Combined Use of *Lachancea thermotolerans* and *Schizosaccharomyces pombe* in Winemaking:

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Introduction

The combined use of *Lachancea thermotolerans* (Benito 2018) and *Schizosaccharomyces pombe* (Benito 2019) is a novel winemaking biotechnology able to solve some modern industrial oenology problems, specially related to warm viticulture regions. Those regions produce musts with high levels of sugar and lack of acidity. The proposed biotechnology was reported for the first time in 2015 (Benito *et al*. 2015), and since then, several scientific articles have been published regarding this topic. These reported scientific studies follow an evolution similar to that performed in the past for *Saccharomyces cerevisiae* and *Oenococcus oeni*. They started by reporting results for basic winemaking parameters emphasizing on the influence of the biotechnology on the acidity improvements and malic acid stability (Benito *et al.* 2015). Later studies focused on volatile aroma and amino acid composition (Benito *et al*. 2016a), influence on colour parameters of red wines (Benito *et al*. 2017) and manoproteins improvements (Benito *et al*. 2019). A recent review summarizes all the advances regarding this biotechnology comparing the results obtained by several different researchers when they applied this biotechnology (Benito 2020). The different studies show that the new biotechnology can solve specific oenology problems such as lack of acidity, biogenic amines, ethyl carbamate or undesirable color losses.

L. thermotolerans increases wine acidity in warm viticulture that commonly suffer from lack of acidity due to the production of L-lactic acid during alcoholic fermentation that is able to reduce the pH down to 0.2-0.4 units (Benito *et al.* 2016b; Benito 2018). Other secondary quality improvements related to *L. thermotolerans* use are low volatile acidity production, aroma complexity or increase in color intensity (Benito 2018). However, the main disadvantage related to the use of *L. thermotolerans* at the real industry scale is its low fermentative capacity that does not allow fermenting over 10% (v/v) ethanol concentration. For that reason, it must be combined to a more powerful fermenter such as *S. cerevisiae* or *S. pombe*.

S. pombe was initially applied to de-acidify high acidic sharp wines with high malic acid concentrations over 5 g/L (Benito 2019). Nevertheless, it is also nowadays use in warmer areas in order to avoid performing malolactic fermentation under problematic situations such as wines with high pH over 4 and with high sugar concentrations over 250 g/L (Benito 2019). Under these scenarios, lactic acid bacteria could metabolize fermentable sugar into acetic acid during the alcoholic fermentation or during difficult slow alcoholic fermentation endings (Figure 1). Those undesirable deviations could deteriorate the quality of the final wine.

