

## Effects of using mixed wine yeast cultures in the production of Chardonnay wines

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### ABSTRACT

The effect of using mixed cultures of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts in the physicochemical and sensory qualities of the wines were analyzed in this study. Based on growth curves, sugar consumption and glycerol production in synthetic must, *Candida membranifaciens* L1805 was selected from a group of four *Candidas* spp. isolates from Chile and Argentina. This yeast was subsequently used in combination with *S. cerevisiae* in Chardonnay must. A monoculture of *S. cerevisiae* was used as control. The wines fermented with mixed cultures had lower volatile acidity and ethanol concentration than the control. Furthermore, the chromatographic analysis showed that the wines from mixed cultures presented differences in the concentration of esters and propanol. These characteristics positively influenced the sensory qualities of the wines produced with mixed cultures, which was reflected in the preference for these wines by a panel of enologists. This study shows that the use of non-*Saccharomyces* yeasts could be a strategy to obtain distinctive wines using the native microorganisms from each winemaking area.

**Key words:** non-*Saccharomyces*, wine, aroma, flavor, co-fermentation

### RESUMEN

**Efecto del uso de cultivos mixtos de levaduras en la producción de vinos Chardonnay.** En este estudio se analizó el efecto del uso de cultivos mixtos de levaduras no-*Saccharomyces* y *Saccharomyces cerevisiae* en las cualidades fisicoquímicas y sensoriales de los vinos. *Candida membranifaciens* L1805 fue elegida de un grupo de cuatro *Candida* spp. aisladas de Chile y Argentina, sobre la base de las curvas de crecimiento, el consumo de azúcar y la producción de glicerol en mosto sintético. Posteriormente, esta levadura fue usada en cultivo mixto con *S. cerevisiae* en mosto Chardonnay. Como control se utilizó un monocultivo de *S. cerevisiae*. Los vinos producidos por cultivos mixtos tuvieron menor acidez volátil y producción de etanol que los correspondientes al control. Los análisis cromatográficos mostraron que estos vinos presentaron diferencias en la concentración de ésteres y de propanol. Estas características afectaron positivamente las cualidades sensoriales de los vinos, lo cual se reflejó en la preferencia del panel de enólogos. El estudio muestra que el uso de levaduras no-*Saccharomyces* puede ser una estrategia para obtener vinos diferentes usando microorganismos nativos de cada área vitivinícola.

