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Review

Lachancea thermotolerans, the Non-*Saccharomyces* Yeast that Reduces the Volatile Acidity of Wines

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Abstract: To improve the quality of fermented drinks, or more specifically, wine, some strains of yeast have been isolated, tested and studied, such as *Saccharomyces* and non-*Saccharomyces*. Some non-conventional yeasts present good fermentative capacities and are able to ferment in quite undesirable conditions, such as the case of must, or wines that have a high concentration of acetic acid. One of those yeasts is *Lachancea thermotolerants* (*L. thermotolerans*), which has been studied for its use in wine due to its ability to decrease pH through L-lactic acid production, giving the wines a pleasant acidity. This review focuses on the recent discovery of an interesting feature of *L. thermotolerans*—namely, its ability to decrease wines' volatile acidity.

Keywords: pioneering winemaking techniques; peculiar yeasts; volatile acidity; fermented drinks

1. Introduction

The pioneering of winemaking techniques and new yeast strains contributes to improving the quality of wines worldwide and offering solutions to various problems, such as increased sugar concentrations at grape maturity, or excessively acidic wines. Some non-*Saccharomyces*, as well as some non-conventional species of *Saccharomyces*, present good fermentative capacities and, are able to produce wines with lower levels of ethanol and higher concentrations of glycerol [1]. They are also able to avoid stuck fermentations, as they can grow at lower temperatures [2,3] as well as being nitrogen [4] and salt tolerant [5]. Moreover, mixed inoculations of non-*Saccharomyces*, *S. cerevisiae* yeasts, and lactic acid bacteria (LAB) in sequential fermentations are of great interest to the wine industry for various technological and sensorial reasons [6]. In addition, a peculiar microbial footprint that is characteristic of a particular wine region may be imprinted onto a wine if inoculation with autochthonous yeast is performed [3].

A non-*Saccharomyces* species not yet well-explored with huge biotechnological potential is *Lachancea thermotolerans* [7], formerly known as *Kluyveromyces thermotolerans* [8]. The genus *Lachancea* was proposed by Kurtzman in 2003 to accommodate a group from several different genera showing similarities at the rRNA level. According to Lachance and Lachancea [9], the genus continues to anchorage 11 other species to this day: *L. cidri*, *L. dasiensis*, *L. fantastica*, *L. fermentati*, *L. kluyveri*, *L. lanzarotensis*, *L. meyersi*, *L. mirantina*, *L. nothofagi*, *L. quebecensis*, and *L. walti*. As so-called protoploid *Saccharomycetaceae*, the *Lachancea* species has diverged from the *S. cerevisiae* lineage prior to the ancestral whole genome duplication, and as such, offers a complementary model for studying evolution and speciation in yeast [10].

Another peculiarity of *L. thermotolerans* is its ability to produce L-lactic acid during alcoholic fermentation [11]. Although lactic acid production is uncommon among yeasts, it is of great biotechnological interest in regard to fermentation processes where alcoholic fermentation with concomitant acidification is a benefit, such as winemaking [12].

Sometimes, to select a certain strain with specific enological features, scientists choose to use a genetic engineering approach. However, these techniques are unfortunately quite time-consuming and expensive. Moreover, metabolic engineering based on recombinant technology has some regulatory issues, such as the use of genetically modified organisms (GMO) in the food and wine industry [13]. The alternative solution to genetic manipulation is evolutionary engineering [14,15], which allows for improvements to phenotypes of choice. This methodology is based on the combination of confined environmental selection and natural variability. Evolutionary engineering aims to create an improved strain based on the selection of behavioral differences between individual cells within a population. The reason why non-recombinant strategies based on evolutionary engineering are eye-catching is that they may be able to generate better-quality strains that are not considered to be GMOs. Evolutionary engineering has long been used for generating new industrial strains [16,17], and of course, the first step is always to be well-focused on the selection criteria.

Our starting point for this research was the following question: Are indigenous yeasts—or more specifically, *L. thermotolerans*—able to reduce volatile acidity from musts and wines?

