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Review

Application of Non-*Saccharomyces* Yeasts to Wine-Making Process

José Juan Mateo * and Sergi Maicas

Departament de Microbiologia i Ecologia, Universitat de València, E-46100-Burjassot, Spain; Sergi.Maicas@uv.es
* Correspondence: Jose.J.Mateo@uv.es; Tel.: +34-96-3543008

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Abstract: Winemaking is a complex process involving the interaction of different microbes. The two main groups of microorganisms involved are yeasts and bacteria. Non-*Saccharomyces* yeasts are present on the grape surface and also on the cellar. Although these yeasts can produce spoilage, these microorganisms could also possess many interesting technological properties which could be exploited in food processing. It has been shown that some of the metabolites that these yeasts produce may be beneficial and contribute to the complexity of the wine and secrete enzymes providing interesting wine organoleptic characteristics. On the other hand, non-*Saccharomyces* yeasts are the key to obtain wines with reduced ethanol content. Among secreted enzymes, β-glucosidase activity is involved in the release of terpenes to wine, thus contributing to varietal aroma while β-xylosidase enzyme is also interesting in industry due to its involvement in the degradation of hemicellulose by hydrolyzing its main heteroglycan (xylan).

Keywords: non-*Saccharomyces* yeasts; wine; flavor; β-glucosidase; β-xylosidase

1. Introduction

Since Louis Pasteur elucidated the conversion of grape juice into wine, this process and the role of the yeast therein has been studied extensively [1]. More than 130 years later, there are many areas that are still not well understood [2]. This is especially the case for the roles of the numerous non-*Saccharomyces* yeasts normally associated with grape must and wine. These yeasts, present in all wine fermentations, are metabolically active and their metabolites can impact on wine quality. In the past, the influence of non-*Saccharomyces* yeasts in wine was restricted and even eliminated by inoculation with pure *S. cerevisiae* cultures because they have been regarded as spoilage yeasts [3,4]. However, in the past three decades, great interest has grown in the beneficial role of non-*Saccharomyces* yeasts in wine biotechnology [5,6]. It has been shown that some of the metabolites that these yeasts produce may be beneficial and contribute to the complexity of the wine when they are used in mixed fermentations with *S. cerevisiae* cultures [7,8]. Evidence supporting this fact has been published [9] and the role of the non-*Saccharomyces* yeasts in wine fermentation is receiving increasingly more attention by wine microbiologists in wine-producing countries [10].

Non-*Saccharomyces* yeasts are found on the grapes, but also in lesser numbers on the cellar equipment [11]. The initial environment that affects the microbial makeup of a wine fermentation is that of the vineyard. Although a drastically different environment than juice or wine, the types of microbes present on grapes will have an impact on the ensuing ecology in the wine fermentation, particularly in the early stages. Microorganisms appear to colonize around the grape stomata where small amounts of exudate are secreted [12,13]. The apiculate yeasts, *Hanseniaspora* and *Kloeckera*, its asexual anamorph, are the most prevalent vineyard yeasts and typically represent over half the yeast flora on grapes [14]. Other yeast genera present on berries include: *Metschnikowia*, *Candida*, *Pichia*, *Wickerhamomyces*, *Zygosaccharomyces*, and *Torulaspora* [15]. Also present in the vineyard are

numerous other yeasts, some of which have an impact on wine: *Sporidiobolus*, *Kluyveromyces*, and *Hansenula* [16]. *Saccharomyces* species are relatively scarce among healthy berries (Table 1) [17,18]. Before inoculation with *S. cerevisiae*, they are the yeasts present in the highest numbers in the grape must. During the fermentation there is a sequence of dominance by the various non-*Saccharomyces* yeasts, followed by *S. cerevisiae*, which then completes the fermentation [19]. This is especially evident in spontaneously fermenting grape must, which has a low initial *S. cerevisiae* concentration. Research has shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation [20] and their dominance during the early part of fermentation can leave an imprint on the final composition of the wine [21].

Table 1. Main non-*Saccharomyces* yeasts isolated from grape musts and wines.

