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Review

Yeast Metabolism and Its Exploitation in Emerging Winemaking Trends: From Sulfite Tolerance to Sulfite Reduction

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Abstract: Sulfite is widely used as a preservative in foods and beverages for its antimicrobial and antioxidant activities, particularly in winemaking where SO_2 is frequently added. Thus, sulfite resistance mechanisms have been extensively studied in the fermenting yeast *Saccharomyces cerevisiae*. Nevertheless, in recent years, a negative perception has developed towards sulfites in wine, because of human health and environmental concerns. Increasing consumer demand for wines with low SO_2 content is pushing the winemaking sector to develop new practices in order to reduce sulfite content in wine, including the use of physical and chemical alternatives to SO_2, and the exploitation of microbial resources to the same purpose. For this reason, the formation of sulfur-containing compounds by wine yeast has become a crucial point of research during the last decades. In this context, the aim of this review is to examine the main mechanisms weaponized by *Saccharomyces cerevisiae* for coping with sulfite, with a particular emphasis on the production of sulfite and glutathione, sulfite detoxification through membrane efflux (together with the genetic determinants thereof), and production of SO_2-binding compounds.

Keywords: *Saccharomyces cerevisiae*; sulfur metabolism; *SSU1*; SO_2; acetaldehyde; low-sulfite wine

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1. Introduction

Sulfur dioxide or sulfite (SO_2) is one of the most common, inexpensive, and effective chemical additives in foods and beverages. In the wine industry, sulfur dioxide addition represses the growth of many non-*Saccharomyces* yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [1]. Moreover, SO_2 can counteract both enzymatic and chemical oxidations of wine, thus stabilizing its sensorial properties during storage and aging [2]. Finally, SO_2 improves the release of phenolic compounds from grape skins and seeds during maceration [3].

The flip side of the coin is that the addition of SO_2 presents several disadvantages. High residual quantities in wine may result in unpleasant flavors [3]. The excessive intake of SO_2-containing foods is responsible for adverse reactions in human beings, including bronchospasm, bradycardia, gastrointestinal symptoms, headaches, as well as skin rashes, hypotension, and in rare cases anaphylactic reactions [4,5]. These adverse effects make it mandatory to include the sentence "containing sulfites" on the label of wines in which the concentration of SO_2 is higher than 10 mg/L (Directive 2003/89/EC).

Notwithstanding the World Health Organization recommended an SO_2 daily allowance (RDA) of 0.7 mg SO_2/kg of body weight [1], the European law set the maximum allowed concentrations as high as 150 mg/L and 200 mg/L in red and white wines, respectively (EU Regulation No. 606/2009). These limits are further increased by 50 mg/L if reducing sugar concentrations are equal to or higher than 5 g/L, such as in dessert wines (EU Regulation No. 606/2009). Thus, it has been estimated that wine is one of the main

contributors to SO_2 intake in adults [6]. Indeed, even the consumption of half a bottle of wine can provide an amount of SO_2 higher than the RDA.

Recently, the emergence of the above-mentioned adverse reactions together with environmental concerns has led consumers to turn toward "healthy" products and to choose wines with lower levels or without additional sulfites, even if they are sold at a higher price [4,7]. The increasing demand for such wines is pushing the winemaking sector to develop new practices to reduce the content of sulfites in wines while at the same time retaining the desired organoleptic characteristics [8,9]. However, some researchers and oenologists are still convinced that SO_2 is the only additive that can provide a comprehensive solution to obtain quality wines with a proper shelf-life [10].

1.1. Physical and Chemical Alternatives to SO_2

Suitable SO_2 alternatives to reduce or control spoilage microorganisms in winemaking include both physical methods and chemical additives. Besides, proper preharvest practices can reduce the risk of grape spoilage, thus helping in the reduction of SO_2 addition [11].

Thermal treatments and filtration are the main physical methods used to reduce the microbial load of musts and wines. However, these methods could negatively affect the organoleptic properties of the final product [12]. Thermal treatments in red wines may result in cooked flavors and the degradation of heat-sensitive polyphenols and other bioactive components, while in white wines they lower the concentration of volatile aromatic compounds [13]. Filter fouling from polysaccharides and polyphenols results in the reduction of color intensity, anthocyanins, and tannins in red wines [12]. In white wine stabilization, small aromatic compounds and large flavor precursor could also be removed during filtration, resulting in a poorer aromatic profile [12]. Innovative physical methods, such as high hydrostatic pressure, ultrasound, ultraviolet irradiation and pulsed electric fields have been recently reviewed [14]. Even though promising for the control of spoilage microorganisms and the preservation of wine quality, their main limitations are still related to the lack of protection against wine oxidation and the relative scarcity of information about their efficacy at the winery level (Table 1).

Table 1. Main physical methods used to replace/reduce SO_2 addition in wine.

Method	Target Microorganisms	Advantages	Limits/Disadvantages	Reviewed in
Thermal treatments	Highly active against yeast and bacteria	During vinification favors the extraction of anthocyanins and other polyphenols (thermovinification) Inhibition of oxidative enzymes Stabilization of sweet wines	Changes in wine chemical composition and sensorial properties Appearance of cooked flavors Thermal degradation of polyphenols May require wine filtration	[13,14]
Microfiltration	Active against LAB, AAB and yeast depending on membrane porosity	Clarification, stabilization and sterile filtration combined in a single operation Easily to automate	Very oxidative process could reduce the sensorial quality of the wine. Risk of recontamination after the treatment High cost of disposable materials (filters) Decreases in flavor and color compounds of wine	[14,15]

