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Review

Biocontrol and Enzymatic Activity of Non-*Saccharomyces* Wine Yeasts: Improvements in Winemaking

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Abstract: Wine fermentation is a biochemical process carried out by a microbial consortium already present in the vineyard, including different species of fungi and bacteria that are in an ecological relationship with each other, so that their sequential growth causes the transformation of grape must into wine. Among the fungi, the unicellular ones, yeasts, stand out, including *Saccharomyces cerevisiae*, which is mainly responsible for driving alcoholic fermentation, as do other species present from the beginning of fermentation, known as non-*Saccharomyces* yeasts. These yeasts were previously considered harmful and undesirable; however, their role has recently been re-evaluated, mainly because they can provide products and effects that are of great value in achieving a quality final product. In this review, we discuss the role of non-*Saccharomyces* wine yeasts, firstly with regard to their biocontrol activity both on the grapes and during the vinification process and secondly with regard to their ability to produce enzymes, especially depolymerising ones. In this context, the possible biotechnological applications of these non-*Saccharomyces* yeasts to improve the health and quality of grape and wine production are addressed.

Keywords: biocontrol; depolymerising enzymes; non-*Saccharomyces* wine yeasts; wine

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1. Introduction

Wine is defined as "the beverage resulting exclusively from the partial or complete alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must" [1]. The *Vitis vinifera* L. grapevine is one of the most cultivated and valued fruit crops, covering an estimated surface area of 7.3 million hectares and having diverse uses ranging from fresh consumption (table grapes and raisins) to wine and juice production. World wine production in 2022 was estimated at 258 million hectolitres, with a global wine exports value at around EUR 37.6 billion, the highest figure ever recorded [2]. In terms of environmental sustainability, the wine sector is responsible for about 0.3% of annual global greenhouse gas (GHG) emissions from anthropogenic activities, mainly related to the use of fertilisers, pesticides, and heavy metals in vineyard management but also to the contribution of winemaking from grape harvest to bottling [3]. Additionally, the Intergovernmental Panel on Climate Change [4] suggested that temperatures will rise, and there will be more extreme fluctuations in wet and dry periods, coupled with increased atmospheric CO_2 levels. In the current context of climate change and environmental awareness, improving the quality and health of grape and wine production in a sustainable manner is therefore one of the main challenges facing viticulture today.

Saccharomyces cerevisiae is the primary microorganism involved in the alcoholic fermentation of grape must. However, many other species belonging to non-*Saccharomyces* genera, both dominant grape epiphytes and weakly fermentative, are present during this

process [5]. In the past, non-*Saccharomyces* wine yeasts were considered to be of secondary importance or undesirable spoilage yeasts. Recently, the role of these yeasts has been reassessed, and it is generally accepted that selected strains can have a positive impact on the winemaking process and significantly contribute to wine quality [6]. Consequently, this has led to a growing interest in non-*Saccharomyces* yeasts, because of the wide range of enzymes and other metabolites that are not commonly produced by typical *Saccharomyces* yeasts [7].

Non-*Saccharomyces* wine yeasts, isolated from grape and/or wine fermentation environments, have different abilities, among them the potential to act as bioprotectants and biocontrol agents against phytopathogens [8–12] and wine spoilage organisms [13–15]. Biocontrol in wine grapes and wine comprises the use of selected microorganisms with antagonistic activity and/or their metabolites against other deleterious ones to reduce the need for chemical pesticides or wine preservatives, which can have negative effects on human health and the environment [3,16,17]. Therefore, non-*Saccharomyces* wine yeasts have potential as environmentally friendly tools to improve the sustainability aspects of winemaking. At the same time, non-*Saccharomyces* yeasts have the ability to produce enzymes of oenological relevance. Pectinases are the most common and widely used enzymes in winemaking, which breaking down the pectin and pectic substances present in grape berry cell walls [18]. These enzymes together with other related polysaccharidases assist in improving juice yield, clarification, and filterability, as well as the release of polyphenolic, colour, and flavour compounds trapped in the grape skins, contributing positively to the winemaking process and the global quality of wine [19,20]. To date, commercial pectinases have traditionally been obtained from fungi [21]; however, in the last two decades, yeast pectinases have attracted much attention from different research groups around the world as an alternative to fungal pectinases [22]. Interventions in the main stages of wine production using biocontrol agents and enzymes are illustrated in Figure 1.

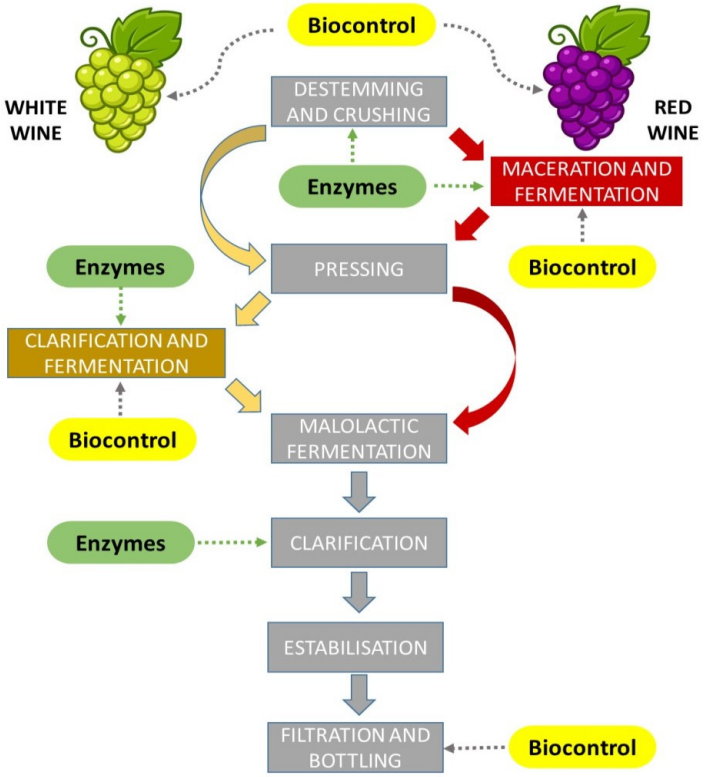

Figure 1. Schematic showing the use of biocontrol agents and enzymes in the main steps of wine production.

In this review, we discuss how non-*Saccharomyces* wine yeasts can help to address some of the modern challenges in oenology: the control of vineyard diseases and wine spoilage, and the production of enzymes of oenological interest, particularly polysaccharide-degrading enzymes, to improve winemaking.

2. Biocontrol Exerted by Non-*Saccharomyces* Wine Yeasts

2.1. Biocontrol in Wine Grapes

Fungal infection can affect not only grape health and productivity, causing major grape diseases involving the decay of fruit, but also the quality of the final product, producing off-flavours and phenolic modifications that impair the aroma, taste, and colour of wine [23]. More worryingly, the occurrence of mycotoxins in wine, linked to the development of several mycotoxigenic fungi in grapes, is a threat to human health [24]. Among the filamentous fungi of major concern, we find *Botrytis cinerea*, responsible for severe grey rot; some *Aspergillus* and *Penicillium* species, causing black or green/blue rots and producing aflatoxin B1, fumonisin B2, patulin, citrinin, and ochratoxin A (OTA); and *Alternaria*, contributing to bunch rot and the production of alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TA) [25–28]. Furthermore, the presence of certain yeasts and bacteria responsible for wine deterioration is favoured by the availability of nutrients in mouldy wine grapes [29]. Therefore, controlling fungal decay in the vineyard is crucial for maintaining the quality and safety of wine.

To avoid fungal infections, viticulture mostly relies on the use of synthetic chemical fungicides in the field during grape ripening. In addition to its many detrimental effects on the environment, as well as on animal and human health, confidence in this method as the only method used for fungi control has waned, being especially questioned when resistant pathogen populations emerged [30]. In this scenario, microbial biocontrol agents (BCAs), which are bacteria or fungi with the ability to prevent fungal plant diseases pre- and postharvest, have attracted scientists' attention. Among the microbial BCA candidates, yeasts have demonstrated remarkable features, such as high antagonistic capacity, implantation ability, and ability to survive for long periods in dry conditions and in other hostile conditions present in the field (thermal amplitude, UV, oxidative stress, etc.) [31]. Moreover, their natural environment is the optimal source of antagonistic yeast strains, and they have already shown the aptitude to successfully outcompete other microorganisms [32]. Therefore, the selection of native non-*Saccharomyces* wine grape yeasts as BCAs against the major fungal pathogens of wine grapes as well as the use of commercial non-*Saccharomyces* yeast-based BCAs in vineyards have been addressed by numerous researchers. Next, we analyse them under the lens of previous pathogen knowledge and the environmental conditions that influence antagonist–pathogen–host interactions, determining both microbial BCA efficacy and resilience in the field. Lastly, possible improvements for non-*Saccharomyces* BCAs applied to wine grapes are addressed.

2.1.1. The Importance of Studying Pathogens and the Influencing Environmental Conditions of Non-*Saccharomyces* BCA Applied to Wine Grapes

The main hurdle that microbial BCAs encounter is the expectation that they should behaves as a chemical fungicide in the field, with the same routine phenological application as synthetic fungicides. Under field conditions and depending on how microbial BCAs are formulated and applied, their consistency and efficacy may be severely compromised [16,33]. Microbial BCAs are living organisms with advantages such as specificity against certain pathogens, distinct modes of actions, and resilience potential, providing a better method of combatting pathogens. However, the complexity of interactions among pathogens, hosts, and antagonists, which is modulated by the environment, requires a deeper knowledge to achieve biological control success [34].

Routine control methods are commonly applied in a generic manner, avoiding any rot, regardless of the causal pathogenic fungi [35]. Even though this may seem advantageous or practical at first look, the lack of specificity of the control actions derives in the affection

to other microbiota already present, such as beneficial endophytes or natural antagonists, and the result or ecological impact cannot be easily predicted. On the contrary, microbial BCAs are specific against certain pathogens, and searching for the appropriate one among the possible candidates requires not only the previous isolation of several strains of a determinate pathogen but also prior research on the pathogen and its importance in the ecosystem of interest. *Aspergillus* and other ochratoxin A (OTA)-producing fungi are a major concern in the wine industry worldwide. OTA is assigned to the 2B group (potential carcinogen) and its levels in wines are legislated, especially in the European Union (IARC, 1993; EC, 2005). In a first study carried out in a wine-grape-growing region located in the east of Mendoza (Argentina) during the 2004 vintage, only 24% of *Aspergillus* section *Nigri* isolates showed OTA production potential, and OTA presence was not noted in the survey [36]. However, a further study in different wine-grape-growing regions in Argentina during the 2007 vintage reported the highest incidences of ochratoxigenic *Aspergillus* strains and OTA occurrence, mostly due to *Aspergillus carbonarius* presence, were located in the northernmost viticulture zone (La Rioja), where the climate conditions (high temperature) were conducive [37]. Accordingly, several non-*Saccharomyces* wine grape yeasts were selected against OTA-producing strains from the previous studied ecosystems [38]. On the other hand, *Alternaria alternata*, the principal component of the wine grape mycobiota in numerous viticulture regions worldwide, was also found to be the main fungus in southern Mendoza during the 2011, 2012, and 2013 vintages [39–46]. More worrying, high incidences of toxicogenic (AOH, AME, and TA producers) (97%) and pathogenic (86%) strains were found, and natural TA occurrence was reported in Malbec, Cabernet Sauvignon, and Syrah wine grape varieties in the same viticulture region during the 2015 and 2016 vintages [11,44,47]. Although not yet regulated, *Alternaria* toxins are in the spotlight of the European Food Security Authority (EFSA) because of their hazardous effects on animals and humans [48–51]. Considering the above, the most toxicogenic and pathogenic *A. alternata* strains previously isolated were employed and resulted in the selection of 15 native non-*Saccharomyces* wine yeasts that completely antagonised *Alternaria* growth and mycotoxin production in wine grapes [11].