Aureobasidium pullulans	*Hansenula* sp
Brettanomyces sp	*Issatchenkia terricola*
B. anomalus	*Kluyveromyces thermotolerans*
Candida guilliermondii	*Lachancea thermotolerans*
C. molischiana	*Metschnikowia pulcherrima/C. pulcherrima*
C. stellata	*Pichia angusta*
C. utilis	*P. anomala*
C. zemplinina	*P. capsulata*
Debaryomyces castellii	*P. guilliermondii*
D.hansenii	*P. kluyvery*
D.polymorphus	*P. membranifaciens*
D.pseudopolymorphus	*Saccharomycodes ludwigii*
D. vanriji	*Schizosaccharomyces pombe*
Hanseniaspora sp. *(Kloeckera)*	*Sporidiobolus pararoseus*
H. guilliermondii	*Torulaspora delbrueckii*
H. osmophila	*Trichosporon asahii*
H. vineae	*Wickerhamomyces anomalus*
H. uvarum	*Zygosaccharomyces bailii*

In the past, the influence of non-*Saccharomyces* yeasts in wine was restricted and even eliminated by inoculation with pure *S. cerevisiae* cultures because they have been regarded as spoilage yeasts [17]. However, in the past three decades, great interest has grown in the beneficial role of non-*Saccharomyces* yeasts in wine biotechnology [18,19]. It has been shown that some of the metabolites that these yeasts produce may be beneficial and contribute to the complexity of the wine when they are used in mixed fermentations with *S. cerevisiae* cultures [20,21].

It is believed that when pure non-*Saccharomyces* yeasts are cultivated with *S. cerevisiae* strains, their negative metabolic activities may not be expressed or could be modified by the metabolic activities of the *S. cerevisiae* strains [22]. Several strains belonging to different non-*Saccharomyces* species have been extensively studied in relation to the formation of some metabolic compounds affecting the bouquet of the final product. Diverse studies on the growth and metabolic interactions between non-*Saccharomyces* and *Saccharomyces* yeasts in mixed cultures have shown that their impact on ethanol content, wine flavor, aromatic profile, and quality and control of spoilage yeasts depends on the strains and the inoculation strategies [23,24]. In addition, a great number of studies inform about enzyme activities in winemaking and fermentations [25,26]. However, there are no known reports that associate the production of enzymatic activities in mixed cultures of *Saccharomyces* and non-*Saccharomyces* during the fermentation with the final aromatic profile of wines.

2. Contribution of Non-*Saccharomyces* Yeast Reduction in the Ethanol Content of Wines

Consumer and market demand for wines containing lower ethanol has shaped research to develop and evaluate strategies to generate low-ethanol wines [27]. Numerous studies have reported lower ethanol yields when using non-*Saccharomyces* yeast [23,28]. Another alternative is to exploit the oxidative metabolism observed in some non-*Saccharomyces* species [29]. Nevertheless, only one study has reported the use of aerobic yeast for the production of reduced alcohol wine. Wines containing 3% v/v ethanol were obtained after fermentation of grape must by *Williopsis saturnus*

and *Pichia subpelliculosa* under intensive aerobic conditions. These reduced alcohol wines were considered to be of an adequate quality [30].

Microbiological approaches for decreasing ethanol concentrations take advantage of the differences in energy metabolism among the wine yeast species. Several strategies that use genetically-modified yeasts have been proposed for the production of low-alcohol wine. Recently, Tilloy and co-workers [31] using evolution-based strategies together with breeding strategy showed that evolved or hybrid strains produced an ethanol reduction of 0.6%–1.3% (*v*/*v*). Another approach to reduce the production of ethanol could be the use of non-*Saccharomyces* wine yeasts, in combination with *S. cerevisiae*, to improve the quality and enhance the complexity of wine. Following numerous studies on the influence of non-*Saccharomyces* yeast in winemaking, there has been a re-evaluation of the role of these yeasts. Indeed, some non-*Saccharomyces* yeast can enhance the profile of the wine, and for this reason the use of controlled multi-starter fermentation using selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains has been encouraged [32]. Indeed, nowadays one of the most recent technological advances in winemaking is the practice of co-inoculation of grape juice with selected culture of a non-*Saccharomyces* coupled with a *S. cerevisiae* starter strain [25]. In this context, non-*Saccharomyces* wine yeasts in multi-starter fermentations could be an interesting way to reduce the ethanol content in wine. In addition, different respiro-fermentative regulatory mechanisms of some non-*Saccharomyces* yeasts compared to *S. cerevisiae* could be a modality to reduce the ethanol production through partial and controlled aeration of the grape juice. Indeed, in this way sugar is consumed via respiration rather than fermentation. Both of these approaches have indicated the promising use of non-*Saccharomyces* wine yeast to limit ethanol production [33,34].

