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ORIGINAL ARTICLE

Screening of enzymatic activities within different enological non-*Saccharomyces* yeasts

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Abstract Ninety-seven non-*Saccharomyces* wine yeast strains belonging to ten different genera and species (*Candida* spp. and *Criptococcus* spp.; *Debaryomyces hansenii*, *Lachancea thermotolerans, Metschnikowia pulcherrima, Pichia kluyveri*, *Sporidiobolus salmonicolor*, *Torulaspora delbrueckii*, *Williopsis pratensis* and *Zygosaccharomyces bailii)* were screened for 13 enzymes related to wine aroma, color and clarity. Understanding the yeasts' influence in these wine characteristics provides a platform for selecting strains for their development as starter cultures and for the management of alcoholic fermentation. Most of the strains showed the presence of one or more enzymes of biotechnological interest. Our screening demonstrated several intraspecific differences within the yeast species investigated, indicating that strain selection is of great importance for their enological application, and also that some non-*Saccharomyces* that have not been thoroughly explored, may deserve further consideration. This research represents the first stage for selecting non-*Saccharomyces* strains to be used as a starter along with *Saccharomyces cerevisiae* to enhance some particular characteristics of wines.

Keywords Enzymes · Winemaking · Non-*Saccharomyces* · Aroma · Color

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Introduction

During the last decade, yeast species other than *Saccharomyces cerevisiae* have been proposed for winemaking as they could positively impact on wine quality by acting in mixed fermentation with *S. cerevisiae*. Traditionally, yeasts have been selected for their fermentative power and low acetic acid production, but nowadays new selection criteria are being used to obtain inocula that improve the technological properties and sensorial features of wines. Nowadays it is becoming ever more important to select yeasts that are good for each kind of wine, region and even microclimate (Suárez-Lepe and Morata 2012).

Knowledge about non-*Saccharomyces* yeasts has greatly increased over the last few years, which has led to some of them being used commercially as starters (*T. delbrueckii*, *M. pulcherrima*, *P. kluyveri*, *L. thermotolerans*) (Masneuf-Pomarede et al. 2015), while several other species are being subjected to studies to assess their effects on wine quality (Bely et al. 2013; Maturano et al. 2015). However, there are other species that remain unconsidered, although they have been repeatedly isolated from winemaking-related ecosystems. With a better understanding of the properties of yeasts, selection procedures could be adapted to obtain strains that could improve wine quality (e.g. enhancing positive aroma compounds and reducing those with negative impact, improving flavor and clarity, modulating color, etc.). Non-*Saccharomyces* wine yeasts could provide different desired characteristics (Jolly et al. 2014). High fermentation efficiency, high sulfite tolerance and killer activity for example, might not be needed with the new technology of wine production, (many consumers prefer more natural and healthier wines with less quantity of additives and low alcohol degree).

Enzymes play an important role in winemaking. They come from grapes, microorganisms and can also be added from commercial preparations. They help to address problems related to fining, filtration and increased aroma and color in wines (Merín and Morata de Ambrosini 2015; Belda et al. 2016b). In a selection process of microorganisms involved in the winemaking process, it is important to analyze the potential of strains for producing extracellular enzymes in order to change the composition of musts and for enhancing the sensory attributes of the wines (Van Resburg and Pretorius 2000). Maturano et al. (2012) determined the ability of different non-*Saccharomyces* yeasts to produce extracellular enzymes of enological relevance (β-glucosidases, pectinases, proteases, amylases and xylanases) during fermentation, and observed that although non-*Saccharomyces* populations were not detected at the end of vinification, their secreted enzymes remained in the fermenting media. The secretion of each enzyme is not characteristic of a particular genus or species, but depends on the yeast strain analyzed (Ganga and Martínez 2004). It is therefore interesting to characterize every isolate so as to determine its potential as an enzyme producer. Selection within each genera or species could help the wine industry to develop the biotechnological use of non-conventional yeasts to improve the quality and differentiation of wines.

The aim of this study was the enzymatic characterization of yeast clones belonging to different non-*Saccharomyces* genera, as the first step in selecting wild strains with beneficial technological properties which can be potentially used as starters in the production of different wines.

