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Research Paper

Characterization of pectinase activity for enology from yeasts occurring in Argentine Bonarda grape

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Abstract

Pectinolytic enzymes are greatly important in winemaking due to their ability to degrade pectic polymers from grape, contributing to enhance process efficiency and wine quality. This study aimed to analyze the occurrence of pectinolytic yeasts during spontaneous fermentation of Argentine Bonarda grape, to select yeasts that produce extracellular pectinases and to characterize their pectinolytic activity under wine-like conditions. Isolated yeasts were grouped using PCR-DGGE and identified by partial sequencing of 26S rRNA gene. Isolates comprised 7 genera, with *Aureobasidium pullulans* as the most predominant pectinolytic species, followed by *Rhodotorula dairenensis* and *Cryptococcus saitoi*. No pectinolytic activity was detected among ascomycetous yeasts isolated on grapes and during fermentation, suggesting a low occurrence of pectinolytic yeast species in wine fermentation ecosystem. This is the first study reporting *R. dairenensis* and *Cr. saitoi* species with pectinolytic activity. *R. dairenensis* GM-15 produced pectinases that proved to be highly active at grape pH, at 12 °C, and under ethanol and SO_2 concentrations usually found in vinifications (pectinase activity around 1.1 U/mL). This strain also produced cellulase activity at 12 °C and pH 3.5, but did not produce β-glucosidase activity under these conditions. The strain showed encouraging enological properties for its potential use in low-temperature winemaking.

Key words: *Aureobasidium pullulans*, *Cryptococcus saitoi*, pectinolytic activity, *Rhodotorula dairenensis*, winemaking.

Introduction

Pectinolytic enzymes are polysaccharidases that degrade pectins present in middle lamella and primary cell walls of plants. This ability is widely used in winemaking as pectinases can help to improve liquefaction, juice yield, clarification, filterability, and to increase the release of color and flavor compounds entrapped in grape skins (Fleet, 2008; Alimardani-Theuil *et al.*, 2011; Martín and Morata de Ambrosini, 2014).

Grape skin forms a physical barrier to diffusion of anthocyanins, tannins and aroma contained in the skin cells. The permeability of skin cell walls to these compounds can be increased by partial hydrolysis of their structural polysaccharides (pectins, cellulose and hemicelluloses), a process that can be facilitated by pectinolytic preparations. These enzymes are complex mixtures mainly comprised of pectinases, but also other desirable activities like cellulases, hemicellulases and acid proteases. Cellulases and hemicellulases are also responsible for an increase in the extraction of grape juice, and the improvement in the clarification of wines (Romero-Cascales *et al.*, 2008).

Nowadays, commercial pectinase preparations are obtained from fungi and apart from desirable activities, they generally contain undesirable enzymes like pectinesterase (Alimardani-Theuil *et al.*, 2011) and β-glucosidase (Romero-Cascales *et al.*, 2008) that can negatively affect

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the quality of wines. Yeasts are known to produce mostly polygalacturonases (Alimardani-Theuil *et al.*, 2011), and they could therefore be a preferable source of pectinases. Considering the key role of yeasts in winemaking, selection of pectinolytic yeasts for enology is an alternative approach to fungal pectinases. Although some pectinolytic wine yeasts have already been described (Fernández *et al.*, 2000; Fernández-González *et al.*, 2004), further studies are needed.

"San Rafael" Designation of Origin (DO), an area in central-west Argentina, represents an important wine region in South America. Despite its long viticulture and enology history, very little is known about the microorganisms involved in spontaneous fermentations. Currently, Argentine Bonarda is one of the red grape varieties with increasing enological interest in the country.

Cold-active enzymes are attractive for usage in food industry since low-temperature conditions favor conservation of sensory and nutritional properties in the product (Sahay *et al.*, 2013). A previous study reported cold-active pectinase activity by yeasts isolated from wine grapes (Merín *et al.*, 2011). However, the presence of pectinolytic yeasts during fermentation and their enzymatic activities under winemaking conditions have not been described.