2. The Vinegar Taint Problem in Wine

In excessive quantities, volatile acids are considered to be a spoilage characteristic of wines, as they confer an unpleasant vinegary aroma along with an acrid taste. The main component of the volatile acidity of wines and musts is acetic acid. The maximum acceptable limit for volatile acidity in most wines is 1.2 g L^{-1} of acetic acid [18], but the aroma threshold for acetic acid depends on the variety and style of the wine, being the vinegary smell recognizable at acetic acid concentrations of 0.8–0.90 g L^{-1} [19]. Acetic acid can be formed at any time during the wine-making process. It can appear on the grapes or the grape-must due to a myriad of yeasts (*Hansenula* spp. and *Brettanomyces bruxellensis*), filamentous fungi (*Aspergillus niger*, *Aspergillus tenuis*, *Cladosporium herbarum*, *Rhizopus arrhizus*, and *Penicillium* spp), and bacteria (LAB-like indigenous *Lactobacilli*, otherwise known as "ferocious", and acetic acid bacteria). It can also appear during the alcoholic fermentation process as a by-product of *S. cerevisiae* sugars' metabolism, or due to some contamination by spoilage yeasts (*Pichia anomala*, *Candida krusei*, *Candida stellate*, *Hansaniaspora uvarum/Kloeckera apiculate*, and *Saccharomycodes ludwigii*) or bacteria (*Acetobacter pasteurianus* and *Acetobacter liquefaciens* that survive during fermentation); after MLF (malolactic fermentation), due to heterofermentative species of *Oenococcus* and *Lactobacillus* that have the potential to produce acetic acid through the metabolism of residual glucose (usually no more than 0.1–0.2 g L^{-1} of acetic acid); or in the bottled wine, due to spoilage by contaminating yeasts and/or bacteria [20,21].

3. *L. thermotolerans*' Main Features in Alcoholic Drinks

In recent years, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) has been studied for its use in wine and beer, due to its ability to decrease pH through lactic acid production [22]. *K. thermotolerans* was alienated from the other species of *Kluyveromyces* and placed in *Lachancea* due to its distinct genetic and metabolic differences, as well as its genetic similarity to other members of the *Lachancea* genus [8].

This yeast is often found in a selection of fruits, such as on the surface of grapes. Consequently, it is present at the beginning of many fermentations before *Saccharomyces*' domination. Due to the production of L-lactic acid (from 0.23 to 9.6 g L^{-1}, depending on the different trial conditions [23,24]), wine produced by fermentation with *L. thermotolerans* is considered to display some different sensory properties—mainly in terms of mouth-feel—with an increased acidic taste [23–25]. Some winemakers desire to have varying degrees of these traits in their wines, and now, *L. thermotolerans* is present in a few commercial yeast inoculates.

For instance, strain 617 of *L. thermotolerans* was selected amongst other non-*Saccharomyces* yeasts to perform combined fermentations with *S. cerevisiae*, in order to increase the acidity and quality of Spanish Airén wine [25]. Although this Spanish grape variety is considered to be very neutral and

productive, the wines it is used in are usually considered to be low quality due to its high sugar content and lack of acidity [25].

During wine fermentation, *L. thermotolerans* also causes an increase in levels of ethyl lactate [19]. Due to these metabolic features (lactic acid and ethyl lactate production), this yeast is currently being studied for the purposes of producing beer without the necessity of LAB inoculation [26]. Thus, using *L. thermotolerans* to produce beer with a sourer taste may be simpler than trying to maintain a co-fermentation with yeasts and bacteria.

However, the metabolic pathway of converting sugars to lactic acid by *L. thermotolerans* is not completely understood. Recent discoveries to date have shown that levels of lactic acid between 1–9 g L^{-1} were found in wine which was fermented with this yeast species [23]. The metabolism of sugars into lactic acid is also a way to reduce the level of alcohol in wines, and a reduction of up to 0.5 to 1% (*v*/*v*) of alcohol is possible [23].

