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A new method for monitoring the extracellular proteolytic activity of wine yeasts during alcoholic fermentation of grape must

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ABSTRACT

The existing methods for testing proteolytic activity are time consuming, quite difficult to perform, and do not allow real-time monitoring. Proteases have attracted considerable interest in winemaking and some yeast species naturally present in grape must, such as *Metschnikowia pulcherrima*, are capable of expressing this activity. In this study, a new test is proposed for measuring proteolytic activity directly in fermenting grape must, using azocasein, a chromogenic substrate. Several yeast strains were tested and differences in proteolytic activity were observed. Moreover, analysis of grape must proteins in wines revealed that protease secreted by *Metschnikowia* strains may be active against wine proteins.

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1. Introduction

Current testing methods for proteolytic activity use solid or liquid laboratory media. Milk, casein, or gelatine plates are frequently used and proteolytic activity is estimated by the appearance of a clearance zone around the colony (Charoenchai et al., 1997; Fernández et al., 2000; Mateo et al., 2015; Reid et al., 2012; Strauss et al., 2001). This requires quite a long incubation time and, moreover, the results obtained by these methods are qualitative and do not differentiate among the various strains' proteolytic activity. Quantifying proteolytic activity requires the use of laboratory buffers, such as phosphate or citrate–phosphate, containing proteins, generally BSA (Lagace and Bisson, 1990; Mateo et al., 2015). The presence of active proteases leads to an increase in the optical density of the solution. The Cd-ninhydrin method (Maturano et al., 2012; Mendoza et al., 2007) is also used to measure proteolytic activity. Cells are incubated with proteins and Cd-ninhydrin reagent in the presence of citrate-citric acid buffer. Proteolytic activity can be determined by measuring optical density. Oxidized insulin is also used as a protease substrate for measuring proteolytic activity in wine. NH_2 products released by protein hydrolysis are measured by ninhydrin (Humbert-Goffard, 2003). In these methods, pH is not always adjusted to a value representative of white wine (between 3.0 and 3.5). Moreover, while incubation times are shorter than those required by plate methods, this analysis remains time-consuming and quite difficult to perform.

Wine haze forms when proteins become unstable and insoluble. The mechanism is not entirely understood, but is hypothesized to result from thermal denaturation of proteins under unfavourable storage conditions. It may be induced by several physicochemical factors, including pH, ethanol concentration, etc. (Dawes et al., 1994; Sarmento et al., 2000). This phenomenon reduces the commercial value of the wine. Some pathogen-related proteins (β-glucanases, chitinases, and thaumatin-like proteins) are responsible for haze formation. They are characterised by a molecular mass between 13 and 30 kDa and an isoelectric point between 4.1 and 5.8 (Hsu and Heatherbell, 1987; Lamikanra and Inyang, 1988; Waters et al., 1992). Bentonite is frequently used to reduce the risk of haze formation in wine, but this treatment presents some disadvantages: loss of volume, non-optimum efficiency against all protein classes at a single concentration, the use of mineral material non-intrinsic to wine, and the elimination of some aroma compounds. Wine treatments with acid yeast proteases may offer a microbial alternative, making it possible to reduce the use of bentonite (Jolly et al., 2003; Lagace and Bisson, 1990; Pocock et al., 2003). Besides reducing haze formation, proteases release peptides and amino acids from proteins, thus increasing the assimilable nitrogen for yeast in must, with a positive impact on wine aroma. Proteases may also be useful for releasing mannoproteins in wine via lees autolysis, thus enhancing wine protein stability (Moine-Ledoux and Dubourdieu, 1999). Proteases

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need to be active at the low pH (3.0–3.5) of wine, in the presence of SO_2, and at winemaking temperatures.

Several yeast genera, including *Saccharomyces*, *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora*, and *Issatchenkia* are frequently isolated from fermenting grape must (Baleiras Couto et al., 2005; Combina et al., 2005; Fleet et al., 2002; Li et al., 2010; Zott et al., 2008). As the environment changes (increase in ethanol content, presence of inhibitors, competition for nutrients, etc.), *Saccharomyces cerevisiae* species become predominant and complete the alcoholic fermentation.

S. cerevisiae is not known for abundant production of extracellular proteases. Recently, Younes et al. (2013) characterised some proteases but they are not active under oenological conditions. Several studies have shown that some non-*Saccharomyces* species, particularly *M. pulcherrima*, have an interesting potential in this regard (Charoenchai et al., 1997; Fleet, 1992; Theron and Divol, 2014).

This article proposes a simple method for assessing and monitoring the proteolytic activity of yeasts during the alcoholic fermentation of grape must.

2. Materials and methods

2.1. Yeast strains

Seven yeast strains were used: 4 *M. pulcherrima*, 1 *Metschnikowia* spp. (99% identity with *Metschnikowia andauensis*), 1 *S. cerevisiae*, and 1 *Torulaspora delbrueckii* (Table 1).