Materials and methods

Yeast isolates

Ninety-seven non-*Saccharomyces* wine yeast strains belonging to 10 different genera taken from ICVV (Instituto de Ciencias de la Vid y el Vino) collections were used. These strains have been previously identified as being present in different ecosystems of the Rioja Qualified Designation of Origin (D.O.Ca. Rioja) wineries, such as on grapes, during fermentation, in wine, winery facilities and in the air (Ocón et al. 2010a, b, 2013). The wine yeast strains belonged to species used commercially as starters (*Lachancea thermotolerans, Metschnikowia pulcherrima, Pichia kluyveri* and *Torulaspora delbrueckii)*, and to species that still remain unconsidered and that have been isolated in D.O.Ca. Rioja enological ecosystems (*Candida* spp. and *Criptococcus* spp. genera, *Debaryomyces hansenii*, *Sporidiobolus salmonicolor*, *Williopsis pratensis* and *Zygosaccharomyces bailii)*. *Criptococcus* spp. and *Sporidiobolus salmonicolor* were isolated in the air of all the wineries where this medium was studied. However, *Williopsis pratensis* was detected only in the air of the bottling area of one winery during two consecutive years. *Debaryomyces hansenii* and *Candida* spp. were isolated in most of the studied wineries, the first one in winery air and facilities, and the second one in alcoholic fermentations and facilities. Finally, *Zygosaccharomyces bailii* has been detected in approximately 15% of vinifications studied by our research group during alcoholic fermentation. The study of enzymatic activities was carried out with the yeast grown for 48 h at 24 °C on a GYP medium (20 g/L dextrose, 5 g/L yeast extract, 5 g/L peptone and 20 g/L agar).

Enzymatic characterization

Enzymes related to aroma

Sulfite reductase (SR) activity The H_2S-production potential of the yeasts was determined by plating them onto a solid juice indicator agar (250 mL grape juice, 15 mL succinate pH 5.1, 735 mL distilled water, 11 g/L bismuth citrate and 30 g/L agar) (Strauss et al. 2001). After 24–48 h of incubation at 30 °C, a low H_2S-producing colony was identified by its white color whereas a high H_2S producing colony was characterized by a black color.

Hydroxycinnamic acid decarboxylase (HCDC) activity Decarboxylation of ferulic and p-coumaric acids by yeasts was determined following the protocol described by Viana et al. (2008). The detection of the activity was performed using YPD plates containing 0.01% (w/v) bromocresol purple (Sigma Aldrich) supplemented with 0.145% (w/v) p-coumaric acid (Sigma Aldrich). Aliquots (10 µL) of cell extracts prepared in a 10 mM phosphate buffer pH 7 (0.869 g/L K_2HPO_4, 0.519 g/L KH_2PO_4) from 24-h must cultures were laid on the surface of the plates and incubated for 24 h at 37 °C. Hydroxycinnamic acid decarboxylase activity can be detected by a color shift from yellow to purple as a result of a pH increase due to the decarboxylation of the hydroxycinnamic acid which leads to an alkalization of the sample environment.

API-ZYM Seven enzymatic activities were measured using an API-ZYM test system (BioMérieux, France), according to the manufacturer's instructions (esterase, esterase-lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase and ß-glucosidase). API-ZYM is a semi-quantitative test system used for screening 19 different enzyme activities. Yeast cultures were previously grown on YPD agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract and 20 g/agar) at 30 °C for 24 h. Subsequently, the cultures were suspended in distilled water until suspensions reached 5–6 McFarland turbidity. Suspensions were inoculated in the microwells on the API-

ZYM strip at a level of 65 µL for each cupule. The strips were incubated at 37 °C for 4–5 h. Later ZYM A and ZYM B reagents were added to each cupule. Enzyme activity was measured comparing the color produced with the APY-ZYM color reaction chart.

Enzymes related to color and fining

Pectinase activity The yeast replica were plated onto a polygalacturonate agar medium (12.5 g/L polygalacturonic acid, 6.8 g/L potassium phosphate (pH 3.5), 6.7 g/L Yeast Nitrogen Base (YNB, Sigma), 10 g/L glucose and 20 g/L agar). The plates were incubated at 30 °C for five days. The colonies were rinsed off with distilled water before staining the plates with 0.1% ruthenium red. Colonies showing a purple halo were identified as positive activity.

Cellulase activity Cellulase production was determined by plating the yeast replica onto YPD plates containing 0.4% de carboxymethilcellulose (CMC, Sigma). The plates were incubated for five days at 30 °C. The colonies were rinsed off with distilled water before staining with 0.03% congo red, followed by destaining with 1 M NaCl. Cellulase activity was determined as positive when a clear halo appeared around the colony.

