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Review

Lachancea thermotolerans Applications in Wine Technology

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Abstract: *Lachancea (kluyveromyces) thermotolerans* is a ubiquitous yeast that can be naturally found in grapes but also in other habitats as soil, insects and plants, extensively distributed around the world. In a 3-day culture, it shows spherical to ellipsoidal morphology appearing in single, paired cells or short clusters. It is a teleomorph yeast with 1–4 spherical ascospores and it is characterized by a low production of volatile acidity that helps to control global acetic acid levels in mixed or sequential inoculations with either *S. cerevisiae* or other non-*Saccharomyces* species. It has a medium fermentative power, so it must be used in sequential or mixed inoculations with *S. cerevisiae* to get dry wines. It shows a high production of lactic acid able to affect strongly wine pH, sometimes decreasing wine pH by 0.5 units or more during fermentation. Most of the acidification is produced at the beginning of fermentation facilitating the effect in sequential fermentations because it is more competitive at low alcoholic degree. This application is especially useful in warm areas affected by climatic change. pH reduction is produced in a natural way during fermentation and prevents the addition of tartaric acid, that produces tartrate precipitations, or the use of cation exchangers resins highly efficient reducing pH but with undesirable effects on wine quality. Production of lactic acid is done from sugars thus reducing slightly the alcoholic degree, especially in strains with high production of lactic acid. Also, an improvement in the production of 2-phenylethanol and glycerol has been described.

Keywords: *Lachancea thermotolerans*; *Kluyveromyces thermotolerans*; acidification; wines; sequential fermentations; non-*Saccharomyces*

1. Introduction

Lachancea thermotolerans was formerly known as *Kluyveromyces thermotolerans*, but it was recently reassigned in the genera *Lachancea* according to multigene sequence analysis [1]. *L. thermotolerans* (LT) is a global yeast species that can be usually found in grapes but also in other habitats as soil, insects and plants [2] and extensively distributed around the world [3]. It can be found in natural spontaneous wine fermentations with a low prevalence on days 2–4 of fermentation [4]. Morphologically, it is globous or ellipsoidal, undistinguishable from *S. cerevisiae* (Figure 1) and can be found as single cells in liquid media or in small groups. It is a teleomorph yeast presenting sexual reproduction with the formation of spherical ascospores (1–4). Asexual reproduction is produced with multilateral budding. LT forms creamy colonies with butyrous texture in solid media.

LT is able to ferment glucose and sucrose [5] and weakly galactose. It shows variable capacity to ferment maltose, trehalose and raffinose [6]. Nitrogen nutrition is similar to *S. cerevisiae* being

necessary a minimum of 200 mg/L of YAN (yeast assimilable nitrogen) to avoid sluggish or stuck fermentations [7]. Serine as N-source also has shown an improvement in the fermentation performance of LT [8]. Strains of LT can express the following extracellular enzymatic activities with effect in wine aroma or phenol extraction: Esterase, Esterase-Lipase, ß-glucosidase, Pectinase, Cellulase, Xylanase, Glucanase [9].

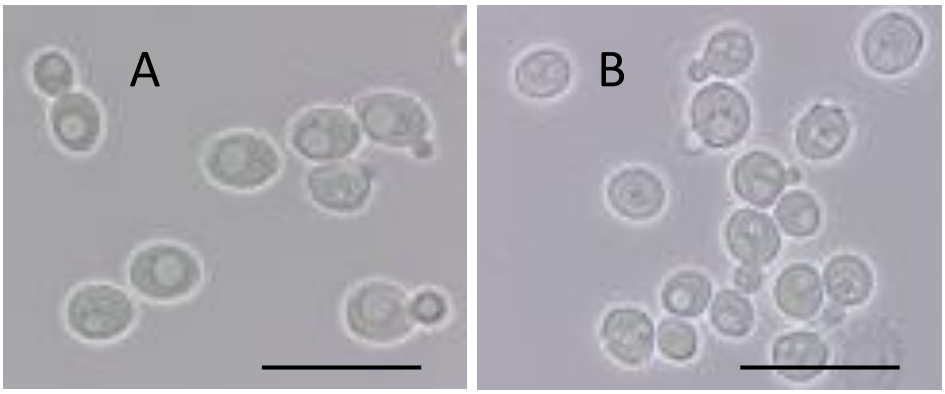

Figure 1. Optical microscopy (**A**) *Saccharomyces cerevisiae* (**B**) *Lachancea thermotolerans*. Scale 10 µm.

