

Review

Non-*Saccharomyces* in Winemaking: Source of Mannoproteins, Nitrogen, Enzymes, and Antimicrobial Compounds

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Abstract: Traditionally, non-*Saccharomyces* yeasts have been considered contaminants because of their high production of metabolites with negative connotations in wine. This aspect has been changing in recent years due to an increased interest in the use of these yeasts in the winemaking process. The majority of these yeasts have a low fermentation power, being used in mixed fermentations with *Saccharomyces cerevisiae* due to their ability to produce metabolites of enological interest, such as glycerol, fatty acids, organic acids, esters, higher alcohols, stable pigments, among others. Additionally, existing literature reports various compounds derived from the cellular structure of non-*Saccharomyces* yeasts with benefits in the winemaking process, such as polysaccharides, proteins, enzymes, peptides, amino acids, or antimicrobial compounds, some of which, besides contributing to improving the quality of the wine, can be used as a source of nitrogen for the fermentation yeasts. These compounds can be produced exogenously, and later incorporated into the winemaking process, or be uptake directly by *S. cerevisiae* from the fermentation medium after their release via lysis of non-*Saccharomyces* yeasts in sequential fermentations.

Keywords: non-*Saccharomyces*; winemaking; aging-on-lees; yeast assimilable nitrogen

1. Introduction

The genus *Saccharomyces* has been the most industrially used in the production of wine. This aspect has been changing in recent years due to an increased interest in the use of non-*Saccharomyces* yeasts [1]. The high potential of these yeasts makes them a useful tool for improving oenological parameters such as nutrients content, stability, aromatic profile, or bioactive profile, in spite of their low fermentative power, and, in some cases, their high production of certain metabolites with negative connotations in the wine [2,3].

Currently, species such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Schizosaccharomyces pombe*, *Pichia kluyveri*, among others, are sold for wine production [4]. These yeasts are often isolated from the grape, grape-must, and wine, as well as from the soil, winery surfaces, harvesting machinery, and other objects associated with the wine production. In the case of grape-must/wine, the majority are isolated during the first stages of fermentative process given their low fermentation ability and low tolerance to ethanol, with respect to *S. cerevisiae*, so that its use would mainly be associated with the contribution of the specific enological metabolites such as enzymes, aromatic compounds, glycerol, organic acids, fatty acids, proteins, amino acids, polysaccharides, among others [3].

Figure 1 summarizes some of the potential applications of non-*Saccharomyces* yeasts, taking as reference the available commercial preparations for wine production. These preparations are sold in the form of (a) dry yeast, inactivated through thermal treatment and then dried; (b) cell autolysates,

which include soluble and insoluble yeast components, partially degraded by the endogenous enzymes; (c) soluble cell extracts, derived from the cytoplasm; and (d) insoluble cell hulls, mainly containing cell walls [5]. These preparations may be the source of compounds of interest such as polysaccharides, proteins, enzymes, or other metabolites with the potential for use in wine production and can be produced exogenously [6].