Xylanase activity Yeast replica were screened for hemicellulase activity by plating onto SC plates (6.7 g/L YNB, 20 g/L agar) containing 0.2% Remazol Brilliant Blue Xylan (RBB-Xylan, Sigma). The plates were incubated for five days at 30 °C. The colonies were washed off the plates with distilled water. Colonies showing activity were identified by a clear zone around the colony.

Glucanase activity Production of β-glucanase activity was determined by replica plating the yeast onto YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) containing 0.2% Yeast Beta Glucan (Megazyme). The plates were incubated for five days at 30 °C. Colonies were rinsed off with distilled water before staining the plates with 0.03% congo red (Merck) for 15 min. Yeasts showing a clear halo around the colonies were considered as glucanase positive.

Results and discussion

Screening of enzymatic activities

Enzymes involved in off-flavors

The volatile sulfur compounds in wines come mainly from the metabolism of yeast and have a significant role in the flavor of wines associated with the reduction character (Mendes-Ferreira et al. 2001; Shinohara et al. 2000). This production has been related to the presence of sulfite reductase activity in yeasts. In this work, the SR activity was highly present in *T. delbrueckii* (100%) and *M. pulcherrrima* (94%) (Table 1). In two other yeasts (*Cryptococcus* spp. and *D. hansenii*) SR activity was present to a lesser extent (20 and 33% respectively). The six remaining species did not show this activity (*Candida* spp., *L. thermotolerans P. kluyveri*, *S. salmonicolor*, and *W. pratensis*). Comitini et al. (2011) found that 100% of *T. delbrueckii* and *M. pulcherrima* tested (nine and seven strains respectively) were SR positive. However, unlike in our study, they also found that 100% of *L. thermotolerans* and *Candida* spp. (five and thirteen strains respectively) were also positive for SR. These differences could be explained by the low number of strains analyzed in both studies, or as a strain-related characteristic (strains isolated in different zones).

Vinyl- and ethylphenols can produce phenolic off-flavors in wine (Suárez et al. 2007; Wedral et al. 2010). The ability of wine yeasts to decarboxylate ferulic and p-coumaric acids by HCDC activity is related to the production of phenolic off-flavors in winemaking. Ethylphenol producers are yeasts belonging to the genus *Brettanomyces/Dekkera*, while the synthesis of vinylphenols varied among yeast species. Hence, the importance of controlling phenolic off-flavor production by using wine yeast strains with low HCDC productivity. In our study, only 50% of *M. pulcherrima* strains were HCDC positive (Table 1), whereas the rest of the genera and species did not show this activity. Shinohara et al. (2000) observed that 78–83% of non-*Saccharomyces* yeasts studied (23 strains) produced phenolic off-flavors in wine fermentation. These yeasts belonged to the *Rhodotorula*, *Candida*, *Cryptococcus*, *Pichia*, *Hansenula* and *Brettanomyces* genera, whereas in the *T. delbrueckii*, *Z. bailii* and *M. pulcherrima* species the productivity was absent. Differences in *Candida*, *Cryptococcus* and *M. pulcherrima* were found in comparison to our work. Those differences could be explained, as in the SR activity, by the number of strains studied, or as a strain-related characteristic (strains coming from different locations). The fact that only 50% of *M. pulcherrima* strains studied showed production of HCDC suggested that this is not characteristic of a particular genus or species, but depends on the yeast strain, as Ganga and Martínez (2004) had already pointed out. Results found in this research were quite good for a selection process, because most of the strains studied were unable to release HCDC activity.

Enzymes related with aroma

All the enzymes shown in Table 1 are involved in the final aroma of wines: the group of esterases, esterases-lipases

Table 1 Production of extracellular enzymes

Yeast Species	N	Enzymatic activities											
		SR	HCDC	Esterase	Esterase-Lipase	Lipase	LeucineA	ValineA	CystineA	ß-glucosidase	Pectinase	Cellulase	Xylanase	Glucanase
Candida spp.	12	0	0	83	100	0	100	75	0	25	50	0	0	0
Cryptococcus spp.	10	20	0	100	100	0	100	0	0	60	60	60	0	60
Debaryomyces hansenii	5	33	0	67	67	0	0	0	0	0	100	33	0	67
Lachancea thermotolerans	3	0	0	94	100	12	100	94	6	6	12	75	62	81
Metchnikowia pulcherrima	6	94	50	100	100	0	94	44	0	63	69	37	0	6
Pichia kluyveri	4	0	0	100	100	0	100	0	0	0	100	0	0	0
Sporodiobulus salmonicolor	7	0	0	100	100	33	100	100	0	0	67	0	0	0
Torulaspora delbrueckii	18	100	0	56	56	0	67	0	6	0	83	11	0	0
Williopsis pratensis	16	0	0	100	100	0	100	100	90	0	0	0	0	0
Zygosaccharomyces bailii	16	43	0	71	86	0	100	0	0	14	71	29	0	0