Understanding the ecological window of microbial BCA action is required to ensure effective biocontrol. This implies an overlap of the microbial BCA's environmental parameters with those of the pathogen for effective microbial BCA competence to be developed and hence disease reduction [52]. In this sense, water activity (aW) and temperature have been underlined as the factors most influencing survival and interactions concerning pathogens and microbial BCAs [53,54]. In a previous work on a synthetic nutrient (SN) medium with a composition similar to that of grapes, temperature (15, 25, and 30 °C) and aW (0.95, 0.96, 0.97, 0.98, and 0.99) were found to affect the mycelial growth and mycotoxin production (TA, AOH, and AME) of *A. alternata* strains isolated from wine grapes, being temperature the most important factor on growth [55]. More concerning, those temperature and aW conditions conducive for growth and mycotoxin production, are naturally present during grape development. In addition, most of the tested *A. alternata* strains produced TA in artificially inoculated grape berries under the different conditions assayed (15, 25, and 35 °C with 100% relative humidity), and TA peaks were present at 15 °C and 25 °C after 24 days of incubation [28]. Therefore, the effectiveness of the antagonistic non-*Saccharomyces* wine grape yeasts previously selected against *A. alternata* in detached berry tests (25 °C, RH 100%) was assessed in SN medium under aW and temperature conditions conducive for *A. alternata* growth and toxin production (aW: 0.99; temperature: 15, 25, and 30 °C) [11,56]. Among the different yeast strains evaluated (six *Metschnikowia* sp., three *Starmerella bacillaris,* and five *Hanseniaspora uvarum*), only the *Metschnikowia* sp. ones successfully prevented the growth and mycotoxin production of *A. alternata* at all assayed temperatures, demonstrating both the influence of temperature and the need for its consideration during antagonist selection.

Additionally, the application of commercial microbial BCAs, some composed of non-*Saccharomyces* yeasts, against *B. cinerea* in vineyards highlights the major influence of environmental conditions on BCA efficacy. The parameterisation of a generic model for the

biocontrol of a *B. cinerea*–grapevine pathosystem predicted that both the temperature and humidity requirements for BCA growth and the ability of BCA to survive most affected biocontrol efficacy [57]. Thereafter, experimental research in detached berries showed that the response of *Botrytis* bunch rot (BBR) control to different temperatures (T; 15, 20, 25, and 30 °C), relative humidity levels (RH; 60, 80, 90, and 100%), and BCA colonisation periods differed among the commercial BCAs assayed [58]. In particular, Botector® (containing *Aureobasidium pullulans* strains DMS 14940-14941, Bio-Ferm, Tulln, Austria) activity was more affected by temperature, relative humidity, as well as the timing between BCA application and *B. cinerea* inoculation. In congruence, Calvo-Garrido et al. [59] reported varying reductions in severity of BBR in experimental vineyards in France, resulting from treatment with commercial microbial BCAs during the 2015, 2016, and 2017 vintages. Although higher reductions in severity rates were achieved with Candifruit® (based on *Candida sake* strain CPA-1, IRTA-Lleida, Lleida, Spain), Serenade Max® (based on *Bacillus subtilis* strain QST713, Bayer S.A. Cropscience, Lyon, France), and Amylo-X® (based on *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747, Certis Europe, Utrecht, The Netherlands) and although their efficacy was consistent throughout the studied seasons, the overall results suggested different responses of BCAs to weather conditions. In addition, Altieri et al. [16] reported differences in the relative efficacies of grey mould control (berries collected and artificially inoculated with conidia of *B. cinerea*) of commercially available microbial BCAs, including *A. pullulans* strains DMS 14941-14940 (Botector®) and *Metschnikowia fructicola* strain NRRL Y-27328 (Noli®, Koppert, Bussolengo, Italy) in field trials during three vintages. The BCA was more effective during dry periods and when the number of days between BCA application in the vineyard and *B. cinerea* inoculation increased, being less effective during rainfall and an associated drop in temperature occurred. It is important to mention that the *C. sake* strain CPA-1 (Candifruit®), first selected from apples as a BCA against *Penicillium expansum*, *B. cinerea,* and *Rhizopus nigricans*, also showed good performance against *B. cinerea* and sour rot in wine grapes, being strongly influenced by temperature and aW [59–64]. Additionally, *A. pullulans* strains DSM 14940 and DSM 14941 (Botector ®) isolated from apple leaves, first developed to be employed as BCA against *Erwinia amylovora* (fire blight) on pome fruit in the field, seemed effective only under certain conditions in vineyards [59,65]. In a recent study, an indigenous *A. pullulans* strain was tested as a BCA against *B. cinerea* and compared with Botector® used during early defoliation in vineyards [66]. The results showed lower *B. cinerea* incidence when the native *A. pullulans* strain was applied despite the fact that the differences were not statistically significant, and biocontrol efficacy was more affected by meteorological conditions than the defoliation practice. In addition, the non-*Saccharomyces* yeast *M. fructicola* strain NRRL Y-30752 (Noli®), originally isolated from grapes grown in central Israel for postharvest protection in stone fruits against *Monilinia* and in strawberries and grapes against *B. cinerea*, showed a good performance in vineyards [16].

Non-*Saccharomyces* wine yeasts were also successfully employed for black aspergilli and ochratoxin A control in glasshouse and vineyards [67]. Importantly, the *Lachancea thermotolerans* strains tested were previously selected owing to their biocontrol performance against *Aspergillus niger* aggregate and *A. carbonarius* strains in a must extract agar that simulated the environmental conditions during grape ripening (aW 0.97, 0.98, and 0.995; temperature 20 and 28 °C), allowing the knowledge of the conditions jointly conducive for pathogen growth and OTA production [38]. Therefore, the good results in the field trials may be explained by the climate conditions during the evaluated vintages (RH 40–60% and temperature 22–26 °C), which favoured biological control and not the OTA production by *A. carbonarius*. In congruence, temperature and humidity influenced the biocontrol activity of a strain of *Metschnikowia pulcherrima* and two strains of *A. pullulans* against *A. carbonarius*, with 60% RH and 20 °C being the most favourable conditions, with higher temperature and RH being the most detrimental. Similarly, other previous works have reported the good performance of *A. pullulans* strains for the biological control of black aspergilli and ochratoxin A [68–70].

Altogether, these findings demonstrate that although non-*Saccharomyces* wine yeast could represent an effective alternative for the control of fungal pathogens in vineyards, the prior knowledge of the pathogen and the particular environmental conditions for its development as well as for BCA response is indispensable. Additionally, understanding the environmental responses of BCA will allow for a better overcoming of current difficulties, such as climate change. Therefore, resilience among microbial BCA candidates is not only desirable but necessary under the present and future challenges.

2.1.2. Improvements in Non-*Saccharomyces* BCAs Applied to Wine Grapes

To overcome vineyard limitations, formulations of microbial BCAs need to be developed that conserve viability with the aid of effective adjuvants, which, in turn, allow BCAs to colonise the host and compete effectively with the fungal pathogen. Therefore, appropriate formulations can improve the efficacy of and reduce the variability in microbial BCAs [16]. Among the different developed formulations of *C. sake* strain CPA-1, film-forming formulations elaborated with a fluidised-bed spray-drying system including biodegradable coatings showed higher viability than liquid-based formulations on grapes [61,62,71]. Additionally, these improved formulations showed a good response to the stressful conditions projected for climate change (temperature 35 °C, RH 40%, and 1000 ppm CO_2), and their efficacy against grey and sour rot in vineyards as well as their resilience in relation to environmental factors and rainfall episodes were confirmed [72–74]. It is worth mentioning that an optimal BCA formulation should rely on the previous fine-tuning of microbial mass production. Therefore, the development of microbial BCA candidates requires sufficient knowledge of organism-specific research methods for mass production and conceptualisation [34]. *C. sake* strain CPA-1 was successfully obtained from a medium prepared with a by-product of the sugar industry [75]. Interesting work was performed by Pelinski et al. [76], who used a statistical model to optimise a simple medium for the biomass production of a biocontrol strain of *L. thermotolerans*. Moreover, to develop appropriate formulations and methods of application, it is critical to understand how microbial BCAs work, their overall mechanisms of action against pathogens, and the employed mechanisms for a certain pathogen under defined environmental conditions. The biocontrol mechanisms employed by antagonistic yeasts against fungal pathogens include nutrient and space competition, mycoparasitism, and the induction of host resistance, which have been extensively reviewed by several authors [31,34,77–79].

Some native non-*Saccharomyces* wine grape yeasts have shown antagonistic ability against more than one fungal pathogen, opening the possibility of broadening the spectrum of action of microbial BCAs, another bottleneck in their application but of usefulness against diseases caused by multietiological complexes. Nally et al. [80] reported three *Saccharomyces* (two *S. cerevisiae* and one *Saccharomyces kluyveri*) and four non-*Saccharomyces* (*Candida catenulata, Dekkera anomala*, and two *Issatchenkia orientalis*) from 234 yeasts from viticultural environments in San Juan (Argentina) capable of reducing infection (less than 40%) by more than one of the phytopathogenic fungi involved in grape sour rot (*Aspergillus caelatus*, *A. carbonarius*, *Aspergillus terreus*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Penicillium comune*, *Rhizopus stolonifer*, and *Ulocladium* sp.). In another work, only 1 *Wickerhamomyces anomalus* strain among 69 non-*Saccharomyces* yeasts isolated from viticulture regions in Spain showed reduction in disease severity caused by the two soil-borne fungi *Verticillium dahliae* (up to 40%) and *F. oxysporum* (up to 50%) [81]. Out of 31 South African grape-must-derived non-*Saccharomyces* yeast strains representing 21 species, 12 strains displayed a broader antagonistic activity, showing inhibition against three strains of *B. cinerea*, a strain of *A. niger*, and *A. alternata* on dual and mixed cultures in different solid and liquid media [82]. Additionally, intraspecific variabilities were noticed among some of the tested species (*W. anomalus, Candida oleophila and Zygoascus meyerae*). A recent work carried out with 397 wine yeasts belonging to 32 species isolated from Portuguese wine regions found only 3 strains (*H. uvarum*, *L. thermotolerans*, and *S. bacillaris*) displaying strong or very strong inhibition against the most common grape phytopathogenic fungal genera tested (*Aspergillus*, *Botrytis*, *Mucor*,

and *Penicillium*) [32]. Although more studies are needed to obtain a conclusion, it seems that broad-spectrum antifungal activity is not a common feature among viticultural yeasts and raises the question as to whether a single microorganism-based biofungicide could provide sufficient protection against numerous different pathogens. In such a scenario, a mixture of compatible non-*Saccharomyces* wine yeast BCAs, with diverse pathogen targets, which exert different antagonistic mechanisms and complementarily cover the entire environmental range of conditions conducive for pathogens, could be a clever strategy, providing a solution not only to broaden the antifungal spectrum but also to reduce variability and improve efficacy in the field. Therefore, further research is needed to achieve the optimal combination of non-*Saccharomyces* wine yeast BCAs.