Table 1. *Cont.*

Method	Target Microorganisms	Advantages	Limits/Disadvantages	Reviewed in
High hydrostatic pressure (HHP)	Active against LAB and yeast	Minimal effects on the physiochemical and organoleptic properties of the wine	Limited activity against AAB Possible activation of oxidative enzymes Impossibility to use in a continuous process. Requires flexible packaging	[13–16]
Ultrasounds	Active against LAB and yeast	Acceleration of wine maturation Increased extraction of phenolic compounds in red wines Reduction of the activity of oxidative enzymes	Possible effects on wine sensory properties	[13,16]
Ultraviolet irradiation	Active against LAB, AAB and yeast	Lower energy requirements than thermal processes Increased extraction of phenolic compounds in red wines Applied to grapes increases the content of bioactive compounds (stilbenes)	Less effective in red than in white wines, as phenolic compounds reduce the antimicrobial activity High turbidity of wine reduces the effectiveness of the treatment High residence times and small volumes	[13,15,16]
Pulsed electric fields (PEF)	Active against LAB and yeast	Minimal effect on wine quality Reduction of maceration time Acceleration of wine maturation Inactivation of yeasts in sweet wine Reduction of the activity of oxidative enzymes	Bacteria are more resistant than yeasts Reduction of the amount of anthocyanins in pink wines	[14–16]

Lysozyme, sorbic acid, dimethyl dicarbonate, phenolic compounds and chitosan are chemical additives with antimicrobial activity admitted by the OIV (Organisation Internationale de la Vigne et du Vin-International Code of Oenological Practices) and permitted in winemaking, according to EU legislation (Reg. EC No. 606/2009 and further modifications). Other substances, such as bacteriocins and silver nanoparticles, showed promising antimicrobial properties [3,14,15]. However, their use is not yet permitted in winemaking (Table 2).

Table 2. Main chemical compounds with antimicrobial activity used to replace/reduce SO_2 addition in wine.

Compound	Target Microorganisms	Application	Advantages	Limits/Disadvantages	Recommended Dosage	Reviewed in
Lysozyme	Highly active against LAB	Delay or inhibition of malolactic fermentation	Negligible effects on wine aroma Lower volatile acidity and biogenic amine content of wine Alcoholic fermentation not inhibited	Allergenic (requires specific label indications above 0.25 mg/L) Does not affect yeast growth Low activity against Gram-negative bacteria *Lactobacillus* and *Pediococcus* are more resistant than *Oenococcus* Binds with polyphenols and produce color loss in red wine Leads to the formation of haze in white wine	500 mg/L (Regulation EC No 606/2009).	[14–16]
Sorbic acid	Active against *S. cerevisiae*	Prevent refermentation of sweet wines	Neutral taste and odor Biodegradable	Not effective against LAB, AAB and some spoilage yeast genera (*Brettanomyces*, *Saccharomycodes* and *Zygosaccharomyces*) If metabolized by LAB causes geranium off-odor Possible negative effects on human health	200 mg/L	[14]
Dimethyl dicarbonate	Very effective against yeasts	Microbiological stabilization of sweet, semi-sweet and semi-dry wines	Increase in color intensity of red wines More effective against yeast than SO_2	Less effective against bacteria Not recommended during wine storage (ephemeral effect) Hydrolyzes into methanol	200 mg/L (Regulation EC No 643/2006)	[14,16]
Phenolic compounds	Active against LAB and pathogenic bacteria	Used in wine ageing	Supposed health benefits Antioxidant activity Increased color intensity Lower production of acetaldehyde Fermentation process not negatively affected	At low concentrations stimulate LAB growth Increased yellow color value in red wines (gallotannins and procyanidins) Less active than SO_2 Hydroxycinnamic acids may be metabolized by *Brettanomyces*	-	[13–16]
Chitosan	Prevent wine spoilage by AAB and the yeast *Brettanomyces* spp.	Suggested as fining agent and for the reduction of *Brettanomyces* spp. (Reg EC 606/2009, Reg EC 53/2011, Reg EC 315/2012)	Biodegradable, biocompatible and nontoxic Reduces the oxidation of polyphenols and thiols Alcoholic fermentation not inhibited Reduces wine contamination by heavy metals and mycotoxins	Could affect color parameters of red wines (intensity and hue value) Little activity against Gram-positive bacteria (LAB)	Approved by OIV (OIV-OENO 336A-2009).	[13–15]
Bacteriocins	Active against LAB	Control of undesirable MLF Control of spoilage LAB	Nisin has the GRAS status Do not affect wine color or smell Nontoxic Do not affect yeast growth	Little or no activity against Gram-negative bacteria (AAB)	-	[14]
Colloidal silver complex (CSC)	Active against LAB, AAB and yeasts	Control of malolactic fermentation Reduction of microbial load prior to starter addition Reduction of wine off-odors	Added at the dose of 1 g/kg control AAB and LAB development, while not affecting the growth of *S. cerevisiae*	Wine composition was slightly affected by CSC treatment Reduction of polyphenol concentrations	Ag residues should not exceed 0.1 mg/L (EU Regulation 1576/201)	[13,14]

In general, it should be noted that the use of all these chemical preservatives is recommended in association with SO_2 to supply the limited antimicrobial efficacy against specific microbial taxa and to ensure appropriate antioxidant protection. Further, chemical preservatives have been recently questioned, given the above-mentioned concerns of consumers. Among natural substances, resveratrol represents an interesting and innovative alternative to SO_2, given its antioxidant and antimicrobial activity against spoilage microorganisms, such as AAB, LAB, and the yeast genera *Dekkera*, *Zygosaccharomyces*, and *Hanseniaspora* [17]. In contrast to sulfites, it has also positive effects on human health, particularly in the prevention of atherogenesis and cardiovascular diseases [18]. Even though resveratrol and related biophenols are naturally present in wines, their concentrations are very limited. Thus, the fortification of wines with resveratrol is required to replace SO_2.