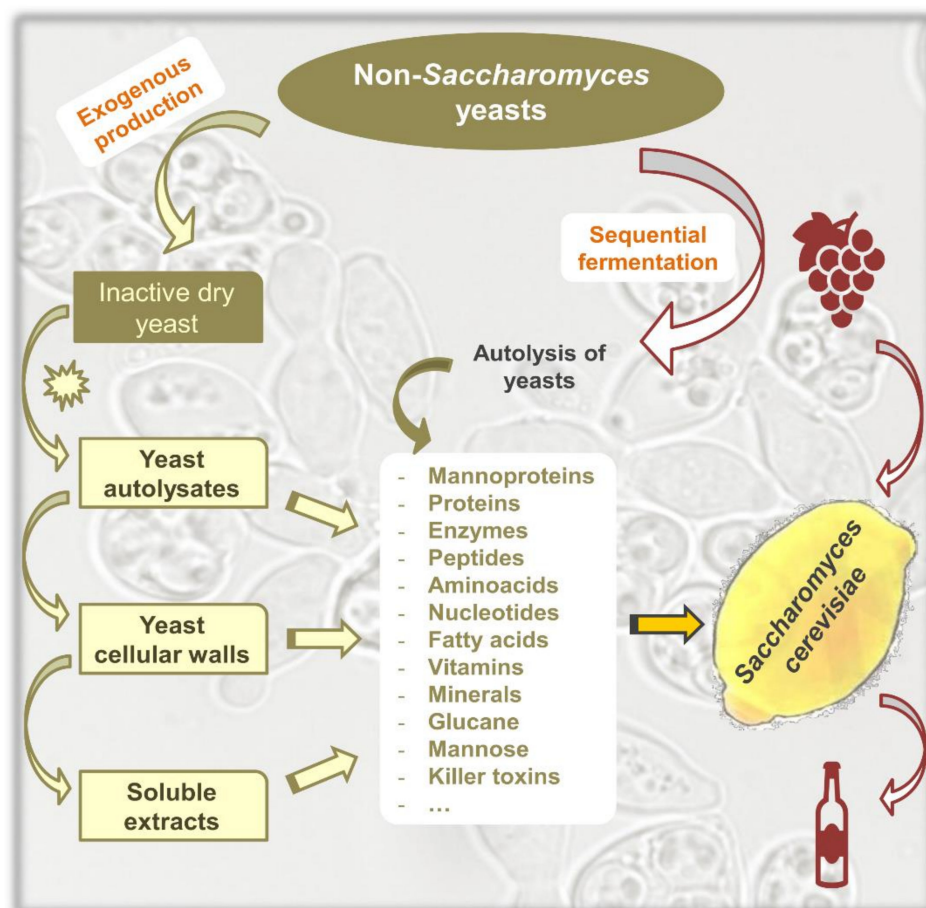


Figure 1. Main cell components of non-*Saccharomyces* yeasts with the potential for use in wine production.

In addition to these preparations, compounds of interest can be obtained directly from the fermentation process as a result of their release from the yeast during cell lysis, for example, in mixed cultures.

2. Non-*Saccharomyces* Yeasts as a Source of Polysaccharides

During aging-on-lees (AOL), a series of changes take place with a direct impact on the wine's properties, due to the interaction between the wine's components, the enzymatic activity, and the polysaccharides released by the lees [7]. The use of non-*Saccharomyces* yeasts for the exogenous production of polysaccharides [6], which can be added to wine, is an interesting alternative. The addition of yeast polysaccharides is a practice authorized by the International Code of Oenological Practice of the International Organization of Vine and Wine (OIV).

These polysaccharides, particularly in red wines, can improve the mouthfullness and body [8], sweetness and roundness [9], aromatic persistence [10], protein and tartaric stability [11,12], and interact with tannins and reduce astringency [13], as well as have an antioxidant effect (due to glutathione) that protects the aromatic compounds and anthocyanins [14], enabling its antioxidant and anti-inflammatory

potential to be maintained [15]. They also interact with the tertiary aromatic compounds, reducing the perception of woody aromas in long-aged wines [16], encourage malolactic fermentation [17], and adsorb undesirable and dangerous compounds such as ochratoxin A (OTA) [18]. In sparkling wines, they improve the quality of the foam [19]. However, during AOL, a reduction of aromatic compounds such as terpenes, esters, and aldehydes [20], and, in some cases, anthocyanins in red wines [16,21], has also been reported.

The most important polysaccharides released during AOL are the mannoproteins (Table 1), which are fixed on a three-dimensional network of glucan and chitin in the cell wall [21,22]. The mannoproteins represent between 80% and 100% of the fraction of polysaccharides founded in the wine (molecular mass 100–2000 kDa: 90% mannose and 10% protein), while glucomannoproteins (molecular mass 20–90 kDa: 25% glucose, 25% mannose, and 50% protein) represent between 10% and 20% of the total [23]. Furthermore, α -galactomannose rather than mannose has been found as part of the structure of polysaccharides in *S. pombe* [22].

The enzymes involved in the cell autolysis and subsequent releasing of polysaccharides into the wine are endo β (1→3)-glucanases, endo β -(1→6)-glucanases, exo-(1→6)- α -D-mannose, exo-(1→2)- α -mannose, and α -D-mannosidase [23].

2.1. Non-Saccharomyces Species as a Source of Polysaccharides

Even though *S. cerevisiae* is the most commonly used yeast as a source of polysaccharides, the literature reports different non-*Saccharomyces* species with the potential to produce and release polysaccharides during AOL: *S. pombe*, *Saccharomyces ludwigii*, *Wickerhamomyces anomalus*, *Hanseniaspora vineae*, *L. thermotolerans*, *M. pulcherrima*, *Starmerella bacillaris*, *T. delbrueckii*, *Zygosaccharomyces rouxii*, and *Zygorhizula florentina* (formerly *Zygosaccharomyces florentinus*), among others.

The genus *Schizosaccharomyces* has shown high rates of release of polysaccharides during alcoholic fermentation, of up to seven times higher than *S. cerevisiae* [24]. During AOL, *S. pombe* together with *S. codes ludwigii* are found among the species with high potentials for releasing polysaccharides [21]. *S. pombe* primarily releases galactomannoproteins (Table 1), at concentrations up to 10 times higher than some strains of *Saccharomyces* and *Pichia* [21]. This potential could mean an advantage with regard to accelerating aging processes.

S. codes ludwigii has also shown a high potential for releasing polysaccharides during fermentation and AOL [21,25,26]. Palomero et al. [21] obtained higher rates of polysaccharide release from *S. codes ludwigii* (110.51 gm/L) and *S. pombe* (103.61 mg/L) with respect to *S. cerevisiae* (36.65 mg/L). Furthermore, the polysaccharides from these non-*Saccharomyces* yeasts show a greater molecular size and may potentially impact the wine's palatability.