Finally, the impact of microbial BCAs applied to wine grapes should be assessed in the following winemaking process. While exploring the antagonism and modes of action of the *S. bacillaris* strains isolated from the wine musts of the viticulture region in north-east Italy against *B. cinerea* on grapes, Lemos Junior [30] assessed their effects on alcoholic fermentation. As a result, the sequential inoculation of *S. bacillaris* 48 h before adding *S. cerevisiae* led to an increase in glycerol and a reduction in ethanol as well as acetic acid parameters. In addition, two *L. thermotolerans* strains, previously chosen for inhibiting *Aspergillus* and OTA in wine grapes, were assessed according to their niche overlap indices with *S. cerevisiae* (defined by the index of overlapping in the carbon sources employed by each of the strains) to exclude any potential competition and for their impact on winemaking [83]. None of the non-*Saccharomyces* wine grape yeasts evaluated occupied the same niche as *S. cerevisiae* and one of them did not negatively affect *S. cerevisiae* growth during fermentation even at high proportions (99% BCA:1% *S. cerevisiae*), producing wines with better characteristics (low acetic acid and high total acidity) than pure *S. cerevisiae* derived ones. Recently, the concept of bioprotection has re-emerged, which consists of adding a yeast biomass to the grapes or must from the moment of harvesting in order to limit the development of indigenous microbiota and thus avoid microbiological alterations in the early stages of winemaking [84]. Future work should be carried out to integrate such a function within the non-*Saccharomyces* wine yeast BCA applied in vineyards as a holistic approach to improve wine quality.

The requirements for biocontrol by non-*Saccharomyces* wine yeasts in wine grapes are summarised in Figure 2.

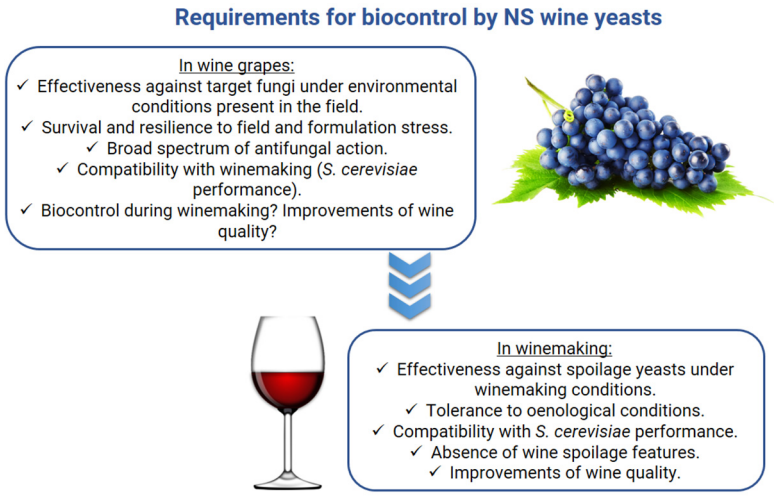

Figure 2. Main requirements for biocontrol by non-*Saccharomyces* wine yeasts in wine grapes and in winemaking.

2.2. Biocontrol in Winemaking

Wine fermentation is a complex process involving grapes, microorganisms, and oenological practices. Yeasts are the most important microorganisms, as they are responsible

for alcoholic fermentation, the main reaction in the transformation of grape must into wine [85], where *S. cerevisiae* is the protagonist species. However, several other species present in grape must coexist and interact with their environment and with each other during fermentation, affecting the analytical profile of the wine [86]. Some of these species are particularly important, especially in terms of spoilage, as they can cause significant economic winemaking losses [87].

The most common microbiological problems affecting wine quality are those caused by yeasts [87]. The main yeasts involved in wine spoilage belong to the genera *Brettanomyces/Dekkera*, *Candida*, *Kloeckera/Hanseniaspora*, *Meyerozyma*, *Pichia*, *Schizosaccharomyces*, and *Zygosaccharomyces* [88–92]. According to Malfeito-Ferreira and Silva [87], spoilage yeasts can be classified as follows: (i) apiculate yeasts (including species of the genus *Kloeckera/Hanseniaspora*), associated with excessive production of ethyl acetate and acetic acid; (ii) film-forming yeasts (including species of the genera *Candida* and *Pichia*), involved in the formation of pellicles on wine surface and the production of off-flavour compounds (oxidised odours and volatile phenols); (iii) sensu stricto spoilage yeasts (including the species *Brettanomyces/Dekkera bruxellensis*, *S. cerevisiae*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe,* and *Zygosaccharomyces bailii*), associated with visible sediment formation and cloudiness in dry wines and refermentation in sweet wines, often with the production of off-flavours (e.g., acetic acid, acetaldehyde, and volatile phenols). Among the sensu stricto spoilage yeasts, *Brettanomyces/Dekkera bruxellensis* represents the main microbiological threat to red wine quality due to its ability to produce volatile phenols, mainly 4-vinylphenol and 4-ethylphenol from p-coumaric acid and 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid, associated with off-odours described as 'horse sweat', 'medicinal', 'phenolic', 'rancid', 'smoke', and 'barnyard' [93]. Its peculiar metabolic features, high stress resistance, as well as its ability to adhere to and colonise inert materials (Dimopoulou et al., 2019) are crucial for its survival during the production and storage phases and for its subsequent development when the environment becomes favourable (e.g., reduction in free sulphite during ageing) [88], also for the emerging problem of biofilm production by some *Brettanomyces* spp. strains [93,94].

Moreover, climate change, which is exerting an increasingly deep influence on the composition of grapes, is generating an increase in sugars in musts and in ethanol and pH (related to low total acidity) in the wine, which indirectly promote undesired microbial proliferation in the grapes, from the first fermentative stages (e.g., lactic acid bacteria, spoilage yeasts) up to the finished wine (e.g., *Dekkera/Brettanomyces*) [95], leading to the production of wines that are more prone to spoilage with a consequent loss of quality [96].

Traditionally, various methods have been used to prevent wine spoilage, including appropriate winery hygiene, filtration, and the use of chemical preservatives. To date, sulphur dioxide (SO_2) has been the most widely used additive in winemaking due to its antimicrobial properties, in addition to its antioxidant function [97]. However, there is an increasing tendency to reduce its concentration in wines due to its harmful effects on human health, especially hypersensitivity in some wine consumers [98]. Furthermore, the increasing tolerance of *Brettanomyces* spp. to this compound [93,99,100] indicates the need to use other preservatives. The search for valid alternatives to sulphites in wine is a real challenge as, in addition to microbiological issues, protection against oxidation and the preservation of organoleptic properties have to be considered [96,101]. Consequently, several emerging technologies have been proposed in recent years to control spoilage yeasts, mainly *Brettanomyces* spp. [93,102], most of them with limited efficiency in controlling microbial contamination. Additionally, the current trend toward healthier lifestyles, linked to the consumption of food and drink without chemical additives, as well as increasing environmental awareness are leading consumers and producers worldwide to seek products with a reduced carbon footprint, which in turn demands wines that are produced and preserved in a more natural way. In this context, the biological control by antagonistic microbial species is a promising strategy to replace or minimise the use of chemical preservatives [93,103]. Yeasts have been proposed as potential biocontrol agents

in oenology because, in addition to their antagonistic abilities against different microbial species [10,11,83,104], they are the predominant and most relevant microorganisms in winemaking. There are a wide variety of non-*Saccharomyces* yeast genera that are now being used to enhance wine flavour or modulate its composition [5]. In addition, biocontrol yeasts can be applied in winemaking through two different approaches: those in which a product of the microbial metabolism is added as a biopreservative [105,106] or those in which the microorganism is added as a starter/protective culture [107–109]. In this section, we review the most relevant research and current knowledge on biocontrol in winemaking, highlighting the role of non-*Saccharomyces* wine yeasts as sources of antimicrobial metabolites or as selected strains to control wine spoilage yeasts during fermentation. Finally, the impact of these yeasts on the fermentation process and the quality of the final product is discussed.

2.2.1. Non-*Saccharomyces* Wine Yeasts as Sources of Antimicrobial Metabolites for Biocontrol in Winemaking

Biocontrol in winemaking consists of the inoculation of microorganisms or their metabolites during the fermentation process or in the final product in order to prevent wine spoilage through different antagonistic mechanisms, which can be classified into passive (competition for space, nutrients, and oxygen) and active (secretion of antimicrobial molecules) antagonistic strategies [103]. Among the latter strategies, active competition includes those used to disadvantage unwanted yeasts by active and direct interference through the production of antimicrobial compounds [110]. The first and most studied antimicrobial compounds produced by yeasts during fermentation, mainly *S. cerevisiae* species, are ethanol and SO_2; however, undesirable yeasts, especially the sensu stricto wine spoilage yeasts, may be resistant to them. In recent decades, numerous studies have shown that other antimicrobial compounds are produced by yeasts and bacteria of oenological interest, including non-*Saccharomyces* wine-related yeasts [103,111,112]. These molecules include the nitrogenous antimicrobial compounds killer toxins, antimicrobial peptides, and bacteriocins. Bacteriocins are proteinaceous antimicrobial molecules produced by lactic acid bacteria, and antimicrobial peptides are secreted by prokaryotes and eukaryotes, mainly by *S. cerevisiae* among the microorganisms of oenological interest, although an antimicrobial peptide with antifungal activity against *B. bruxellensis* was recently described for the first time in *Candida intermedia* [113]. The most widespread and effective antimicrobials among the wine-related yeasts known to date are killer toxins.

Killer toxins (KTs) are antimicrobial proteinaceous compounds that inhibit susceptible yeast strains, but not their own producers [114]. They are heterogeneous in size and complexity, ranging in molecular weight from about 10 to 70 kDa, and from small simple proteins to multimeric protein complexes [103,106,115]. Killer toxins may be associated with double-stranded RNA viruses and linear double-stranded DNA plasmids or may be chromosomally encoded [116]. The modes of action of KTs are highly variable, from rRNA fragmentation to the hydrolysis of β-1,3- and/or β-1,6-glucans of the cell wall, among others [108,116,117]. Despite the variety of mechanisms involved, it has been demonstrated that all known KTs first bind to primary specific receptors on the cell wall of their target microorganism [118,119].