LT has a moderate fermentative power, and an ethanol tolerance around 5–9% *v*/*v* has been published [10–14]. Some effect can be observed in the reduction of the alcoholic degree of wines (0.7% *v*/*v*; [15]). Concerning resistance in the time, it was observed that it is able to survive several days in the presence of 9% *v*/*v* of ethanol [11,16], and it also has a good persistence even when the fermentation is dominated by *S. cerevisiae* [17]. These metabolic properties make it appear during the intermediate phase of the fermentative process before the full prevalence of high fermentative *S. cerevisiae* strains. The use of LT in sequential or mixed fermentations has some tendency to produce sluggish fermentations with more difficulties fermenting the fructose fraction of the grape sugars [7]. Moreover, an oxygen availability requirement for LT persistence seems to be higher than for *S. cerevisiae* [18]. The tolerance to temperature is similar to average strains of *S. cerevisiae* showing a good growth at 25–30 °C, but slower growth below 20 °C [5].

Comitini et al. [12] identified 5 isolates that are able to resist 10–20 mg/L of free SO_2, but it is possible to find strains resistant to more than 100 mg/L of total SO_2 [14]. The production of H_2S is variable from medium to high (25 isolates). Comitini et al. [12] also observed in 5 strains of LT a production of SH_2 ranging from 3–5 in a 0–5 scale. Resistance to DMDC is low, from 25 to 100 mg/L for populations ranging log2–log6 CFU/mL, while typical values for *S. cerevisiae* are 100–300 mg/L with same population [19].

LT has been produced at the commercial level as dry yeasts since 2012 (CONCERTO™ and MELODY™) and recommended to increase flavor complexity and intensity, to improve total acidity and to reduce volatile acidity [20,21]. It is well established that LT is capable of producing wines with higher 'spicy' and acidic notes, thus improving the overall quality of wine [13,22]. It is also described as producer of ethyl isobutyrate (strawberry nuances). Improvements in fruitiness, probably favored by the increase in acidity, are typical sensory descriptors when LT is used to ferment neutral varieties [23].

Some LT strains have been used as fungal biocontrol agents in grapes and vines to inhibit the growth of *Aspergillus* [24]. These strains do not affect the metabolic properties and performance of *S. cerevisiae* during alcoholic fermentation [25].

2. Isolation and Selection

As most of other non-*Saccharomyces* yeasts, LT can be distinguished from *S. cerevisiae* using lysine media (Figure 2; [26] and culturing temperatures in the range 25–28 °C. The use of chromogenic media is quite useful (Figure 2) for the initial isolation of yeasts belonging to this species. In CHROMagar®

they show a characteristic red-brown color that can be distinguished easily from the purple colonies of *Saccharomyces* or the creamy colors of most of the other yeasts.

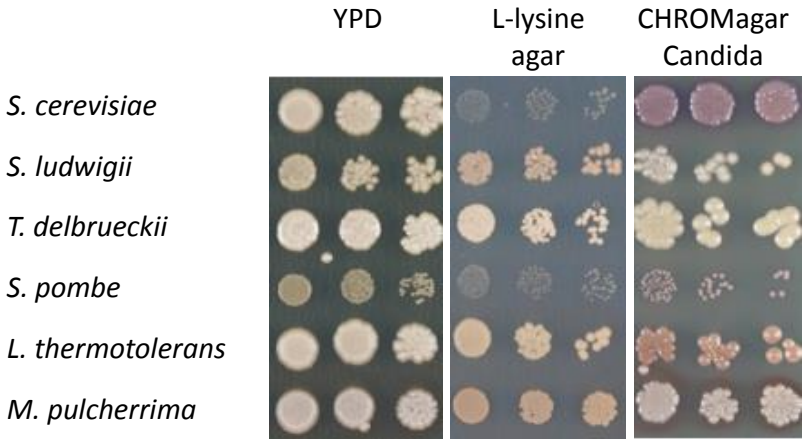

Figure 2. Colony shape and color in YPD, L-lysine specific to isolate non-*Saccharomyces* and Chromogenic media (Adapted from Loira et al. [26]).

