 36.0 billion € in 2023, the second highest value ever [1].

In this context of a highly internationalised and extremely competitive market, combined with consumers getting more educated and confident about their wine preferences, there is an important push towards distinguished wines that meet the favoured styles at a certain quality level and price point. Besides the economic issues regarding wine distribution and marketing, wine producers worldwide are also facing many challenges affecting viticulture and winemaking, including climate change, evolving consumer trends, new regulations, health concerns, social and environmental sustainability.

Investing in technology and innovation is one of the options for grape-growers and winemakers to keep up with those challenges and thrive in the wine business. Among

the many factors that impact wine sensory quality, style, safety, sustainability, and sense of place, the climate, aspect, soil, and viticultural and winemaking practices are normally factored in. Nevertheless, the contributions of biodiversity are widely becoming more recognized, among which the microbial ecology stands out.

Besides driving fermentation, microorganisms also play important roles in the grapevine health and during wine ageing. The conversion of sugars into alcohol and carbon dioxide is a very straightforward process, but throughout winemaking there are multiple biochemical reactions performed by a myriad of different species who are interacting in a lot of different ways. With so many variables and possible outcomes, microbiology is one of the best platforms to develop innovative practices [2].

Hence, the aim of this article is to explore the current use and potential developments of non-conventional yeasts to diversify and improve wine fermentations. An overall perspective will be given on the most important species involved in winemaking and their metabolism affecting the wine profile. In particular, the strategies that take advantage of yeasts' antioxidant and antimicrobial activities will be discussed in further detail, with a view to

reduce the addition of sulphur dioxide (SO<sub>2</sub>) in grape must and wine.

## 2. Spontaneous Fermentations and Non-*Saccharomyces* in Winemaking

The biochemical transformation of grape berries into wine would not be possible without the intervention of microorganisms. A huge diversity of bacteria, yeasts, and moulds naturally colonize the soil, the interior of vine trunks, the surface of leaves and grape bunches. In the spontaneous grape must fermentation, some vineyard species stick around, joined by winery equipment resident microorganisms, and the process is carried out by a succession of metabolic active yeasts and bacteria, each one better adapted to different stages of the biotransformation [3].

In most cases, strains of the species *Saccharomyces cerevisiae*, “the wine yeast”, dominate the microbiota and lead the alcoholic fermentation, thanks to its excellent fermentative performance and better tolerance to the stresses found in this environment, such as low pH and high concentrations of ethanol and SO<sub>2</sub>. Due to the unpredictability and irregularity of spontaneous fermentations, there is a high risk of finding a stuck/sluggish process or high levels of undesired metabolites and toxic compounds. By the end of the 19<sup>th</sup> century, researchers started to study the inoculation of grape must with pure cultures of fermentative yeasts. More than 100 years later, the use of *S. cerevisiae* starter cultures became a well-established technology, with a wide range of strains available to winemakers [4].

Notwithstanding the consistency, safety, and quality of a more controlled fermentation process, some producers claim that the suppression of the autochthonous microbiota by widely used commercial strains could presumably reduce a wine’s uniqueness and typicity. A biotechnological alternative that is gaining traction more recently is the promotion of mixed culture fermentations, i.e. the simultaneous or sequential inoculation of strains from different species, thus increasing the diversity of biochemical pathways without the risk of losing control over undesired activities [5].

The role of other yeast species during fermentation has been neglected or seen as spoilage for a long time, of only secondary importance compared with *S. cerevisiae*. In the last decades, this belief was re-evaluated thanks to a renewed interest and more in-depth research on the yeast diversity. My first research with non-conventional yeasts, also called “non-*Saccharomyces*”, built a collection of more than 20 different species isolated across Italian vineyards, showing different genetic background and species- and strain-dependent varied characteristics, such as stress tolerance, enzymatic activities, metabolic pathways, nutritional needs, and fermentation performance [6]. Table 1 illustrate the huge diversity of the main non-*Saccharomyces* species and their most interesting properties that can be explored to expand the technological toolkit available to winegrowers.

**Table 1.** List of the most studied and commercially explored wine-related non-*Saccharomyces* species (adapted from [7-9]).