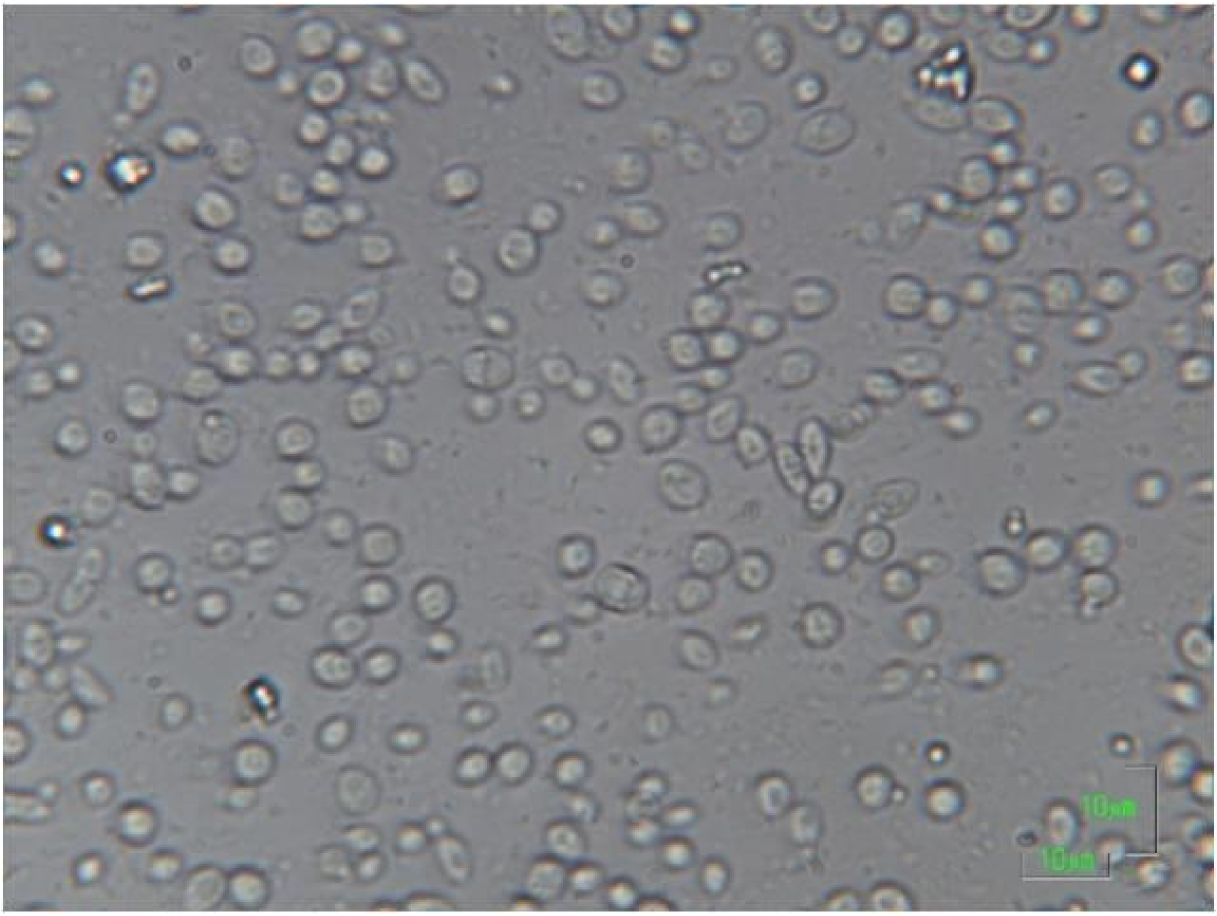

Figure 1. Microscopic observation of an alcoholic fermentation ending in a low acidic must/wine with high content in sugar. *Saccharomyces cerevisiae* are the cells between 5 and 10 µm, while *Oenococcus oeni* are the small cells of about 1 µm. In this difficult alcoholic fermentation ending, S. cerevisiae cells are still difficultly fermenting sugar in an already high alcohol concentration of about 15 % v/v after 20 days of fermentation. A high population of lactic bacteria (*Oenococcus oeni*) start to be evident although the alcoholic fermentation is not properly finished yet.
Source: https://www.mdpi.com/2304-8158/9/10/1423 Open access (Benito 2020)

Although the main winemaking use of *S. pombe* is the degradation of malic acid in order to de-acidify or to achieve microbiological stabilization before bottling. Last studies show other interesting abilities such as polysaccharides release, colour improvements or stabilization against biogenic amines and ethyl carbamate formations (Benito 2019). The main disadvantage related to *S. pombe* use is the high production of acetic acid of most strains; the problem is nowadays solve with proper strain selection processes (Benito *et al.* 2016c).

The achievement of microbial stable wines from a malic acid point of view is achieved performing alcoholic fermentation by *S. cerevisiae* followed by malolactic fermentation by *Oenococcus oeni*. This traditional methodology is the most suitable for moderate climates. However, modern oenology try to adapt to new problems such as climate change that increase the incidence of warm areas where vines are cultivated. One of the key problems of these warm areas is the common production of grapes with sugar concentrations over 250 g/L. This fact makes it difficult for regular yeast to end the fermentation of sugars into ethanol. This fact increases the risk of fermentation stops or very long fermentation endings. Another key problem is the low acidity of very ripe grapes. This fact may unbalance wines from a sensory point of view and generates technical problems related to high pH management. Under high pH, malolactic fermentation may result in high levels of volatile acidity or biogenic amines, especially in uncontrolled spontaneous processes.