However, one aspect to take into account during AOL in red wines is color loss, due to the interaction between the lees and anthocyanins, mainly through adsorption of pigments by the lees [16] and through degradation due to anthocyanin- β -glucosidase activity [27]. The lees from *M. pulcherrima*, *S. codes ludwigii*, or *S. pombe* have shown a low adsorption of anthocyanins with respect to the lees of *S. cerevisiae* and other non-*Saccharomyces* yeasts such as *T. delbrueckii* or *L. thermotolerans* [21,28]. Furthermore, there may be less color loss in wines in the presence of higher pyranoanthocyanins content, which are more stable due to its chemical structure [29], with *S. pombe* being one of the yeasts with the greatest synthesis of these pigments [30].

Additionally, *S. pombe* has also shown the ability to decrease the biogenic amine content in sparkling wines, with better results than *S. cerevisiae* [30], which would be related to the adsorption of the amines on the lees during second fermentation and subsequent aging in the bottle [31]. However, considering the short duration of AOL studies, it is necessary to evaluate the evolution of amines over longer aging periods, such as those carried out in traditional sparkling wine production, given that other studies have reported an increase in the biogenic amines content in wines in contact with lees [32].

In terms of other non-*Saccharomyces* yeasts, *Hanseniaspora vineae* has shown a higher rate of cell lysis with respect to commercial *S. cerevisiae* strains [33]. Other yeasts that have shown a notable polysaccharide contribution are *L. thermotolerans* [34], *Z. rouxii* [26], and *Z. florentina*, particularly in mixed fermentations with *S. cerevisiae* [35].

Table 1. Some non-*Saccharomyces* yeasts with the potential to release mannoproteins.

Yeast	Protein and Monosaccharide Content in Mannoproteins [25]				Nitrogen Requirement
	Protein (%) ^a	Mannose (%) ^b	Glucose (%) ^b	Galactose (%) ^b	
<i>Saccharomyces cerevisiae</i>	24	88	12	-	Slow ammonium uptake [4].
<i>Metschnikowia pulcherrima</i>	25	86	14	-	Weak or no growth in nitrate agar, and unable to develop in YPD agar at 37 °C [28].
<i>Wickerhamomyces anomalus</i> ^c	9	74	26	-	Capable to uptake nitrate [36].
<i>Saccharomycodes ludwigii</i> ^d	12	93	7	-	Unable to uptake nitrate [37].
<i>Schizosaccharomyces pombe</i> ^e	11	55	22	23	Capable to uptake cadaverine and ethylamine [37].
<i>Starmerella bombicola</i> ^f	14	73	27	-	
<i>Pichia fermentans</i>	15	87	13	-	
<i>Hanseniaspora uvarum</i> ^g	23	81	19	-	
<i>Hanseniaspora valbyensis</i>	20	75	25	-	
<i>Lachancea thermotolerans</i>	16	82	18	-	
<i>Torulaspora delbrueckii</i>	18	85	15	-	
<i>Zygosaccharomyces bailii</i>	29	79	21	-	
<i>Brettanomyces bruxellensis</i>	16	88	12	-	

^a Percentage of dry matter. ^b Sugars (%) in the polysaccharide fraction. ^c *Wickerhamomyces anomalus* (formerly *Pichia anomala*). ^d *Saccharomycodes ludwigii*: high autolytic activity: polysaccharide-releasing [21,25]. ^e *Schizosaccharomyces pombe*: high autolytic activity: polysaccharide-releasing [21,38]. ^f *Starmerella bombicola* (formerly *Candida stellata* DBVPG 3827). ^g *Hanseniaspora uvarum* (formerly *Kloeckera apiculata*).

For its part, the information regarding the use of other yeasts such as *W. anomalus* as a source of mannoproteins is scarce [26]. This yeast has a high potential for the production of polysaccharides and other metabolites of interest, given its ability to metabolize a large variety of nitrogen sources, including nitrate [36], which would allow production costs to be reduced.

2.2. Accelerated Release of Polysaccharides

One disadvantage of AOL is that it is a very slow process, which requires up to 9 months to obtain the desired effects in the treated wine. Among the strategies to improve AOL, existing literature reports (a) the selection of yeast species and strains with rapid autolysis [21,39], (b) acceleration of the cell autolysis through mixed cultures involving sensitive and killer yeasts [40], mixed cultures among different yeast species, which enable the regulation of cell death [41], the addition of β -glucanase [42], and the application of ultrasound [39,43].

Several studies have reported a higher rate of polysaccharide release with ultrasound (US) treatments [39,43,44]. Lees from *S. codes ludwigii*, *S. pombe*, *M. pulcherrima*, and *S. cerevisiae*, among others, were evaluated over a 7-week aging period in a hydroalcoholic medium, applying US for 10 min a day, with *S. codes ludwigii* lees showing the highest rate of polysaccharide release after the third week (around 460 mg/L) [39]. In the same study [39], a decrease in anthocyanins content was observed in the treated red wine, without affecting the pyranoanthocyanins content [21,29], particularly with *S. codes ludwigii* lees, which also allowed to reduce proanthocyanidins content, and consequently, astringency and bitterness.