3. Contribution to Wine by Non-*Saccharomyces* Yeast

According to Fleet [35], yeast influences wine aroma by different mechanisms; of these, novo biosynthesis of aroma compounds is probably the most important [36]. The variety of odor compounds produced by non-*Saccharomyces* yeasts is known [37]. The contribution of non-*Saccharomyces* yeasts to wine quality can take various forms. Production of glycerol by *Candida stellata* and esters by *C. pulcherrima* has been reported [15]. Other non-*Saccharomyces* yeasts are also widely recognized for producing glucosidase enzymes [38], which, by hydrolyzing such bonds, are capable of releasing volatile compounds linked to sugars, giving greater complexity to the wine's aromatic profile [39]. Conversely, others such as *Kloeckera apiculata* are associated with the production of acetic acid, which lowers wine quality [40]. Therefore, to determine the potential of non-*Saccharomyces* yeasts to be used in the wine industry, it is necessary to check that their activity in mixed culture does not affect the development of *Saccharomyces*, or produce compounds that may harm wine quality. These metabolic products include terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid, and succinic acid [41].

Therefore, sensory differences were found [42]. Over 160 esters have been distinguished in wine [43]. These esters can have a helpful effect on wine quality, especially in wine from varieties with neutral flavors [44]. Non-*Saccharomyces* can be divided into two groups, neutral yeasts (producing little or no flavor compounds) and flavor-producing species. Flavor-producing yeasts included *P. anomala* (*Hansenula anomala*) and *K. apiculata*. *Candida pulcherrima* is also known to be a high producer of esters [41]. The accumulation of esters in wine is determined by the balance between the yeast's ester-synthesizing enzymes and esterases (responsible for cleavage and in some cases, formation of ester bonds) [45]. Although extracellular esterases are known to occur in *S. cerevisiae* [46], the situation for non-*Saccharomyces* needs further investigation.

Different non-*Saccharomyces* yeasts produce different levels of higher alcohols (n-propanol, isobutanol, isoamyl alcohol, and active amyl alcohol) [44]. This is important during wine production, as high concentrations of higher alcohols are generally not desired, whereas lower values can add to wine complexity.

Glycerol, the next major yeast metabolite produced during wine fermentation after ethanol, is important in yeast metabolism for regulating redox potential in the cell [47]. Glycerol contributes to smoothness, sweetness, and complexity in wines, but the grape variety and wine style will govern the extent to which glycerol impacts on these properties [48]. Several non-*Saccharomyces* yeasts, particularly *C. zemplinina*, can consistently produce high glycerol concentrations during wine fermentation [25]. Unfortunately, increased glycerol production is usually linked to increased acetic acid production [49], which can be detrimental to wine quality. Spontaneously-fermented wines have higher glycerol levels, indicating a possible contribution by non-*Saccharomyces* yeasts [14].

Other compounds that are known to play a role in the sensory quality of wine include volatile fatty acids, carbonyl, and sulfur compounds [44]. Volatile thiols greatly contribute to the varietal character of some grape varieties, particularly Sauvignon Blanc [50]. Some non-*Saccharomyces* strains, specifically isolates from *C. zemplinina* and *P. kluyveri*, can produce significant amounts of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexan-1-ol acetate (3MHA), respectively, in Sauvignon Blanc wines [51]. Similarly, *T. delbrueckii*, *M. pulcherrima*, and *Lachancea thermotolerans* have also been described as able to release important quantities of 3MH from its precursor during Sauvignon Blanc fermentation [52].

However, the use of some non-*Saccharomyces* yeast in mixed fermentations with *S. cerevisiae* can generate wines with increased volatile acidity and acetic acid concentration [25]. Some non-*Saccharomyces* yeasts are able to form succinic acid [48]. This correlates with high ethanol production and ethanol tolerance. Succinic acid production could positively influence the analytical profile of wines by contributing to the total acidity in wines with insufficient acidity. Nevertheless, succinic acid has a "salt-bitter-acid" taste and excessive levels will negatively influence wine quality. Other non-*Saccharomyces* metabolites can act as intermediaries in aroma metabolic pathways. Acetoin is considered a relatively odorless compound in wine [53]. However, diacetyl and 2,3-butanediol (potentially off-flavors in wine) can be derived from acetoin by chemical oxidation and yeast-mediated reduction, respectively. This indicates that acetoin can play a role in off-flavor formation in wines. Definitely, high concentrations of acetoin produced by non-*Saccharomyces* yeasts can be utilized by *S. cerevisiae* in mixed and sequential culture fermentations [54].