Benito et al. [27] investigated the application of *L. thermotolerans* and *Schizosaccharomyces pombe* as an alternative to the classic malolactic fermentation in a wine made with *Vitis vinifera L.* cultivar Tempranillo. While *S. pombe* totally consumed the malic acid, *L. thermotolerans* produced lactic acid, which allowed excessive deacidification to be avoided. In addition, the results from the fermentation trails showed positive differences in several parameters, such as acetic acid, glycerol, acid profile, sensory evaluation, color, and anthocyanin profile. Moreover, Benito et al. [24] also demonstrated that wines crafted through this technique had biogenic amines (BAs) levels lower than 2 mg L^{-1}. The combined use of two non-*Saccharomyces* strains allowed for a reduction of the value of all measured BAs, in comparison with the use of *Saccharomyces* and malolactic fermentation, from 0.44 *vs*. 1.46 mg L^{-1} and 1.71 *vs*. 2.18 mg L^{-1}, respectively. The authors [24] also stated that these differences should be attributed to the ability of *Schizosaccharomyces pombe* to metabolize urea [28].

4. Strain Isolation and Wine Biodeacetification

Several approaches have been developed in which regards to the deacetification of wines, including "empirical" enological techniques, where acidic wines are refermented by mixing them with marc from a finished wine fermentation, or by mixing them with freshly-crushed grapes or musts. More modern techniques have been explained and studied in enological, biochemical, and microbiological terms [20,29–31]. Under aerobic conditions, acetate can be used as a sole source of carbon and energy for the purposes of energy generation and cellular biomass [32]. This feature is not just present in *S. cerevisiae* strains—some *Zygosaccharomyces bailii* strains also display biphasic growth in media containing mixtures of glucose and acetic acid [33].

In previous works, such as a study by Vilela et al. [34], several yeast strains have been isolated (e.g., *Saccharomyces* and non-*Saccharomyces*) in Wallerstein Laboratory Nutrient Agar (WL) media using the refermentation processes of acidic wines, at winery scale [34]. Among all isolates, a group of yeasts was selected for testing for their ability to consume acetic acid in the presence of glucose, using a differential medium containing acetic acid and glucose adapted from Schuller et al. [35], shown in Figure 1.

Four of those isolates in this medium were obtained and characterized by fingerprinting with primer T3B, then identified by the amplification of the D1–D2 variable domain at the 5′ end of the 26S rDNA (nucleotides 63–642 for *S. cerevisiae*) with primers NL-1 and NL-4. The amplified fragments were subsequently sequenced. As shown in Figure 1, this method confirmed the presence of *L. thermotolarans*, coded in our work as strain number 44C [29].

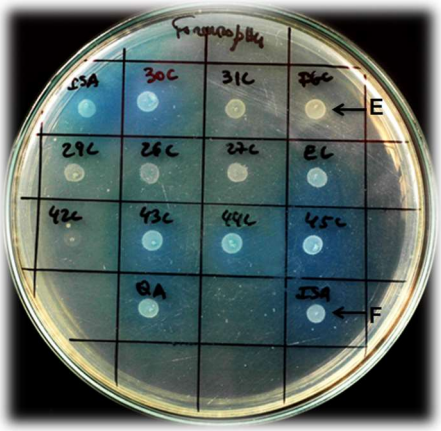

Figure 1. Growth and color change (due to pH changes) of the differential medium with 0.5% (*v*/*v*) acetic acid, 0.05% (*w*/*v*) glucose, and bromocresol green (0.005% (*w*/*v*)) at pH 4.0, indicating the simultaneous consumption of glucose and acetic acid by the isolated strains, namely "44C". E: *S. cerevisiae* PYCC4072 (negative control); F: *Z. bailii* ISA1307 (positive control). Retrieved from Vilela et al. [34].

Subsequently, the effect of glucose and acetic acid concentrations and aeration conditions, on the consumption of acetic acid by the previously mentioned strain, were studied at laboratory scale. The strain *Z. bailii* ISA1307 was used as a reference strain. The results showed that *L. thermotolerans* 44C was able to degrade 28.2% of the initial acid when grown under limited aerobic conditions in a mixed substrate medium which contained glucose (5.0%, *w*/*v*) and acetic acid (5.0 g L^{-1}). Moreover, strain 44C also presented the ability to degrade acetic acid in media with 5.0% or 0.75% (*w*/*v*) of glucose, under limited aerobic conditions. Although the higher initial concentration of glucose did not alter the rate of acetic acid consumption by strain 44C, this strain did decrease the rate of glucose consumption [29].