Numbers indicate the percentage of isolates with positive activity into each species

N, number of isolates studied; SR, sulfite reductase; HCDC, hydroxycinnamic acid decarboxylase

and lipases, the group of aminopeptidases (leucine arylamidase, valine arylamidase and cystine arylamidase), β-glucosidase and the group of carbohydrolases (pectinase, cellulase, xylanase, glucanase). Besides, HCDC and SR are also aroma-related enzymes, although with negative effects.

Lipases contribute to an increase in the concentrations of free fatty acids from grapes or microorganisms, and esterases catalyze the synthesis of esters that contribute to the secondary aroma of wines. Esterase and esterase-lipase activities were present in all the species studied (Table 1), even in some of them in all the strains. 100% of the strains of *Cryptococcus* spp. genera and *W. pratensis*, *S. salmonicolor*, *P. kluyveri* and *M. pulcherrima* species showed esterase activity, and in the remaining ones this activity was variable (from 56% in *T. delbrueckii* to 94% in *L. thermotolerans*). Something similar happened with esterase-lipase activity which was positive for 100% of *Candida* spp., *Cryptococcus* spp., *W. pratensis*, *S. salmonicolor*, *P. kluyveri*, *M. pulcherrima* and *L. thermotolerans*. In the three remaining species (*Debaryomyces hansenii*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*) more than 50% of strains displayed this activity. Viana et al. (2008) also revealed the potential of some non-*Saccharomyces* to produce acetate esters, specifically for the *Hanseniaspora* and *Pichia* genera. However, lipase activity was scarcely detected and only 33% of *S. salmonicolor* strains and 12% of *L. thermotolerans* showed this activity. Charoenchai et al. (1997) revealed that several wine yeasts (*Candida stellata, Candida pulcherrima, Candida krusei* and *T. delbrueckii*) had the potential to show extracellular lipolytic activity, degrading lipids and releasing free fatty acids. The liberation of medium chain fatty acids, such as decanoic or octanoic acids, could inhibit the growth of *Saccharomyces cerevisiae* and malolactic bacteria, leading to stuck fermentations. In contrast, in the current study no lipase activity was found for *Candida* spp. studied, or *T. delbrueckii,* which would result positive by preventing fermentation depletions.

The proteolytic enzymes of arylamidases or aminopeptidases (A) catalyze the hydrolysis of N-terminal amino acids from peptides. The increase in the amino acids content enhances the nutrient content in must and the production of aroma compounds in wine (Trinh et al. 2010). Some amino acids such as valine, leucine, isoleucine and phenylalanine (Santamaría et al. 2015), are known for being precursors of active aroma compounds produced by the yeasts. These enzymatic activities are also involved in the reduction of the proteic instability in wines (Dizy and Bisson 2000). In relation to these groups of enzymes it was found that *D. hansenii* did not show any activity, while *W. pratensis* was positive for the three activities in most of the strains (100% for leucine, 100%

for valine and 90% for cystine). *L. thermotolerans* strains also produced the three activities but with lower percentages of positives (100, 94 and 6% respectively). In the remaining species' strains, one or two activities could be found (One in *Criptococcus* spp., *P. kluyveri* and *Z. bailii* and two in *Candida* spp., *M. pulcherrima*, *S. salmonicolor* and *T. delbrueckii*). Other authors (Charoenchai et al. 1997; Strauss et al. 2001) found *Kloeckera apiculata*, *M. pulcherrima*, *Pichia anomala*, *C. stellata* and also *D. hansenii* strains with protease activity, and Ganga and Martínez (2004) showed that this activity was present in a greater percentage of isolates from *M. pulcherrima* (78,4%) and *Candida* spp. (56,7%). The methodologies employed to determine the proteolytic activity in the previous works differed from the present methods, which could explain the different results obtained in the current study. Belda et al. (2016a), detected absence of proteolytic activity in the majority of *S. cerevisiae* strains and highlighted the potential of *Wickerhamomyces anomalus*, *M. pulcherrima* and *Kluyveromyces marxianus* as proteolytic enzyme producers. Within the group of three enzymes included in proteolytic activity or aminopeptidases in this work, the most frequent one was leucine arylamidase, present in nine species with a high percentage (average of 96% from those nine species, with the minimum percentage found being 67% in *T. delbrueckii*), and the lowest cystine arylamidase, which was only detected in three species. The only species in which this last activity was detected to a great extent was *W. pratensis*.