Non-*Saccharomyces* yeasts have also been reported to affect the concentration of polysaccharides in wine [55]. Polysaccharides can positively influence wine taste and mouth-feel by increasing the perception of wine "viscosity" and "fullness" on the palate [56]. The early death of some non-*Saccharomyces* yeasts during fermentation can also be a source of specific nutrients for *S. cerevisiae* enabling it to ferment optimally. These nutrients include cellular constituents such as cell wall polysaccharides (mannoproteins). For this method of nutrient supply to be effective, any killer or other inhibitory effects by the non-*Saccharomyces* yeasts against *S. cerevisiae* should be known [57] so that the subsequent *S. cerevisiae* fermentation is not adversely affected.

Other non-*Saccharomyces* extracellular enzymatic activities, such as proteolytic and pectinolytic (polygalacturonase) enzymes, might also be beneficial to winemaking [58]. For example, proteolytic activity of some non-*Saccharomyces* yeast could lead to a reduction in protein levels with accompanying increase in protein stability of the end-product. Species found to produce the greatest number of extracellular enzymes are *C. stellata*, *H. uvarum* and *M. pulcherrima* [59].

Certain flavor and aroma compounds are present in grapes as glycosidic precursors with no sensory properties [60]. These compounds may be hydrolyzed by the enzyme D-glucosidase to form free volatiles that can improve the flavor and aroma of wine, but this enzyme is not encoded by the *S. cerevisiae* genome [61]. However, certain non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces*, *Hansenula*, *Candida*, *Pichia*, and *Hanseniaspora* possess various degrees of D-glucosidase activity [21] and can play a role in releasing volatile compounds from non-volatile precursors. An intracellular D-glucosidase has also been isolated and purified from *Debaryomyces hansenii*. This enzyme, which is not inhibited by glucose and ethanol, was used during fermentation of Muscat grape juice, resulting in an increase in concentration of monoterpenols in the wine [62].

4. Non-*Saccharomyces* Strains as Glycosidase Producers for Vinification

4.1. β-Glucosidases

Glycosidically-bound volatiles are highly complex and diverse, especially regarding the aglycone moiety. The sugar parts consist of β-D-glucopyranosides and different diglycosides: 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides, 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranosides (vicianosides), 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside (rutinosides), 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides, 6-*O*-β-D-glucopyranosyl-β-D-glucopyranosides, and 6-*O*-β-D-xilopyranosyl-β-D-glucopyranosides (primeverosides). The aglycon part is often formed with terpenols, but other flavor precursors can occur, such as linear or cyclic alcohols, C-13 norisoprenoids, phenolic acids, and probably volatile phenols such as vanillin [63].

If we consider only glycosides with the most flavorant aglycons the most abundant in grape juice are apiosylglycosides (up to 50% according to grape variety) followed by rutinosides (6% to 13%) and, finally, glucosides (4% to 9%). All glycosides are not present in all cultivars and their proportions also differ according to grapes [64]. The glycoside flavor potential from grapes remains quite stable during winemaking and in young wines as well. These findings opened a new field of intensive research on the chemistry of glycoconjugated flavor compounds to exploit this important flavor source. Some aglycones are already odorous when released from glycosides and can contribute to the varietal aroma of wines [65].

Terpene glycosides can be hydrolysed by an enzymic way [66] to enrich wine flavor by release of free aromatic compounds from natural glycoside precursors. Enzymatic hydrolysis of glycosides is carried out with various enzymes which act sequentially according to two steps: firstly, α-L-rhamnosidase, α-L-arabinosidase, or β-D-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose, or apiose and the corresponding β-D-glucosides are released; subsequent liberation of monoterpenol takes place after action of a β-D-glucosidase (Figure 1) [67]. Nevertheless, one-step hydrolysis of disaccharide glycosides has also been described; enzymes catalysing this reaction have been isolated from grapes [67]. Enzymic hydrolysis of glycoside extracts from Muscat, Riesling, Semillon, Chardonnay, Sauvignon, and Sirah varieties have provoked the liberation not only of terpenes, but also C-13 norisoprenoids, such as 3-oxo-β-ionol and 3-hydroxy-β-damascenona [68].