This study reports the occurrence of pectinolytic strains among representative yeasts isolated at different stages during spontaneous fermentation of Argentine Bonarda grape. And it focuses on selection and characterization of their enzymatic behavior under wine-like conditions (pH 3.5, 12 and 28 °C, ethanol and SO_2) for their potential use in winemaking.

Materials and Methods

Study area, sampling and fermentation

Grape samples (*Vitis vinifera* L.) of cv. Argentine Bonarda were collected from Cuadro Benegas viticultural region (lat. 34.62° S, long. 68.45° W) in San Rafael DO, Mendoza, Argentina, during the 2009 vintage.

Representative bunches of healthy grapes were aseptically hand harvested, transported to the laboratory and kept cold until their study. The collected grapes were crushed to conduct the spontaneous fermentation in 1 L Erlenmeyer flasks containing 800 mL of must (reducing sugars 220 g/L; titratable acidity 4.6 g/L; yeast assimilable nitrogen (YAN) 310 mg/L; pH 4.2). Progress of the alcoholic fermentation was monitored daily by measuring weight loss using flasks with stoppers containing a Müller valve that allows only CO_2 to escape from the system. Fermentations were carried out in duplicate at 25 ± 1 °C until constant weight for two consecutive days.

Yeast isolation

Before the grape crushing, 10 berries were placed in a flask containing 10 mL of sterile peptone-water (0.1%, w/v) and were shaken at 165 rpm during 1 h at room temperature. Aliquots of adequate dilutions of both berry washing solution and grape must, sampled at 0, 3, 5, 8 and 12 days of fermentation, were plated onto WL Nutrient Agar. This medium allows presumptive discrimination among yeast species by colony morphology and color (Pallmann *et al.*, 2001). Plates were incubated at 25 °C for 5 days for colony development. A representative number of each colony type was recovered. Isolates were purified by streak plating and subcultured onto Yeast extract Peptone Dextrose (YPD: yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L; agar, 20 g/L) for subsequent identification.

Molecular yeast identification

Yeast isolates were examined with PCR-DGGE to group them according to their DGGE mobility and representative strains of each group were subsequently sequenced as described by Rantsiou *et al.* (2013). Isolates were subjected to DNA extraction and a DNA fragment from the D1-D2 loop region of the 26S rRNA gene was amplified by PCR using primers NL1GC/LS2 following the protocol proposed by Cocolin *et al.* (2000).

The DCode™ Universal Mutation Detection System (Biorad, USA) was used for DGGE analysis according to Cocolin *et al.* (2000) with minor modifications in the denaturing gradient (30 to 50% urea-formamide) and the electrophoresis conditions (constant voltage of 130 V for 4 h). To obtain species identification, PCR amplification of representative colonies from each migration-specific group was conducted by using primers NL1/NL4 (Kurtzman and Robnett, 1998). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Germany) and sequencing was carried out at MWG Biotech (Germany) and CERELA (Argentina). Strains were identified by searching the GenBank database with the BLAST program (http://www.ncbi.nlm.nih.gov).

Screening of pectinolytic activity among isolated yeasts

The ability of isolated yeasts to hydrolyze pectin was assayed using the Petri dish method according to Merín *et al.* (2011). The isolates were point-inoculated onto a mineral medium containing 0.2% (w/v) citric pectin as carbon source, at pH 4.5, and incubated at 28 °C for 48-72 h. Enzyme activity was evidenced by clear halos around the colonies against a purple-brown background after addition of Lugol's solution.

Enzyme assays

Production of extracellular enzymatic extracts

For enzyme production, yeasts showing pectinolytic activity on plates were inoculated in a basal liquid medium (containing per L of 50 mM citric-citrate buffer: dextrose, 20 g; soy peptone, 10 g; meat peptone, 10 g; yeast extract, 10 g) at pH 3.5, proximate to wine pH. The cultures were

incubated under shaking conditions (100 rpm) at 12 and 28 °C for 5 or 3 days, respectively. Cells were removed by centrifugation (5000x *g*, 15 min at 4 °C) and supernatants were filtered (0.22 μm) to obtain cell-free supernatants (enzymatic extracts) on which all the enzymatic activities were assayed throughout this study.