**Palabras clave:** no-*Saccharomyces*, vino, aroma, sabor, cultivos mixtos

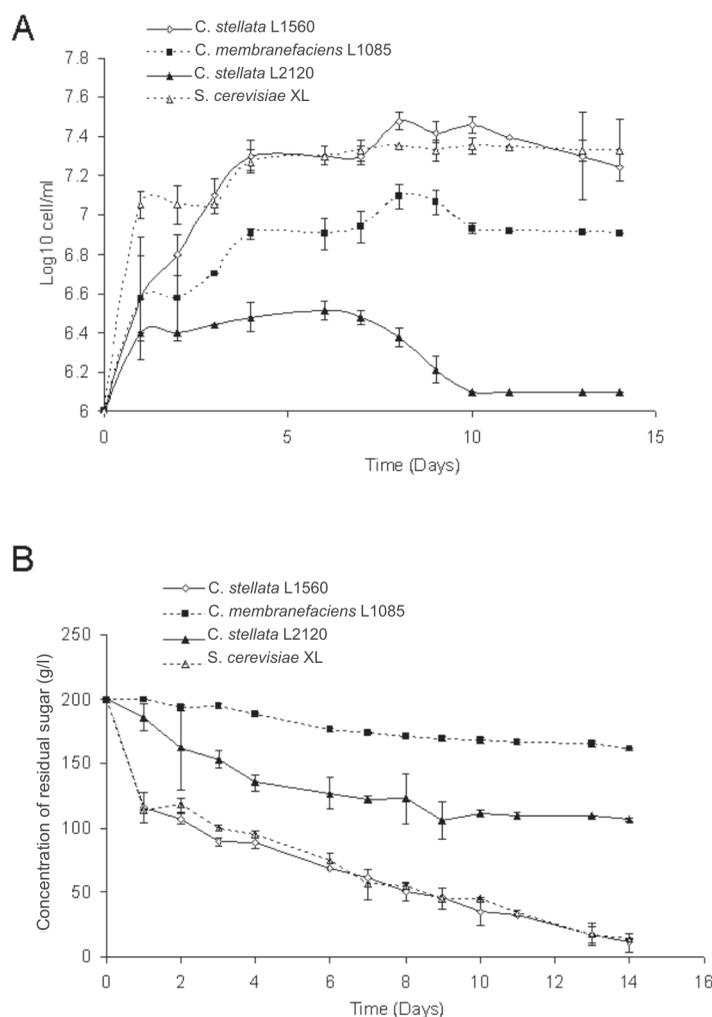
In wine production, yeasts are responsible for transforming the sugar present in the grape must into ethanol, carbon dioxide and hundreds of secondary products that collectively contribute to the qualities of the product (7). Hence, these microorganisms may have a positive or negative influence in the sensory traits of the product. Although non-*Saccharomyces* yeasts were long considered harmful, evidence in recent years has shown that their use may give complex organoleptic characteristics to the wine, thus increasing its quality (3, 6, 8, 9), because they produce compounds such as glycerol, isoamilic alcohol, succinic acid, acetic acid and propanol that affect the sensory characteristics of the product (4, 8). Experiments carried out by Ciani and

Picciotti (5) with white must, using 6 non-*Saccharomyces* yeasts independently, showed that fermentation with *Candida stellata* DBVPG4124 produced higher glycerol concentration compared to the other microorganisms used, including *Saccharomyces cerevisiae*. Moreover, there was also a variation in the concentration of isobutanol and isoamilic alcohol. On the other hand, Toro and Vazquez (14) using *Candida cantarelli* in mixed cultures with *S. cerevisiae* obtained wines with 40% more glycerol than those fermented only with *S. cerevisiae*. In addition, the use of mixed cultures of *C. stellata* and *S. cerevisiae* or *Debaryomyces vanriji* and *S. cerevisiae* have produced wines with a fruity aroma (3, 6, 8, 9, 12). Based on these promising results, there are already dry yeast

mixtures of *S. cerevisiae* / *Kluyveromyces thermotolerans* / *Torulaspora delbrueckii* (Vinifloras® Harmony.n.sac, CHR Hansen) and *T. delbrueckii* / *S. cerevisiae* (Level 2TD, Lallemand Inc.) in the market. With the aim of determining the potential of non-*Saccharomyces* yeasts from South America, in particular those of the genus *Candida*, we studied the effect of using mixed cultures of native isolates of *Candida* spp. and *S. cerevisiae* in the physicochemical and sensory traits of the wines. *C. stellata* L1560 and *Candida membranifaciens* L1805 isolated from Cauquenes (Chile) and *C. stellata* L2120 isolated from Mendoza (Argentina) were used. Commercial yeasts of *S. cerevisiae* XL were also used, obtained from DSM Food Specialties. Fermentations were carried out in triplicate in 300 ml of synthetic must (5 g/l tartaric acid, 5 g/l malic acid; 0.3 g/l CaCl<sub>2</sub>; 1.3 g/l MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 1.2 g/l NH<sub>4</sub>PO<sub>4</sub>; 2.5 g/l KOH; 100 g/l fructose; 5 g/l sucrose; 100 g/l glucose; 2 mg/l thiamin; 9 µg/l biotin; 4.6 mg/l nicotinic acid; 400 µg/l pyridoxine hydrochloride; 2 mg/l calcium pantonate) and inoculated with 1x10<sup>6</sup> cells/ml, which

were incubated for 14 days at 25 °C without shaking. A culture of *S. cerevisiae* was used as control. The fermentation was followed by count in a Neubauer chamber and quantification of sugar reduction (13). The concentration of glycerol was quantified at the end of the process using the enzymatic kit from Roche (Germany).