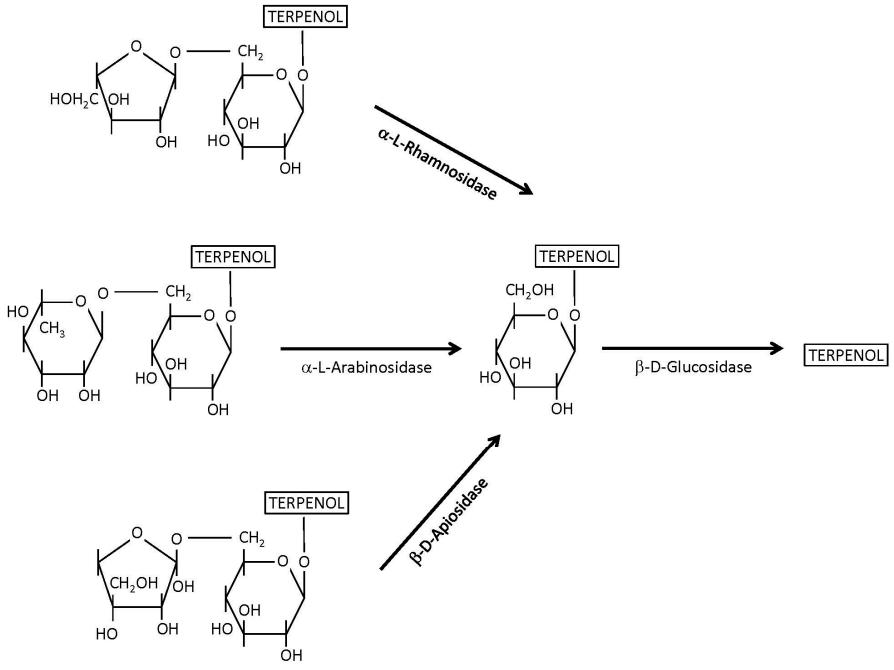

Figure 1. Sequential enzymatic hydrolysis of dissacharidic flavor precursors [66].

Few data are available regarding glycosidase activities of oenological yeast strains and the technological properties of the enzymes. Low α-rhamnosidase, α-arabinosidase, or β-apiosidase activities were detected in S. cerevisiae [69], but different efforts have been made to clone these genes obtained from differents microorganisms in S. cerevisiae [70]. Nevertheless, data on β-glucosidase activity on Saccharomyces are contradictory. First, results showed that these yeasts had a very low activity [71] but Delcroix et al. [69] found three enological strains showing high β-glucosidase activity. On the other hand, Darriet et al. [72] have shown that hydrolases located in the periplasmic space of a strain of S. cerevisiae were able to hydrolyse monoterpene glucosides of Muscat grapes; they also found that the activity of this β-glucosidase was glucose independent. Mateo and Di Stefano [73] detected β-glucosidase activity in different Saccharomyces strains on the basis of its hydrolytic activity on p-nitrophenyl-β-D-glucoside (pNPG) and terpene glucosides of Muscat juice. This enzymatic activity is induced by the presence of bound β-glucose as carbon source in the medium and seems to be a characteristic of the yeast strain. This β-glucosidase is associated with the yeast cell wall, is quite glucose independent but is inhibited by ethanol. This β-glucosidase is associated with the yeast cell wall is quite glucose independent but is inhibited by ethanol. These results could open new pathways regarding other glycosidase activities in S. cerevisiae; α-rhamnosidase, α-arabinosidase or β-apiosidase activities could be induced in wine yeast by changing the composition of the medium including inductive compounds, as well as in filamentous fungi [74].

Several flavor and aroma compounds in grapes are present as glycosylated flavorless precursors [2]. These compounds may be hydrolyzed by the enzyme β-glucosidase to form free volatiles that can increase the flavor and aroma of wine, but this enzyme is not encoded by the S. cerevisiae genome [46]. In contrast, non-Saccharomyces yeasts belonging to the genera Debaryomyces, Hansenula, Candida, Pichia, and Hanseniaspora possess various degrees of β-glucosidase activity and can play a role in releasing volatile compounds from non-volatile precursors [75]. Co-fermentation of Chardonnay grape juice with D. pseudopolymorphus and S. cerevisiae resulted in an increased concentration of the terpenols: citronellol, nerol, and geraniol in wine [76]. Similarly, cofermentation of Muscat grape juice with D. vanriji and S. cerevisiae produced wines with increased concentration of several terpenols [77]. Equally, mixed cultures of Sauvignon Blanc grape juice with *C. zemplinina* / *S. cerevisiae* and *T. delbrueckii* / *S. cerevisiae* produced wines with high concentrations of terpenols compared to wines only fermented with S. cerevisiae [23].