More recently, Del Fresno et al. [43] evaluated the effect of AOL (lees from *S. cerevisiae*) in the presence of oak chips in red wines. The evaluation period was 135 days at 14°C, under dark conditions, agitating the samples once a week to simulate “bâtonnage”. The samples were treated with US twice a week for 5 min for the first 5 weeks. From this moment, the process was accelerated increasing to two US treatments a week (15 min per treatment). In a parallel experiment, a hydroalcoholic medium was used to adequately quantify the polysaccharides released from the lees. In general, the polysaccharide release increased after using US for 135 days, releasing around 11.8 mg/L, more than double that of the untreated samples (approx. 5.3 mg/L). Additionally, an increase in the protein content of the US-treated samples was observed after 120 days of AOL.

However, the same authors [43] reported the increase in dissolved oxygen in the treated red wines as a disadvantage, whose effect was evident in the lower anthocyanins content in the US-treated wines in the absence of lees. This finding reveals the protective effect of polysaccharides against oxidation.

3. Non-Saccharomyces Yeasts as a Source of Nitrogen for *Saccharomyces cerevisiae*

Yeast assimilable nitrogen (YAN) is nitrogen that yeasts can assimilate and metabolize, preferably from sources such as ammonium (NH_4^+), amino acids, and peptides of up to five amino acids [45]. Its content in the grape-must varies between 50 and 500 mg/L [46].

Among YAN's functions are reproduction and cell growth, the protein for sugar transport synthesis, and enzyme synthesis, as well as the functions accomplished by the amino acids as the precursors of aromatic compounds, mainly higher alcohols, produced by deamination and decarboxylation [47]. The most predominant amino acids as NH_4^+ transporters in grape-must are α -alanine, serine, arginine, proline, glutamic acid, and glutamine [48]. During fermentation, their use varies, with higher uptake of glutamic acid, glutamine, and arginine [4], whereas proline, a proteinogenic amino acid not metabolized by *S. cerevisiae*, is among the least used [4]. Additionally, the demand for arginine is increased by lactic acid bacteria during malolactic fermentation [48].

S. cerevisiae has shown a preference for NH_4^+ and amino acids, thanks to its nitrogen catabolite repression (NCR) mechanism [49], through which the genes involved in the transport and metabolism of NH_4^+ and glutamine are activated and, once they are depleted, the genes involved in the transport and metabolism of other sources such as arginine, glutamate, and alanine, among others, become activated.

Concentrations of YAN below 150 mg/L in the grape-must carry the risk of sluggish or stuck fermentation [4], as well as a low synthesis of some aromatic compounds such as of esters, volatile fatty acids, and higher alcohols [50]. This N deficiency is remedied in the winery through the addition of extra N sources such as diammonium phosphate (DAP). However, the excessive use of DAP can lower phenylpropanoid production (affecting the complexity of the wine) [51,52], increase the wine acidification, produce high levels of residual phosphate, stimulate the production of esters such as ethyl acetate, and increase the levels of hydrogen sulfide (H_2S), especially when there is a deficit of other essential nutrients such as vitamins, minerals, and lipids; further, excessive levels of DAP can increase turbidity, promote microbial instability, and facilitate the production of unpleasant aromas and harmful compounds such as ethyl carbamate and biogenic amines [53–56].

3.1. Non-Saccharomyces Species as a Source of Nitrogen

As an alternative N source, yeast cell structures can be used in the form of hulls, hydrolyzed, or extracts, which can be produced exogenously [6], and then added to the fermentative medium. Additionally, this source of N can be obtained from the rest of the cells after the death and lysis of non-Saccharomyces yeasts used at the beginning of the sequential fermentations with *S. cerevisiae*.

Aureobasidium pullulans (an yeast-like fungus) is a potential source of essential amino acids [57], and it can grow in low-cost carbon sources like agricultural and food waste, due to its amylase [58], cellulase [59], lipase [60], xylanase [61], laccase [62], mannanase [63], and protease [64] activities. However, the use of *A. pullulans* for the production of protein on a large scale has still not been explored [65].

Similarly, the use of species such as *S'codes ludwigii*, *S. pombe*, *Candida stellata*, *M. pulcherrima*, *W. anomalus*, *H. vineae*, *Z. rouxii*, *Zygosaccharomyces bailii*, *L. thermotolerans*, and *Z. florentina*, among others, as potential N sources is limited, considering their high capacity for production and release of mannoproteins [21,25,26,33–35], which is up to 29% of protein (Table 1). From the little background information available, *W. anomalus* has been used as a source of single cell protein at industrial level, specifically to produce protein for fish farming [66]. This demonstrates the potential of *W. anomalus* as source of N, taking into account the ability of this yeast to use a wide range of N sources, including nitrate [36].

One aspect to consider is the depletion of nutrients during fermentation with different yeasts. Difficulties have been reported in the introduction of *S. cerevisiae* after *Hanseniaspora/Kloeckera* in sequential fermentations [67], an effect that is associated with the depletion of thiamine and calcium pantothenate [68,69], reducing the availability of these nutrients for *S. cerevisiae* (second inoculum).

Similarly, sequential fermentations can cause a depletion of YAN by first phase yeasts, especially those with high nutrient demands and a low ability to release nitrogenous compounds. This situation can be overcome with the use of yeasts such as *M. pulcherrima*, with high proteolytic activity and amino acid release as a source of N for *S. cerevisiae* (second inoculum) [70,71].