1.2. Microbiological Alternatives to SO_2

An alternative strategy to the chemical and physical methods for the control of wine spoilage microorganisms comes from the proper exploitation of microbial interactions and metabolism.

The first approach, bioprotection, relies on the deliberate inoculation of yeast species able to quickly colonize the environment, thus preventing the development of spoilage microorganisms [19,20]. The most obvious yeast species for the bioprotection of grape must is *Saccharomyces cerevisiae*, given its ability to start and complete the alcoholic fermentation [21,22]. Indeed, early inoculation of *S. cerevisiae* has been recommended by the International Organisation of Vine and Wine (OIV) to avoid wine spoilage. Recently, also non-*Saccharomyces* yeasts have been suggested for the bioprotection of musts, given the re-evaluation of their role in winemaking, particularly in consideration of their positive influence on the complexity and sensorial quality of wines [23,24].

Another approach is based on the use of yeast starters able to produce antimicrobial metabolites, such as mycocines, antimicrobial peptides, and short-chain fatty acids (Table 3).

Table 3. Yeast metabolites with antimicrobial activities proposed to replace/reduce SO_2 addition in wine.

Metabolites	Target Organisms	Advantages	Limits/Disadvantages	Ref.
Killer toxins (mycocines)	Active against spoilage and contaminant yeast species	Safe for human consumption Starter yeasts producing killer toxins are commercially available Killer toxins from selected non-*Saccharomyces* yeasts do not inhibit *S. cerevisiae* and LAB	Killer toxin activity is affected by pH and ethanol concentration of wine Studies on the application of purified toxins in winemaking are scarce Direct addition of killer non-*Saccharomyces* yeasts may negatively affect wine quality	[14,25–30]
Short-/medium-chain fatty acids	Inhibition of yeast growth Active against LAB	Monolaurin has GRAS Status Approved by OIV as antifoaming agents in winemaking Fatty acid esters contribute to wine fruitiness	Responsible for cheesy, fatty, and rancid odors	[14,31–34]
Antimicrobial peptides (AMP) from wine yeasts	Active against bacteria (LAB), spoilage and biofilm forming yeast	Selected AMP does not affect *S. cerevisiae* growth Nontoxic	Lack of biotechnological tools for larger production at an industrial scale Effects of pH and sugar concentrations on AMP activity should be clarified Studies in actual winemaking are scarce	[14,35–40]

Among microbial alternatives and microbial-based practices aimed at reducing SO_2 content in wine, the formation or consumption of sulfur-containing compounds by wine yeast has become a crucial point of research during the last years. In this context, the relative importance of specific traits of yeast metabolism, such as sulfite tolerance and sulfite production, is also changing. The following paragraphs of the present review will narrow in on the main mechanisms weaponized by *Saccharomyces cerevisiae* for coping with sulfite, bringing into focus the perspective of their potential interest within the emerging scenario of low-SO_2 wine production. These mechanisms span from metabolic pathways, such as sulfite and glutathione production, to sulfite detoxification through membrane efflux (together with genetic determinants thereof), towards production of SO_2-binding compounds.

2. The Changing Role of Sulfur Metabolism in Yeast: From (High) Sulfite Tolerance to (Low) Sulfite Production

In the identification of the most suitable strategies to reduce or substitute SO_2 and to obtain low-sulfite wines, a crucial point is the management of the sulfur-containing metabolites produced by yeasts during fermentation (Figure 1). Indeed, even in the case of unsulfited musts, relatively high amounts of SO_2 can be found at the end of alcoholic fermentation, due to yeast metabolism. It has been observed that the majority (80%) of *S. cerevisiae* wine strains produce less than 10 mg/L SO_2, but also that some strains can release up to 30 mg/L SO_2 [41,42].