The assessment of enzymatic activities in the present work was always carried out at the same temperature of the enzymatic production, at 12 or 28 °C, as appropriate.

Pectinolytic activity

Pectinolytic activity was assayed by measuring the amount of reducing sugars released from a pectin dispersion (0.25% pectin in 50 mM citric-citrate buffer, pH 3.5) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). Galacturonic acid was used as standard (Sigma, USA). The reaction mixtures (enzymatic extract/substrate 1/10) were incubated at 12 or 28 °C, at the corresponding enzyme production temperature, for 30 min as previously described (Merín *et al.*, 2011). One pectinase unit (U) is defined as the enzymatic activity that releases 1 μmol of reducing sugar per min under the assay conditions.

Other cold-active hydrolytic activities of enological interest

Cellulase and xylanase activities were assayed in enzymatic extracts obtained at 12 °C as described above by measuring the reducing sugars according to Miller (1959). Cellulase was measured using microgranular cellulose (Whatman CC41) and xylanase using birchwood xylan (Sigma, USA) as substrates at a concentration of 0.25% (w/v) in 50 mM citric-citrate buffer (pH 3.5). The enzymatic reactions were carried out at 12 °C for 30 min. One cellulase or xylanase unit is defined as the enzymatic activity that releases 1 μmol of reducing sugar (as glucose or xylose, respectively) per min under the assay conditions.

β-Glucosidase activity was assayed by incubating 100 μL of enzymatic extract with 100 μL of 15 mM D-(+)-cellobiose (Fluka, USA) solution in citric-citrate buffer (pH 3.5) at 12 °C for 30 min. Glucose produced was quantified using the enzymatic colorimetric test (GOD-POD) (Arévalo-Villena *et al.*, 2007). One β-glucosidase unit is defined as the enzymatic activity that releases 2 μmol of glucose from cellobiose per min under the assay conditions.

Extracellular protease activity was assayed qualitatively by point-inoculation of yeasts on plates of skim milk agar and gelatin agar at pH 4.5, according to the method described by Charoenchai *et al.* (1997). Skim milk agar plates were directly examined for clear zones surrounding yeast growth after incubation at 12 °C for 3-5 days, whereas gelatin agar plates were flooded with 10 mL acetic acid (50 g/L) before examination for clear zones around the yeast cells.

Appropriate enzyme and substrate blanks, as well as calibration curves, were included in all quantitative enzymatic assays.

T. delbrueckii BTd259 (Maturano *et al.*, 2012) was used as positive control for pectinolytic and other hydrolytic activities.

Influence of enological parameters on pectinolytic activity

The effects of ethanol, sulfur dioxide (SO_2) and a combination of ethanol and SO_2 on pectinase activity were evaluated in cell-free supernatants (enzymatic extracts produced as described in previous section) under standard enzymatic assay conditions. The substrate was supplemented with ethanol and total SO_2 at final concentrations of 15% (v/v) and 120 mg/L, respectively, and reactions were carried out at 12 and 28 °C, as appropriate. Reaction mixtures assayed under the same conditions at their respective temperatures but in absence of ethanol and SO_2 corresponded to the reference activity.

Statistical analysis

Analysis of variance (ANOVA) and Fisher's LSD test ($p < 0.05$) were applied to all experimental data, using STATGRAPHICS Plus 5.1 (Manugistics, Rockville, MD, USA). Data normality and variance homogeneity in the residuals were verified by modified Shapiro-Wilks and Levene's test, respectively.

Nucleotide sequence and yeast strain accession numbers

Partial sequence of the 26S rRNA gene of the most representative strains were submitted to the GenBank database available at NCBI under accession numbers: JF414133 (*Saccharomyces cerevisiae*), JF414134 (*Candida zemplinina*), JN637171 (*Cryptococcus saitoi*), JN637172 (*Rhodotorula dairenensis*).

The latter two strains were also deposited at the Banco Nacional de Microorganismos (BNM) Culture Collection (Buenos Aires, Argentina) under accession numbers: BNM 538 (*Cryptococcus saitoi* GM-4) and BNM 539 (*Rhodotorula dairenensis* GM-15).