Species	Technological features	Effects on wine
<i>Torulaspora delbrueckii</i>	↑ esters, terpenes, thiols, polysaccharides ↓ volatile acidity, alcohol content, volatile phenols, acetaldehyde	increased aromatic complexity, purity, and intensity; smoother mouthfeel, fuller body
<i>Lachancea thermotolerans</i>	↑ lactic acid, total acidity, esters, glycerol ↓ pH, volatile acidity, alcohol content, SO <sub>2</sub>	increased freshness and balance; increased aromatic complexity
<i>Metschnikowia pulcherrima</i>	↑ higher alcohols, acetate and ethyl esters, terpenes, thiols, glycerol, glutathione ↓ alcohol content, spoilage microorganisms, acetaldehyde	biocontrol and bioprotection; increased aromatic complexity, purity, and intensity
<i>Starmerella bacillaris</i>	↑ glycerol, preference for fructose, glutathione ↓ alcohol content	increased aromatic complexity, purity, and intensity
<i>Schizosaccharomyces pombe</i>	↑ glycerol, polysaccharides ↓ malic acid, volatile acidity	increased roundness and body; improved stability
<i>Pichia kluyveri</i>	↑ thiols, varietal aromas, glycerol ↓ volatile acidity, volatile phenols	increased aromatic complexity
<i>Hanseniaspora vineae</i>	↑ acetate esters	increased aromatic complexity

As it can be seen, most non-*Saccharomyces* applications are focussed on the organoleptic profile of wines, modulating characteristics such as the aromatic composition, body, and mouthfeel. Those yeasts produce enzymes that hydrolyse some non-aromatic precursors, releasing aroma-active molecules, and have different pathways to metabolize sugar and nitrogen nutrients and modify non-volatile and volatile compounds, with some biochemical activities that are less pronounced or not present *S. cerevisiae* [10]. Nevertheless, as non-*Saccharomyces* yeasts normally have a much lower fermentation power than *S. cerevisiae*, they cannot ferment a grape must to dryness, and the sequential or co-inoculation of a reliable *S. cerevisiae* strain is imperative for completing the alcoholic fermentation. In microvinification trials with sequential inoculations of Pinot Grigio [11] and Corvina [12], a remarkable variability was found among diverse native strains of the species *L. thermotolerans*, *M. pulcherrima*, and *S. bacillaris*, with a differentiation and positive impact on the wine profile compared to the single *S. cerevisiae*.

Besides aroma modulation, non-*Saccharomyces* yeasts use alternative metabolic routes to deviate the production of ethanol from the grape sugars and favour other

molecules, such as lactic acid and glycerol, thus reducing the alcohol content and increasing wine freshness, balance, and mouthfeel. Hence, multistarter fermentations with combined non-*Saccharomyces* and *S. cerevisiae* are being proposed to address some current issues for winegrowers, such as the consequences of climate change [13]. Global warming, probably the biggest concern for viticulture worldwide, is causing the shortening of the growing season with a disruption of phenological times and ripening in warmer conditions, resulting in grapes harvested with more sugar and less acidity, or unripe anthocyanins and flavour precursors. Musts with high sugar and potential alcohol might be stressful to many wine yeasts, while high pH is more prone to spoilage microbes that could produce off-flavours [14].

Several yeast metabolic activities can also be explored to promote a higher efficacy of sulphites, helping to meet the goals of making healthier and more sustainable wines, in line with consumers needs and expectations. Achieving increased wine stability with a reduced need of SO<sub>2</sub> addition is an important field of investigation, as discussed in the next sections.

### 3. Yeast Alternatives to Reduce Sulphites Addition

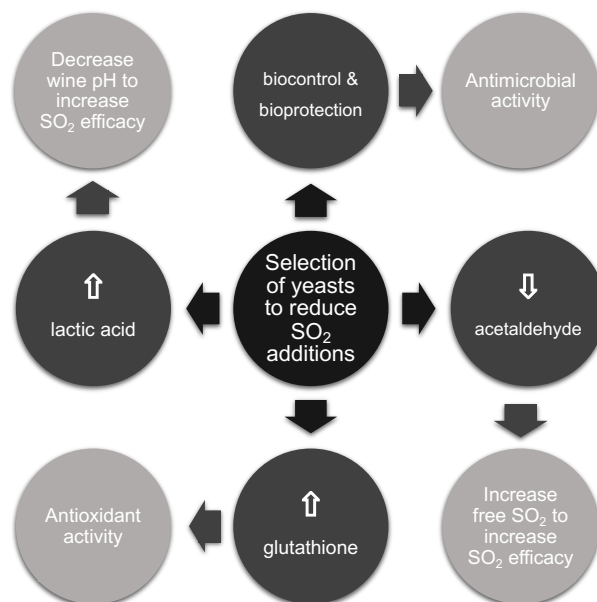
Sulphites are naturally produced by yeasts in small quantities during wine fermentation. Thanks to the antioxidant, antioxidasic, and antimicrobial properties, ease to use, and a low cost, they became also the most used preservatives in winemaking, usually added pre-fermentation or post-bottling. Nevertheless, too high quantities could cause off-flavours and aromatic faults and trigger adverse reactions in sensitive individuals, which led to the establishment of maximum allowed limits in wine and the warning “Contains Sulphites” to appear on bottles worldwide [15, 16].