The use of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been proposed to identify LT during the study of the ecology of wine grapes, but with a low sensibility, needing a population of at least log2 CFU/mL [27]. Microsatellite markers and a multilocus SSR analysis have been developed to assess the genetic diversity of LT isolates [28]. Identification of LT isolates can be performed by sequencing of the D1/D2 region of the 26S rRNA gene and RAPD fingerprinting what allows yeast identification at species level [29,30]. Moreover, restriction patterns of amplified regions of 26S rDNA can be used as a routine methodology to identify non-*Saccharomyces* yeast species during red wine fermentation [31] (Figure 3). Finally, specific PCR primer pairs for the intron 2 of the mitochondrial COX1 gene, allow detect *L. thermotolerans* in wine at 10^4 cells/mL and with a *S. cerevisiae*/*L. termotholerans* ratio of 1000/1 [32].

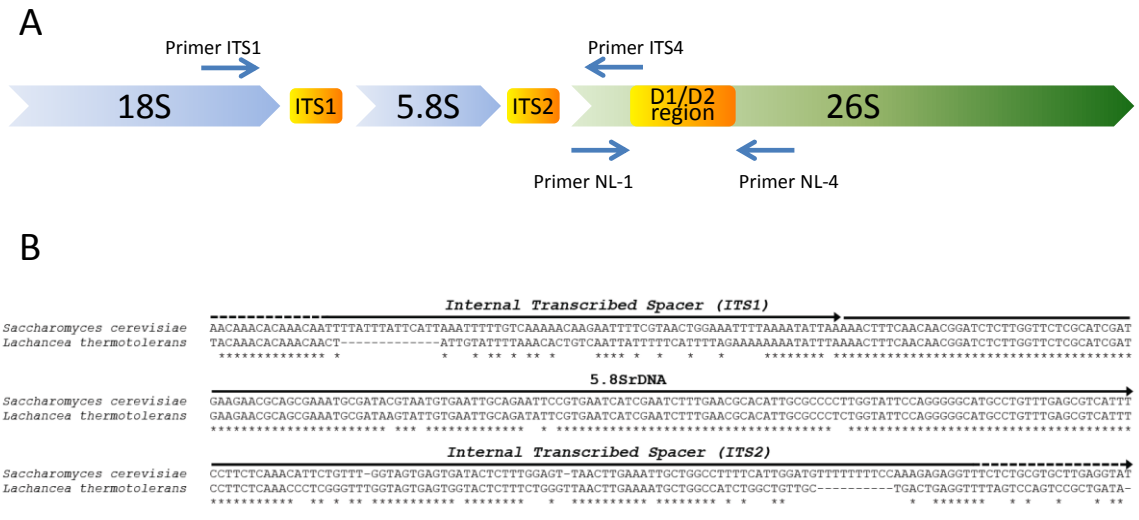

Figure 3. (**A**) Schematic diagram of the yeast rDNA gene cluster. The 18S, 5.8S and 25–28S rDNA genes are separated by the internal transcribed spacers 1 (ITS1) and 2 (ITS2). Primers for routine sequencing are shown; (**B**) Alignments of complete 5.8S rDNA sequence and partial sequences of Internal Transcribed Spacer 1 and 2. The marked sequences are identical regions. GenBank accession numbers: for *S. cerevisiae*, KT958553.1 and for *L. thermotolerans*, CU928180.1.