It has been reported that non-*Saccharomyces* yeasts can produce β-glucosidase [58]. The β-glucosidases from non-*Saccharomyces* species, such as *C. molischiana*, *C. wickerhamii*, and *P. anomala* were found to be more tolerant of winemaking conditions (for example, low pH values, low temperatures, high sugar, or ethanol levels) and tend to be more specific for glycosides than those from other yeast species [78]. Attempts have previously been made to enhance wine aroma using non-*Saccharomyces* yeasts and their glycosidases [79] because there is substantial yeast diversity in grapes and wines. Screening indigenous yeasts with glycosidases and their application in winemaking may allow wineries to make wines with more pleasant, typical, varietal aroma characteristics. Therefore, it is important to explore the potential of indigenous yeast biodiversity from specific enological ecosystems for specific and abundant β-glucosidases. β-Glucosidase-producing strains can be screened in Petri dishes with media containing cellulose-congo red, p-nitrophenyl-β-D-glucopyranoside (pNPG), or 4-methylumbelliferyl-β-dglucuronide (4-MUG) [80,81].

Yeasts of the *Hansenula* species isolated from fermenting must were reported to have an inducible β-glucosidase activity, but this enzyme was inhibited by glucose [82]. Other yeast strains, such as *C. molischiana* and *C. wickerhamii*, also possess activities towards various β-glucosides and they were little influenced by the nature of aglycon [83]. β-Glucosidase from *C. molischiana* was immobilized to Duolite A-568 resin, showing similar physicochemical properties to those of free enzyme. The immobilized enzyme was found to be very stable under wine conditions and could

be used repeatedly for several hydrolyzes of bound aroma [84]. *Endomyces fibuliger* also produces extracellular β-glucosidase when grown in malt extract broth [85].

Screening 370 strains belonging to 20 species of yeasts, all of the strains of the species *D. castelli*, *D. hansenii*, *D. polymorphus*, *K. apiculata*, and *H. anomala* showed β-glucosidase activity [86]. A strain of *D. hansenii* exhibited the highest exocellular activity and some wall-bound and intracellular activity and its synthesis, occurred during exponential growth, was enhanced by aerobic conditions and repressed by high glucose concentration. The optimum condition for this enzyme was pH 4.0–5.0 and 40 °C. This enzyme was immobilized using a one-step procedure on hydroxyapatite. The immobilized enzyme exhibited a lower activity than the purified free enzyme, but was much more stable than the enzyme in cell-free supernatant [87]. Their studies have shown the ability of several wine yeasts to hydrolyse terpenoids, norisoprenoids and benzenoids glycosides; among wine yeasts *H. uvarum* was able to hydrolyze both glyco-conjugated forms of pyranic and furanic oxides of linalool [88]. Other authors have also shown the important role of non-*Saccharomyces* species in releasing the glycosidic-bound fraction of grape aroma components [89].

A total of 17 *Pichia* (*Wickerhamomyces*) isolates obtained from enological ecosystems in the Utiel-Requena Spanish region were characterized by physiological and molecular techniques (PCR-RFLP and sequencing) as belonging to the species *P. fermentans*, *P. membranifaciens*, and *W. anomalus*. In order to characterize their enzymatic abilities, xylanase, β-glucosidase, lipase, esterase, protease, and pectinase qualitative and quantitative assays were made. *W. anomalus* and *P. membranifaciens* showed to be the most interesting species to be used as sources of enzymes for the winemaking industry. Glycosidase enzymes show a high degree of tolerance to high levels of glucose and ethanol, making them of great interest to be used in enological procedure [90].

The sensorial characteristics of the wines produced with Muscat grapes are related to the level of terpene alcohols, so an improvement of such a level, as a result of hydrolytic processes conducted by *Hanseniaspora*, is expected. Isolates from *H. uvarum* and *H. vineae* have been proved to be good candidates to be used in commercial vinification processes to enhance wine properties. Wine inoculated with yeasts showed an increase in the level of aromatic compounds (Table 2) [91].

Table 2. Terpene compounds in Muscat wine. Concentration expressed as μg/L [a] [91].