Terpenes are a type of varietal compound that contributes to improve wine aroma. Most of them are bound to sugar molecules to form odorless compounds. β-glucosidase breaks them down to release free terpenes, thus enhancing the wine aroma. The β-glucosidase activity was absent in half of the studied species, and its presence was only significant in *Cryptococcus* spp. and *M. pulcherrima* (60% and 63% respectively). β-glucosidase activity was detected in other species, although in low ratios (25% in *Candida* spp., 14% in *Z. bailii* and 6% in *L. thermotolerans*). Mendes Ferreira et al. (2001) indicated that *Kloeckera apiculata* and *M. pulcherrima* were potential candidates for releasing terpenes due to their high β-glucosidase activity. Moreover, Rossouw and Bauer (2016) found isolates of non-conventional yeasts (*Cryptococcus* spp. and *Candida* spp.) producing high amounts of terpenes. In the current research, the strains of *Cryptococcus* spp., *M. pulcherrima* and *Candida* spp. were the ones that most displayed this enzymatic activity. Other studies have shown that species from the genera *Debaryomyces*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Dekkera*, *Hansenula*, *Hanseniaspora* species and *Zygosaccharomyces* genera (Ganga and Martínez 2004) and even *Pichia* (Charoenchai et al. 1997) displayed this activity. However, Strauss et al. (2001) did not find β-glucosidase activity in 245 isolates of *K. apiculata*, *D. hansenii* and *Candida* spp. analyzed, and Fernández et al. (2000) indicated that the enzyme β-glucosidase was mainly linked to *M. pulcherrima* species. These latter results were in agreement with those described in this report.

The glycosidically bound terpenols are located mainly in the grape skins. So, enzymatic degradation of the cell wall by carbohydrolases (pectinase, cellulase, xylanase and glucanase) contributes to the release of those precursors from the berries to the must. It has been proposed that the cell wall degradation of the grapes allows many aromatic or potential aromatic compounds to form part of the must, resulting in a better aromatic profile (Pérez-González et al. 1993). Our results showed that the only non-*Saccharomyces* species where the four carbohydrolases activities were present was *L. thermotolerans*, and this species was the only one showing xylanase activity. On the other hand, within the *W. pratensis* no strain showed activities in this group. Interestingly, within *Cryptococcus* spp. and *D. hansenii*, both non-conventional yeasts in fermentation processes, a high percentage of the tested strains exhibited three activities which could be relevant in a selection process in order to improve the aromatic profile of wines.

Enzymes related to color and fining

Some of the enzymes, such as pectinase, cellulose, xylanase and glucanase, related to aroma were also directly involved in color and in fining. Indeed, the degradation of the structural polysaccharides by these carbohydrolases can result in higher juice extraction, improvement in fining and filterability during winemaking, increasing the extraction of substances that enhance color and aroma during maceration. The rise in the extraction of phenolic compounds led to the formation of more polymeric pigments in aged red wine, resulting in an increase in color intensity and stability. Louw et al. (2006) carried out vinifications with a recombinant *Saccharomyces* strain able to degrade glucan and xylan, which resulted in significant increases of free-run wine, significant differences in color intensity, color stability and volatile composition. In the screening of pectinase, cellulose, xylanase and glucanase activities that have been carried out in 10 different species, differences among them were found. Pectinase was the most widespread activity among the non-*Saccharomyces* yeasts studied in this work, with nine out of ten species having it, and in most cases, with high percentages (average of 68% from those nine species). Besides, this enzyme was the only one from this group of enzymatic activities present in *Candida* spp., *S. salmonicolor* and *P. kluyveri*. Some of these carbohydrolases have been detected in different genera (*Kluyveromyces, Metschnikowia, Candida,*

Dekkera, Torulaspora, Zygosacharomyces, Pichia and *Hanseniaspora*) (Ganga and Martínez, 2004; Manzanares et al. 1999). Belda et al. (2015) demonstrated the impact of one *M. pulcherrima* strain producer of pectinolytic enzymes on wine traits: color, anthocyanin and polyphenol content of wines increased when that strain was used in mixed fermentations.