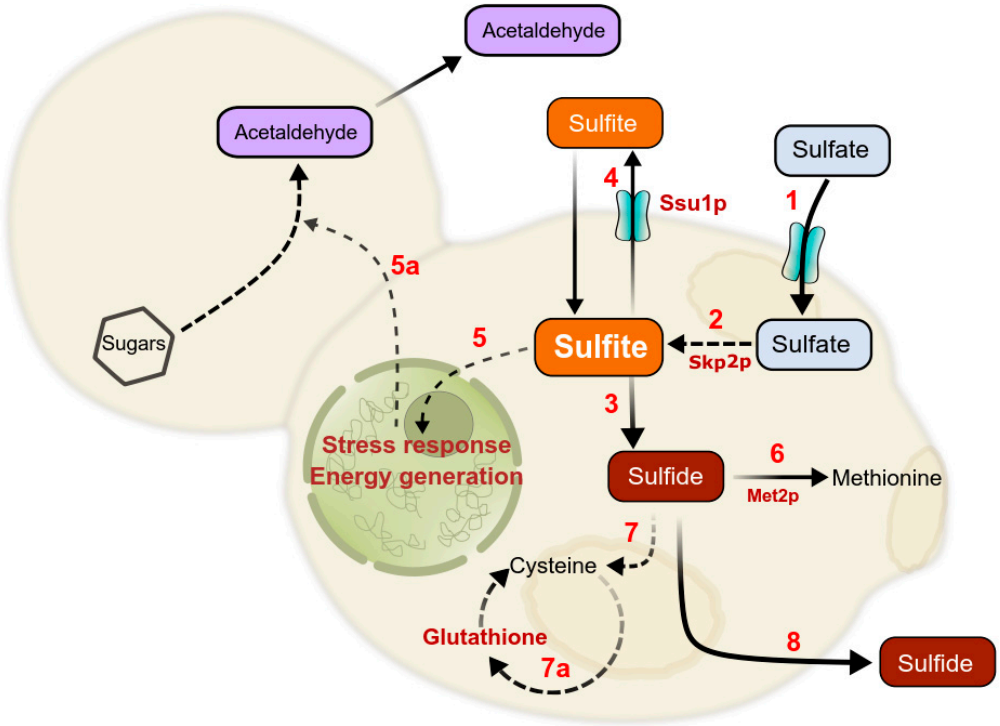

Figure 1. Simplified overview of sulfite-related pathways. (1) Extracellular sulfate is transported into the cell through the sulfate permease. (2) After activation by adenylation, sulfate is reduced to sulfite by the activity of different enzymes coded by *MET3*, *MET14*, and *MET16*; *SKP2* affects the activity of *MET14*. (3) Sulfite is further reduced to sulfide by the sulfite reductase enzyme. (4) Sulfite is transported outside the cells by the sulfite transporter Ssu1p. (5) Sulfite triggers multiple stress responses in the cells, among which (5a) an increased acetaldehyde production. Sulfide is incorporate directly into methionine (6) and indirectly (7) into cysteine. *MET2* influences the availability of O-acetylhomoserine, required to synthesize methionine. (7a) Glutathione is formed through the reaction of cysteine with glutamate and glycine. (8) Excess sulfide diffuses from the inside to the outside of the cell.

2.1. Sulfite Production by Yeast and Its Importance in New Winemaking Trends

In forthcoming winemaking scenarios, yeast strains producing low concentrations of SO_2 (but not overproducing sulfide) are useful potential tools. Therefore, an enhanced sulfur assimilation pathway is probably the most relevant trait for yeast selection and improvement for low-sulfite wine production.

In this frame, a valuable study [43] approached this goal by firstly examining the kinetics of SO_2 production by two yeast strains, one high and one low sulfite producer. Transcriptomic analysis revealed that the low-sulfite producer strain overexpressed genes of the sulfur assimilation pathway, which is the mark of a lower flux through the pathway consistent with a lower intracellular concentration in cysteine. Through a QTL (quantitative trait loci) mapping strategy [44,45], *MET2* and *SKP2* were recognized as the genes responsible for these phenotypic differences between strains. New variants of these genes were identified in the low-sulfite producer strain. *MET2* influences the availability of a metabolic intermediate, *O*-acetylhomoserine, whereas *SKP2* affects the activity of a key enzyme of the sulfur assimilation branch of the pathway, the APS kinase, encoded by *MET14*. Furthermore, these genes also affected the production of propanol and acetaldehyde. These pleiotropic effects are probably linked to the influence of these genes on interconnected pathways and the chemical reactivity of sulfite with other metabolites [43].

Afterward, the same research group patented a selection process [46] based on the QTL technology for recombining the above-mentioned genes (linked to the desired traits: nonproduction of SO_2, acetaldehyde, and H_2S) to select another yeast combining those desired traits and other interesting oenological properties. This selection method involved repeated crosses (backcrossing) between the low-SO_2 yeast and the target, a technologically relevant fermenting yeast.

Indeed, the robust low sulfite-producing phenotype associated with the combination of the selected alleles suggested that their transfer to any high producer strain of wine yeast would be sufficient to control sulfite/sulfide and acetaldehyde production in most cases. The transfer of these alleles via a non-GMO route was chosen, therefore backcrossing approaches were applied, previously used to improve wine yeasts. Furthermore, *MET2* and *SKP2* were demonstrated to be genetically linked, and therefore simultaneously transferrable during backcrossing cycles [43]. The target wine yeast was crossed once with the low SO_2, H_2S, and acetaldehyde producing yeast, and then several times with "daughter" yeasts possessing interesting characteristics. This gave a more precise cross (93.75% of the target yeast's genome preserved) [47]. By applying the above-mentioned patented technology [46] ("Method of control on the production of sulfites, hydrogen sulfur and acetaldehyde by yeast (Variants MET_2/SKP_2)", filed by INRA and Montpellier SupAgro, France), industrial yeast-producers have already obtained different yeasts producing very low concentrations of SO_2, H_2S, and acetaldehyde, by changing the "technological" parental strain for backcrossing [47].

2.2. Glutathione Production

One of the major compounds derived from the sulfur metabolism in yeast is glutathione (GSH), formed through the reaction of cysteine with glutamate and glycine. GSH represents more than 95% of the low-molecular-mass thiol pool and 0.5–1% of the dry weight of *S. cerevisiae* [48]. In yeast cells, GSH acts as a sulfur and nitrogen reservoir, being transported to the vacuole when cells are subjected to nitrogen starvation [49]. Besides, it protects yeast cells from heavy metals and other toxic compounds and shows scavenging activity towards free radicals [48,50]. Given its antioxidant activity, the addition of GSH in concentrations up to 20 mg/L of wine, has been proposed as a means to confer chemical oxidative stability during wine aging and storage (Resolutions OIV-OENO 445-2015 and OIV-OENO 446-2015) [51]. However, exogenous GSH supplementation can lead to sulfur off-flavors, particularly when the treated wines have low phenolic and nitrogen contents [52]. Given this limitation, the selection of *S. cerevisiae* strains with increased GSH production represents an alternative strategy to increase the glutathione content of wine

and to reduce the need to add exogenous SO_2 [53]. Genetic strategies such as random mutagenesis, metabolic engineering, and hybridization, as well as evolution-based strategies, have been applied to this end, as recently reviewed by [54]. Particularly, the exposure of yeast cells to a high toxic concentration of Mo (VI) as the specific selective pressure, resulted in the activation of resistance mechanisms in yeast, among which the production of high levels of GSH [55]. More in detail, the evolved strains enhanced the GSH content of wine up to 120%, compared with the initial GSH content in must. Further research is required to evaluate the impact of oenological factors and winemaking conditions, other than yeast strains, on the release of glutathione and its effect on wine quality.