More recently, those concerns about consumers’ health affected the perception of wine safety and sustainability, and there is a growing trend of producing wines with low or no added sulphites. Around 50% of respondents in a recent survey in the USA think that sulphites in wine can cause headaches [17]. Another survey about consumers’ perceptions, wine preferences, and willingness to pay showed an association between wines with no added sulphites and health benefits, and those wines were perceived as differentiated products among other wines identified as sustainable [18].

Nevertheless, as hotly debated after the publication of Sophie Parker-Thomson’s MW’s research paper [19], the strong reduction or altogether elimination of SO<sub>2</sub> in winemaking might lead to the rising of biogenic amines, the most likely cause for symptoms of wine intolerance. Biogenic amines are mainly produced through decarboxylation of certain amino acids by spoilage bacteria, which may grow undisturbed in must and wine without antiseptic SO<sub>2</sub> [20]. Furthermore, the absence of sulphites in stored wines, especially in unsuitable conditions, can accelerate the hydrolysis and modification

of phenolic and volatile compounds with negative consequences to the organoleptic profile [21, 22].

Hence, to successfully achieve the goal of reducing SO<sub>2</sub> additions and not risk the wine quality, integrity, and stability, the wine industry must actively look for alternatives to replace its effects. The following diagram show some of the strategies that target the activity of wine yeasts (Figure 1).



**Figure 1.** Alternative solutions using non-conventional yeasts to increase the efficacy or substitute the action of SO<sub>2</sub>, to reduce sulphites addition in grape must and wine.

#### 3.1. Acetaldehyde

The total sulphites naturally present or added to wine can be divided in “bond” or “free” SO<sub>2</sub>, as they react with other molecules forming stable bonds. One of the strongest SO<sub>2</sub>-ligands is acetaldehyde, an important volatile compound produced by yeasts during fermentation. Acetaldehyde has a perception threshold between 100 and 125 mg/L in wine, with levels above that potentially contributing with green, grassy, nutty, or apple-like aromas in wine, more or less desirable depending on the style. Its final levels may depend on the yeast strain, but also on fermentation conditions.

Binding with SO<sub>2</sub> can limit acetaldehyde’s sensory impact, but bond SO<sub>2</sub> has a limited antimicrobial and antioxidant effect. Therefore, a reduction of acetaldehyde levels in wine would help to keep a higher fraction of free SO<sub>2</sub> available [23].

Notably, in our studies of multistarter fermentations, some selected non-*Saccharomyces* yeasts produced less acetaldehyde than *S. cerevisiae*. In particular, the sequential inoculation of *S. bacillaris* with *S. cerevisiae* reached the strongest decrease of acetaldehyde release, an average of 30.33 mg/L against 54.25 mg/L with the singly inoculated *S. cerevisiae*. Thus, those selected strains represent a promising alternative to increase free SO<sub>2</sub> and reduce sulphites addition [11, 12].

### 3.2. Glutathione

Other molecules that show similar activities in wine can replace SO<sub>2</sub>, such as glutathione, the most abundant low molecular weight thiol in biological systems, a compound naturally produced by grape berries and yeasts to prevent oxidation inside the cells. Glutathione is a biologically active sulphur tripeptide composed of L-γ-glutamyl-L-cysteinyl-glycine, which inside the cells is prevalent in its reduced form (GSH). When released in the fermenting musts, it can limit oxidative phenomena in wine.

Glutathione addition is a valued strategy, especially in white wine production technology, to control detrimental colour changes and impaired aromatic profile associated with uncontrolled oxidation reactions. Due to this activity, a maximum of 20 mg/L pure glutathione addition was approved by the OIV [24]. As other important metabolic activities in wine, the production of glutathione is also greatly variable among different yeast species and strains.

A promising alternative approach considers the use of yeast starter cultures high producers of this compound *in situ*, during the fermentation process, in substitution of external GSH addition. Recent research showed that multistarter fermentations with non-*Saccharomyces* yeasts produce even higher concentrations of GSH compared to single *S. cerevisiae*. In our microvinification trials, some strains of *L. thermotolerans* and *M. pulcherrima* caused an increase of 10 mg/L of glutathione at the end of wine fermentation [25].