In a recent study, Prior et al. [4] carried out sequential fermentations of *L. thermotolerans*/*S. cerevisiae* and *T. delbrueckii*/*S. cerevisiae* to evaluate whether the rest of these non-*Saccharomyces* yeasts (first phase) can be used as a source of N for *S. cerevisiae*. After 48 h of fermentation with non-*Saccharomyces*, the medium was filtered and then inoculated with *S. cerevisiae*. A reduction in sugar consumption by *S. cerevisiae* was observed in the filtered medium, in other words, fermentative yield decreased.

The results obtained in the aforementioned studies show that in sequential fermentations, non-*Saccharomyces* yeast rests (first phase) after death and lysis can release various cell components into the fermentative medium to be used as N sources for *S. cerevisiae* [4]. This strategy can be used more efficiently with non-*Saccharomyces* species with high ability to release mannoproteins (Table 1) and with high β -glucanase and protease activities.

3.2. Nitrogen Requirements for Sparkling Wines Production

The selection of yeasts with high rates of nitrogenous compounds release can ensure an adequate supply of N. Two of the non-*Saccharomyces* species which have shown the greatest ability to release amino acids during the second fermentation are *S'codes ludwigii* and *S. pombe* [30], increasing the amino acids content with respect to the base wine. The advantage of these yeasts over *S. cerevisiae* is related to their amino acid release mechanisms, with their different consumption rates and their cell composition [21,47].

One of the most important properties in sparkling wine is the ability to form foam or foamability. Mannoproteins improve foam formation and stability thanks to their hydrophobicity, high glycosylation, and high molecular mass, which enable them to surround and stabilize the gas bubbles in the foam [72]. The proteins also participate in this process which, together with the peptides and amino acids, are released mainly during aging in the bottle as a result of the enzymatic degradation of the cell walls and other cell structures [7,73], contributing to the complexity of sparkling wines.

The higher production of mannoproteins in sequential fermentations by *S. bacillaris*/*S. cerevisiae* [74], *L. thermotolerans*/*S. cerevisiae*, and *T. delbrueckii*/*S. cerevisiae* can also be utilized, which also contributes to reducing volatile acidity and increasing 2-phenylethanol [75,76], and improving foamability and foam stability (sequential *T. delbrueckii*/*S. cerevisiae* and *M. pulcherrima*/*S. cerevisiae* fermentations) [77,78].

Finally, during AOL (in the bottle), it has been observed that the content of free amino acids and peptides depends on the yeast species and strains [73], which have an influence on the flocculation ability to facilitate lees movement during riddling [79]. A potential field for future studies is the optimization of aging and disgorging operation in sparkling wines, for example, by inserting magnetic nanoparticles to accelerate sedimentation and lees removal [80].

4. Non-Saccharomyces Yeasts as a Source of Exogenous Enzymes

Another potential application of non-*Saccharomyces* yeasts is enzyme production. In general, enzymes of microbial origin are considered to have greater activity and stability than those of plant and animal origin [81]. Considering that the genus *Saccharomyces* is not characterized as being a good producer of exogenous enzymes [82], there is increasing interest in finding sources of enzymes of enological interest among non-*Saccharomyces* species, some of which are summarized in Table 2.

Table 2. Summary of the enzymes produced by non-*Saccharomyces* yeasts for wine production.

Enzyme	Yeast	Application
β -glucosidase	<i>Lachancea thermotolerans</i>	Release of terpenes and thiols from their precursors: improvement of the aromatic profile [83–85].
	<i>Torulaspora delbrueckii</i>	Release of thiols from their cysteinylated precursors: improvement of the aromatic profile [84].
	<i>Wickerhamomyces anomalus</i>	- High stability of β -glucosidase excreted by <i>W. anomalus</i> MDD24 [86].
		- Release of terpenes from their glycosylated precursors: improvement of the aromatic profile [85,86].
	<i>Metschnikowia pulcherrima</i>	- Release of terpenes from their glycosylated precursors: improvement of the aromatic profile [71,83].
		- Release of thiols from their cysteinylated precursors: improvement of the aromatic profile [84].
		- Foaming and release of aromatic compounds in sparkling wines [87].
	<i>Candida stellata</i>	Release of terpenes (β -myrcene, limonene, linalool, α -terpineol, and farnesol) from their glycosylated precursors: improvement of the aromatic profile [88].
	<i>Hanseniaspora uvarum</i>	- Release of terpenes and C13-norisoprenoids from their precursors: improvement of the aromatic profile [89,90].
		- Activity up to 6.6-fold higher than some <i>S. cerevisiae</i> strains [90].
Protease	<i>Saccharomyces ludwigii</i>	Activity up to 46% higher than <i>S. cerevisiae</i> at 30 °C [91].
	<i>Aureobasidium pullulans</i>	Release of terpenes from their glycosylated precursors: improvement of the aromatic profile [92,93].
	<i>Wickerhamomyces anomalus</i>	Aspartic protease WaAPr1 excreted by <i>W. anomalus</i> 227 [94].
	<i>Metschnikowia pulcherrima</i>	- Aspartic protease MpAPr1 excreted by <i>M. pulcherrima</i> IWB T Y1123 [95].
		- Degradation of proteins: improvement of clarification and stabilization [95].
		- Degradation of proteins and peptides: source of N for fermentative yeast [70,71,83].
		- Improvement of grape-must extraction and clarification, wine filtration [71], and stabilization [83].
		- Improvement of foam stability in sparkling wines [71,87,96].
		- Increase in the amino acids content: production of aromatic compounds [83,95].
	<i>Candida stellata</i>	- Degradation of proteins: improvement of clarification and stabilization [97].
		- Degradation of proteins and peptides: source of N for fermentative yeast [97].