3. Sulfite Detoxification through Membrane Efflux

3.1. Genetics and Strain Distribution of the Sulfite Transporter Ssu1p

The cellular and molecular mechanisms of resistance to sulfur dioxide were only initially investigated around the turn of the millennium, mostly in *S. cerevisiae*, and every newly published study reveals a further level of complexity and interaction. In the following paragraph, we will focus on the sulfite transport through the plasma-membrane protein Ssu1p, which is an important mechanism of detoxification and, at once, an important marker of adaptive evolution.

SO_2 detoxification through Ssu1p is one of the most efficient resistance mechanisms in *S. cerevisiae* [56,57]. Although wine yeasts also cope with SO_2 by means of other systems, such as acetaldehyde production and the upregulation of sulfite reduction or whole sulfur metabolism, this sulfite pump is required for efficient sulfite efflux. Ssu1p is encoded by the gene *SSU1*: this gene shows a high level of polymorphism [58] and deleterious mutations in its coding sequence cause SO_2 susceptibility [56,57].

Nowadays, it is known that the *SSU1* promoter sequence is involved in three different chromosomal rearrangements (CR) (i.e., XV-t-XVI, VIII-t-XVI, and inv-XVI) that increase its expression leading to a more efficient sulfite pumping over [59–61]. Interestingly, these three independent events have been generated by parallel evolutionary routes driven by human selection: this makes the genomic organization of the *SSU1* locus an interesting marker for the study of the adaptive evolution of *S. cerevisiae* to the winemaking environment, which is, in turn, a subject deeply studied over time [62,63].

The first described mechanism of an *SSU1*-linked adaptive advantage was the reciprocal translocation that occurs between chromosomes VIII and XVI, widespread among wine yeasts, which was identified in the early 2000s [59,64]. This translocation generates an allele of the sulfite pump, *SSU1*-R, with higher expression levels than *SSU1*, and confers greater resistance to sulfites [59,64]. By investigating in deep detail the possible mechanisms for regulation of the expression of the *SSU1* gene of a wine yeast strain, Pérez-Ortin and coworkers [59] found that the *SSU1*-R allele, which confers sulfite resistance to yeast cells, is the result of a promoter change generated upon the reciprocal translocation between chromosomes VIII and XVI, due to unequal crossing over which puts the *SSU1* coding region under the control of the ECM34 promoter. Indeed, the *SSU1*-R promoter contains up to six repeats of a 76 bp enhancer sequence from the promoter of ECM34, a highly expressed protein of unknown function. *SSU1*-R is expressed at much higher levels than *SSU1* [65]. It has also been shown that at least two repeats of the 76 bp enhancer sequence are required for *SSU1*-R expression [59,66] and that *SSU1*-R is not directly regulated by the transcription factor Fzf1p [66], which instead regulates *SSU1*. It was also demonstrated that the number of 76-bp repeats influences the expression of *SSU1* [66] and that number of repeats increases in wine yeasts that display stronger SO_2 resistance.

This translocation was initially found only in wine yeast strains [59], suggesting that the use of sulfite as a preservative in wine production over millennia could have favored its selection. This was the first time that a gross chromosomal rearrangement was shown to be involved in the adaptive evolution of *S. cerevisiae*, because of the selective pressures imposed by winemaking procedures [62]. Moreover, a high level of polymorphism has

been observed in the *SSU1* gene among vineyard-isolated strains [58], suggesting that this transport system is important in the evolution of SO_2 resistance mechanisms overall.

To make things more complicated, wine strains of *S. cerevisiae* exhibit different degrees of ploidy and different levels of heterozygosity, therefore the number of *SSU1* and *SSU1*-R could potentially explain the diverse range of resistance observed between strains. Indeed, although chromosomal rearrangements lead in most cases to *SSU1* constitutive overexpression [67,68], in some works the *SSU1* translocation located between chromosome XVI and VIII was shown to play only a minor role in SO_2 resistance, being present only in few strains with an intermediate sulfite resistance level [69].

More recently, another translocation—between chromosomes XV and XVI—has been identified both in genome-sequencing studies [70] and in works investigating the relationship to quantitative trait loci (QTL) of the lag phase duration during wine fermentation [60]. This translocation also increased the expression of the gene *SSU1*. In the XV-t-XVI translocation, the upstream region of *SSU1* is placed head to tail with the ADH1 promoter from chromosome XV [60]. Both translocations have been traditionally observed only in wine yeasts: the VIII-t-XVI translocation was the more frequent and the XV-t-XVI form was found only in commercially selected wine strains, which suggested a more recent event [63].

In 2019, a third, novel chromosomal rearrangement that triggers wine yeast sulfite adaptation was identified: an inversion in chromosome XVI (inv-XVI) probably due to sequence microhomology, involves *SSU1* and *GCR1* regulatory regions and was shown to increase the expression of *SSU1* and the sulfite resistance of a commercial wine yeast strain [61].

All three translocations were separately associated with resistance in a medium containing SO_2. Therefore, the widespread use of sulfites in winemaking likely causes a convergent evolutionary rearrangement that confers a growth advantage to the strains carrying the *SSU1* recombinant forms [63]. Nevertheless, most of the previously mentioned studies about CR focused on some specific yeast strains (though gradually shifting, over years, from laboratory to winemaking strains). Therefore, the strain dependency of the *SSU1*-driven SO_2 resistance, although established [68,69], was not easy to assess overall. Some new insights, valuable for a vision at a glance, come from recent research surveys examining wider populations of *Saccharomyces cerevisiae* strains, variously related to the wine environment.