The killer character, first discovered in *S. cerevisiae* in 1963 [120], has been described in a variety of genera other than *Saccharomyces*, including *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Wickerhamomyces*, *Williopsis*, *Ustilago*, and *Zygosaccharomyces* [13,92,108,118,121,122]. The use of non-*Saccharomyces* KTs has been extensively studied to control the development of wine spoilage yeasts, given that these toxins typically have a broader spectrum of inhibition and, in some cases, greater stability than *S. cerevisiae* KTs [15,92,105,123]. Following a report of the extensive anti-*Hanseniaspora* killer activity produced by *Kluyveromyces phaffii* (reclassified as *Tetrapisispora phaffii*) [124], most studies have focused on killer toxins active against *Dekkera*/*Brettanomyces*, the main wine spoilage yeast present during wine fermentation and in postfermentative

ageing processes. *Kluyveromyces whickerhamii* and *Pichia anomala* (reclassified as *Wickerhamomyces anomalus*) [125] produce killer toxins (named Kwkt and Pikt, respectively) that were shown to control the growth of *D. bruxellensis* and *B. bruxellensis* under conditions similar to those used for wine fermentation (pH 3.5–4.5 and 20–25 °C). Moreover, the toxins applied in small-scale fermentation showed efficient antispoilage effects, controlling the growth and ethyl-phenol production of the spoilage yeast without inhibiting the population of *S. cerevisiae* [106]. Moreover, the killer toxins Kwkt and Pikt were shown to cause damage to *Dekkera*/*Brettanomyces* cells through cell membrane permeability and cell metabolism, demonstrating dose-dependent fungistatic and fungicidal activity [126]. Their effect was compared with that of sulphur dioxide, which induced a viable but nonculturable state in *Brettanomyces*, whereas the killer toxins caused irreversible death of the sensitive yeast, ensuring the complete loss of its viability.

Over the past decade, there has been a significant increase in research on the killer toxins secreted by various non-*Saccharomyces* wine-related yeasts with activity against *Brettanomyces* but also with a broader spectrum of activity against other spoilage yeasts. In 2014, Mehlomakulu and colleagues [127] identified and partially characterised two killer toxins, CpKT1 and CpKT2, from the wine yeast *Candida pyralidae*, which showed killer activity against different strains of *Brettanomyces*, especially in grape juice. The toxins were active and stable at pH 3.5–4.5 and temperatures between 15 and 25 °C, and the activity was not affected by the ethanol and sugar concentrations commonly found in grape juice and wine. Furthermore, the killer toxins did not inhibit either *S. cerevisiae* or the lactic acid bacteria strains tested. Subsequently, cells of *Brettanomyces* were exposed to the killer toxin CpKT1 in red grape juice to determine its mode of action. The killer toxin caused cell membrane and cell wall damage in *B. bruxellensis* [128]. *Torulaspora delbrueckii* was also reported to produce killer toxins with a wide killer spectrum against wine spoilage yeasts. Villalba et al. [15] evaluated the ability of indigenous wine strains of *T. delbrueckii* to release killer toxins against spoilage yeasts belonging to *B. bruxellensis* and other volatile phenol-producing species, *Pichia guilliermondii* (reclassified as *Meyerozyma guilliermondii*), *Pichia manshurica* and *Pichia membranifaciens*. The KT of the NPCC1033 strain, named TdKT, showed the broadest spectrum of action against the spoilage yeasts tested, without inhibiting the growth of the fermenting yeast *S. cerevisiae*. The TdKT was, therefore, biochemically characterised and demonstrated to be active under the oenological conditions (pH, temperature, ethanol, glucose, and SO_2 concentrations) typical of both the initial and final stages of wine fermentation.

Recently, Comitini et al. [115] reported another mycocin (named WA18) produced by a *W. anomalus* strain isolated from a natural underground cheese ripening pit, which was able to inhibit the wine spoilage yeast *B. bruxellensis* with a broad spectrum of activity. The partially purified WA18 protein showed high stability under the physicochemical conditions typical of the winemaking process, with maximum killer activity at pH 4.2 and 20 °C. Indeed, the WA18 toxin was able to counteract the *B. bruxellensis* in wine and reduce the production of ethyl-phenols. Although the WA18 strain was demonstrated to be compatible with *S. cerevisiae* under coculture conditions, the WA18 mycocin was proposed as a biocontrol tool with good potential to inhibit spoilage yeasts in winemaking, especially during wine storage. Recently, three killer toxins previously investigated (named Pikt, Kwkt, and WA18), two secreted by two different *W. anomalus* strains and one excreted by *K. wickerhamii* yeast, were produced at the pilot scale, lyophilised, and characterised [105]. All preparations greatly reduced *B. bruxellensis* populations at different doses; in particular, the lyophilised killer toxin D15 (Kwkt) was also efficient during the industrial winemaking stage, achieving the long-term control of *Brettanomyces* yeasts and, at the same time, the significant reduction in 4-ethylphenol production. Furthermore, safety aspects were evaluated in human intestinal cells (Caco-2), which showed that the three lyophilised formulations had no harmful effects on human epithelial cells, paving the way for their possible commercial application.

Taken together, the above results suggest the potential use of killer toxins as biological agents to control the development of wine spoilage yeasts, primarily *B. bruxellensis*, in grape juice during fermentation and wine ageing. In this approach, the biochemical characterisation of the toxin and the evaluation of its stability under winemaking conditions, as well as the development of commercial formulations at low production cost, will be crucial to ensure its application as a biocontrol tool in wine production.

2.2.2. Non-*Saccharomyces* Wine Yeasts as Selected Strains for Biocontrol in Winemaking

Some of the killer toxins described in the literature to date are produced by yeast strains from ecological niches different from the place where they are proposed to be used, such as from olives, in soils from different environments, cheese ripening pits and, in some cases, obtained from culture collections without specifying their origin [105,115,118,122,125,129]. This is not necessarily a limitation, especially if the killer toxin is considered as a bioprotective additive in the winemaking process. However, if the killer yeasts are proposed as biocontrol agents against wine spoilage yeasts during vinification, it may be a competitive advantage to look for yeasts from the same niche as that in which they will be applied. The best sources of antagonistic strains are probably their natural environments, where they are well adapted and able to survive because they have developed strategies to inhibit coexisting microorganisms [32]. Thus, they are likely to be better able to compete with spoilage yeasts that are also typical of that environment.

Killer activity has ecological relevance in yeast populations in natural habitats [130]. In the wine fermentation ecosystem, they are involved in yeast defence mechanisms against their coexisting microorganisms [103]. Therefore, the killer phenotype could potentially be exploited to inhibit the growth of unwanted microorganisms within a microbial ecosystem such as that occurring in wine [127]. Indeed, the widespread presence of killer activity has been demonstrated in yeasts isolated from various oenological sources, including grape berries, fermenting grape must, wine, and wineries in different viticulture regions around the world. In Patagonia (Argentina), several killer yeasts have been isolated from wineries, mainly belonging to the species *P. anomala* (*W. anomalus*), *S. cerevisiae* and, to a lesser extent, *Kloeckera apiculata*, *Kluyveromyces thermotolerans* (reclassified as *Lachancea thermotolerans*), *T. delbrueckii*, and *Candida albidus* [131], as well as *Kluyveromyces lactis* [92]. Killer yeasts have also been isolated from wineries in the northwestern region of Argentina, with killer activity detected in *S. cerevisiae* and several species of the genera *Candida* and *Pichia* [121]. In Spain, a new killer toxin (Kbarr-1) was found in *T. delbrueckii* isolated from the spontaneous fermentations of grapes collected from different vineyards in the "Ribera del Guadiana" region [132]. Moreover, several isolates belonging to the species *H. uvarum*, *M. pulcherrima*, *M. guilliermondii*, *Pichia occidentalis*, and *W. anomalus*, previously isolated from oenological environments in San Juan and Mendoza (Cuyo region), Argentina, were shown to have antagonistic activity against eight strains of two of the most relevant wine spoilage yeasts, *B. bruxellensis* and *Zygosaccharomyces rouxii* [13]. Although no further study of the antagonistic mechanisms was carried out, the authors suggested a killer mechanism, except for *M. pulcherrima*, whose antagonistic activity was attributed to iron depletion from the medium by binding to pulcherrimic acid rather than to a killer factor. The yeasts selected in this work showed a wide range of inhibition (intra- and interspecific inhibition of spoilage target yeasts). It is worth mentioning that many killer isolates from the studies described here were further evaluated for their killing properties and oenological characteristics, showing promising results for their use as biocontrol yeasts for winemaking, confirming that grape–wine ecosystems represent an exceptional source of potential killer yeasts.

Another consideration when selecting non-*Saccharomyces* killer yeasts is the spectrum of activity against various spoilage yeasts. Killer toxins have mainly been tested against *Brettanomyces/Dekkera bruxellensis*, the dominant wine spoilage microorganism worldwide that is responsible for significant losses in wine quality [93]. Nevertheless, other regionally specific spoilage yeasts should also be considered. In this sense, some research has investigated a wider range of spoilage yeasts as the targets for inhibition. The killer activity of *W.*

anomalus and *M. pulcherrima* against wine spoilage yeasts of the species *M. guilliermondii* and *P. membranifaciens*, in addition to *Dekkera* spp., has been reported [92,108,133,134]. Particularly, *T. delbrueckii* has been shown to produce killer toxins with a broad killer spectrum against, in addition to *Brettanomyces* strains, different species of the genus *Pichia*, which are able to produce undesirable volatile phenols [15,92,134]. *M. guilliermondii* tolerates the elevated sugar concentration typically found in fresh must and spoils the early stages of winemaking, whereas *P. manshurica* and *P. membranifaciens* can grow in wine due to their resistance to ethanol [133]. The killer yeast *T. delbrueckii* Kbarr-1 has been shown to have a broad antifungal spectrum [132,135]. Kbarr-1 toxin killed all previously known *S. cerevisiae* killer strains (K1, K2, K28 and Klus) and other non-*Saccharomyces* yeasts such as *Hanseniaspora* sp., *K. lactis*, *S. pombe*, and several *Candida* spp., although they did not kill the wine spoilage yeast *Brettanomyces*. It is therefore of great importance to select a broad-spectrum control system that is effective against a wide range of spoilage species or against multiple regional spoilage yeasts, as well as *B. bruxellensis*, the most common and damaging wine spoilage species in the world.

Currently, no product based on killer toxins is commercially available [93,136]. Despite the relatively high number of non-*Saccharomyces* killer toxins reported so far, their practical application is generally limited, on the one hand, due to their low stability under physicochemical stress conditions of must and wine [15,122] and, on the other hand, due to high production costs of purified product formulations to be added at concentrations able to guarantee efficacy comparable to that of conventional chemical analogues [93,105]. Therefore, a biological control approach in which non-*Saccharomyces* yeasts are added as a protective culture to produce a killer toxin during the winemaking process is an attractive alternative. It can meet the current demand for healthier beverages with environmentally friendly strategies and at low production costs. Consequently, in addition to controlling undesirable microorganisms and their wine spoilage, these yeasts can even improve the complexity, the aroma profile, and the overall sensory quality of wine [5,137]. As these yeasts are generally not capable of completing alcoholic fermentation, they are most commonly used for co- or sequential inoculation with *S. cerevisiae* [86].