Compound	Control [b]	*Hanseniaspora* Inoculated		
		H. uvarum H107	*H. vineae* G26	*H. vineae* P38
Oxide A [c]	29.7 (1.2)	30.4 (2.1)	33.7 (3.2)	26.9 (3.4)
Oxide B [d]	nd	nd	nd	nd
Linalool	20.0 (0.9)	40.4 * (3.9)	47.4 * (3.4)	38.2 * (5.3)
Ho-trienol	24.0 (3.2)	51.3 *(5.3)	35.1 * (4.2)	24.9 * (0.6)
2-Phenylethanol	1890.2 (43.4)	3057.5 * (39.8)	2747.8 * (26.8)	2568.5 * (45.6)
Oxide C [e]	nd	nd	nd	nd
Oxide D [f]	nd	nd	nd	nd
Terpineol	53.3 (3.4)	67.2 * (4.7)	65.1 *(1.2)	54.5 (3.9)
Nerol	24.6 (2.8)	25.8 (1.1)	23.4 (3.1)	26.3 (1.2)
Geraniol	59.8 (5.0)	61.3 (3.7)	56.9 (1.7)	62.8 (1.7)
Diol 1 [g]	43.2 (4.7)	87.9 * (2.1)	80.2 * (2.1)	81.2 * (3.2)
4-Vinylphenol	63.2 (1.2)	89.7 * (2.4)	75.7 * (5.8)	62.1 (0.9)
Endiol [h]	nd	58.8 * (2.1)	52.0 * (3.4)	34.1 * (4.2)
Diol 2 [i]	12.0 (0.6)	13.4 (0.9)	7.8 (2.6)	10.1 (0.9)
2-Phenylethyl acetate	28.0 (4.1)	56.2 * (7.2)	23.3 (1.2)	25.8 (4.7)
2-Methoxy-4-vinylphenol	89.0 (6.1)	103.0 * (5.3)	105.4 * (6.5)	94.1 (2.9)

[a] Values in brackets represent standard deviation (n = 3). ANOVA one factor, significant difference is indicated as * ($p < 0.05$); [b] Wine produced only with *Saccharomyces cerevisiae*; [c] *cis*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol; [d] *trans*-5-vinyltetrahydro-1,1,5-trimethyl-2-furanmethanol; [e] *cis*-6-vinyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol; [f] *trans*-6-vinyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol; [g] 2,6-Dimethyl-3,7-octadien-2,6-diol; [h] 2,6-Dimethyl-7-octene-2,6-diol; [i] 2,6-Dimethyl-2,7-octadien-1,6-diol; nd: not detected

The potential applications of wild yeast strains with β-glucosidase activity have been investigated under simulated oenological conditions, coupled with the exploration of the potential applications of the β-glucosidases by studying the enzymatic activity and stability under similar oenological

conditions [92]. The effects of different oenological factors on β-glucosidase production indicated that one isolate from the *T. asahii* strain had higher β-glucosidase production than the other strains under low pH conditions. However, isolates from *H. uvarum* and *S. cerevisiae* strain showed higher β-glucosidase production under high-sugar conditions. Furthermore, the influence of oenological factors on the activity and stability of the β-glucosidases revealed that the enzyme from the *T. asahii* strain had a stronger low-pH-value resistance than the other yeast β-glucosidases [92].

Hu et al. [93] applied a semiquantitative colorimetric assay to screen yeasts from three different regions of China. Among 493 non-*Saccharomyces* isolates belonging to eight genera, three isolates were selected for their high levels of β-glucosidase activity and were identified as *H. uvarum*, *P. membranifaciens*, and *Rhodotorula mucilaginosa*. β-Glucosidase from the *H. uvarum* strain showed the highest activity in winemaking conditions among the selected isolates. For aroma enhancement in winemaking, the glycosidase extract from *H. uvarum* exhibited catalytic specificity for aromatic glycosides of C13-norisoprenoids and some terpenes, enhancing fresh floral, sweet, berry, and nutty aroma characteristics in wine.