Interesting results arise from a recent study about the impact of XVI-VIII/XV-XVI translocations on copper and sulfite tolerance in vineyard *Saccharomyces cerevisiae* strain populations [71] (253 strains representing three vineyard-populations from two different countries and 20 industrial starters). Firstly, the results of this work surprisingly evidenced that the levels of SO_2 tolerance in vineyard strains were comparable or higher than those in industrial strains. This could suggest a widespread SO_2 tolerance in the vineyard as well as in the cellar. The VIII-t-XVI was found in 69% of the strains, while the XV-t-XVI was present in 13%. The industrial group had the highest number of strains (80%) carrying the translocation VIII-t-XVI, which was also widespread through the vineyard populations, as 68.4% of the remaining strains carried it [71]. These results also demonstrated for the first time the diffusion of the XV-t-XVI among vineyard strains, although at a low level (13.4%), probably due to a recent translocation event. XV-t-XVI was present in 13.4% of the vineyard strains analyzed in this work, with the largest variation between Brazilian strains (that showed the lowest values) and European. Moreover, the number of VIII-t-XVI homozygous strains was always higher than that of XV-t-XVI

A recent research work examined for the first time all three chromosomal rearrangements (VIII-t-XVI, XV-t-XVI, and inv-XVI) in a population of nearly 600 yeast strains, including commercial starters and natural isolates collected from grapes and fermented musts, and provides new insights into the allele frequency of rearranged *SSU1* forms [72]. Essentially, industrial strains were significantly enriched in translocations VIII-t-XVI and XV-t-XVI compared to natural isolates. At the same time, the translocated *SSU1*-forms turned out, once again, to be nonunique to wine strains as previously proposed but spread

among natural isolates (which in this study came both from cellar and vineyard environments). In contrast, the inv-XVI allele was rarer and never found in industrial strains.

In earlier papers focused on genomic variations involving the *SSU1* gene translocations VIII-t-XVI and XV-t-XVI [59,60], the authors speculated that the most probable origin of the translocations could have been in the yeast's adaptation to the large use of sulfites in cellars, which is a very common practice in the winemaking process. According to the most recent findings [71,72], the vineyard environment seems to contribute significantly to the diffusion of these genomic variations: Crosato and coworkers also suggested a possible association with the use of copper sulfate in the vineyard as a plausible explanation [71]. At the same time, Marullo et al. [72] showed that in the subpopulation of unique naturally isolated strains, strains having inherited at least one rearranged chromosome XVI were significantly more frequent in the cellar group compared with the vineyard group. Therefore, the occurrence percentages observed could reflect that among wine-related yeast isolates, cellar strains undergo a selective pressure even stronger than vineyard strains likely due to winemaking operations [72].

3.2. Sulfite Efflux and Its Importance in New Winemaking Trends

The cellular efflux through Ssu1p can be considered a neutral mechanism for SO_2 homeostasis in wine, as sulfite is not produced nor consumed by the yeast in this case, but just pumped out. Therefore, in the context of winemaking trends aiming at lowering the final SO_2 content in wines, this might not represent a target for direct yeast selection: strains that allow a sulfite reduction through consumption would ideally be preferred.

On the other hand, this mechanism might be interesting in the future because it potentially allows the yeast to operate an efficient sulfite-detoxification without recurrent acetaldehyde production. Indeed, acetaldehyde production is usually undesired, since this compound binds SO_2 and enhances the gap between its bound and free fractions. Winemakers, in the presence of high acetaldehyde concentrations, are often obliged to add SO_2 at the end of the process to guarantee antioxidant protection to the wine, after alcoholic fermentation and independently from yeast metabolism which is, at this stage, over. Therefore, yeasts that cope with SO_2 through an efficient efflux rather than with acetaldehyde production might be interesting for this indirect reason.

Looking at this subject from an adaptive evolution point of view, the translocated forms of *SSU1* which are currently very common in starters and widespread in winery-issued strains will possibly show to be less essential in new winemaking scenarios. An interesting finding on this point comes from the most recent work on *SSU1* rearrangements [72], where the winery-isolated strains included in the *SSU1* survey were split into three groups depending on the type of grape juice matrix: sweet, white, and red. Strains isolated from white juice were enriched in the VIII-t-XVI rearrangements. In contrast, strains isolated from sweet grape juices were strongly enriched in the native chromosome form. These results were considered by the Authors to be consistent with the traditional enological practices used in the Bordeaux area, where the addition of sulfite in the musts is routinely used for dry white wine fermentation, but mostly avoided at the beginning of sweet wine fermentation to limit SO_2 binding phenomena.

This suggests that the selection of chromosomal rearrangements is strongly influenced by the winemaking practices used in cellars [72]. Therefore, it is likely conceivable that a decreasing selective pressure coming from lower must sulfitation might change the landscape of chromosomal-rearrangement diffusion among winery-related strains. In parallel, changes in the use of copper sulfate in the vineyard, which is currently more and more limited by regulations for copper-related environmental issues [73], may also change the scenery of chromosomal-rearrangement diffusion among vineyard-related strains [71].

4. Inducible Response Triggered by SO_2

Despite the great importance of sulfite efflux through Ssu1p, it has been observed that in some cases *SSU1* mRNA levels did not correlate with sulfite tolerance, probably due to the contribution of other factors to yeast sulfite resistance [74].

Therefore, much work has been done over time to decipher the transcriptional response triggered by sulfite exposure in wine yeasts [68,74–76], with a particular focus on inducible genes.