This antagonistic approach poses new challenges for researchers and oenologists because, in addition to the characteristics that a killer yeast must have in terms of its killing capacity and toxin characterisation, it must also meet specific requirements in order to be used as a bioprotective culture during winemaking, such as showing effectiveness against spoilage yeasts under winemaking conditions, tolerance to oenological conditions (high sugar concentration, ethanol, SO_2, and growth at low temperature), compatibility with the performance of *S. cerevisiae* (nutritional competition with *S. cerevisiae*, killer phenotype against *S. cerevisiae*), the absence of wine spoilage traits (H_2S, volatile acidity, off-flavours, undesirable growth characteristics), and the possibility of improving wine quality (production of enzymes of oenological interest). Several studies have evaluated the oenological properties of killer yeasts to meet these requirements. Two biocontrol strains, *W. anomalus* BWa156 and *M. pulcherrima* BMp29, showed potential to dominate in the early stages of fermentation due to their high specific growth rates and short lag phases, also due to their efficient fermentation under grape must conditions of 30°Brix, 10% and 12% ethanol, respectively, 0.4 ppm molecular SO_2, and low temperature (15 °C) [13]. The biocontrol yeasts *L. thermotolerans* RCKT4 and RCKT5 were able to ferment in 25–250 mg/L SO_2 media and tolerate high ethanol (7–11% *v*/*v*) and sugar (21 and 30°Brix) concentrations [83]. In addition, many studies have tested the interaction of killer yeasts with *S. cerevisiae* in small-scale mixed fermentations in grape juice. The killer yeasts *P. membranifaciens* CYC1086 [122] and *Ustilago maydis* CYC 1410 [118], producing the killer toxins PMKT2 and KP6, respectively, which have been shown to control the growth of *B. bruxellensis* and its production of 4-ethylphenol under fermentation conditions [118], did not affect the growth parameters of *S. cerevisiae*. Also, strain *W. anomalus* Cf20 was compatible with *S. cerevisiae* under coculture conditions in Malbec must [108]. Similarly, nutritional profiles have been evaluated to determine nutrient competition with *S. cerevisiae* using the niche overlap index.

The non-*Saccharomyces* yeasts tested did not occupy the same niche as *S. cerevisiae*, leaving a wide range of wine grape nutrients available to the fermentative yeast [83]. Additionally, the absence of undesirable traits (production of H_2S, volatile acidity and other off-flavours) was assessed. Several antagonistic yeasts belonging to *W. anomalus*, *H. uvarum* and *M. pulcherrima* showed low levels of H_2S production [13]. In another work, the killer strain *W. anomalus* Cf20 produced high levels of ethyl acetate (434 mg/L) in pure culture on grape must, whereas, in mixed cultures with *S. cerevisiae*, ethyl acetate production was moderate (80–160 mg/L) [108]. Finally, the production of enzymes of oenological interest, such as pectinases, proteases, and β-glucosidase, was tested to determine whether the killer strains had the ability to improve the aroma profile of wines. The killer strain *W. anomalus* BWa156 was shown to have pectinase and protease activities [13].

Figure 2 summarises the requirements for biocontrol exerted by non-*Saccharomyces* wine yeasts in winemaking.

The biocontrol potential of killer non-*Saccharomyces* wine yeasts against wine spoilage microorganisms and some of their oenological properties have been extensively studied in vitro [13,108,118,125]. However, few works have evaluated the killing activity of non-*Saccharomyces* wine yeasts against the target yeasts during must fermentation or the microbial interactions during this process and their impact on wine quality. Recently, Fernández de Ullivarri et al. [109] evaluated the fermentative and biocontrol properties of two indigenous killer yeast strains from the northwestern wine region of Argentina, *S. cerevisiae* (Sc) Cf8 and *W. anomalus* (Wa) Cf20, with the aim of controlling the growth of the spoilage strain *M. guilliermondii* (Mg) Cd6 under red winemaking conditions. Sc Cf8 and its combination with Wa Cf20 effectively controlled the growth of Mg Cd6 at low SO_2 concentration (50 mg/L potassium metabisulphite) during Malbec must fermentation. The killer strain Wa Cf20 did not affect the Sc Cf8 population or the fermentative kinetics, whereas Wa showed a loss of viability after 6 days of fermentation. Mg Cd6 lost viability after 3 days in both mixed fermentations (Sc + Mg and Sc + Wa + Mg), probably due to the presence of killer toxins but also due to the ethanol in these wines. Despite the fact that the killer strain Sc Cf8 alone had lower inhibitory activity than in combination with Wa Cf20, the former modulated the growth of Mg Cd6 and its production of volatile compounds, mainly ethyl acetate, which positively influenced the aroma profile and complexity of the wine. The judges preferred the Malbec wine produced by a mixed Sc + Mg culture in the descriptive sensory analysis. The authors suggested that modulation of the metabolism of the spoilage yeast occurred when it was present in mixed culture with killer strains. Several authors have shown that the metabolism of yeasts in grape must may be reciprocally modulated in the presence of other yeast species [85,138]. Overall, the study suggested that killer yeasts could be used as starter cultures in the production of regional wines where low concentrations of SO_2 are used.

Another strategy that has been explored is the use of killer non-*Saccharomyces* wine yeasts to promote their dominance in wine fermentation in the presence of *S. cerevisiae*, with the aim of improving the aromatic complexity of wines. The killer *T. delbrueckii* wine strain producing the Kbarr-1 toxin has been applied in white [135] and red [139] winemaking at the industrial scale. The killer strain had the advantage of dominating must fermentation in the presence of *S. cerevisiae* with respect to the nonkiller isogenic strains. However, total domination was not guaranteed when a relatively large population of *S. cerevisiae* yeasts was present in the grape must. Killer-mixed fermentations showed reduced levels of the major ethyl and acetate ester compounds, which are responsible for the fresh fruit aroma in white wines, but increased levels of some minor ethyl ester and lactone compounds, which may be responsible for dried-fruit/pastry aromas, such as γ-ethoxy-butyrolactone [135]. In another work, the killer *T. delbrueckii* strain was also able to dominate red wine fermentation in the presence of wild *S. cerevisiae*, promoting malolactic fermentation [139]. A subsequent study proposed the use of *T. delbrueckii* killer yeasts in sparkling wines [140]. Although single *T. delbrueckii* inoculation was not able to complete the second fermentation of sparkling wine, mixed inoculation with *S. cerevisiae*

finalised the second fermentation, obtaining dry sparkling wines with high pressure that showed an improved sensory quality compared to that produced with single *S. cerevisiae* inoculation, mainly due to the increases in ethyl propanoate, isobutyric acid, and butanoic acid detected in the mixed fermentations.

Finally, it is worth mentioning that the studies carried out with killer yeasts as biocontrol cultures in winemaking may suggest that the antagonistic effect observed on sensitive yeasts or their dominance over other species during fermentation is due to a killing mechanism, but other mechanisms cannot be excluded, beyond the fact that killer toxins have been produced under winemaking conditions, since living organisms are added to the fermentation process. Other mechanisms, both active (antimicrobial metabolites, cell-to-cell contact) and passive (competition for nutrients, oxygen, space), not addressed in this review, may also be involved in the antagonistic effect. All microorganisms can exert passive mechanisms, although only some can exert active ones [103]. As suggested by Simonin et al. [141], the decrease in biodiversity and the limitation of spoilage microorganisms observed in the prefermentative phase when a *T. delbrueckii* strain was added early in the white winemaking process to evaluate its bioprotective effect could be related to active compounds produced by this strain, such as killer toxins; however, it could also be due to competition for oxygen and nutrients, strongly suggesting that the observed effect is due to the combination of several of these mechanisms.

3. Grape Cell Wall Depolymerising Enzymes

Enzymes are "eco-friendly" biocatalysts widely used in the modern wine industry. Oenological enzymes and their specific functions have recently attracted the attention of researchers worldwide [142–147]. The microbial enzymes relevant for vinification include pectinases, cellulases, glycosidases, laccases, lipases, esterases, proteases, and glucanases. In winemaking, pectinases are the most common and widely used enzymes [18], representing about 25% of the total commercial enzymes used in food processing [148].

Pectinolytic enzymes, which are usually referred to as "pectinases" by winemakers, act upon the pectin and pectic substances present on grape berry cell walls. These enzymes are of two main types: methylesterases and depolymerases. The former release the methoxyl groups from the pectin, while the latter break the bonds between the galacturonate units, either by hydrolysis of the α-(1,4)-glycosidic bonds (hydrolases) or by β-elimination (lyases). Pectinases are widely used in the beverage industry for the clarification of fruit juices, and, particularly in the winemaking process, they have shown a great influence on both the sensory and technological properties of wines. They can help to improve liquefaction, clarification, and filterability, releasing more colour and flavour compounds entrapped in the grape skins, thereby positively contributing to the wine bouquet and facilitating the release of phenolic compounds [19,20,149–154].

The enzymes used in oenology do not consist of a single enzyme: they are complex mixtures of pectinases (mainly composed of polygalacturonase, pectinesterase, and pectin lyase), in addition to secondary activities such as cellulases, hemicellulases, and acid proteases [143,155,156]. The latter also help to increase grape juice extraction and improve the colour and clarity of wines and related parameters such as viscosity and filterability.

It is essential to know the substrate on which these enzymes act. The cell wall of the grape berry is composed of cellulose, noncellulosic polysaccharides, and, to a lesser extent, proteins. The cellulose is in the form of microfibrils embedded in a matrix of hemicelluloses and pectins and stabilised by a network of proteins. Pectin acts as a cement for the other structural polysaccharides. On this basis, the main pectinase activity is not sufficient, and the complete degradation of the plant cell wall requires the cooperation of several enzymes to break down its various polymeric components.

Oenological enzyme preparations can be used for different purposes, depending on their main enzymatic activities. The pectinolytic-based clarification enzymes mainly comprise pectin methylesterase, polygalacturonase, and pectin-lyase, which facilitate the removal of pectin particles in suspension from the cell walls of the grape pulp during the

settling phase in white and rosé wine production [157]. The so-called maceration enzymes are cocktails primarily composed of *endo*-polygalacturonase, pectin methyl esterase, and pectin-lyase as well as cellulase and hemicellulase. Numerous studies have shown that the use of these enzymes improves colour and increases the concentration of tannins in wines [152,158–163].

Particularly, in red wines, the process of must maceration is crucial for the extraction of phenolic compounds and thus for achieving healthier wines with better colour [164,165]. These compounds add colour and flavour to the wine, contributing to the mouthfeel, quality, and palatability of red wines [166]. In addition, according to several health studies, the long-term consumption of a diet rich in plant polyphenols protects against cancer, cardiovascular disease, diabetes, osteoporosis, and neurological diseases [165,167]. Polyphenols are mainly located inside the skin cell vacuoles and are partially extracted from berry skin into the must/wine during winemaking. However, the grape skin's cell walls constitute a barrier against the diffusion of these compounds. Thus, an increase in cell wall permeability to polyphenols by partial hydrolysis of their constituent structural polysaccharides could be achieved by the use of depolymerising enzymes.

