Proteolytic activity under white wine fermentation by *Hanseniaspora vineae* yeast strains

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Abstract. Apiculate yeasts from *Hanseniaspora* genus are predominant on the native flora of grapevines, while *Hanseniaspora uvarum* is well known for its abundant presence in grapes, it results generally, in detrimental quality effects on wine due to high production of acetic acid. By contrast, *Hanseniaspora vineae* is better adapted to fermentation, increasing flavor complexity in wines as it has been demonstrated in winemaking practices during the last decade. We obtained a collection of more than 22 different *Hanseniaspora vineae* strains from which we could detect, by a plating screening method, high and moderate protease activity. In this study, we tested these strains under real winemaking conditions on a Sauvignon blanc white wine must. Results obtained demonstrated that there is an interesting protease active diversity in all *H. vineae* strains when compared to conventional fermentations conducted by *Saccharomyces cerevisiae*. Four *H. vineae* strains showed a decrease in wine protein turbidity, up to three times lower than that observed in *S. cerevisiae*, which imply a significant decrease in the demand for bentonite before bottling. This attribute of some *H. vineae*strains should be very attractive at the commercial level to reduce manipulations and flavor removal in the production of delicate white wines such as Sauvignon blanc.

1 Introduction

In contrast to what has been observed for *Saccharomyces* yeasts, non-*Saccharomyces* species can produce and release different enzymes into the medium [1,2]. The presence of these enzymes depends in part on the carbon and nitrogen sources present in the must. In the work of Buerthet al. [3] it was found that small changes in the concentration of these nutrients can affect the nature, quantity and diversity of the enzymes secreted. The enzymes most studied for their role during winemaking are protease, β-glucosidase and pectinase because they are involved in sensory attributes such as color, aromas and wine stability [4]. Proteases are enzymes responsible for the hydrolysis of proteins present in musts and wines.

Concentration of proteins in wine will depend on the grape variety from which the juice is made, ripening conditions of the grapes, fermentation process, as well as the environmental conditions prevailing during the vegetative growth of the vine [5].

Proteins are responsible for the appearance of sediments or floccules that can produce turbidity affecting the stability of wines before or after bottling, thus causing economic losses to wine producers [5–8]. The occurrence of these defects has repercussions especially in white and rose wines, since the lower concentration of tannins leave increased levels of free proteins, which could be unstable after bottling affecting limpidity and visual aspect of this type of wines [8].

Currently, the most widely used mechanism to remove proteins and prevent the precipitation of these compounds in bottled wine is the use of bentonite [5,8], a mineral clarifier, which has some disadvantages. It affects the sensory quality of the wines as it can remove compounds that contribute to color, as well as aromatic compounds and finally generates losses of wine on the bentonite lees [5,8]. This in turn goes against the trend of "low input winemaking", where it is intended that the winemaking process develop through the minimum number of interventions possible to avoid losing color and flavor [9].

Due to the disadvantages of using bentonite, yeasts with proteolytic activity are desirable since they will have the capacity to hydrolyze proteins to small peptides and amino acids that can be easily consumed by yeasts as a source of nitrogen [4]. In this way, they favor the enrichment of nutrients in the medium, thus avoiding the stuck or sluggish of fermentations that may occur due to a possible nitrogen deficiency in grape musts.

In particular, yeasts of the species *Hanseniaspora vineae* (*H. vineae*), already tested for their fermentative capacity and aroma contribution [10–15] have preliminarily been demonstrated to reduce the haze induced in white wines of Sauvignon blanc must [16]. Therefore, they might be a useful alternative to bentonite and other treatments, for use during vinification of white wines, especially in varieties with high protein content such as Sauvignon blanc [17–20].

In this sense, the objective of this work was to study the capacity of different yeast strains of the species *H. vineae* to reduce the haze of final wines produced with Sauvignon blanc and confirm the association of this capacity to proteolytic activity.

2 Material and methods

2.1 Yeast strains

Twenty-two yeasts of the species *H. vineae* (Table 1), from the collection of native yeasts belonging to the Área de Enología y Biotecnología de lasFermentaciones (Faculty of Chemistry-UdelaR, Montevideo, Uruguay),

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were used for this work. The commercial yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) Lalvin BM 4 × 4 (Lallemand) was used as a reference strain for the assays.