On another note, some metabolites produced by yeasts during fermentation may condense with grape anthocyanins to produce highly stable adducts such as vitisines A and B. So, the yeast strains used in vinification have a substantial influence on the formation of stable pigments and, therefore proper yeast selection is important to ensure the stability of the wine coloring matter (Morata et al. 2016). Yeasts with HCDC activity can also be used to decarboxylate hydroxycinnamic acids and form vinylphenols, that condense with grape anthocyanins to produce vinylphenolic pyroanthocyanin adducts, molecules that show great color stability. Benito et al. (2011) showed that during mixed fermentations using non-*Saccharomyces* with high HCDC activity, the content of stable pigments increased without sensorial modifications. Besides this, the content of p-coumaric acid was also reduced, and the formation of 4-ethylphenol (off-flavor) caused by massive *Dekkera/Brettanomyces* contamination in the wines was also minimized (Benito et al. 2009). Taking into account this theory, non-*Saccharomyces* yeasts having HCDC activity could increase the color stability and avoid the accumulation of vinylphenols in wines. In our study, strains with HCDC activity were only found for *M. pulcherrima*. So, displaying HCDC activity could be positive if the purpose of the yeast selection were to improve the red wine color due to the accumulation of vinylphenol adducts, and negative if the objective were to improve the aroma of the wine because of the risk of ethylphenol synthesis by *Dekkera/Brettanomyces*. Something similar happens with β-glucosidase activity, which is a desired enzymatic activity for aroma improvement in wines, but its presence could be harmful for color in red wines due to the degradation of anthocyanins (Suárez-Lepe and Morata, 2015). Manzanares et al. (2000) indicated that some non-*Saccharomyces* included in *Dekkera*, *Rhodotorula* and *Schizosaccharomyces* genera were not able to produce β-glucosidase. In all the species studied in this research, there were strains that did not show this activity (meaning that β-glucosidase activity is a strain-dependent trait), which could be chosen for inocula in red vinifications. In fact, its presence was significant only in *Cryptococcus* spp. and *M. pulcherrima* (60 and 63% respectively).

Proteolytic enzymes hydrolyze proteins improving fining of musts and wines and could reduce the proteic instability in wines (Dizy and Bisson 2000). As previously indicated, *D. hansenii* did not show any of the three aminopeptidases assayed in this study, and in *W. pratensis* and *L. thermotolerans* we found strains with the three activities. The most frequent proteolytic enzyme detected was leucine arylamidase which was present in nine species in a high percentage (>67%).

Enzymatic profiles and preselection within species

All the strains studied showed several enzymes of biotechnological interest, but only in *L. thermotolerans* were all the enzymatic activities with positive influence in wine quality found. However, it was quite complicated for all of the activities to converge in the same strain. So, because the secretion of each enzyme depends on the yeast strain analyzed (Ganga and Martínez 2004), the strains have been grouped using their enzymatic profiles (Tables 2, 3) in order to choose the best strains within each species according to their final use (white or red wines), or the characteristic we want to modulate (aroma or color).

In Table 2 the strains were gathered according to each species taking into account the enzymatic activities that could be involved in the aroma of finished wines. So, we found different enzymatic profiles in each species and each of them was made up of a different number of strains. Only one profile was described in *P. kluyveri*, two different profiles in *W. pratensis* and *S. salmonicolor*, three in *Cryptococcus* spp. and *D. hansenii*, five in *Candida* spp., *Z. bailii* and *T. delbrueckii* and six in *M. pulcherrima* and *L. thermotolerans*. Surprisingly, one *D. hansenii* profile (DA1) did not show any enzymatic activity and the TA2 profile in *T. delbrueckii* only showed the SR activity. The main differences in profiles within each species were mainly due to esterase, lipase and β glucosidase activity. Taking into account that information, for the next stage in the selection process based on aroma characteristics, we would choose the strains included in the following profiles, because they had all three enzymatic activities: CA5 of *Candida* spp., CCA1 of *Cryptococcus* spp., LA5 of *L. thermotolerans,* MA4 of *M. pulcherrima*, ZA3 of *Z. bailii*, and DA2 of *D. hansenii*, PKA1 of *P. kluyveri*, SPA2 of *S. salmonicolor*, TA5 of *T. delbrueckii*, WA1 of *W. pratensis*, and ZA5 of *Z. bailii*, as having two of the activities.