In other works, however, the use of these enzymes very slightly enhanced the phenolic composition [151,168]. These discrepancies are probably due to the different enzyme activities of the commercial preparations, as well as varietal and vineyard effects on the content and composition of anthocyanins and the composition and morphology of the skin cell wall. However, the lack of positive results in some experiments does not seem to be because of a deficient enzymatic cell wall degradation, since several studies have demonstrated that these enzymes effectively degrade cell walls. Gao et al. [169] and Osete-Alcaraz et al. [170,171], using comprehensive microarray polymer profiling (CoMPP), found that when purified grape skin cell walls are exposed to pure commercial pectinolytic enzymes, a significant depectination of the cell walls occurs, opening the cell walls to the diffusion of phenolic compounds. Other authors found similar results by studying changes in grape skin composition during actual vinification in the presence of enzymes, and they observed improved depectination and unravelling of the cell walls in the presence of enzymes [172].

A few scientific advances have been reported regarding the chemical and structural composition of the grape cell wall and the effects of hydrolytic action on its polysaccharide components by commercial enzymes [173,174]. These advances revealed a particular cell wall composition in the different cell layers and the need for unique enzyme activities to achieve its complete degradation. Gao et al. [174] proposed a schematic model of the grape berry skin cell wall, in which two main fractions can be distinguished: one rich in pectin, in deeper layers close to the pulp, and the other rich in hemicellulose, in epidermal cell layers. The authors reported that changes in the structural composition of grape cell walls during ripening, the enzyme action during the maceration phase, and the deconstruction process that occurs during alcoholic fermentation are factors that significantly influence the extractability and release of favourable compounds from the wine [174].

The method of adding enzymes with different activities leads to different effects on the release of bound polyphenols. In one study carried out with both purified and combined enzymatic activities from Monastrell grapes (from a vineyard in Murcia, S.E. Spain), Apolinar-Valiente et al. [175] concluded that cellulase acts sooner in grape skin degradation than polygalacturonase. Additionally, they observed a synergistic effect when a polygalacturonase plus cellulase enzymatic combination was added. In subsequent studies, Osete-Alcaraz et al. [170,171] demonstrated that the use of polygalacturonase and pectinlyase enzymes in combination had a marked efficacy on the depectination of cell walls and promoted the highest release of tannins into the solution. This enzyme mixture improved the exposure of grape skin hemicelluloses and released the highest quantity of soluble polysaccharides of all treatments. Benucci et al. [173] reported that the effectiveness of enzymes applied individually and/or in multienzyme blends in improving the extractability of phenolic compounds from postharvest withered grape skins (wine

grapes from Piedmont region, northwest Italy) were variety-dependent. For the Nebbiolo variety, polygalacturonase, either as single enzyme or in multienzyme blends, increased skin softening during simulated maceration as a result of the degradation of cell walls and led to a higher extraction of anthocyanins, oligomeric flavanols, and polymeric flavanols, without affecting the anthocyanin profile. Nevertheless, in the Barbera wine grape variety, no remarkable advantages were revealed by applying macerating enzymes, either using enzymes alone or combinations. Río-Segade et al. [153] also found a varying effect on the anthocyanin profiles of Cabernet Sauvignon and Nebbiolo grapes by macerating enzymes.

Moreover, using pectinolytic enzymes in combination with other technologies, several authors have studied the efficiency of the extraction of phenolic compounds using pectolytic enzymes and high-power ultrasound [162,176]. Increases of 18% in colour intensity, 21% in total phenolic content, and, in particular, 30% in tannin extraction at the end of alcoholic fermentation were observed when pectinase and ultrasound were used alone and in combination [162]. More recently, more precise studies were conducted on the application of the appropriate technology in combination with a particular pectinolytic preparation, with the objective of improving both the extraction of phenolic compounds from grapes and their maintenance in the final wine: Osete-Alcaraz et al. [145] compared the effect of favouring phenolic extraction with a pectinolytic-based maceration enzyme and the partial elimination of the suspended material using a pectinolytic-based clarification enzyme. Both enzymes increased wine colour intensity and phenolic content compared to a wine produced without enzymes, but the best results were found with the clarification enzyme. The authors argued that the precipitation of lees rich in polyphenols probably created a pronounced gradient of phenolic compounds from the grapes to the must, and this improved the colour characteristics in the final wine compared to the wine made using a traditional enzymatic maceration. However, the results could also have been due to the particular composition of the enzyme system of the clarifying enzyme preparation used.

Therefore, the effectiveness of enzymatic maceration in red wine production depends on the use of an optimal combination of specific enzymes to achieve the complete degradation of the grape cell walls, thus optimising the extraction of phenolic compounds and promoting their stabilisation in the wine [169,175]. Figure 3 shows a scheme of the action of the enzymes degrading the polysaccharides on the grape berry cell wall and, consequently, their effect on the wine.

3.1. Non-Saccharomyces Wine Yeasts as Alternative Sources of Enzymes

Currently, the microbial strains normally used for pectinase production are of fungal origin. *A. niger* and *Trichoderma* sp. (i.e., have "generally regarded as safe" (GRAS) status) are conventionally used as enzyme-producing organisms, which are regulated by the International Organisation of Vine and Wine (OIV) [177]. In the last two decades, yeast pectinases have attracted a great deal of attention from various research groups worldwide as an alternative to fungal pectinases [19,20,22,146,178–180]. Yet, reports of carbohydrase enzymes from wine-related yeasts are particularly scarce. Several reviews have recently been published on commercial pectinases or enzymes for the oenological industry, mainly produced by fungi [18,21,142]; nevertheless, there is a gap regarding reviews on the pectinases secreted by yeasts for winemaking, or this subject was only reviewed as a tiny part of a more general issue [22,142].

Unlike filamentous fungi, yeasts and yeast-like organisms generally display more versatile enzymes, with shorter enzyme production times and greater technological stability [22]; they display desirable polysaccharidase activities and do not usually secrete pectinesterase, which releases toxic methanol into the wine [181,182]. Moreover, considering their predominance during fermentation, those with beneficial enzymatic endowment could be directly used as starter cultures without the application of enzyme preparations [5,19,142,183]. Here, the yeasts naturally found in grapes are rich sources of enzymes of oenological interest, and selected yeasts can be used during winemaking, entailing a suitable alternative to fungal pectinases. Therefore, these yeasts could produce the exocel-

lular enzymatic activities required during vinification, contributing to the depolymerising of the grape cell wall components (Figure 3).

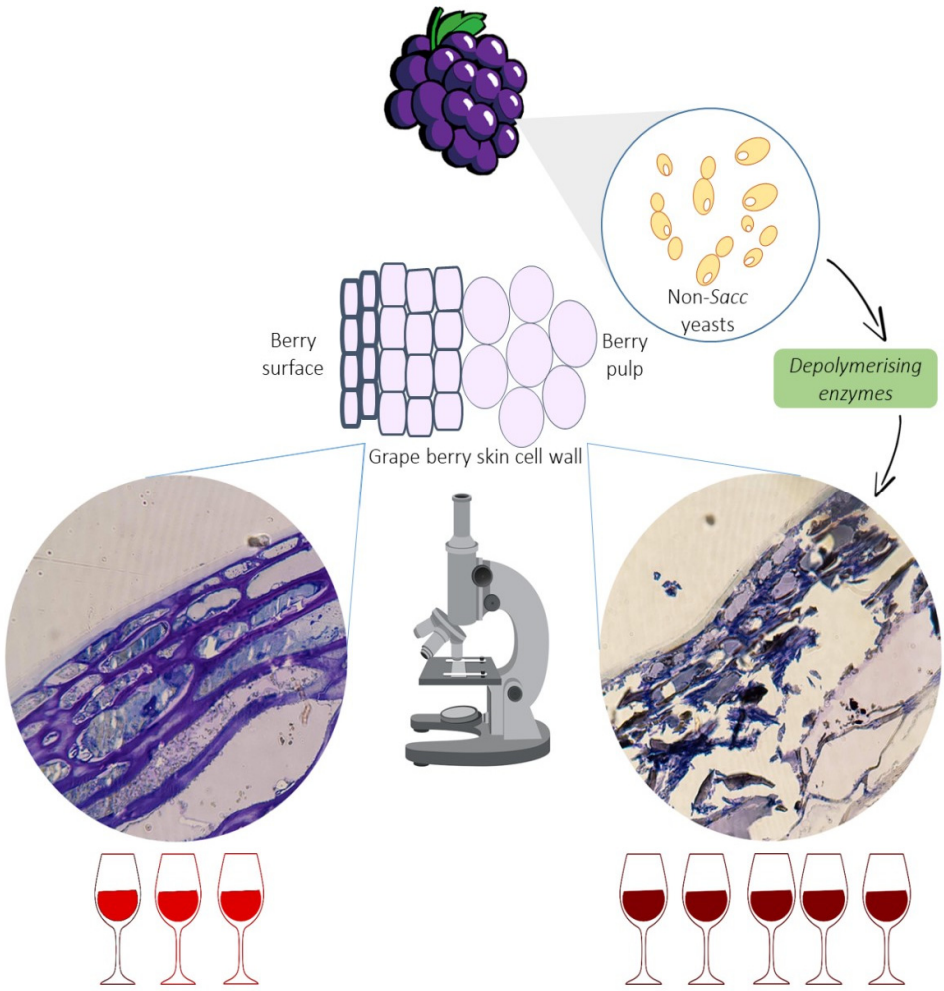

Figure 3. Scheme of the polysaccharide-degrading enzyme action on the grape skin cell wall and its effects on wine.

Pectinolytic enzymes from yeasts have been studied as processing aids in winemaking since the early 1990s. Gainvors et al. [184] proved that a crude pectolytic extract from a *S. cerevisiae* strain added to grape must had the same effect on turbidity as the same quantity of a commercial pectinase. Blanco et al. [185] showed that fermentation conducted by pectinolytic strains of *S. cerevisiae* produced wines displaying filtration times about 50% lower. A few native strains have been reported to have the ability to degrade pectin during fermentation [182,185]. Despite the efforts made to find wine strains of *S. cerevisiae* species with pectinolytic ability, most strains do not show the capacity to degrade pectic substrates or have low levels of activity [178,183,185].

Recently, there has been interest in the application of non-*Saccharomyces* wine yeasts [166,186,187]. Special attention has been paid to non-*Saccharomyces* yeast species naturally present as the epiphytic microbiota of wine grapes, which could also be relevant in terms of new activities of oenological interest or higher levels of activities that can help the wine industry address future technical and consumer challenges [142]. In addition to the enhancement in the sensory profile of wines, these yeasts stand out for their ability to produce a large variety of extracellular enzymes, among which pectinases, cellulases, xylanases, and glycosidases are included [19,20,142,146,183,188,189]. These enzymes improve the technological process and impact the sensory characteristics of wine by releasing aroma

precursors, increasing the colour intensity and colour stability of red wines and facilitating the clarification process.