Table 2. Cont.

Enzyme	Yeast	Application
Glucanase	<i>Hanseniaspora uvarum</i>	Degradation of proteins: improvement of clarification and stabilization [89,97].
	<i>Lachancea thermotolerans</i> , <i>Torulaspora delbrueckii</i> , <i>Zygosaccharomyces bailii</i> , <i>Pichia kluyveri</i>	- Increase in the amino acids content: source of N for fermentative yeast [83]. - Increase in the amino acids content: production of aromatic compounds [83]. - Degradation of proteins: improvement of clarification and stabilization [83].
	<i>Wickerhamomyces anomalus</i>	- β-1,3-glucanase excreted by <i>W. anomalus</i> AS1: viscosity reduction in grape-musts [98,99]. - β-1,3-glucanase excreted by <i>W. anomalus</i> AS1: improvement of bioactive profile by release of <i>trans</i>-resveratrol from its glycosylated precursor polydatin [98]. - Antimicrobial control against <i>Dekkera/Brettanomyces</i>: attack at the cell wall level [100].
	<i>Schizosaccharomyces pombe</i> <i>Saccharomycodes ludwigii</i> <i>Lachancea thermotolerans</i> , <i>Metschnikowia pulcherrima</i> , <i>Debaryomyces hansenii</i> <i>Metschnikowia pulcherrima</i> , <i>Hanseniaspora guilliermondii</i> , <i>Hanseniaspora opuntiae</i> , <i>Hanseniaspora vineae</i> , <i>Hanseniaspora clermontiae</i> , <i>Pichia guilliermondii</i>	High mannoproteins-releasing during AOL [21,38,99]. High mannoproteins-releasing during AOL [21,25]. Release of mannoproteins [25,83].
Hydroxycinnamate decarboxylase (HCDC)		Involved in the synthesis of vinylphenolic pyranoanthocyanins: improvement of color stability in red wines [83,101–103].
Urease	<i>Schizosaccharomyces pombe</i>	- High urease activity [104]. - Urea hydrolysis: reduced synthesis of ethyl carbamate (carcinogenic [105]).
Carboxypeptidase	<i>Aureobasidium pullulans</i>	- Degradation of ochratoxin A (OTA) [106]. - Drawback: possible action against fermentative yeasts [107].
Pectinase	<i>Wickerhamomyces anomalus</i>	Degradation of pectins: improvement of clarification and turbidity reduction [85].
	<i>Metschnikowia pulcherrima</i>	- Degradation of pectins: improvement of clarification and turbidity reduction [87,97]. - Improvement of extraction of anthocyanins and polyphenols from the skins of the grape berries [108].
	<i>Candida stellata</i>	Degradation of pectins: improvement of clarification and turbidity reduction [85,97].
	<i>Hanseniaspora uvarum</i>	Degradation of pectins: improvement of clarification and turbidity reduction [89,97].
	<i>Aureobasidium pullulans</i>	- Degradation of pectins: improvement of clarification and turbidity reduction [92,93]. - Production of cold-active and acid-tolerant pectinases, suitable for low-temperature winemaking [92].

Table 2. Cont.

Enzyme	Yeast	Application
Cellulase	<i>Lachancea thermotolerans</i> , <i>Metschnikowia pulcherrima</i> , <i>Candida stellata</i> ,	- Degradation of cellulose released from the grape cell walls: improvement of extraction, filtration and clarification [83,92,97].
	<i>Hanseniaspora uvarum</i> , <i>Aureobasidium pullulans</i> , <i>Debaryomyces hansenii</i>	- Improvement of extraction of pigments and aromatic compounds from the skins of the grape berries [83,92,97].
Xylanase	<i>Lachancea thermotolerans</i> , <i>Candida stellata</i> , <i>Hanseniaspora uvarum</i> , <i>Aureobasidium pullulans</i> <i>Torulaspora delbrueckii</i> ,	Degradation of hemicellulose: improvement of wine aroma by increasing of monoterpenyl diglycoside precursors in the grape-must [83,92,97].
β -lyase	<i>Kluyveromyces marxianus</i> , <i>Meyerozyma guilliermondii</i> (formerly <i>Pichia guilliermondii</i>)	Release of thiols from their cysteinylated precursors: improvement of the aromatic profile [109,110].
Lipase	<i>Lachancea thermotolerans</i>	Increase on free fatty acids concentration [83].
	<i>Aureobasidium pullulans</i>	Improvement of wine aroma: synthesis of ethyl esters and ethyl acetates from lipid cleavage [111].