2.2. Preculture and alcoholic fermentation conditions

2.2.1. Precultures

Except for *S. cerevisiae* and *T. delbrueckii*, two preculture steps were necessary before alcoholic fermentation. First, strains were grown in YPD-based medium containing 1% yeast extract (*w*/*v*), Difco Laboratories, Detroit, MI), 1% Bacto peptone (*w*/*v*, Difco), and 2% glucose (*w*/*v*) at 24 °C for 24 h. Then, the cultures were transferred to half-diluted grape must with agitation at 24 °C for 24 h. Culture in half diluted grape must was only necessary for *S. cerevisiae* and *T. delbrueckii*.

2.2.2. Alcoholic fermentations

Alcoholic fermentations were carried out in 125 mL of Sauvignon Blanc grape must from the Bordeaux region (reducing sugars: 203 g/L, pH: 3,3) at 18 °C (usual temperature for white grape must fermentation), in 140 mL flasks, with agitation. All fermentations were performed in triplicate. The different strains were inoculated as pure cultures at 5×10^6 viable cells/mL. Fermentation kinetics were monitored by regular measurements of the weight loss due to CO_2 release. Several growth and fermentation parameters were calculated for each species: growth rate ($\text{division}\cdot h^{-1}$), maximum population size ($\text{CFU}\cdot mL^{-1}$), fermentation lag phase (time between yeast inoculation and the beginning of alcoholic fermentation in h), maximum fermentation rate ($g\cdot L^{-1}\cdot h^{-1}$), and maximum CO_2 release ($g\cdot L^{-1}$).

Table 1
Strains used in alcoholic fermentation.

Species	Strain	Collection
Metschnikowia pulcherrima	CRBO L1329	Centre de Ressources Biologiques Oenologique, Bordeaux, France
	Y6259	Northern Regional Research Laboratory
	NZ366	Centre de recherche Pernod-Ricard, Créteil, France
	IWBT Y1123	Institute for Wine Biotechnology, Stellenbosch University, South Africa
Metschnikowia spp.	CRBO L0563	Centre de Ressources Biologiques Oenologique, Bordeaux, France
Saccharomyces cerevisiae	Zymaflore X5	Laffort, Bordeaux, France
Torulaspora delbrueckii	Zymaflore Alpha	

2.3. Cell enumeration

Yeast growth during fermentation was monitored by plate counting on a YPD-based medium: 1% yeast extract (*w*/*v*), 1% Bacto peptone (*w*/*v*), 2% glucose (*w*/*v*), and 2% agar (*w*/*v*). Plates were incubated at 24 °C.

2.4. Proteolytic activity

A chromogenic protease substrate, azocasein (Sigma Aldrich), was used to monitor proteolytic activity directly in fermenting grape must. Azocasein proteolysis released a free dye into the supernatant, which was quantified by measuring optical density at 440 nm. Several concentrations of azocasein stock solution, prepared in NaOH 0.1 M, were tested, as well as various final concentrations in bioreactors, to determine the optimum quantity of azocasein needed to be in excess of the substrate without markedly increasing must pH (data not shown). The optimum concentration for stock solution was determined to be 20 $mg\cdot L^{-1}$, giving a final concentration of 1.5 $mg\cdot L^{-1}$ in the bioreactors.

Samples were taken throughout alcoholic fermentation. The proteolysis reaction was stopped with trichloroacetic acid (10% final concentration, *v*/*v*, Sigma). Samples were centrifuged at 21, 693 *g* for 10 min and then the optical density of the supernatant was measured to evaluate the proteolytic activity of the species tested. Initial assays were carried out with or without azocasein to check that this substrate did not impact yeast growth or alcoholic fermentation.

2.5. Analysis of grape must proteins

At the end of alcoholic fermentation, 50 mL samples from *S. cerevisiae*, *Metschnikowia* spp. CRBO L0563, *M. pulcherrima* IWBT Y1123, and *M. pulcherrima* Y6259 were centrifuged at 6000 rpm for 5 min. The molecular weights of the supernatant macromolecules were determined by HPLC on a TSKgel® G2000 SW column (Phenomenex), using the protocol described by Dubourdieu et al. (1986) for the fractionation of molecules from 10 to 70 kDa. Three molecular masses were separated by retention time, corresponding to three fractions: P1 (>50 kDa), P2 (40 kDa) and P3 (<30 kDa). The grape must proteins responsible for haze formation are characterised by molecular masses between 13 and 30 kDa (Waters et al., 1992), associated with the P3 fraction.

2.6. Data analysis

R software was used for statistical analysis. Variance heteroscedasticity made it possible to apply the Kruskal–Wallis test to determine the difference between the growth and alcoholic fermentation parameters with and without azocasein and the effect of *M. pulcherrima* protease on grape must protein.

3. Results

3.1. Azocasein did not impact yeast growth or alcoholic fermentation

In this part we focused on the effect of azocasein on the growth and alcoholic fermentation kinetics of several wine yeast species: *S. cerevisiae* (X5), *T. delbrueckii* (Alpha), *M. pulcherrima* (IWBT 1123), and *Metschnikowia* spp. (CRBO L0563). Several growth and alcoholic fermentation parameters were calculated for each species, as described in materials and methods (Table 2). All fermentations with *S. cerevisiae* were completed whereas, as expected, *T. delbrueckii* and *Metschnikowia* cultures stopped at 85% and 65%, respectively.

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