2.2 Proteolytic activity on skim milk medium

Milk medium at pH 6 (optimal for enzymatic activity) and pH 4.5 (closer to grape must pH) [4]. For this, to 70 mL of 0.05M citrate phosphate buffer (44.2 mL disodium phosphate in 25.8 mL of 0.1M citric acid) Skim Milk (Difco) is added at a final concentration of 100 g/L. To the Skim Milk solution, 60 mL of phosphate buffer is added and heated, but not boiled. For pH 4.5 plates a sterile minimal medium is prepared separately, containing 4.8 g of glucose, 3.36 g YNB (yeast nitrogen base) without amino acids and 9.6 g of bacteriological agar in 480 mL of distilled water. A yeast strain of *Metschnikowia pulcherrima* was used as a positive control and *S. cerevisiae* as a negative control. Each yeast to be tested was replicated in triplicate. Plates were incubated at 28°C for 5 days. The presence of enzyme activity was determined by the formation of a translucent halo around the colonies. The size of halo was considered as positive protease activity with three levels as indicated [12].

2.3 Fermentations in grape juice

To determine the effect of yeasts on wine haze, fermentations were carried out with the 22 yeast strainsof *H. vineae*. Each fermentation was carried out in triplicate on Sauvignon blanc grape must. The musts were inoculated with an initial concentration of 10^6 cells/mL of *H. vineae* and supplemented with a solution of yeast extract (2.5 mg/L) and thiamine (0.4 mg/L). Each trial was carried out with a control of *S. cerevisiae* yeast at a concentration of 10^6 cells/mL.

Fermentations were carried out in 125 mL flasks, with a must volume of 60 mL. The fermentations were monitored by weight loss, due to CO_2 release. Halfway through fermentation (when the weight loss value reached a value close to 6 g/100 mL), *S. cerevisiae* was co-inoculated at a concentration of 10^5 cells/mL. In this way we ensured the ending of fermentation. Fermentations were considered to have ended when the weight loss was constantand sugars below 3 g/L.

2.4 Heat Stability Test (HST)

At the end of the fermentations, the wines obtained were filtered through a 0.22 µm membrane and dispensed into 4 tubes containing 10 mL of wine each. One set of 2 tubes was immersed in a bath at 80ºC for 30 minutes and a control (of 2 tubes) was left unheated. When they were cooled, the haze was measured in a turbidi-meter (PRO Turbidity Meter MI415, Milwaukee) and expressed in nephelometric turbidity units (NTU) [21].

2.5 Protein concentrations in wine

Extracellular proteins were measured in wines after fermentation with the Braford method [22]. A calibration curve was prepared with bovine serum albumin (BSA) as the standard, using the following concentrations: 0 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1 mg/mL. Protein samples (100 µL aliquots) mixed with an equal volume of deionized water, to which 200 µL of the Bio-Rad Protein assay reagent was added. The absorbance at 620 nm was measured using an automatic plate reader (Tecan, Männedorf, Switzerland) and data were acquired with the Magellan software for further statistical analyses. All measurements were performed in triplicate.

2.6 Statistical analysis

The variation of the results obtained from the HST was analyzed by analysis of variance (ANOVA). Differences between mean values were determinate by LSD test. All the ANOVA analyses were performed with Statistica v. 7.0 software.

Tabla 1. Identification code of the yeasts used in this work. Protease activity at pH 6 where (+) indicate presence of activity, (++) higher activity, (-) no activity evidence and (ND) not determined.

Specie	Code	Proteaseactivity at pH6
H. vineae	T02-05F	++
H. vineae	T02-19F	+
H. vineae	T02-25F	++
H. vineae	TE11-24F	+
H. vineae	TE11-48F	+
H. vineae	M12-111F	++
H. vineae	T12-151F	+
H. vineae	T12-184F	+
H. vineae	M12-196F	+
H. vineae	C12-219F	+
H. vineae	TE 18-63F	+
H. vineae	CH 18-72G	+
H. vineae	CH 18-87F	+
H. vineae	MO 18-90Fa	+
H. vineae	L 18-91F	+
H. vineae	M 18-102G	+
H. vineae	M 18-113F	+
H. vineae	M18-128F	+
H. vineae	T18-152G	+
H. vineae	M18-159F	+
H. vineae	M18-164F	+
H. vineae	T18-169F	+
Saccharomyces cerevisiae	**BM 4X4**	-

3 Results and Discussion

3.1 Proteolyticactivity on skim milk medium

As observed in previous studies [12] at pH 4.5 no proteolytic activity could be observed on the plate, while at pH 6 the formation of transparent halos around the colonies was observed, indicating the presence of exocellular enzymatic activity. These results would indicate that at pH 4.5 the conditions of the skim milk medium on the plate would not be ideal for the development of enzymatic activity. At pH 6 it could also be observed that there is a different intensity of activity, given the difference in the size of the halos, thus indicating that it is a strain-dependent characteristic. This is in agreement with what was previously observed byus [12]. In Table 1, three strains showed greater evidence of enzymatic activity (T02-05F, T02-25F, and M12-111F). In the rest of the strains, activity was also evidenced, but with less intensity.