3. Biotechnological Application: Wine Acidification

Selected LT strains have been used for acidification of fermented beverages as wines [13,33,34] and beers [35–37]. LT is a strong producer of lactic acid [33] with significant influence in wine pH, also in other fermented beverages (Table 1). Lactic acid is stable after fermentation and ageing because it is neither chemically degraded nor microbiologically metabolized under enological conditions. Concentrations can range 1–16.8 g/L [28]. Most of the acidification is produced at the beginning of fermentation (Figure 4A), which facilitates the acidification in sequential fermentations with *S. cerevisiae* at low-medium ethanol levels (<6% *v*/*v*) when LT is competitive with *S. cerevisiae*. The acidification strongly influences pH being possible to reduce more than 0.5 pH units from an initial pH of 3.8–4 (Figure 4B). This has interesting implications in wines of warm areas because at pH near 4 the wine is unprotected and many spoilage microorganisms can grow even in absence of residual sugars and high alcoholic degree. Molecular SO_2 levels at pH 4 are very low (<0.5 mg/L) even when free SO_2 concentration can be higher than 50 mg/L. These values are unsuitable for conflictive spoilage yeasts like *Brettanomyces*/*Dekkera* that needs 0.8 mg/L of molecular SO_2 to be controlled [38]. The use of LT in mixed or sequential fermentations with *S. cerevisiae* when pH is decreased to 3.5–3.7 promotes better levels of molecular SO_2 with low contents of total sulfites, making the fermentations and especially the ageing process safer.

Table 1. Acidification biotechnologies using *Lachancea thermotolerans* in wine fermentation suitable to be used in high pH musts from warm regions.

Biotechnology	LT Fermentation Time	Initial pH	Final pH	Comments
Sequential fermentation LT → *S. Cerevisiae* LT → *S. pombe*	0–4 days Most of the acidification is performed in the 3 first days	3.9–4.2	3.5–3.7 depending on LT strains and implantation success	Population inoculated of LT must be >log6 CFU/mL
Mixed fermentation LT + *S. Cerevisiae* LT + *S. pombe*	0–6 days	3.9–4.2	3.5–3.7 depending on LT strains and implantation success	Population inoculated of LT must be >log6 CFU/mL Ratio between LT + *S. cerevisiae* (or *S. pombe*) must be log6/log2 including wild Sacch.
Coinoculation LT + LAB (*O. oeni*) and subsequent inoculation of *S. cerevisiae*	0–6 days	3.9–4.2	3.3–3.5 depending on LT strains, implantation success and lactic acid production by LAB	Strong pH reduction. Light alcohol degree reduction

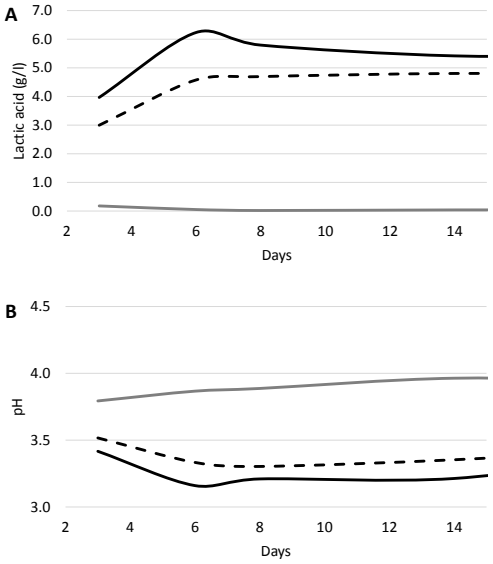

Figure 4. Lactic acid production (**A**) and pH (**B**) evolution during the fermentation of a red grape must with 240 g/L of sugar by *Lachancea thermotolerans* strain L3.1 (black line), *L. thermotolerans* and sequentially *S. cerevisiae* (dashed line) and *S. cerevisiae* (grey line).