Under this new climate change scenario, the combined use *L. thermotolerans* and *S. pombe* is proposed. In this combination, when applied to warm viticulture areas, *S. pombe* consumes all of the malic acid, converting it into small amounts of ethanol and carbon dioxide. Thus, achieving the desired industrial microbiological stabilization based in total sugar and malic acid consumption that reduces the risk of re-fermentation by lactic bacteria due to the lack of nutrients. In addition, *L. thermotolerans* generates lactic acid that compensates for the lack of acidity produced by the disappearance of malic acid and generates natural wines of optimal acidity from low-acidic grape juices.

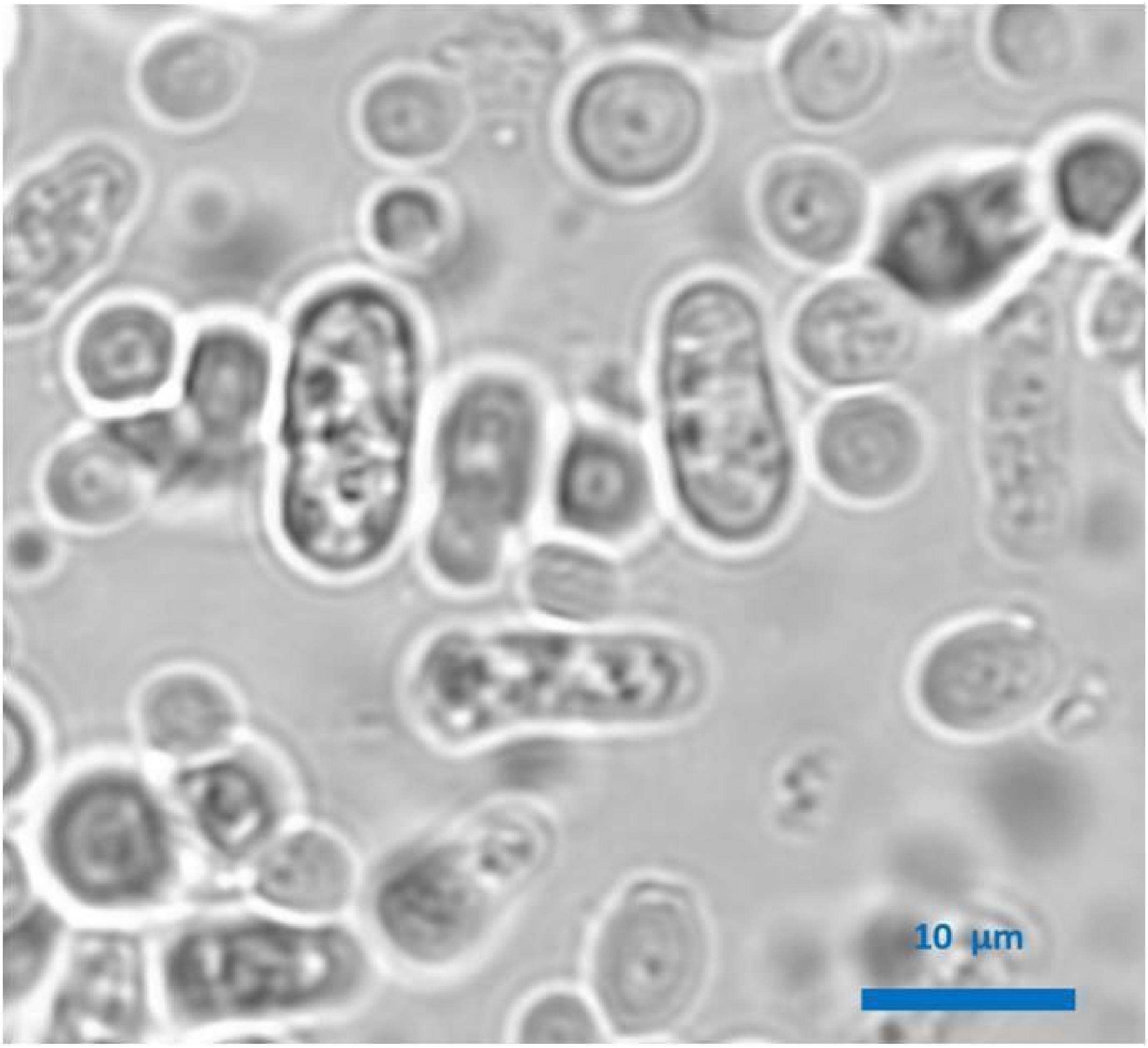

Figure 2. Microscopic observation of a wine produced with combined *Lachancea thermotolerans* and *Schizosaccharomyces pombe* alcoholic fermentation. *S. pombe* cells are rectangular, while *L. thermotolerans* cells are spherical.
Source: https://www.mdpi.com/2304-8158/9/10/1423 Open access (Benito 2020)

Results of basic parameters

Table 1 shows the results in basic parameters of the last trial we published (open access) regarding basic parameters for the proposed biotechnology. Fermentation trials involving *S. pombe* degraded all malic acid during alcoholic fermentation while *S. cerevisiae* trials degraded just about 5 % . *O. oeni* metabolized the remaining malic acid to lactic acid during malolactic fermentation in order to get stable wines from a microbiological point of view in trials fermented by *S. cerevisiae*. Fermentations involving *L. thermotolerans* produced L-lactic acid during alcoholic fermentation. The final