In a competitive oenological sector and bearing in mind that enzymes play a pivotal role in the winemaking process, it is worth developing new enzymatic technological strategies. The use of native non-*Saccharomyces* wine yeasts as alternative sources of depolymerising enzymes is a promising oenological approach. Therefore, their enzymatic activities can be exploited in form of new enzyme products or directly as exoenzyme-producing starter cultures for winemaking. Both the exogenous grape skin cell wall depolymerising enzymes from yeasts and enzyme-producing yeasts as starter cultures are presented in this review (Table 1).

Table 1. Enzymes for the depolymerisation of the grape skin cell wall from several native non-*Saccharomyces* wine yeasts used in winemaking.

Enzymes	Enzyme-Producing Yeast Strain (Origin)	Grape Variety Used in Winemaking	Biotechnological Impacts	References
Exogenous enzymes				
Multienzymatic extract (mainly carbohydrolases)	*Aureobasidium pullulans* m11-2 (DO San Rafael-Mendoza wine region, Argentina)	*Vitis vinifera* L. cv. Malbec	Improvement in clarification and filtration. Increases in colour extraction and antioxidant capacity of wines. Great potential for prefermentative cold maceration.	[180]
Cold-active pectinases with side activities (cellulases and xylanases)	Strains of *A. pullulans*, *Filobasidium capsuligenum*, *Rhodotorula dairenensis*, *Cryptococcus saitoi* and *Saccharomyces cerevisiae* (DO San Rafael-Mendoza wine region, Argentina)	-	* Enzymes resistant to oenological conditions, with potential application for low-temperature winemaking.	[183]
Cold-active pectinolytic activities (pectinmetilesterases, exo- and endo-polygalacturonases)	*Rhodotorula mucilaginosa* PT1 and *Cystofilobasidium capitatum* SPY11 (from soil in different regions in India)	-	* Potential application in wine production and juice clarification at low temperature.	[190]
Pectinases cold-active	Strains of *A. pullulans* (DO San Rafael-Mendoza wine region, Argentina)	-	* Potential for winemaking at low temperature.	[191]
Polygalacturonase activity (PG)	*S. cerevisiae* (from viticultural area of Valdepena, Spain)	-	* Potential application for wine production with a strain *S. cerevisiae* with unusually high PG activity.	[182]
Polygalacturonase activity (PG)	*S. cerevisiae* (C.E.C.T., Colección Española de Cultivos Tipo)	Grape juice (from La Mancha, España)	Reduction in filtration times.	[192]
For “in situ” production				
Pectinases with side activities (cellulases and xylanases)	*Torulaspora delbrueckii* m7-2 in sequential fermentations with *S. cerevisiae* (DO San Rafael-Mendoza wine region, Argentina)	*V. vinifera* L. cv. Malbec	Improvement in clarification and filtration. Increases in colour extraction and antioxidant capacity of wines.	[180]
Pectinase activity	*A. pullulans* GM-R-22 (DO San Rafael-Mendoza wine region, Argentina) in both sequential and simultaneous inoculation with commercial *S. cerevisiae*	*V. vinifera* L. cv. Malbec	High efficiency in low-temperature winemaking and prefermentative cold maceration. Increases colour, total polyphenols, and stability. Wines with better tonality and flavour. Improvement in clarification efficiency.	[20]

Table 1. *Cont.*

Enzymes	Enzyme-Producing Yeast Strain (Origin)	Grape Variety Used in Winemaking	Biotechnological Impacts	References
Pectinase activity (with β-glucosidase and tannase side activities)	*A. pullulans* AWRI4229 and AWRI4231 (Adelaide Hills, South Australia, Australia)	*V. vinifera* L. cv. Chardonnay (grape juice)	Important implications for wine production and quality. Impacts grape juice composition and modulates fermentation kinetics by competition for trace elements.	[179]
Pectinase activity	*A. pullulans* GM-R-22 (DO San Rafael-Mendoza wine region, Argentina) in sequential inoculation with commercial *S. cerevisiae*	*V. vinifera* L. cv. Malbec	Great effectiveness in prefermentative cold maceration. Improvement in chromatic parameters and decreasing of filtration time and turbidity. Enhancement in desirable volatile compounds, such as esters and norisoprenoids.	[19]
Polygalacturonase activity	*Metschnikowia pulcherrima* NS-EM-34 (Spanish Designation of Origin Ribera del Duero) in sequential fermentations combined with commercial *S. cerevisiae*	*V. vinifera* L. cv. Tempranillo	Improvement in clarification process and liberation of phenolic compounds. Impacts sensorial aspects of wines such as their aromatic complexity and alcoholic content.	[193]
Pectinase, cellulase, xylanase, and glucanase activities (plus other aroma-related enzymes)	*M. pulcherrima*, *Zygosaccharomyces bailii*, *Candida zeylanoides*, and *T. delbrueckii* in sequential inoculation with *S. cerevisiae* (the Rioja "Qualified" Designation of Origin, The Rioja, Spain)	*V. vinifera* L. cv. Tempranillo	Improvement in monomeric anthocyanin and stilbene composition. Wines with better colour and likely healthy properties.	[194]
Pectinase activity with glucosidase side activity	*M. pulcherrima* MP 346 and *Metschnikowia fructicola* MF 98-3 in sequential inoculation with *S. cerevisiae* with prefermentative cold maceration (Lallemand SAS, Blagnac, France)	*V. vinifera* L. cv. Sangiovese	Enhancement in properties and stability of wine colour (higher levels of flavonoids and anthocyanins, and preservation of red tone).	[195]
Multiple enzymes (pectinases, β-glucosidases, proteases, amylases, and xylanases)	*S. cerevisiae* BSc562, *Hanseniaspora vineae* BHv438, and *T. delbrueckii* BTd259 in pure and mixed cultures (Culture Collection of Autochthonous Microorganisms at the Biotechnology Institute, Faculty of Engineering-National University of San Juan, Argentina)	*V. vinifera* L. cv. Pedro Giménez	Impacts aromatic characteristics and final quality of the wine by natural precursor hydrolysis of polymers present in grape juice.	[196]

* The effect of these enzymes was tested in vitro.

3.1.1. Exogenous Enzymatic Extracts Produced by Non-*Saccharomyces* Yeasts

Claus and Mojsov [142] and Espejo [21] have extensively reviewed the enzyme preparations used in winemaking as oenological supplies, their effects on process engineering, and the quality of the final product. They have presented vast information on commercial pectinase preparations, including suppliers, predominant enzymatic activity, and the main purpose of the application. However, studies on beneficial enzymes from alternative enzyme microbial sources, such as wine-related yeasts, are scarce.

In the past decade, promising microorganisms for exoenzyme production have been identified from the grape–must–wine ecosystem. In 2011, Merín et al. [191], in a study carried out in a Denomination of Origin San Rafael viticultural region (Mendoza, Argentina), found that *A. pullulans* was the predominant pectinolytic species on wine grape surfaces, which was able to produce pectinase at a low temperature (12 °C). Later works focused on searching for pectinolytic yeasts in other viticulture and oenological environments apart from the grape surface, finding that in addition to this species, *Rhodotorula dairenensis* and *Cryptococcus saitoi* are pectinolytic yeasts on the grape surface, but no pectinolytic activity was detected in yeasts isolated from fermenting must [197]. *Filobasidium capsuligenum* and *S. cerevisiae* were identified as pectinase producers from the winery equipment [198]. These studies mainly focused on yeast selection from the production of pectinolytic and secondary activity under wine-like conditions (pH 3.5, 12–28 °C), with special interest in cold-active enzymes. More recently, the selection of indigenous yeasts from wine grape surfaces in the same viticultural region (San Rafael DO) was pursued for the production of a multienzyme system of carbohydrolases and related enzymes of oenological importance based on their physicochemical and technological performance during short macerations of Malbec must [146]. In this work, 16 strains were selected and identified as belonging to the genera *Aureobasidium*, *Candida, Debaryomyces*, *Hanseniaspora, Metschnikowia, Pichia, Saccharomyces,* and *Torulaspora*, which were compared with five previously selected strains corresponding to different yeast species from the same ecosystem [183]. *A. pullulans* strains had a broader enzyme blend and higher activity, dominated by pectinases and followed by xylanases and cellulases. Moreover, the *T. delbrueckii* m7-2 strain produced high amounts of polysaccharidase and showed prominent performance.

Similar non-*Saccharomyces* screenings from oenological ecosystems in different wine regions worldwide have been undertaken. Belda et al. [188] analysed the metabolic potential of 770 yeast isolates from different oenological origins representing 15 different species, studying their production of enzymes of oenological interest, mainly glycosidase enzymes related to terpene aroma release, and protease, polygalacturonase, and cellulase activities, linking phylogenetic and enzymatic data. Two different groups were established, β-glucosidase and protease activities, as being prevalent in the yeast collection studied, whereas α-L-arabinofuranosidase, polygalacturonase, and cellulase were the less abundant. Escribano et al. [199] carried out a screening of 13 enzymes related to wine aroma, colour, and clarity from 97 non-*Saccharomyces* wine yeast strains belonging to 10 different genera and species (*Candida* spp. And *Cryptococcus* spp., *Debaryomyces hansenii*, *L. thermotolerans*, *M. pulcherrima*, *Pichia kluyveri*, *Sporidiobolus salmonicolor*, *T. delbrueckii*, *Williopsis pratensis,* and *Z. bailii*). Most of the strains showed the presence of one or more enzymes of biotechnological interest. Furthermore, in all these studies, several intraspecific differences within the yeast species have been observed, evidencing the great importance of strain selection for the oenological application [146,188,199].

Recently, Longhi et al. [180] produced a multienzyme extract from the native *A. pullulans* m11-2 strain and used it in a prefermentative cold maceration (PCM) of Malbec must for four days at 8 °C, following by inoculation with native *S. cerevisiae.* The enzymatically treated wines presented better chromatic aspects such as colour intensity, total polyphenol content, and pigment stability than the control wines. Additionally, it was observed that the antioxidant capacity, as measured by the DPPH radical scavenging method, was improved, and the extraction of the trans-resveratrol stilbene was greater in the wine. These results suggested that this method of polysaccharidase supplementation in the vinification is an

effective biotechnological strategy to produce wines with better sensory properties that are also healthier.