Results and Discussion

Isolation and identification of representative yeasts from Argentine Bonarda grape and fermenting must

Alcoholic fermentation of Argentine Bonarda must was completed in 12 days. The red wine had a final pH of 4.1 and a final ethanol concentration of 11.7% (data not shown).

A total of 48 yeast colonies were isolated from grape surface, fresh must and spontaneous fermentation on WL medium. The 22 colonies isolated from grapes and must comprised 8 groups with different morphology. During the fermentation process, the diversity of the yeast colony morphology decreased and only 6 different types were observed. Consequently, the 48 colonies were classified into 14 morphological groups (Table 1), characterized by their

Table 1 - Identification at species level of morphological groups of yeasts isolated from grapes, fresh must and fermenting must, based on the migration of the bands obtained by PCR-DGGE.

Morphological group	Isolation source of yeast groups	DGGE pattern	DGGE gel lane	Species identification[a]
7, 11	Grape, fresh must, fermenting must	I	1, 5	*Hanseniaspora* sp.
10, 12, 13, 14	Fermenting must	II	2, 6, 7, 9	*Saccharomyces cerevisiae*
1, 2	Grape	III	3, 4	*Rhodotorula dairenensis*
9	Fermenting must	IV	8	*Candida zemplinina*
8	Grape, fresh must, fermenting must	V	10	*Metschnikowia* sp.
3, 4, 5	Grape, fresh must	VI	11, 12, 13	*Aureobasidium pullulans*
6	Grape	VII	14	*Cryptococcus saitoi*

[a]One representative isolate from each DGGE pattern was identified by sequencing of the D1-D2 loop region of 26S rRNA gene and comparison with BLAST tool in GenBank.

specific color, consistency and surface, as previously described (Pallmann *et al.*, 2001; Urso *et al.*, 2008).

All the colonies of each morphological pattern were subjected to PCR-DGGE to establish species-specific migration groups. Profiles of each DGGE migration group are presented in Figure 1.

Seven different DGGE profiles were generated. Comigrating DGGE bands were considered to belong to the same species. After sequencing, 7 yeast genera were identified, corresponding to *A. pullulans*, *R. dairenensis*, *Cr. saitoi*, *C. zemplinina* and *S. cerevisiae* species and *Hanseniaspora/Kloeckera* sp. and *Metschnikowia* sp. (Table 1). The identified yeasts are among the most frequently described on grape skins and fresh musts from diverse regions around the world (Merín *et al.*, 2011; Barata *et al.*, 2012; Rantsiou *et al.*, 2013), and during wine fermentation (Fleet, 2008; Urso *et al.*, 2008; Rantsiou *et al.*, 2013).

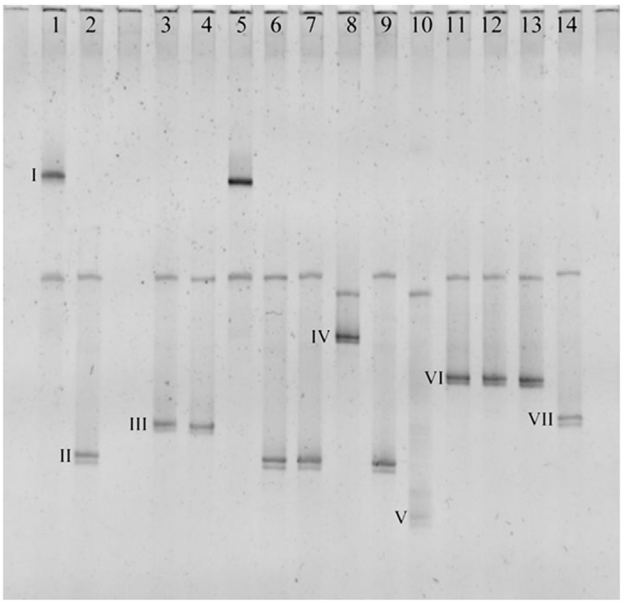

Figure 1 - DGGE profiles of yeasts isolated from Argentine Bonarda grapes and must during spontaneous fermentation. Correlations between lane designations and colony groups as well as Roman numerals and DGGE patterns are indicated in Table 1. The bands common to all isolates are single stranded DNA artifacts that were not influenced differentially by the gradient (Cocolin *et al.*, 2000).