4.2. Xylanases

β-1,4-xylan is a heteroglycan with a backbone of β-(1→4)-linked D-xylopyranose residues that can be substituted with L-arabinofuranose, D-glucuronic acid, and/or 4-*O*-methyl-D-glucuronic acid [94]. It constitutes the major component of hemicelluloses found in the cell walls of monocots and hardwoods, and represents one of the most abundant biomass resources. Recently, xylanolytic enzymes of microbial origin have received great attention due to their possible industrial applications for sustainable fuel-ethanol production from xylan. Two key reactions proceed during hydrolysis of the xylan backbone; endo-1,4-β-xylanases (1,4-β-D-xylan xylanohydrolase) hydrolyze internal β-(1→4)-xylosidic linkages in the insoluble xylan backbone to yield soluble xylooligosaccharides, while 1,4-β-xylosidases are exoglycosidases that cleave terminal xylose monomers from the non-reducing end of short-chain xylooligosaccharides [95]. Additional enzyme activities, such as α-L-arabinofuranosidase, α-D-glucuronidase, and acetyl xylan esterase, remove side-chain substituents. 1,4-β-Xylosidase is important in xylan degradation, considering that xylans are not completely hydrolyzed by xylanases alone. The finding of new isolates of non-*Saccharomyces* yeasts, showing beneficial enzymes (such as β-glucosidase and β-xylosidase) can contribute to the production of quality wines [96]. In a selection and characterization program we have studied 114 isolates of non-*Saccharomyces* yeasts, four isolates were selected because of their both high β-glucosidase and β-xylosidase activities. The ribosomal D1/D2 regions were sequenced to identify them as *P. membranifaciens*, *H. vineae*, *H. uvarum*, and *W. anomalus*. The induction process was optimized to be carried on YNB-medium supplemented with 4% xylan, inoculated with 10^6 cfu/mL and incubated 48 h at 28 °C without agitation. Most of the strains had a pH optimum of 5.0 to 6.0 for both the β-glucosidase and β-xylosidase activities. The effect of sugars was different for each isolate and activity. Each isolate showed a characteristic set of inhibition, enhancement or null effect for β-glucosidase and β-xylosidase. The volatile compounds liberated from wine incubated with each of the four yeasts were also studied, showing an overall terpene increase when wines were treated with non-*Saccharomyces* isolates. In detail, terpineol, 4-vinyl-phenol, and 2-methoxy-4-vinylphenol increased after the addition of *Hanseniaspora* isolates. Wines treated with *Hanseniaspora*, *Wickerhamomyces*, or *Pichia* produced more 2-phenyl ethanol than those inoculated with other yeasts [97].

An ethanol-tolerant 1,4-β-xylosidase was purified from cultures of a strain of *P. membranifaciens* grown on xylan at 28 °C. The enzyme was purified by sequential chromatography on DEAE-cellulose and Sephadex G-100. The relative molecular mass of the enzyme was determined to be 50 kDa by SDS-PAGE. The activity of 1,4-β-xylosidase was optimum at pH 6.0 and 35 °C. The activity had a Km of 0.48 ± 0.06 mmol·L^{-1} and a Vmax of 7.4 ± 0.1 μmol·min^{-1}·mg^{-1} protein for p-nitrophenyl-β-D-xylopyranoside. The enzyme characteristics (pH and thermal stability, low inhibition rate by glucose and ethanol tolerance) make this enzyme a good candidate to be used

in enzymatic production of xylose and improvement of hemicellulose saccharification for production of bioethanol [98].

5. Conclusions

It is generally accepted that the wealth of yeast biodiversity with hidden potential, especially for oenology, is largely untapped. In order to exploit the potential benefits of non-*Saccharomyces* yeasts in wine production and to minimize potential spoilage, the yeast populations on grapes and in must, as well as the effect of wine-making practices on these yeasts, must be known, as must the metabolic characteristics of non-*Saccharomyces* yeasts. Strain selection will be very important, as not all strains within a species will necessarily show the same desirable characteristics. For example, significant variability is found in the formation of β-glucosidase amongst strains within some non-*Saccharomyces* yeast species.

Whatever the outcome of the search for non-*Saccharomyces* yeasts for use in wine production, the accepted list of desirable characteristics as pertaining to the wine yeast *S. cerevisiae* will not necessarily apply to non-*Saccharomyces* yeasts. High fermentation efficiency, sulfite tolerance and killer properties, for example, might not be needed in the new technology of wine production. The new non-*Saccharomyces* wine yeasts will necessarily have a different list of desired characteristics: efficient sugar utilization, enhanced production of desirable volatile esters, enhanced liberation of grape terpenoids and production of glycerol to improve wine flavor and other sensory properties can be met by selected non-*Saccharomyces* wine yeasts.

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