There is little information regarding the application of purified β -glucosidase, β -lyase, xylanase, cellulase, among others enzymes, produced by non-*Saccharomyces* yeasts for winemaking processes, leaving open the possibility of future research which focuses on improving the release of terpenes, thiols, norisoprenoids, and/or their precursors, with positive impacts on the aromatic profile, especially in white wines, considering that approximately 90% of these compounds are conjugated in the grape skin [99]. This is in addition to the selection of suitable strains, because high levels of β -glucosidase can increase the synthesis of undesirable volatile phenols [101], as well as hydrolyze the anthocyanins in red wines, exposing them to oxidation and/or transformation into colorless forms [27,112].

In contrast, one problem in white wines is the protein haze (wine turbidity), which is usually corrected by removing the proteins from the grape-must with bentonite, with the disadvantage of removing other compounds of enological interest, mainly aromatic compounds. The protease activity of non-*Saccharomyces* yeasts can be used (Table 2) and thus reduce the protein content in the grape-must and therefore prevent the wine haze. The proteases hydrolyze the proteins from the grape-must into smaller molecules like peptides and amino acids, with the consequent clarification and subsequent stability of the wine obtained, and with the additional advantage of providing YAN for the fermentative yeasts [87,89].

5. Non-*Saccharomyces* as Biocontrol Agents Against Contaminating Yeasts

5.1. Antimicrobial Peptides

Some peptides produced by yeasts have shown antimicrobial effects against several grape-must/wine contaminating yeasts. In general, these peptides show lengths of up to 100 amino acids, sorted into variable sequences [113]. Their mechanism of action would be related to changes in the integrity of the cell wall of the target yeasts [114]. Peptides with molecular mass below 10 kDa have shown greater antimicrobial effects [115], such as those produced by *Candida intermedia*, especially the LAMAP1790 strain, with an effect on several strains of *Brettanomyces bruxellensis* and without affecting the growth of *S. cerevisiae* [115].

S. cerevisiae CCMi885 is another yeast that produces antimicrobial peptides with molecular mass lower than 10 kDa and with an effect on *B. bruxellensis*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *C. stellata*, *L. thermotolerans*, *Kluyveromyces marxianus*, and *T. delbrueckii* [116]. However, as these peptides have not shown total inhibition over *B. bruxellensis*, their application may require the use of other usual winemaking treatments, such as the addition of SO₂.

According to Peña et al. [117], to achieve the application of these peptides at an industrial level, it is necessary to understand their behavior in mediums with different pH values and sugar levels, as well as high alcohol levels and in the presence of other winemaking yeasts and/or bacteria. Additionally, the implementation of procedures that enable high production and, therefore, satisfy a potential industrial level demand is needed.

5.2. Killer Toxins

Some yeasts produce molecules called “killer toxins”, which are glycosylated proteins with effect against sensitive yeast strains [118]. For instance, the action of killer toxins CpKT1 and CpKT2 produced by *Candida pyralidae* (YWB T Y1140 strain) on the cell wall of *B. bruxellensis* have been reported [119]. These toxins have a molecular mass of over 50 kDa, are stable at acidic pH values (3.5–4.5), temperatures between 15 and 25 °C, at high alcoholic content, and at different sugar concentrations. In other words, they are stable under normal winemaking conditions.

In the same way, a killer toxin produced by *T. delbrueckii* NPCC 1033 (TdKT) has shown potential to control yeasts such as *Brettanomyces bruxellensis*, *Pichia guilliermondii*, *Pichia mandshurica*, and *Pichia membranifaciens* [120], being the mechanism of action, the attack at the cell wall level, related to their glucanase and chitinase enzymatic activities. The toxin has a molecular mass of over 30 kDa, its killer activity is stable at pH values of 4.2–4.8, and is inactivated at temperature above 40 °C, confirming their potential use as a biocontrol tool at oenological conditions.

Similarly, Kwkt toxins produced by *Kluyveromyces wickerhamii* [121] and PMKT2 produced by *P. membranifaciens* have shown effects on *B. bruxellensis* [122], although in the case of PMKT2, effects on *S. cerevisiae* have also been observed.

W. anomalus also produces killer toxins with effects on *Dekkera/Brettanomyces*, especially the Pikt toxin [121,123] produced by the D2 and DBVPG 3003 strains, whose fungicidal effect on wine can be sustained for 10 days [121]. The mechanism of action would be the attack at the cell wall level, specifically of β -1,6 glucans. [124], in a similar manner to the *W. anomalus* Cf20 toxin (KTCf20) that binds to the β -1,3 and β -1,6 glucans. β -1,3-glucanase activity has been previously reported in toxins secreted by *W. anomalus* [100]. Additionally, several toxins secreted by this yeast has shown stability and high activity at pH values of 3.0–5.0 and temperatures up to 30 °C, i.e., these toxins are compatible with the winemaking conditions [100,125].