Figure 1A shows the growth curves of each isolate. It indicates that the *C. stellata* L1560 isolate had the greatest growth of the non-*Saccharomyces* isolates, reaching an average population of 1.8 x 10<sup>7</sup> cells/ml at the end of the fermentative process, close to the average yeast population of the *S. cerevisiae* isolate (2 x 10<sup>7</sup> cells/ml). The *C. stellata* L2120 isolate grew slowly in the first days of the process, reaching its maximum average population of 4.8 x 10<sup>6</sup> cells/ml on the eighth day. The *C. membranifaciens* L1805 isolate had an average population of 1.2 x 10<sup>7</sup> cells/ml on the eighth day of fermentation, with an average of 8.3 x 10<sup>6</sup> cells/ml by the end of the process. The sugar consumption in the cultures (Figure 1B) showed that the *C. stellata* L1560 isolate had



**Figure 1.** Growth of yeasts (A) and sugar concentration (B) during fermentation of *Candida* spp. isolates in synthetic must. *S. cerevisiae* XL was used as control.

a similar behavior to *S. cerevisiae* XL, obtaining a final concentration of residual sugar of 10.9 g/l on day 14. The use of this non-*Saccharomyces* isolate is questionable given that in a mixed culture of *C. stellata* L1560 and *S. cerevisiae* it may influence the growth of *S. cerevisiae*. The *C. stellata* L2120 isolate had a final glycerol concentration of  $6.6 \pm 0.1$  g/l, while the *C. stellata* L1560 culture obtained a concentration of  $8.8 \pm 0.16$  g/l. The *C. membranifaciens* L1805 culture had a final concentration of  $5.3 \pm 0.1$  g/l. All the non-*Saccharomyces* isolates showed a higher glycerol concentration in the media than that produced by *S. cerevisiae* ( $3.6 \pm 0.1$  g/l). Based on the glycerol production, the growth kinetics and the fact that *C. membranifaciens* L1805 does not predominate with *S. cerevisiae*, this isolate was used in subsequent studies of natural must fermentations. This fermentation was carried out in triplicate in Chardonnay must maintained at  $-20$  °C for 6 months. The microbiological analysis of the must prior to its use showed a yeast concentration below 10 cfu/ml. For natural must fermentation, *C. membranifaciens* L1805 was grown in 500 ml of sterilized natural must (heated at 60 °C for 10 min) and incubated at 28 °C for 24 h. The final yeast population was determined by count in a Neubauer chamber. Based on previous works on mixed fermentations (14), the initial cultures were added at a final concentration of  $1.5 \times 10^6$  cells/ml at 3.5 l of must. Additionally,  $2.5 \times 10^4$  cells/ml of *S. cerevisiae* were added, resulting in a 1:60 ratio of *S. cerevisiae*: non-*Saccharomyces*. The fermentation control containing only *S. cerevisiae* was used. Fermentation was carried out at 16 °C for 25 days, with daily homogenization and monitoring by variations in density. When the fermentations were completed, the wines were stored at 4 °C. The wines were analyzed for sugar reduction (13), pH (2), alcohol concentration (2), volatile acidity concentration (2), density (2), sensorial analysis (1) and quantification of aromatic compounds (15). All the