3.2 Heat Stability Test (HST)

As for the measurement of fermentation haze, as with the proteolytic activity on the plate, we observed different behaviors in each strain studied. This indicates that the behavior of each yeast to reduce the haze in the wines, associated with protease activity, will depend on each strain.

Of the 22 strains studied, four of them (T02-05F, TE11-48F, T12-151F, and M12-184F) showed a significant decrease of haze in wines after heat treatment compared to the control wine fermented only with *S. cerevisiae* (Fig. 1). As we can also see in this figure there are two strains, T02-25F and M12-111F that show significant increased levels of turbidity compared to *Saccharomyces*. Both strains were highly considered producers of protease activity by the skim milk agar medium.

From these results it is also clear that what is observed in skim milk plates on the behavior of the proteolytic enzymatic activity of the yeasts, will not necessarily be fulfilled in the real wine conditions of a fermentation. Furthermore, protein levels found in the final wines were also not correlated to the reduction of haze in the four successful strains (data not shown). From the practical point of view the heat stability test is essential to reach protein stability. Although protease activity might be high in some of the strains, these results showed that protein stability might be affected by other strain characteristics, such as polysaccharide or mannoprotein production, or by the fast cell lysis process that some*H. vineae*have [23,24] and might balance the extracellular total protein content.

Interestingly, strain T02-05F (HV205) has been used previously in many real winemaking processes with highly positive organoleptic results from flavor and color point of view [13,16,23,25-27]. Although it was used in many grape varieties as it can be seen in these works, it was never study and related to protein haze in varieties such as Sauvignon blanc. Further studies with this commercial strain should be done to evaluate this aspect in white haze prevention reducing bentonite treatments by its protease capacity or by other extracellular phenomena such as fast cell lysis or increase polysaccharide or mannoprotein synthesis [28]. Therefore, we can conclude that in addition to the before mentioned characteristics of *Hanseniaspora vineae* yeasts, this yeast can reduce haze in wines. This makes it a very attractive alternative yeast for winemaking.

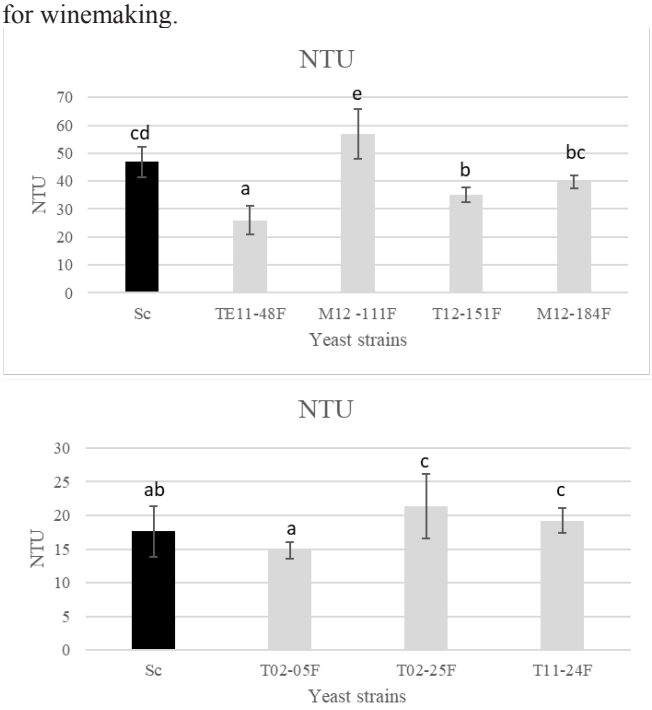

Figure 1. Difference in turbidity between untreated and heat-treated wines from fermentations inoculated with *H. vineae* (grey) and comparison with a control inoculated with *S. cerevisiae* (black).

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