Other studies have reported the antimicrobial effect of *W. anomalus* on species such as *Pichia guilliermondii* or *P. membranifaciens* [125,126] during the first stages of fermentation, and even on *S. cerevisiae* [125,127]. This indicates the need for more studies to evaluate the compatibility of the *W. anomalus* strains that produce these toxins with other yeasts to avoid technological problems such as sluggish and stuck fermentations, as well as take advantage of the potential of the different non-*Saccharomyces* species involved in the fermentation process, to obtain wines with greater complexity and stability.

Although it is not a non-*Saccharomyces* yeast, *Saccharomyces eubayanus* has also shown the ability to produce the killer toxin SeKT, with effect on spoilage yeasts such as *B. bruxellensis*, *P. membranifaciens*, *Meyerozyma guilliermondii* and *P. manshurica* [128]. The mechanism of action comprises cell wall disruption through β -glucanase and chitinase activities. The toxin have a molecular mass of around 70 kDa, and has shown stability at high glucose and ethanol concentrations (300 g/L and 16% v/v, respectively), at SO₂ concentrations of up to 100 mg/L, and at temperatures and pH values less than 26 °C and 5.0, respectively.

The results obtained in the aforementioned studies show the potential use of the killer toxins as a biocontrol tool at oenological conditions, especially against the spoilage yeast *B. bruxellensis*, for example, during wine aging and storing.

5.3. Other Molecules as Biocontrol Agents

One feature of *M. pulcherrima* is its potential effect against different contaminating yeast species. In the context of wine production, this activity would be mainly related to the production of the pigment pulcherrimin, from the chelation of Fe in the fermentative medium [129], reducing the availability of this mineral, with harmful effects on *Brettanomyces/Dekkera*, *Pichia*, *Hanseniaspora*, *S'codes ludwigii*, and *Candida* [130].

However, the most significant advantage of this antimicrobial mechanism is the absence of harmful effects on *S. cerevisiae* [130]. In other words, it may be compatible with the main yeast used for wine production, for example, in mixed fermentations, with the consequent reduction of the SO₂ dose, usually used as an antimicrobial agent [2].

6. Future Perspectives

One of the greatest challenges related to the industrial use of non-*Saccharomyces* yeasts for AOL is achieving acceptance from producers, especially in regions with a deep-rooted winemaking tradition and taking into account the unfavorable background of these yeasts, as well as the economic impact of prolonged storage of the wines in AOL and the possible risks of contamination. The optimization of aging conditions is one aspect that requires special attention, especially those conditions which enable the acceleration of the release of polysaccharides from the lees.

Another aspect that requires more research is the use of exogenously produced lees added to the wine [6], especially for the aging of red wines, which involves the identification of species, and especially strains with high rates of polysaccharide release which, in turn, present low anthocyanin adsorption [28] and low expression of anthocyanase activity (anthocyanin- β -glucosidase) that causes hydrolysis of anthocyanins [27]. Additionally, this requires strategies, which can be simultaneously implemented with the selection of yeasts with high capacity to produce pyranoanthocyanins, more stable against degradation [27], thereby minimizing color loss. One of the yeasts that has shown these characteristics is *S. pombe*.

There is also a need for additional studies on fermentation with natural grape-musts to verify the properties of mannoproteins released by non-*Saccharomyces* yeasts in synthetic mediums, given that in these last mediums, there is no interaction between the mannoproteins and the components which are naturally present in the grape-must, and to take advantage of the potential of these yeasts for the production of new products with industrial applicability [74].

Similarly, the effect of the addition of inactive yeasts, hulls, or lees as sources of N on parameters related to protein haze and turbidity formation is still not clear. One possible alternative is the addition of protease-hydrolyzed hulls/lees, which are also produced by non-*Saccharomyces* yeasts. *M. pulcherrima* has shown high protease activity and, therefore, the release of amino acids as a source of N for *S. cerevisiae*, especially in mixed cultures. This activity has also been shown by *W. anomalus*, with the advantage of using a wide range of N sources, including nitrate [36], and by *A. pullulans*, with the ability to use low-cost carbon sources such as agricultural and food waste, and whose protein production on a large scale has still not been studied [65].

It has been reported that the species and strain of the yeast influence the free amino acids and peptide content during AOL [57,58], thus reducing the content of amino acids in aging periods of over 9 months [73]. Therefore, it is necessary to conduct studies, which simulate normal aging conditions (even up to 10 years in sparkling wines) by evaluating the impact of the proteins, mannose, glucose, and galactose present in the lees on the quality of treated wine, which, until now, has only been studied in model mediums [131]. The effect of proteins from non-*Saccharomyces* yeasts on the quality of sparkling wines, their effect on lees movement during riddling, as well as the effect on the sensorial profile must also be studied.

Finally, most of the positive contributions of non-*Saccharomyces* yeasts with regard to *S. cerevisiae* are related to a higher presence of active enzymes, which depends, in part, on the carbon and nitrogen sources present in the fermentative medium. Small changes in the composition of the medium can

affect the nature, quantity, and diversity of the secreted enzymes [132]. Therefore, proper maintenance of N levels in the fermentative medium is of vital importance, which can be achieved by identifying yeast species and strains with high release rates of nitrogenous compounds, mainly amino acids.

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