Occurrence of pectinolytic yeasts isolated during spontaneous fermentation of Argentine Bonarda grape

Out of 48 representative yeast isolates, 11 (23%) belonging to 3 species showed pectinolytic activity on agar plates (Figure 2). According to a recent review by Alimardani-Theuil *et al.* (2011), of approximately 700 yeast species identified to date, only very few produce pectinolytic enzymes, which is in accordance with our results.

The total number of representative yeasts isolated from grapes, fresh must and fermentations is shown in Figure 2A. Among the yeast isolated from grapes and must, *A. pullulans* was the most abundant species with 8 isolates, followed by *Hanseniaspora* sp. with 6, *Metschnikowia* sp. and *R. dairenensis*, both with 3, and *Cr. saitoi* with 2 isolates.

Figure 2B shows that pectinolytic yeasts were only isolated from grape berry surfaces and fresh must. *A. pullulans* was the predominant pectinolytic species, representing 73% of the total number of isolates able to degrade pectin. Besides, all *A. pullulans* isolates produced pectinases. Similar results have previously been observed in wine grapes, with *A. pullulans* as the most frequent cold-active-pectinase-producing species (Merín *et al.*, 2011, 2014), and in tropical environments where this species represented the highest proportion (22%) of pectinolytic microorganisms (Buzzini and Martini, 2002). The results suggest that this pectinolytic microorganism is more prevalent in plant materials, soil and water.

The remaining pectinolytic species corresponded to the basidiomycetous yeasts *R. dairenensis* and *Cr. saitoi*, representing 18% and 9% of the total pectinolytic isolates, respectively (Figure 2B). Two thirds of the representative isolates of *R. dairenensis* and half of the *Cr. saitoi* isolates produced pectinases. Yeasts belonging to these two genera have previously been described as pectinolytic microorganisms (Federici, 1988; Nakagawa *et al.*, 2004; Turchetti *et*