Generally, sulfite itself has been shown not to affect the expression of *SSU1* or *SSU1-R* [66,76,77]. However, in specific strains, it was shown by using quantitative real-time PCR that the level of expression of *SSU1* increased progressively during alcoholic fermentation [68]. Moreover, a study based on RNA-sequencing [69] showed that *SSU1* expression level, when no sulfite was added, was able to discriminate sensitive from resistant strains among four industrial starters. In fact, only the sensitive strain tested showed a very low gene expression level, although, when sulfite was added, the level was comparable to those found in more resistant strains. These results suggested that the basal gene expression level was the main one responsible for sulfite resistance, while the gene induction level had a minor role. These findings agreed with those previously obtained by Divol et al. [67] and confirmed that the adaptation to sulfite, by means of a high basal *SSU1* expression level, was the main mechanism responsible for sulfite resistance at the molecular level, whereas the physical effects of SO_2 triggered multiple stress responses in the cell [69].

Going beyond *SSU1*, the first expression profiling of changes triggered by sulfite in *S. cerevisiae* was performed using the application of a genome-wide yeast DNA microarray, unveiling that genes involved in energy generation and acetaldehyde production may account for the major acquired resistance to sulfite [76]. In particular, after sulfite treatment, genes involved in glycolysis represented the highest proportion of induced genes identified which agreed with the previous findings of sulfite toxicity in yeast, since sulfite was found to cause ATP depletion. Data indicated that maintenance of ATP generation and an increased acetaldehyde production may account for the easily induced resistance to sulfite. Sulfite also appeared to lead to the metabolic shift from oxidative to fermentative growth, allowing the cell to excrete more acetaldehyde. In addition, the presented results showed the evidence at the gene expression level that sulfite represses genes involved in transcription, protein biosynthesis, and cell growth [76]. It is worth noting, though, that this first pioneering work was performed on laboratory strains in non-oenological conditions, also due to the intrinsic limitations of DNA microarrays available at the time.

In this context, a more recent study performed through RNA-sequencing and focusing on four wine strains, provided some more insight into the strain effect and winemaking conditions [69]. Results demonstrated that, at the molecular level, the physical effect of SO_2 triggered multiple stress responses in the cell, and that high tolerance to general oenological stressing conditions increased SO_2 resistance. Adaptation mechanism due to high basal gene expression level rather than specific gene induction in the presence of sulfite seemed to be responsible for modulating strain resistance. This mechanism involved a higher basal gene expression level of specific cell wall proteins, enzymes for lipid biosynthesis, and enzymes directly involved in the SO_2 assimilation pathway and efflux. In particular, when the transcriptome profile of strains in the presence of sulfite was compared with that under control conditions, the most resistant strain showed only a few genes differentially expressed, evidencing a very limited general stress response due to the SO_2 presence. This work also evaluated whether any adaptation mechanism, due to constitutive gene expression and working independently from the SO_2 presence, could be involved in high sulfite resistance in the tolerant strains. Transcriptome profile comparison between the most resistant and the most sensitive strains growing in unsulfited conditions evidenced a gene expression related to general stress response higher in strains with low or intermediate resistance than in the most SO_2-resistant one, and its intensity was proportional to the sulfite sensitivity level [69].

Concerning gene expression profiles specifically triggered by SO_2, a recent work demonstrated that the poorly characterized transcription factor Com2 was essential for the tolerance and response of *S. cerevisiae* cells to SO_2 at pH 3.5 [75]. Com2 encodes an orphan homolog of the environmental stress-responsive transcription factors Msn2 and Msn4 [78]. Transcriptomic analysis revealed that Com2 controls, directly or indirectly, the expression of more than 80% of the genes activated by SO_2, a percentage much higher than the one that could be attributed to any other stress-responsive transcription factor. Large-scale phenotyping of the yeast haploid mutant collection led to the identification of 50 Com2-targets contributing to the protection against SO_2 including all the genes that compose the sulfate reduction pathway (*MET3, MET14, MET16, MET5, MET10*) and most of the genes required for the biosynthesis of lysine (*LYS2, LYS21, LYS20, LYS14, LYS4, LYS5, LYS1*, and *LYS9*) or arginine (*ARG5,6, ARG4, ARG2, ARG3, ARG7, ARG8, ORT1*, and *CPA1*). Other uncovered determinants of resistance to SO_2 (not under the control of Com2) included genes required for function and assembly of the vacuolar proton pump and enzymes of the antioxidant defense, consistent with the observed cytosolic and mitochondrial accumulation of reactive oxygen species in SO_2-stressed yeast cells [75].

Sulfite Inducible Response and Its Importance in New Winemaking Trends

Sulfite resistance itself is possibly going to lose some importance among yeast selection criteria: in the forthcoming scenario of wines containing limited sulfite concentrations, many winemakers add little (or no) SO_2 at the beginning of fermentation to lower the final sulfite doses in the bottle [19,79]. In this context, inducible responses to sulfite in yeast may become more crucial, if sulfite addition at high dosages becomes an exception rather than a rule. As part of this changing scenario, in the age of high throughput sequencing technologies and omics data, the current challenge is to further unveil the molecular determinants underlying a specific trait of industrial interest such as inducible sulfite response [74]. Indeed, the majority of data about sulfite-triggered inducible stress response [75,76], including those about the newly discovered transcription factor Com2, are still coming from studies performed in laboratory strains and cultures, not mimicking winemaking conditions. Therefore, it would be very promising in the near future to investigate more thoroughly these traits in aneuploid wine strains and under real fermentation conditions.