In the wine industry, low-temperature fermentation (15–20 °C) is commonly applied to increase the production and retention of volatile compounds, thereby improving the aromatic profile of the wine [156,200,201]. It is a common practice in white and rosé wines but has only recently being implemented in young red wines [201]. In the last decade, for the same reason, the production of red wines at low temperatures has increased, with the aim of improving the production and retention of aroma compounds, thus improving the sensory quality of the wine [156,201]. Nevertheless, commercial oenological pectinases, produced by fungi, have significant activities at much higher temperature ranges [202]. Consequently, the selection of new yeast pectinases for winemaking must consider the stability of the enzymes under vinification conditions. Sahay et al. [190] isolated yeasts from the soil in different regions in India and identified a psychrotolerant yeast *Rhodotorula mucilaginosa* PT1 and psychrophilic yeast *Cystofilobasidium capitatum* SPY11, which showed ability to produce cold-active pectinolytic activities (pectinmetilesterases, exo- and endo-polygalacturonases), which exhibited 50–80% of their optimum activity under some major oenological conditions, including a pH of 3.5 and temperatures of 6 and 12 °C, suggesting suitability for application in wine production and juice clarification at low temperature. Merín and Morata de Ambrosini [183] evaluated the influence of the oenological factors that could affect the yeast pectinase production (grape carbon sources and SO_2) and pectinase activity (ethanol and SO_2) of 15 indigenous yeasts belonging to *A. pullulans*, *F. capsuligenum*, *R. dairenensis*, *C. saitoi*, and *S. cerevisiae* species. Pectinase production was not affected by a high glucose concentration (200 g/L), at 12 °C and pH 3.5, or by the presence of SO_2 (120 mg/L). Ethanol (15%) had little effect on the pectinase activity of *A. pullulans* strains but reduced the relative activity to 12–79% of that of basidiomycetous yeasts. In addition, the non-*Saccharomyces* strains showed promise regarding properties of oenological interest in addition to from cellulase and xylanase activities. Moreover, the study indicated that cold-active and acid-tolerant pectinases from non-*Saccharomyces* yeasts were able to remain active under the glucose, ethanol, and SO_2 concentrations usually used in winemaking, suggesting their potential use as processing aids in low-temperature winemaking. This observed natural adaptation to the environment is related to the fact that the strains were selected from the same niche where they were intended to exert their hydrolytic action and therefore showed a tolerance to the conditions of the must and must–wine environment.

It is important to know the enzymatic activity of an enzyme preparation, as the dose to be used depends on it. Additionally, a high international enzyme unit (IU) value does not always ensure a greater beneficial effect on the final quality of the wine. Negative results could be expected in the presence of high enzymatic activity in the medium due to excessive rupture of the plant cell wall, since other off-flavour compounds or proteins could be extracted along with the desired compounds, leading to a precipitation of colour [203]. Indeed, this could explain the contradictory results regarding the use of pectinases in winemaking that have been reported. In this context, Oskay [204] found that the highest pectinase production (8.2 U/mL) by the *A. pullulans* P56 strain was obtained in vitro in the simultaneous presence of ammonium sulphate and yeast extract (ratio 1:1), using citric pectin (10 g/L) as the carbon source. These values can be compared with those reported in the literature, such as by Merín et al. [197], where the extracellular pectinase activities of the enzymes produced by two strains of *A. pullulans* (GM-1 and GM-2) were 0.967 and 1.325 U/mL, respectively, when a basal liquid medium without pectin was used and incubated at 28 °C and pH 3.5. These different production levels could be related to the niche from which the microorganisms came. For example, Oskay [204] used a microorganism that was probably isolated for different purposes and from a different niche than that of Merín et al. [197], where the isolation was performed from the grape surface, selecting the most potent yeast producers from several rounds of screening for the highest pectinolytic activities. This could indicate that enzymes are produced according to the substrates present in the medium to be degraded and that these capacities are due

to not only the enzyme content but also the composition of the multienzyme systems produced in a particular niche. Another special case is the polygalacturonase produced in large quantities by strains of *Aspergillus* spp. isolated from soil, which showed the highest enzyme production over a 48 h incubation period, and the optimum temperature, pH and substrate for enzyme activity were found to be 30 °C (75.4 U/mL), 5.8 (72.3 U/mL), and 0.5% (112.0 U/mL), respectively [205]. This enormous enzyme activity is related to the role of the microorganism in the niche, as in the case of soil microorganisms equipped with enzymes for the complete decomposition and recycling of organic matter. The role of the microorganisms on the surface of a grape would be very different, since they decompose organic matter in a more moderate way, with the aim of obtaining a drink suitable for human consumption from this organic substrate.

3.1.2. Non-*Saccharomyces* Wine Yeasts for In Situ Enzyme Production in Winemaking

A promising alternative to pectinase preparations could be the native yeasts that produce extracellular pectinolytic and other depolymerizing enzymes during the winemaking process. This option would avoid the addition of costly exogenous pectinolytic enzymes and would improve the quality of wines, as these yeasts can produce substantial sensory complexity in wines [5,206].

Maturano et al. [196] evaluated the ability of three native non-*Saccharomyces* yeasts to produce extracellular enzymes of oenological importance (β-glucosidases, pectinases, proteases, amylases or xylanases) in pure and mixed *Saccharomyces*/non-*Saccharomyces* cultures during the microfermentation of grape juice c.v. Pedro Jiménez (*V. vinifera* L.). The assayed *Saccharomyces* and non-*Saccharomyces* isolates produced a broad range of the tested enzymes throughout fermentation, where pure or mixed cultures of *T. delbrueckii* BTd259 showed the highest production of all enzymes assayed except for β-glucosidase. High concentrations of sugars did not affect enzymatic activities, but ethanol seemed to adversely affect β-glucosidase and pectinase activities with increasing fermentation duration. The study contributed to a better understanding of the microbial interactions and enzyme production that occur under fermentation conditions, although the impact of enzymatic activities on the quality and the aroma profile of wine was not evaluated.

Belda et al. [193] performed an exhaustive yeast screening, testing several enzymes of oenological interest using 462 isolates from wineries of Ribera del Duero DO (Spain), to select strains to be applied in combined fermentation with *S. cerevisiae*. Only *A. pullulans*, *M. pulcherrima,* and *M. fructicola* showed polygalacturonase activity. Strain *M. pulcherrima* EM-34, which was evaluated in sequential inoculation with *S. cerevisiae* in both laboratory and semi-industrial scale assays, promoted increases in colour intensity and total polyphenolic index and improved the clarification properties of wines with respect to the control wines fermented only with *S. cerevisiae*.

Additional studies on pectinolytic *Metschnikowia* yeasts were carried out by Benucci et al. [195]. Two commercial *Metschnikowia* strains (*M. pulcherrima* MP 346 or *M. fructicola* MF 98-3) were applied during prefermentative cold maceration (PCM) in order to enhance the properties and stability of Sangiovese wine colour. This grape variety has problems with colour stability caused by its high content of unstable and oxidizable phenols. The commercial pectinolytic enzyme LALLZYME® Cuvée Rouge (Lallemand SAS) was applied as a reference treatment. The chromatic properties and colour stability of Sangiovese wine over time were enhanced by applying *Metschnikowia* strains (MP 346 and MF 98-3) during the PCM; in particular, MF 98-3 significantly increased the colour intensity of 12-month-old bottled Sangiovese wines.

A. pullulans, a dominant pectinolytic species found on grape surfaces and in the very early stages of fermentation [179,197,207], has received particular attention due to its wide range of biotechnological and environmental applications, including in bioproducts such as enzymes (amylases, cellulases, lipases, proteases, xylanases, and mannanases) and polymers (mainly pullulan) [180,183,191,193,208]. However, few studies have been conducted to understand the impact of this species or its enzymes on red grape macerations [146],

even during fermentation. Indeed, the pectinolytic strain *A. pullulans* GM-R-22 was applied in different low-temperature red winemaking approaches, both in the absence [19] and presence of the endogenous microbiota [20] of Malbec grapes (*V. vinifera* L.) from the DO San Rafael (Mendoza, Argentina) wine region. In 2018, Merín and Morata de Ambrosini [19] evaluated the impact of this strain, inoculated during the PCM stage of microvinification trials with pasteurised Malbec must, on the fermentation process and the wine quality. The population and fermentative kinetics of *S. cerevisiae* were no different in the mixed culture compared to those of the pure *S. cerevisiae* culture. High pectinase activity was detected during PCM in the presence of *A. pullulans*, and this wine showed enhanced chromatic parameters and significantly lower filtration time and turbidity compared with the control wine. In addition, an improvement in the production of desirable volatile compounds, such as esters and norisoprenoids, and a reduction in higher alcohols were observed. A subsequent study, carried out on nonpasteurised Malbec must subjected to two low-temperature red winemaking techniques (PCM and low-temperature red fermentation), demonstrated that *A. pullulans* GM-R-22 was able to survive in grape juice and the first days of fermentation, producing high levels of pectinase, and its wines exhibited higher total anthocyanins, total polyphenols, colour intensity, and stability, as well as filtration times that were 30% lower, with high sensory scores for tonality and aroma intensity [20]. The sensory analyses in these studies showed that *A. pullulans* had an effect on red wines, highlighting the violet hue, the plum jam and spicy aromas, the body, and the equilibrium.

Another research group also studied the physiology of *A. pullulans* (AWRI4231 strain) through lab-scale fermentation trials with Chardonnay (*V. vinifera* L.) grape juice [179], demonstrating the aptitude of this species to survive in grape juice while producing polysaccharides, polymers of malic acid (poly β-malic acid), and enzymes with pectinase, β-glucosidase and tannase activity. A possible antagonism of yeast through metal competition including Fe and Zn was also observed. Despite its transient presence during the fermentation process, this work proved the potential of *A. pullulans* to influence wine and the winemaking process.

Despite these promising outcomes using *A. pullulans* as a pectinase producer during winemaking, these vinifications have been conducted at the laboratory scale, so that the specific role of this unconventional species in oenology on wine production and quality at larger-scale production more proximate to real winemaking conditions remains poorly explored. Furthermore, more detailed studies focused on understanding the impact of *A. pullulans* on wine production and characterising the organoleptic properties of the specific compounds produced by this species are needed.

Finally, Longhi et al. [180] examined new approaches to applying microbial enzymes in different winemaking techniques using *V. vinifera* L. cv. Malbec grapes from the DO San Rafael (Mendoza, Argentina) wine region. In one of them, a new strain of *T. delbrueckii* was used in sequential inoculation with an autochthonous *S. cerevisiae* strain. The former strain was first inoculated to produce an enzyme complex in situ during winemaking for four days, and subsequently the latter strain was added. The process was carried out under traditional winemaking conditions at 22 °C. The pectinolytic activity produced by the *T. delbrueckii* strain during vinification increased colour extraction and led to a high-quality Malbec wine with greater colour intensity, enhanced sensory features, and a distinctive varietal aroma compared to the control.

4. Conclusions

Non-*Saccharomyces* wine yeasts could represent an effective alternative for the control of fungal pathogens in vineyards, but prior knowledge of the target pathogen and the environmental conditions in the field are essential for successful biocontrol. Further research is needed to develop non-*Saccharomyces* wine yeast biocontrol agents that are effective, resistant to field and formulation constraints, as well as promising for the winemaking process. In winemaking, non-*Saccharomyces* wine yeasts could also be used as biocontrol agents, either using the selected strains or the derived antimicrobial metabolites (e.g., killer

toxins) to control wine spoilage yeasts during fermentation. Due to the low stability and high production costs of the latter approach, the inoculation with selected strains is an attractive alternative for reducing sulphites in oenology and the production costs. In this regard, future studies focusing on the selection of non-*Saccharomyces* wine yeast biocontrol cultures with high efficacy, compatibility with the fermentation process, and the potential to improve wine quality would be very valuable. Additionally, a promising alternative to pectinase preparations could be non-*Saccharomyces* wine yeasts, which produce extracellular pectinolytic and other depolymerising enzymes during winemaking. This strategy would avoid the addition of costly exogenous pectinases and would enhance the quality of wines, because these yeasts can confer considerable sensory complexity to wines.

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