L-lactic acid concentrations varied from 1.46 to 3.11 g/L. The final pH varied from 3.47 to 3.91 g/L due to malic and lactic acid metabolisms. L-lactic acid concentration levels were higher in the cases were *L. thermotolerans* fermented the grape juice than when *O. oeni* performed malolactic fermentation.
Final ethanol concentrations varied from 13.55 to 13.80 % (v/v). *S. pombe* alone fermentations produced slightly lower ethanol concentrations than *S. cerevisiae* controls. Although studied ethanol concentration differences were significantly different, those differences in our specific case were lower than 0.25% (v/v).
Fermentations involving *S. pombe* ended in final urea concentrations lower than 0.1 mg/L due to the urease enzymatic ability of this species.
Final malolactic fermentations performed by *O. oeni* showed final citric acid concentrations of about 0 mg/L. Slightly higher acetic acid concentrations in about 0.1 g/L appeared in the cases that performed malolactic fermentation. Those acetic acid rises could be due to citric acid consumption by *O. oeni* during malolactic fermentation process.
A decrease in color intensity of about 20 % was observed during the malolactic fermentation while this effect did not take place in the controls that did not perform malolactic fermentation.

Table 1. Final analysis of fermentations from original must of Tempranillo grapes: *S. cerevisiae* alone (SC), sequential fermentation with *S. cerevisiae* and *L. thermotolerans* (LT…SC), sequential fermentation with *S. pombe* and *L. thermotolerans* (LT…SK), *S. pombe* alone (SK), and fermentations after a malolactic fermentation with *Oenococcus oeni* (+ MLF).