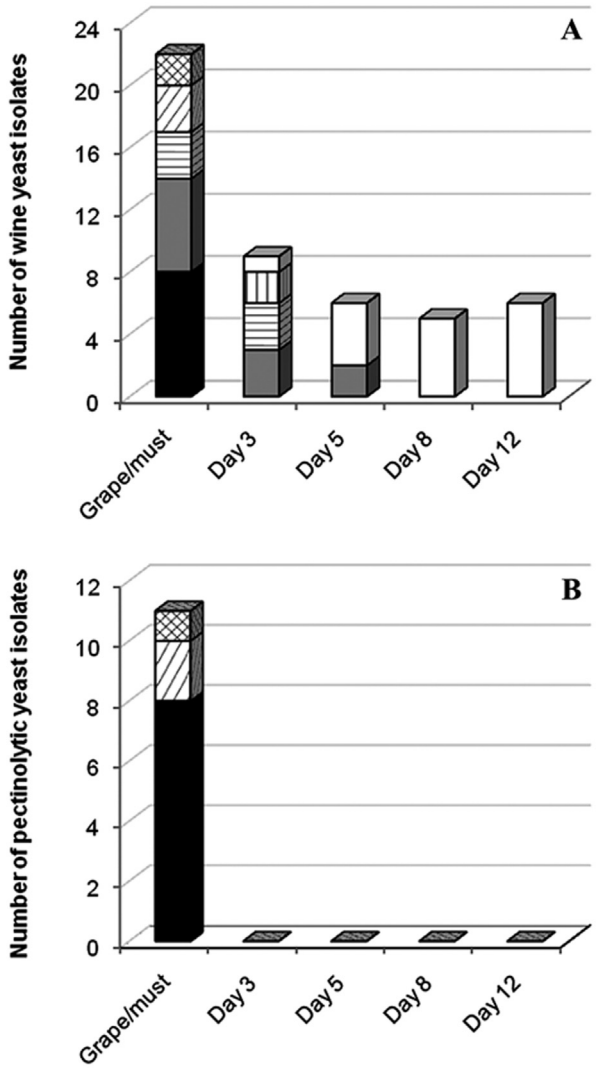

Figure 2 - Occurrence of pectinolytic yeasts among representative yeasts isolated from grapes, fresh must and fermentation (sampling time: days 3 to 12) of cv. Argentine Bonarda. Number of isolates of yeast species (A) and of pectinolytic yeast species (B) found on grapes and during spontaneous fermentation. *Aureobasidium pullulans, Hanseniaspora* sp., *Metschnikowia* sp., *Rhodotorula dairenensis, Cryptococcus saitoi*, *Candida zemplinina* and *Saccharomyces cerevisiae*.

al., 2008), particularly the species *R. mucilaginosa* (Vaz *et al.*, 2011; Sahay *et al.*, 2013) and *R. glutinis* (Taskin, 2013), which are phylogenetically closely related to *R. dairenensis*. Nevertheless, to our knowledge, this is the first study reporting pectinase activity by *R. dairenensis* and *Cr. saitoi* species.

Most yeasts present on wine grape at harvest time and during fermentation belong to ascomycetous species (Fleet, 2008; Urso *et al.*, 2008; Barata *et al.*, 2012). Nevertheless, only a few ascomycetous species have been reported to produce pectinases in wine ecosystem such as *Kluyveromyces*, *Candida, Metschnikowia* and some *S. cerevisiae* strains (Fernández *et al.*, 2000; Fernández-González *et al.*, 2004). In this study, no pectinolytic activity was detected among ascomycetous yeasts, either non-*Saccharomyces* or *S. cerevisiae* isolates (Figure 2). Comparable results have recently been obtained in studies of pectinolytic yeasts isolated from viticultural and enological environments (Merín *et al.*, 2011, 2014), which are in agreement with findings reported by other authors. In an extensive screening survey, Buzzini and Martini (2002) demonstrated that pectinase activity was rarely found within ascomycetes in tropical environments, observing this ability in only 1.5% of studied ascomycetes. Charoenchai *et al.* (1997) did not detect pectinolytic activity in ascomycetous wine yeasts.

The fact that pectinolytic yeasts were only found on grape surfaces and must, whereas non-pectinolytic yeasts dominated the middle and last fermentation stages (Figure 2), suggests that a higher incidence of pectinolytic species may be observed in nutrient-poor environments like the grape surface and other plant surfaces, and in marine and glacial ecosystems (Nakagawa *et al.*, 2004; Turchetti *et al.*, 2008). It seems that pectinolytic yeasts play an ecological role on the grape surface since they can utilize pectin from cell walls releasing intracellular sugars to surface. Conversely, microorganisms in musts do not require pectinolytic activity to acquire carbon sources because of the abundance of readily usable sugars. This is consistent with results reported by Barata *et al.* (2012), who observed that the microbiota of truly intact berries after véraison is dominated by basidiomycetous yeasts (*e.g. Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp.) and *A. pullulans*; while visually intact berries may bear microfissures and softens, increasing nutrient availability and explaining the possible dominance by the oxidative or weakly fermentative ascomycetous populations (*e.g. Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp.) approaching harvest time.

According to our results, the microfissures and softens on visually intact berries could be due to the action of pectinases produced by pectinolytic yeasts on grapes, like *A. pullulans* and basidiomycetous yeasts found in this study, which would release intracellular substrates from grape, thus sustaining the growth of non-pectinolytic ascomycetous yeasts on the grape berry surface.

Characterization of enzymatic activities

Hydrolytic activities under winemaking conditions

All yeasts showing extracellular pectinase activity on plate were also assayed in liquid medium at 28 °C and pH 3.5 to select the best pectinolytic yeasts under winemaking conditions (data not shown). Of the 11 pectinolytic yeasts, two *A. pullulans* strains (GM-1 and GM-2) and two basidiomycetous yeasts (*R. dairenensis* GM-15 and *Cr. saitoi* GM-4) produced the highest activities at grape pH (0.967, 1.325, 1.260, 0.930 U/mL, respectively). These activities are higher than those produced by other yeasts, such as *Saccharomyces* strains: 0.107-0.679 U/mL (Oliveira *et al.*,

2006) and *Zygoascus hellenicus* strains: 0.025-0.220 U/mL (Ahansal *et al.*, 2008). However, the pectinolytic activities cited were displayed at pH 5.0.