experiments were carried out in triplicate and the data were subjected to analysis of variance (ANOVA) with a  $p \leq 0.05$  (LSD Test). Table 1 shows the physicochemical and chromatography analysis of the wines obtained. The volatile acidity and alcohol concentration of the wine with the mixed culture had lower values in both parameters, which is an interesting result considering that volatile acidity is an indicator of microbiological contamination. In relation to the reduction of alcohol concentration in the mixed culture, there could be competition among the inoculated microorganisms which made the production of this metabolite less efficient. The monoculture of *S. cerevisiae* alone had a higher concentration of glycerol, which was contrary to what was expected. The levels of sugar consumption in the co-fermentation of *C. membranifaciens* L1085 / *S. cerevisiae* and the control assays were similar. The similarity in sugar consumption and the lower levels of glycerol concentration obtained in mixed culture may have occurred because the non-*Saccharomyces* yeast did not grow and the *S. cerevisiae* yeast predominated at all times. To verify this observation, 10% of yeast colonies obtained at 2, 10 and 26 days of fermentation were analyzed by RFLP analysis of the ITS region (11). This analysis confirmed that two days after the process began there was only 20% of the *C. membranifaciens* population, which could be insufficient for glycerol production. It is also possible that there are microcomponents in the natural must, not considered in a synthetic must, that could affect microorganism growth and metabolite production. Consequently, it would be interesting in future studies to analyze the relative doses of *S. cerevisiae* and non-*Saccharomyces* to apply. With the aim of determining if this small population of *C. membranifaciens* influences the sensorial characteristics of the wines, a preference test was carried out (1), where enologists preferred the wines obtained from mixed cultures. Parallely, the aromatic compounds of the wines

**Table 1.** Chemical analysis and volatile compounds of the wines obtained with mixed cultures and yeast monocultures<sup>(1)</sup>

	Mixed culture ( <i>C. membranifaciens</i> / <i>S. cerevisiae</i> )	Monoculture ( <i>S. cerevisiae</i> )
Reducing sugar (g/l)	$1.7 \pm 0.2^a$	$1.6 \pm 0.0^a$
Volatile acidity (g/l) <sup>(2)</sup>	$0.13 \pm 0.01^a$	$0.17 \pm 0.01^b$
Alcohol (% v/v)	$12.6 \pm 0.1^a$	$15.6 \pm 0.0^b$
pH	$3.4 \pm 0.0^a$	$3.3 \pm 0.0^b$
Glycerol	$6.0 \pm 0.01^a$	$6.4 \pm 0.01^b$
Ethyl caproate (mg/l)	$0.44 \pm 0.04^a$	$0.64 \pm 0.02^b$
Hexyl acetate (mg/l)	$0.07 \pm 0.00^a$	$0.12 \pm 0.01^b$
Ethyl caprylate (mg/l)	$0.68 \pm 0.19^a$	$1.02 \pm 0.12^b$
1-Propanol (mg/l)	$6.33 \pm 1.80^a$	$1.81 \pm 0.03^b$

<sup>(1)</sup>Means of the same row with the same superscript letters are not significantly different ( $p > 0.05$ ).

<sup>(2)</sup>Expressed as acetic acid.

were analyzed using the technique described by Viana *et al.* (15). The concentrations of isoamilic alcohol, 2-phenylethanol, isobutanol, benzyl acetate, ethyl acetate, isobutyl acetate, isoamyl acetate, diethyl succinate, 2-phenylethanol acetate, 2-phenylethanol, isobutane and ethyl lactate did not show differences in the two types of wine obtained. However, significant differences were observed in the concentrations of ethyl caproate, ethyl caprylate and hexyl acetate, all being higher in the control wine (*S. cerevisiae*). The mixture of ethyl caproate, ethyl caprylate and hexyl acetate has been described as conferring fruity aromas, but an increase of these can also have a negative effect (10). This could be the reason why the enologists preferred wines fermented with mixed cultures.

While the chemical parameters did not show any differences between the control wine and those obtained from mixed cultures, variations were observed in the composition of the aromatic compounds, which could be correlated to the preferences of the enologists. The search for new non-*Saccharomyces* isolates allows to provide wines with distinctive qualities, and our continent has great potential owing to the wide diversity of yet unstudied yeasts that can serve as differentiators of our musts.

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