LT is unable to entirely ferment a grape must as said, reaching maximum ethanol concentrations around 9% *v*/*v*, even when especially selected yeasts are used. So, it is necessary to use sequential or mixed fermentations with *S. cerevisiae* [7,12] or *S. pombe* [39,40] to get completely dry wines without residual sugars. The use of an inoculation ratio log7:log3 cfu/mL (LT/*S. cerevisiae*) is suitable to see a significant effect in pH reduction [12]. Suitable implantation to reach an effective acidification has also been tested according to inoculum size in mixed fermentations with *S. cerevisiae* [13]. However, the best results were reached in sequential inoculation after 48 h, decreasing the pH values from 3.53 in *S. cerevisiae* control fermentation to 3.33 [13]. When the inoculation is performed after 24 h, pH reduction is lower than 0.1 units, statistically significant, but probably without enological repercussions.

The production of lactic acid during fermentations with variable glucose contents in model media (8, 12, 16% *w*/*w*) is stable and independent of sugar levels, ranging from 2.48 ± 0.8–2.66 ± 0.9 g/L for LT strain L3.1 [41]. However, when the same sugar concentrations were tested in the presence of 2 g/L of lactic acid, the production by LT was affected, ranging from 1.25 ± 0.7 g/L (in model media with 8% *w*/*w* glucose) to 3.68 ± 0.3 g/L (with 16% *w*/*w* glucose). Nitrogen contents also affect the production of lactic acid by LT, being reduced below 150 mg/L of yeast assimilable nitrogen (YAN), but also at high concentration (>500 mg/L) (Figure 5). Highest lactic acid production is correlated with suitable YAN levels for yeast nutrition (150–200 mg/L) and subsequently with higher yeast populations (Figure 5).

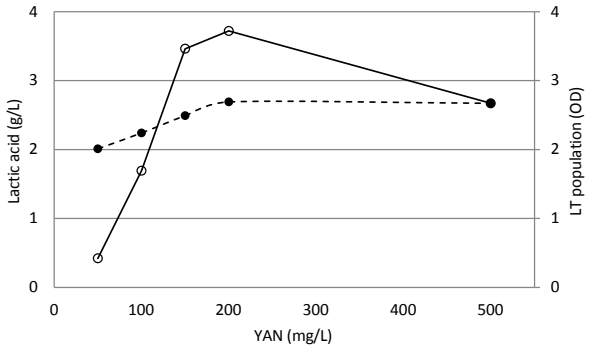

Figure 5. Lactic acid production during fermentation by *L. thermotolerans* strain L3.1 at variable yeast assimilable nitrogen (YAN) concentrations (black line). LT population estimated by OD (dashed line). Adapted from Hernández [41].

The use of LT fermentations together with *S. pombe* has been described as an alternative biotechnological tool to emulate malolactic fermentation (MLF) [42]. However, even when it can be similar in terms of acidification, the sensory profile reached in a typical MLF is more complex [43].

Initial evidence suggests that the use of LT is compatible with MLF [44]. However, lactic acid at high concentration (>4 g/L), as can be produced when LT is used during fermentation, can behave as an inhibitor of lactic acid bacteria (LAB), thus hindering MLF. It is possible to use yeast-bacteria co-inoculations to facilitate the MLF by promoting the simultaneous development of alcoholic and malolactic fermentations. The use of LT-LAB co-inoculations with subsequent *S. cerevisiae* sequential fermentation (LT-LAB-SC) is quite effective to degrade malic acid and at the same time reach lower pH values. When this biotechnology has been used at industrial level in fermentations of 1 ton of crushed grapes, the final pH reached 3.3 while the control only fermented by *S. cerevisiae* remained at pH 4 that was the initial grape value. In this case, some acidification is produced by LT and a complementary amount of lactic acid is produced by fermentation of sugars by LAB. Both microorganisms work promoting the formation of stable acidity in natural enological conditions. Achieving these pH values is only possible by using ion exchange resins, but affecting strongly wine composition and quality.

Traditionally, MLF is a way to obtain microbiologically stable red wines with a better sensory profile, but it can also affect the freshness in wines of warm areas. However, the use of LT with the production of high contents of lactic acid (>4 g/L) because of its inhibitory effect on LAB, can be an

approach to obtain fresh wines in warm regions protecting malic acidity but also increasing lactic acidity and with a suitable stability.