Moreover, preparations of inactivated dry yeasts containing glutathione-rich non-*Saccharomyces* yeasts can be used as a source of nutrients. Hence, yeast strains high producers of glutathione represent an important resource to preserve wine sensory characteristics and stability while reducing SO<sub>2</sub> inputs [26]. Further studies are necessary to evaluate the long-term effect of this GSH increment associated with multistarter fermentations, following the evolution of aromatic compounds and oxidation impact during ageing and storage.

### 3.3. Lactic acid

One of the most important factors affecting the SO<sub>2</sub> efficacy in wine is the pH. A lower pH favours the SO<sub>2</sub> chemical conformation most effective to enter the cells of spoilage microorganisms and disrupt their metabolism [16]. Thus, the production of organic acids by wine yeasts, or biological acidification, increases the microbiological stability without the need of adding more sulphites.

Strains of *L. thermotolerans* produce lactic acid from grape sugars, a very uncommon metabolic activity that attracts a great biotechnological interest. Indeed, *L. thermotolerans* contribution could be useful to address some recent concerns of the wine industry regarding climate change, which is causing an increase of sugar levels (and consequently of ethanol) and loss of acidity of the grapes.

We observed in laboratory trials a remarkable variability among strains, ranging from 0.26 g/L to 10.54 g/L of lactic acid [27]. In real fermentations at winery scale, a significant decrease of the wine pH was found, with 5.35 g/L of lactic acid [28].

Besides acetaldehyde, glutathione, and lactic acid, which have a direct or indirect influence on the need for SO<sub>2</sub> in wine, other molecules released by non-*Saccharomyces* yeasts can help to counteract the development of undesired microorganisms, improving the stability of wines through biocontrol and bioprotection [15].

### 3.4. Biocontrol and Bioprotection

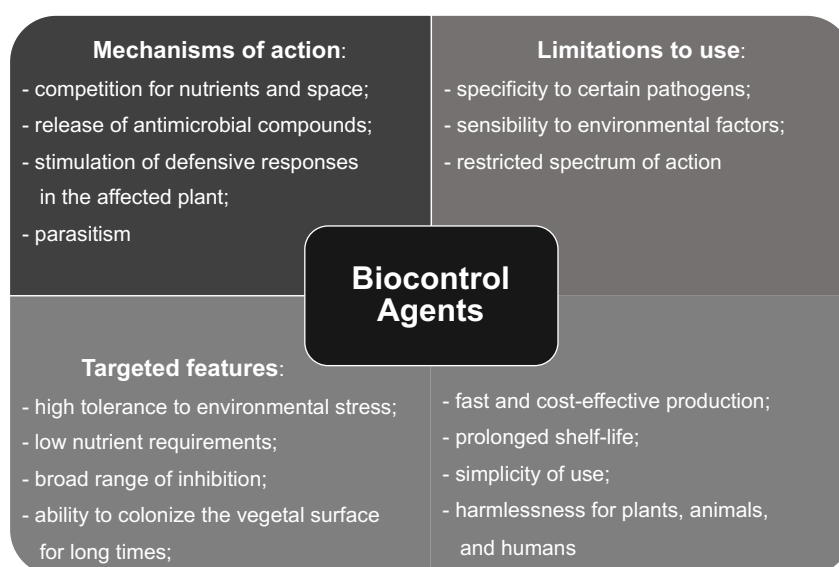
Among the vast biodiversity that develops in grape and wine environments, certain species of yeasts, moulds, and bacteria are considered grapevine pathogens, causing damage to the host plants, or spoilage organisms, negatively affecting the quality of grapes and fermenting musts. Moulds (filamentous fungi) are among the most relevant threats for viticulture, since they are very difficult to control and may infect different parts of the plant, usually being more evident on grape bunches [29].

As bunch rot and other diseases cause significant qualitative and quantitative losses, it is of utmost importance to prevent outbreaks in the vineyards. The vine training system and pruning management should be planned to optimize the aeration and light incidence to limit the conditions that favour mould development, as well as maintaining the soil health and avoiding other environmental hazards.

In some difficult vintages and certain environmental conditions, especially heavy rainfall, fungi might become an increasing threat and the most common practice to combat the infections is the use of chemical fungicides. However, the continuous use of these products builds up environmental pollution and pathogen resistance, ultimately posing risks to human health [30].

Therefore, modern biotechnology proposed alternative methods to protect the vineyards and respect the environmental, economic, and social sustainability of the agri-food system. One such approach considers the use of biological control agents, i.e. the application of microorganisms with the ability to counteract the pathogens. Several mechanisms can be involved in the antagonistic activity of biocontrol agents, but there also some limitations to this approach [31, 32]. The selection of novel microbial species and strains for potential biocontrol activity targets some features, as summarized on the Figure 2. Yeasts are commonly considered ideal biocontrol agents as they meet those criteria and, thus, they have been object of increasing research interest [33, 34].