5. SO_2-Binding Compounds Produced by Yeasts

As for the pH of the wine, only a minor amount of SO_2 is in the molecular and most active form. The vast majority consists of the bisulfite anion, which loses most of its antimicrobial and antioxidant activities when forming complexes with wine constituents [80]. Acetaldehyde, the major carbonyl compound formed during alcoholic fermentation, forms a stable addition product with bisulfite (hydroxysulfonate), which accounts for 40–50% of the total SO_2 in wine [81]. In yeast cells, acetaldehyde acts as the terminal electron acceptor during alcoholic fermentation and is required to maintain the overall redox balance [81]. Given that the formation of hydroxysulfonate makes acetaldehyde unavailable to accomplish this function, yeast reacts to SO_2 addition by increasing acetaldehyde production. It was found that up to 400 µg of acetaldehyde is produced for each mg of added SO_2 [82]. Particularly, these quantities are produced at the onset of fermentation, in correspondence with the yeast lag phase of growth, as a resistance mechanism against sulfur dioxide. Further production of acetaldehyde has been observed during the yeast exponential growth phase. Finally, acetaldehyde is metabolized by yeast during the stationary phase and at the end of fermentation [83]. Given this dynamic, the final amount of acetaldehyde is usually independent of the levels formed during fermentation [81].

A recent analysis of the acetaldehyde kinetics in wine yeasts revealed that *Candida vini*, *Hansenula anomala*, *Hanseniaspora uvarum,* and *Metschnikowia pulcherrima* are low producers (<10 mg/L), while *Candida stellata*, *Zygosaccharomyces bailii*, and *Schizosaccharomyces pombae* release up to 48 mg/L of acetaldehyde during fermentation [83]. Other authors found that *Torulaspora delbrueckii* and *K. apiculata* are low producers of acetaldehyde [82,84]. Thus, in

mixed fermentations with the now available non-*Saccharomyces* starter cultures [85], the *inoculum* of yeast genera that release lower amounts of acetaldehyde may allow a reduction in the quantities of SO_2 needed to maintain an effective antimicrobial and antioxidant activity in wine.

Similarly, the selection of *S. cerevisiae* strains with low production of acetaldehyde goes in the same direction. Li and Mira de Orduna [86] found that acetaldehyde produced by 20 *S. cerevisiae* strains ranged from 14 to 28 mg/L, while Wells and Osborne [87] showed a higher variability, with some strain producing 109 mg/L and others less than 36 mg/L. Particularly high acetaldehyde levels, up to 200–400 mg/L, are produced by the so-called "flor" strains of *S. cerevisiae* during the aging of sherry-like wines [88]. Different approaches have been evaluated to further reduce the acetaldehyde produced by *S. cerevisiae*. A mutant strain, obtained by inhibiting the activity of alcohol dehydrogenase 2 (*ADH2*) through UV mutagenesis and using a competitive ADH2 inhibitor (4-Methylpyrazole) as a selection marker, produced about 10 mg/L of acetaldehyde [89]. Yin et al. [90] obtained an isolate producing 3.9 mg/L of acetaldehyde by using a genome shuffling protocol. Recently, a reverse engineering strategy was used to modify the redox state of the cell (NADH/NAD+), resulting in a strain with 45% lower acetaldehyde production than that of the parental strain [91]. Interestingly, all these results were obtained from brewing strains, while studies regarding *S. cerevisiae* wine strains are still scarce. For instance, only recent works [92] have addressed the investigation of the effect of the initial content of SO_2 in grape juice on yeast metabolism linked to the production of acetaldehyde, showing that the initial content of SO_2 not only affects the synthesis of sulfur metabolites but also impacts the overall sensory profile of wines, also demonstrating that acetaldehyde bound to SO_2 could not be metabolized by the yeast during the time course of fermentation and that only free acetaldehyde could impact metabolism. Nevertheless, future work will be needed to study the genericity of the observed behaviors in yeast strains other than the one investigated.

Similarly, a great deal of information about the production of acetaldehyde by dairy LAB is available, while it is not yet clear the production of this compound by wine LAB [81]. On the other end, the ability of heterofermentative wine LAB, such as *Oenococcus oeni*, to metabolize bound acetaldehyde, thus increasing the levels of free SO_2, is well documented [81].

Another research topic that still needs to be better exploited is the quantification of carbonyl compounds, other than acetaldehyde, produced by yeast and able to bind free SO_2. Wells and Osborne [87] found that *S. cerevisiae* production of pyruvic acid was strain-dependent, while no statistical differences were found for α-ketoglutarate. Interestingly, at low acetaldehyde concentrations, a large portion of the bound SO_2 was as pyruvic acid-bound SO_2. This suggests that not only acetaldehyde, but also pyruvic acid should be the target of future research aiming at selecting yeast strains for SO_2 reduction in wine.

6. Conclusions

The increasing consumer demand toward wines with low sulfite content is pushing the winemaking sector to develop new practices, including fermentation management, to reduce SO_2 content in wine.

Suitable SO_2 alternatives have been proposed for winemaking over years. In this review, we summarized those aimed at controlling spoilage microorganisms, thus contributing to fill in the antimicrobial role of SO_2. Besides innovative physical methods and chemical additives, an alternative strategy for the control of wine spoilage microorganisms is described, arising from the proper exploitation of microbial interactions and metabolism. In this context, the relative importance of specific traits of yeast metabolism, such as sulfite tolerance and sulfite production, is changing. The ability to tolerate high SO_2 concentrations, which is currently very common in starters and widespread in winery-issued strains, will possibly be shown to be a less essential feature in new winemaking scenarios. On the contrary, the formation of sulfur-containing compounds by wine yeasts has become a crucial point of research during the last decades, since relatively high amounts of SO_2 can

be found at the end of alcoholic fermentation, due to strain-dependent yeast metabolism. The selection of *S. cerevisiae* strains with low production of acetaldehyde goes in the same direction, for limiting SO_2-ineffectiveness caused by its main binding compound. Furthermore, yeasts that cope with SO_2 through an efficient efflux rather than with acetaldehyde production might be interesting for this indirect reason.

Therefore, this review examined the main mechanisms weaponized by *Saccharomyces cerevisiae* for coping with sulfite in winemaking, aiming at enlarging the perspective of their potential interest within the emerging scenario of low-SO_2 wine production.

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