To verify the potential application of *L. thermotolerans* 44C in refermentation processes, the strain was inoculated in a mixed medium containing two-thirds of minimal medium [36], supplemented with one-third of an acidic white wine. Once again, *Z. bailii* ISA1307 was used as a control strain. The volatile acidity of the mixture was 1.13 g L^{-1} of acetic acid, corresponding to the values usually found in acidic wines. Two wine-supplemented mineral media (Table 1) were tested: The first medium simulated the refermentation of a wine with freshly crushed grapes or with grape-must [13% glucose (*w*/*v*), 4% ethanol (*v*/*v*)]; the second wine-supplemented mineral medium simulated the refermentation of a wine with the residual marc from a finished wine fermentation [3.3% glucose (*w*/*v*), 10% ethanol (*v*/*v*)]. Acetic acid consumption was again evaluated under aerobic or limited aerobic conditions to assess whether aeration was a limiting factor in the process. Once it was known that higher glucose concentration levels could lead to a decreased rate of glucose consumption, the rate of acetic acid and glucose consumption was evaluated at the end of 48 and 72 h for the first and second medium, respectively [29].

Strains *Z. bailii* ISA1307 and *L. thermotolerans* 44C were efficient in terms of acetic acid consumption in the high-glucose medium and aerobic conditions, as 94.8 and 94.6% of the initial acetic acid was consumed (Table 1). However, the efficiency of *L. thermotolerans* 44C in acetic acid consumption decreased significantly in the high-glucose concentration medium under limited aerobic conditions, as only 15.3% of the initial acetic acid was consumed [29], as shown in Table 1.

Table 1. Percentage of acetic acid (*italic letters*) and glucose (**bold letters**) consumption after refermentation of wine-supplemented culture medium, containing glucose 13% (*w*/*v*) and ethanol 4% (*v*/*v*) or glucose 3.3% (*w*/*v*) and ethanol 10% (*v*/*v*), after 48 and 72 h of incubation, respectively. Results obtained for strains and culture conditions with the same letter are not significantly different ($p < 0.001$) [29].

	Glucose (13%, *w*/*v*) Ethanol (4%, *v*/*v*)		**Glucose (3.3%, *w*/*v*) Ethanol (10%, *v*/*v*)**	
	Aerobic Conditions	Limited Aerobic Conditions	Aerobic Conditions	Limited Aerobic Conditions
Yeast strains	*Acetic acid* **Glucose**	*Acetic acid* **Glucose**	*Acetic acid* **Glucose**	*Acetic acid* **Glucose**
Z. bailii ISA 1307	*94.8 ± 3.30 c* **52.4 ± 2.62 c**	*40.9 ± 9.80 a* **38.8 ± 6.36 b**	*71.2 ± 3.02 b* **23.1 ± 5.60 a**	*41.6 ± 2.64 a* **39.4 ± 2.10 b**
L. thermotolerans 44C	*94.6 ± 4.79 d* **58.5 ± 8.60 c**	*15.25 ± 3.30 a* **31.0 ± 5.69 b**	*28.1 ± 1.70 c* **16.4 ± 1.76 a**	*17.4 ± 7.16 b* **30.4 ± 5.79 b**

5. Conclusions

Climate change has caused increased temperatures for many wine-growing regions around the world. *L. thermotolerans* offers a unique potential to counter this effect of global warming on wine grapes by producing acid during fermentation, which can moderately reduce alcohol levels and produce, also, high concentrations of the fruity-like flavor compound, ethyl lactate (using lactate as a precursor).

L. thermotolarans can also be used to develop a controlled biological deacetification process of wines with high volatile acidity. However, the ability of *L. thermotolerans* 44C to consume acetic acid is a highly oxygen-dependent one, which means that its metabolism must shift more towards respiration than to fermentation. The high amount of sugars present in the grape-must may also inhibit or delay *L. thermotolerans*' acetic acid consumption during refermentation processes.

Consequently, further research is needed before the deacetification of wines will be able to be added to the list of *Lanchancea*'s enological features.

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