We tested the antagonistic activity of *S. bacillaris* strains against *Botrytis cinerea*, in both *in vitro* and *in vivo* assays, reporting a significant inhibition of the fungal growth due to competition of space/nutrients and the production of volatile compounds by the yeast, and a visible decrease of grey rot symptoms on fruits [35].



**Figure 2.** Summary of the characteristics of biological control agents used in the wine production chain.

Bioprotection is another concept that has emerged recently in the oenological field, sometimes used as a synonym to biocontrol, but many researchers and biotechnology companies differentiate: while biocontrol commonly refers to the microorganisms that inhibit pathogens on plants and grapes, bioprotection considers the inoculation of yeast strains in grape must, at pre-fermentative operations such as cold maceration, to occupy the environment and suppress the growth of possible spoilage microorganisms [36]. Some biocontrol agents might persist on the berry surface and survive in the grape must, which means they could also have a bioprotective effect. While *S. cerevisiae* starters normally have efficient competitive mechanisms to inhibit almost completely the native microbiota and rapidly dominate the alcoholic fermentation, the bioprotective non-*Saccharomyces* could ideally be selected “friendly yeasts”, i.e. they combat the undesired species but allow some native non-*Saccharomyces* to share the same environment and positively contribute with the wine sensory diversity [5].

If biocontrol or bioprotection agents arrive metabolically active at the onset of fermentation, they could have a significant influence in the process and the wine quality. In an innovative and holistic view of winemaking, the selection of strains should favour those non-*Saccharomyces* species that naturally occur on the grape environment and show a positive impact on wine quality. Furthermore, it is essential that they are compatible with the native or inoculated strains of *S. cerevisiae* that will ultimately guide the alcoholic fermentation to its successful completion.

In a recent study [37], the combined effects of inoculating a selected strain of *M. pulcherrima* on grapes at the beginning of the post-harvest withering process and in must before alcoholic fermentation to produce a sweet ‘passito’ wine were analysed. *M. pulcherrima* was inoculated in must for multistarter sequential

microfermentation trials with *S. cerevisiae*. Microbiological, chemical, and sensory analyses were carried out to monitor the vinification of treated and control grapes. Grape bunches during withering were a suitable environment for the development and persistence of a high population of *M. pulcherrima*, which effectively prevented growth of moulds. Differences in grape must composition were observed, and the diverse inoculation strategies caused noticeable variations of fermentation kinetics and main oenological parameters. Sensory analysis highlighted a remarkable differentiation of wines due to the modulation of aromatic compounds by *M. pulcherrima*, both in the post-harvest withering process and in the multistarter fermentation. *M. pulcherrima* proved effective to protect grapes against fungal infections during withering and contribute to alcoholic fermentation generating wines with distinguished aromatic characteristics.

#### 4. Conclusion

The number of scientific studies testing non-*Saccharomyces* yeasts in wine fermentation has been steadily growing in the last decades, thanks to the advancement of ecology, molecular, and biochemical sciences leading to a better comprehension of the microbial diversity in vineyards and wineries and of the chemical diversity of non-volatile and volatile molecules interacting in the wine matrix. With the role of non-*Saccharomyces* comprehensively investigated, they will become an asset to overcome the effects of climate change and the challenge to reduce inputs, especially the addition of sulphites. As discussed in this article, besides the modulation of aromatic characteristics, a new generation of non-conventional yeasts can produce organic acids and antioxidant molecules, reduce the generation of ethanol and other compounds, and prevent microbial spoilage.

Nevertheless, the inoculation of industrial yeasts is still seen with suspicion by many producers worldwide, who claim a presumable “standardization” of wines with loss of regional peculiarities. It is important to reiterate the safety and reliability of using selected strains, who are all natural yeasts isolated from grape and wine environments and exhaustively characterized in research trials, and the diversity of strains indicated for very different wine styles and terroirs. Moreover, as the technology transfer of selected yeasts from the laboratory to the industrial-scale production remains an important bottleneck, only a small portion of the studied strains are currently available, with costs that might be impeditive to smaller producers. Many non-*Saccharomyces* struggle to overcome the stressful conditions to grow and dehydrate large volumes of biomass of active dry yeasts. Improved research by oenological companies and more interest by producers should increase the availability of non-conventional yeasts, as innovative solutions become more necessary for the future of the wine sector.

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