In Table 3 the strains were grouped according to the enzymatic activities involved in the color and clarity of the finished wines. The number of different enzymatic profiles found within each species were one in *P. kluyveri*, two in *W. pratensis* and *S. salmonicolor*, three in *Candida* spp., *Cryptococcus* spp., *D. hansenii*, and *Z. bailii*, six in *T. delbrueckii* and *L. thermotolerans* and finally nine in *M. pulcherrima*. In almost every species there were profiles which did not show any carbohydrolase activity: CC3 in *Candida* spp., SPC2 in *S. salmonicolor*, TC2 and TC3 in *T. delbrueckii*, MC8 and MC9 in *M. pulcherrima* and LC4 in

Table 2 Specific enzymatic profiles related to wine aroma

Species/profiles	Enzymatic activities									N	%
	Esterase	Esterase-Lipase	Lipase	LeucineA	ValineA	CystineA	ß-glucosidase	HCDC	SR		
Candida spp.											
CA 1	+	+	−	+	+	−	−	−	−	5	42
CA 2	+	+	−	+	−	−	−	−	−	3	25
CA3	−	+	−	+	+	−	−	−	−	1	8
CA 4	−	+	−	+	+	−	+	−	−	1	8
CA 5	+	+	−	+	+	−	+	−	−	2	17
Cryptococcus spp.											
CCA 1	+	+	−	+	−	−	+	−	−	2	40
CCA 2	+	+	−	+	−	−	+	−	+	1	20
CCA 3	+	+	−	+	−	−	−	−	−	2	40
Debaryomyces hansenii											
DA 1	−	−	−	−	−	−	−	−	−	1	33
DA 2	+	+	−	−	−	−	−	−	−	1	33
DA 3	+	+	−	−	−	−	−	−	+	1	33
Lachancea thermotolerans											
LA 1	+	+	−	+	+	−	−	−	−	11	70
LA 2	+	+	−	+	−	−	−	−	−	1	6
LA 3	−	+	−	+	+	−	−	−	−	1	6
LA 4	+	+	+	+	+	−	−	−	−	1	6
LA 5	+	+	+	+	+	−	+	−	−	1	6
LA 6	+	+	−	+	+	+	−	−	−	1	6
Metchnikowia pulcherrima											
MA 1	+	+	−	+	−	−	−	+	+	5	32
MA 2	+	+	−	+	−	−	+	+	+	2	12
MA 3	+	+	−	−	+	−	−	−	−	1	6
MA 4	+	+	−	+	+	−	+	−	+	5	32
MA 5	+	+	−	+	−	−	+	−	+	2	12
MA 6	+	+	−	+	+	−	+	+	+	1	6
Pichia kluyveri											
PKA 1	+	+	−	+	−	−	−	−	−	4	100
Sporodiobulus salmonicolor											
SPA 1	+	+	−	+	+	−	−	−	−	4	67
SPA 2	+	+	+	+	+	−	−	−	−	2	33
Torulaspora delbrueckii											
TA 1	+	+	−	−	−	−	−	−	+	1	6
TA 2	−	−	−	−	−	−	−	−	+	5	28
TA 3	+	+	−	+	−	−	−	−	+	8	44
TA 4	−	−	−	+	−	−	−	−	+	3	16
TA 5	+	+	−	+	−	+	−	−	+	1	6
Williopsis pratensis											
WA 1	+	+	−	+	+	+	−	−	−	9	90
WA 2	+	+	−	+	+	−	−	−	−	1	10
Zygosaccharomyces bailii											
ZA 1	+	+	−	+	−	−	−	−	+	1	14
ZA 2	+	−	−	+	−	−	−	−	+	1	14
ZA 3	+	+	−	+	−	−	+	−	+	1	14
ZA 4	−	+	−	+	−	−	−	−	−	2	29
ZA 5	+	+	−	+	−	−	−	−	−	2	29

Presence (+) or absence (−) of each activity; SR, sulfite eductase activity; HCDC, hydroxycinnamic acid decarboxylase activity; N, number of isolates showing each profile; %, percentage of each profile within the species