Compounds	SC	SC + MLF	LT…SC	LT…SC + MLF	LT...SK	SK
L-Lactic Acid (g/L)	0.01 ± 0.01 a	1.46 ± 0.05 b	1.63 ± 0.14 c	3.11 ± 0.21 e	1.86 ± 0.19 d	0.01 ± 0.01 a
L-Malic Acid (g/L)	2.43 ± 0.03 b	0.01 ± 0.01 a	2.39 ± 0.05 b	0.01 ± 0.01 a	0.01 ± 0.01 a	0.01 ± 0.01 a
Acetic Acid (g/L)	0.28 ± 0.01 a	0.39 ± 0.02 b	0.25 ± 0.03 a	0.34 ± 0.04 b	0.30 ± 0.04 ab	0.36 ± 0.02 b
Glucose + Fructose (g/L)	1.55 ± 0.19 b	0.07 ± 0.03 a	1.61 ± 0.24 b	0.05 ± 0.02 a	1.72 ± 0.25 b	1.58 ± 0.16 b
Glycerol (g/L)	7.12 ± 0.02 a	7.17 ± 0.05 a	7.14 ± 0.06 a	7.19 ± 0.11 ab	7.39 ± 0.09 b	7.78 ± 0.03 c
pH	3.64 ± 0.02 b	3.73 ± 0.02 c	3.47± 0.03 a	3.58 ± 0.06 b	3.53 ± 0.05 ab	3.91 ± 0.02 d
Urea (mg/L)	1.78 ± 0.06 b	1.97 ± 0.08 c	1.82 ± 0.09 bc	2.11 ± 0.11 d	0.06 ± 0.03 a	0.03 ± 0.01 a
Citric Acid (g/L)	0.29 ± 0.01 b	0.02 ± 0.01 a	0.27 ± 0.02 b	0.04 ± 0.02 a	0.29 ± 0.03 b	0.27 ± 0.02 b
Ethanol (% *v*/*v*)	13.78 ± 0.02 c	13.80 ± 0.05 c	13.72 ± 0.06 cb	13.70 ± 0.09 cb	13.62 ± 0.05 b	13.55 ± 0.04 a
Acetaldehyde (mg/L)	34.16 ± 1.55 c	1.88 ± 0.33 a	29.55 ± 2.13 b	1.79 ± 0.24 a	46.38 ± 2.96 d	58.36 ± 2.55 e
Pyruvic Acid (mg/L)	58.56 ± 3.55 b	13.67 ± 3.79 a	62.42 ± 5.73 b	17.82 ± 6.21 a	122.63 ± 9.15 c	168.82 ± 5.78 d
Color Intensity	0.19 ± 0.01 ab	0.15 ± 0.01 a	0.18 ± 0.01 ab	0.16 ± 0.01 a	0.20 ± 0.01 bc	0.22 ± 0.01 c

Results are the mean ± SD of three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$).

Table Source: https://link.springer.com/article/10.1186/s13568-019-0738-0 Open access (Benito *et al.* 2019)

Conclusion

New biotechnology based on the combined use of *L. thermotolerans* and *S. pombe* is a suitable alternative to ferment musts in warm viticulture areas, where performing malolactic fermentation at high pH levels with possible residual sugars and high ethanol concentrations may produce undesirable deviations in the quality of the wine. Most studies report the combined use of *L. thermotolerans* and *S. pombe* to be faster than classical winemaking methodology that includes alcoholic fermentation and malolactic fermentation, improving the same microbial stability for the final product. *L. thermotolerans* and *S. pombe* fermentations are occasionally described to result in higher acidity, color intensity, polysaccharide and fruity ester contents, with a lower pH, ethanol, higher alcohols, biogenic amines and ethyl carbamate precursors.

Abstract

Commercial red wines use the malolactic fermentation process to ensure stability from a microbiological point of view. In this second fermentation, malic acid is converted into L-lactic acid under controlled steps. However, this process is not free from possible collateral effects able to produce off-flavors, wine quality loss and human health problems. In warm viticulture regions such as the south of Spain, the risk of suffering a deviation during the malolactic fermentation process increases for the high must pH. This contributes to produce wines with high volatile acidity and biogenic amines. The work develops a method that comprises combining the use of two non-Saccharomyces yeast as an alternative to the traditional malolactic fermentation in specific difficult scenarios. In this method, malic acid is consumed by Schizosaccharomyces pombe, thus achieving the microbiological stabilization aim before bottling, while Lachancea thermotolerans produces lactic acid in order not to reduce and even increase the acidity of wines produced from low acidity musts. This technique reduces the risks inherent to the malolactic fermentation process when performed in warm regions with high potential alcohol degree and pH. The result is more fruity wines that contain less acetic acid and biogenic amines. The proposed work is based on several works performed during the last 3 years related to the topic:
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