4. Metabolic Profile and Influence on Wine Aroma and Flavor

The use of LT in wine has been described with a low production of volatile acidity (0.3–0.5 g/L [12,16]). LT was reported as a useful biotechnological tool to decrease volatile acidity [45]. Acetaldehyde levels similarly can be diminished by using LT during fermentation [22,46]. LT has been described as a moderated producer of higher alcohols [22]. The production of ethyl acetate is quite moderate (40–60 mg/L, [13]). Sequential fermentations of LT with *S. pombe* produce similar concentrations than with *S. cerevisiae* (40–50 mg/L, [40]). It is an interesting yeast to control volatile acidity produced by *S. pombe* to make full fermentations in absence of *S. cerevisiae* [39]. The production of ethyl lactate is moderate in sequential fermentations with *S. cerevisiae* (7–8 mg/L) and in mixed fermentations with *S. pombe* (8–32 mg/L) [39,40].

LT have been described as producer of β-D-glucosidase (βDG) [47] and carbon-sulfur lyase (CSL) [48], enzymes involved in the release of aroma compounds from must varietal precursors (Table 2). Usually non-*Saccharomyces* are more effective producing 3-mercaptohexan-1-ol (3MH) than 4-mercapto-4-methylpentan-2-one (4MMP) [49]. However, when LT have been used in must fermentation, significant amounts of 4MMP also moderate amounts of 3MH were released. The production of significant concentrations of 4-methyl-4-sulfanylpentan-2-one (4MSP; box-tree aroma) and 3-sulfanylhexan-1-ol (3SH; grapefruit and passion fruit hints) has also been described [48].

Table 2. Effect of *Lachancea thermotolerans* in wine fermentation.

Acidification pH or Lactic Acid (g/L)	Fermentative Power (Ethanol % *v/v*)	Aroma, Flavor, Polysaccharides and Color	Molecules	Reference
3.5 → 3.2; 5.1 g/L lactic acid	9	Acidity	Lactic acid	[16]
3.2 → 2.9 in coinoculation	4–8			[12]
3.53 → 3.33 sequential 48 h 0.1 units reduction sequential 24 h	10.5			[13]
1–16.6 g/L				[28]
1.2–2.6 g/	9.5–10.4			[14]
		Esters	2-phenylethanol, phenethyl propionate, ethyl salicylate, methyl salicylate, 3-methylthio-1-propanol	[50]
		Enhanced formation of terpenes & Thiols	Nerol, terpinen-4-ol 4MSP & 3SH	[48]
		β-D-glucosidase Carbon-sulfur lyase	Free terpenes and thiols	[47,48]
	7.7	Polysaccharides/ mannoproteins	*N*-acetyl hexosamines	[51]
		Polyalcohols	Glycerol	[12,16]
		Polymeric pigments	malvidin-3-glucoside-ethyl-catechin dimer	[40]

The use of LT in the fermentation of Syrah and Sauvignon blanc musts increased the formation of 2-phenylethanol, phenethyl propionate, ethyl salicylate, methyl salicylate, 3-methylthio-1-propanol [50]. The contents of terpenes nerol and terpinen-4-ol were also positively affected (Table 2). LT general effect in aroma profile is the production of several acetate esters and certain terpenes [52].

One of the most highlighted roles of *L. thermotolerans* is the production of glycerol during wine fermentation. This increase in glycerol is observed during spontaneous fermentation [12,53,54], and sequential inoculations between *L. thermotolerans* and *S. cerevisiae* [12,13,16]. However, in the case of sequential inoculations the main advantage is that glycerol is generated with a decreased volatile acidity and acetic acid concentration [12,55]. The production of glycerol is also highly related to the fermentation temperature [13] and it increases with oxygenation [56]. Generally speaking, the extent of influence that *L. thermotolerans* can exert on a given fermentation is relative to the amount of time it spends alone in contact with the grape must [13,16]. Glycerol, the next major yeast metabolite after ethanol, is associated with the smoothness (mouth-feel), sweetness and complexity in wines [57]. However, the sensory impact of glycerol is also intimately related to the grape variety and wine style [58].