Table 3 Specific enzymatic profiles related to color and fining

Species/profiles	Enzymatic activities								N	%
	Pectinase	Cellulase	Xilanase	Glucanase	LeucineA	ValineA	CystineA	HCDC		
Candida spp.										
CC 1	+	−	−	−	+	+	−	−	3	25
CC 2	+	−	−	−	+	−	−	−	3	25
CC 3	−	−	−	−	+	+	−	−	6	50
Cryptococcus spp.										
CCC 1	−	+	−	+	+	−	−	−	2	40
CCC 2	+	+	−	+	+	−	−	−	1	20
CCC 3	++	−	−	−	+	−	−	−	2	40
Debaryomyces hansenii										
DC 1	+	−	−	+	−	−	−	−	1	33
DC 2	+	+	−	+	−	−	−	−	1	33
DC 3	+	−	−	−	−	−	−	−	1	33
Lachancea thermotolerans										
LC 1	−	+	+	+	+	+	−	−	9	56
LC 2	−	+	−	+	+	+	−	−	2	13
LC 3	−	−	−	+	+	+	−	−	1	6
LC4	−	−	−	−	+	+	−	−	2	13
LC 5	+	−	−	−	+	+	−	−	1	6
LC6	+	+	+	+	+	+	+	−	1	6
Metchnikowia pulcherrima										
MC 1	+	−	−	−	+	−	−	+	5	31
MC 2	+	+	−	−	−	−	−	−	2	13
MC 3	+	−	−	−	+	+	−	−	2	13
MC4	+	+	−	−	+	−	−	+	1	6
MC 5	−	+	−	−	+	+	−	−	2	13
MC6	−	−	−	+	+	+	−	+	1	6
Pichia kluyveri										
PKC 1	+	−	−	−	+	−	−	−	4	100
Sporodiobulus salmonicolor										
SPC 1	+	−	−	−	+	+	−	−	4	67
SPC 2	−	−	−	−	+	+	−	−	2	33
Torulaspora delbrueckii										
TC 1	+	−	−	−	−	−	−	−	5	28
TC 2	−	−	−	−	−	−	−	−	1	6
TC 3	−	−	−	−	+	−	−	−	2	11
TC4	+	+	−	−	+	−	−	−	2	11
TC 5	+	−	−	−	+	−	−	−	7	38
TC6	+	−	−	−	+	−	+	−	1	6
Williopsis pratensis										
WC 1	−	−	−	−	+	+	+	−	9	90
WC 2	−	−	−	−	+		−	−	1	10
Zygosaccharomyces bailii										
ZC 1	+	+	−	−	+	−	−	−	2	29
ZC 2	+	−	−	−	+	−	−	−	3	42
ZC 3	−	−	−	−	+	−	−	−	2	29

Presence (+) or absence (−) of each activity; SR, sulfite reductase activity; *HCDC* hydroxycinnamic acid decarboxylase activity; N, number of isolates showing each profile; %, percentage of each profile within the species

L. thermotolerans. No strain within the *W. pratensis* species showed any of these activities. However nine out of the ten strains of this species showed the three aminopeptidase activities involved in proteolysis. In this last group of enzymes related to natural wine fining, there were also strains which did not exhibit any activity: all the *D. hansenii* strains, TC1 and TC2 in *T. delbrueckii* and MC2 in *M. pulcherrima*. The most surprising result was found in the profile LC6 of *L. thermotolerans*, as it was the only strain that exhibited all the activities studied included in this group; the four carbohydrolases and the three aminopeptidases. So, this strain could be an excellent candidate for improving color and limpidity in red wines. What is more, this strain could even increase acidity level in wines due to the ability of *L. thermotolerans* species to produce lactic acid during alcoholic fermentation (Gobbi et al. 2013).

Taking into account the overall results, the most suitable strains within each species to continue in a selection process based on color and limpidity characteristics would be: CC1 in *Candida* spp., CCC2 in *Cryptococcus* spp., DC2 in *D. hansenii*, LC6 in *L. thermotolerans,* MC4 and MC7 in *M. pulcherrima*, PKC1 in *P. kluyveri*, SPC1 in *S. salmonicolor*, TC4 in *T. delbrueckii*, WC1 in *W. pratensis* and ZC1 in *Z. bailii*,. However, to exploit the benefits of non-*Saccharomyces* yeasts in wine production it is necessary to know the implications of using these yeasts for winemaking practices. Further work is now required to examine both the production and the activity of the observed enzymes and the development of the selected strains under vinification conditions, and to investigate what impact such activities have on the sensory properties of wine.

Conclusion

This study has revealed the potential for non-*Saccharomyces* yeasts to produce extracellular enzymes of enological significance. The screening showed a wide intraspecific difference within the yeast species investigated, indicating that strain selection is of great importance, as not all strains within a species would necessarily show the same desirable or undesirable characteristics. Besides, these data confirmed the need to carry out a selection process according to the different kind of wines the yeasts are destined to produce, or the different wine characteristics they have to improve. However, the potential of these enzymes to modify grape and wine composition and the impact of these enzymatic activities on the aroma and color profile of finished wines needs to be studied further.

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