Other relevant application of LT is the sensory improvement of typical regional wines [59,60]. Recently, it has been observed the key contribution of microbioma influence in terroir finger print of regional wines [61].

5. Effect on Wine Color

Yeasts can affect wine color by pH reduction [62], favoring the formation of stable pigments such as pyranoanthocyanins [63–66] or polymeric pigments [40,67], reducing the adsorption of grape anthocyanins in cell walls [66,68,69], or protecting the anthocyanins from oxidative damage by releasing reductive compounds like glutathione during fermentation and ageing on lees [62].

As for acidification, some yeasts are able to reduce wine pH during fermentation through the release or transformation of certain organic acids. Color intensity of anthocyanins is pH dependent. The production of lactic acid by LT during fermentation can affect strongly the acidity with reductions of 0.3–0.5 pH units in some cases, so it can affect wine color in a significant way. Moreover, as the lactic acid is stable during ageing, this effect can be permanent in wine. Additionally, it should be considered that acidity also helps to protect wine color by producing higher levels of molecular SO_2.

The effect of the formation of pyranoanthocyanins like vitisins during fermentation is promoted by the release of the precursors: Pyruvic acid for vitisin A and acetaldehyde for vitisin B. The correlation between the excretion of these metabolites by *S. cerevisiae* strains and the subsequent condensation with malvidin-3-*O*-glucoside has been previously reported [63]. Not significant effects on the formation of vitisins have been observed when LT has been used in sequential fermentations with *S. cerevisiae* [39,40]. Some improvements can be seen when LT is used sequentially with *S. pombe*, but this is due to the contribution of this last yeast. The selection of appropriate strains can increase the formation of vitisins during fermentation, but it does not seem that LT is a good promoter for fermentative formation of vitisins. Similar results have been published for the formation of vinylphenolic pyranoanthocyanins [39,40], so probably most of the LT strains do not express hydroxycinnamate decarboxylase activity. Concerning the formation of polymeric pigments, sequential fermentations of LT with *S. cerevisiae* and especially with *S. pombe* favors the formation of malvidin-3-glucoside-ethyl-catechin dimer [40] (Table 2).

The absorption of anthocyanins in yeast cell walls can be between 3 and 6% of total content in wines [69]. Adsorption is strain dependent in *S. cerevisiae* [68] but also variable among non-*Saccharomyces* species [62]. Compared with other species, LT has shown a medium-high adsorption capacity.

6. Special Wines

The use of LT could be also interesting in special sweet wines to better balance sweetness and acidity. The fermentative production of lactic acid by LT can help to make the strongly sweet wines like ice wines more pleasant, increasing the freshness when the grape acidity levels are unsuitable. Using LT, it could be possible to ferment at around 8–10% *v*/*v* of ethanol, remaining natural residual sugars together with a well-balanced acidity.

The quality of natural sparkling wines with second fermentation in bottle is strongly dependent on freshness, and therefore, in acidity. When the base wine is produced in warm areas frequently it lacks of enough acidity what affects sensory quality in mouth, but also the stability during second fermentation and ageing. The use of LT in the production of base wines is an interesting biotechnological tool to provide them of enough stable acidity to make safer fermentations in bottle, but also reaching better sensory profiles.

The complementary use of fructophilic non-*Saccharomyces* yeasts, such as *Candida zemplinina* [70], helps to increase sugar consumption enhancing at the same time flavor, what can be useful in some wine types. *C. zemplinina* also improve mouthfeel and roundness due to its high production of glycerol [70].

7. Conclusions

LT is a really interesting non-*Saccharomyces* yeast species to improve the quality of wine fermentation. The natural acidification by production of stable lactic acid facilitates safer wines, less prone to spoilage, with a higher freshness and with lower levels of SO_2. Also, it opens the possibility of new biotechnologies as the simultaneous co-fermentation with lactic acid bacteria reaching even lower pHs and also consuming sugars what can reduce slightly the alcohol content. It would be interesting to go deeper into the selection of LT strains to get a stronger fermenter able to surpass 10% v/v in ethanol content.

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