The studied strains exhibited similar pectinolytic activity to that previously reported for *A. pullulans* at pH 3.5 and 12 °C, which has already been characterized (Merín *et al.*, 2011). Consequently, considering that this is the first study reporting pectinase production by *R. dairenensis* and *Cr. saitoi*, both strains (GM-15 and GM-4) were selected to characterize their enzymatic activity under wine-like conditions.

Because of the interest in cold-active enzymes, pectinolytic and other hydrolytic activities useful in vinification were further assayed in yeast enzymatic extracts produced at pH 3.5 and 12 °C. Pectinase activity in both strains was significantly higher than activity showed by *T. delbrueckii* BTd259 control strain, particularly in *R. dairenensis* GM-15 (1.104 U/mL) (Table 2), and also higher than pectinolytic activities produced by other yeasts, like *Saccharomyces* and *Zygoascus hellenicus* strains (0.100-0.679 U/mL), even at 30 or 50 °C (Oliveira *et al.*, 2006; Ahansal *et al.*, 2008).

Cellulase and hemicellulase enzymes degrade cellulose and hemicelluloses, respectively, present in grape cell walls. Consequently, they are responsible for an increase in the extraction of juice and color, and for improvement in the clarification of wines (Romero-Cascales *et al.*, 2008). Of the secondary enzymes assayed, only cellulase activity was detected in GM-15 enzymatic extract with a considerable activity (0.549 U/mL) at 12 °C (Table 2).

β-Glucosidase enzymes hydrolyze glycosilated complexes releasing volatile compounds that contribute to wine aroma (Rodríguez *et al.*, 2007). However, commercial pectinases containing β-glucosidases may cause a loss of color in red wines because some of these enzymes are able to degrade anthocyanins, glycosilated polyphenols that are mainly responsible for the red color of wine (Romero-Cascales *et al.*, 2008). The fact that β-glucosidase was not detected in the enzymatic systems analyzed (Table 2) is a positive feature when the yeast enzymes are applied to red winemaking.

Protease activity was also assayed on plate at 12 °C. Neither of the strains secreted proteases into the medium, unlike the *T. delbrueckii* BTd259 control strain (Table 2).

Effect of ethanol and sulfur dioxide on pectinolytic activity

Wine is a complex system that presents a combination of factors such as pH, temperature, ethanol and SO_2, among others. Since the sum of the responses of each of the single parameters does not necessarily predict the combined response of such parameters (Grimaldi *et al.*, 2005), the combined effect of ethanol and sulfur dioxide on enzymatic activity should be studied.

To acquire high quality products, the enology sector uses different technologies like low temperature vinification (Gómez-Míguez *et al.*, 2007; Fleet, 2008) and due to the potential value of cold-active enzymes, research on these enzymes is increasing conspicuously in these years. Therefore, the individual and combined effect of 15% (v/v) ethanol and 120 mg/L SO_2 on pectinolytic activity was assessed at 12 and 28 °C (Figure 3). At 12 °C, 15% (v/v) ethanol and 120 mg/L SO_2 as individual parameters hardly affected pectinolytic activity (retaining around 80-90% of relative activity), except for GM-4 pectinase, which was greatly inhibited by ethanol (conserving only 12% of relative activity). The combination of ethanol and SO_2 reduced pectinolytic activity to 50 and 70%, for GM-4 and GM-15, respectively.

At 28 °C, the ethanol concentration assayed reduced the pectinolytic activity of GM-4 by 50% and of GM-15 only by 20%. Nevertheless, emphasis must be laid in the fact that at the SO_2 concentration assayed (120 mg/L, the highest concentration generally observed in must at the beginning of the fermentation), activity increased by 33% (GM-4) and 22% (GM-15), compared with the reference activity. The combination of ethanol and SO_2 assayed at 28 °C slightly reduced GM-4 activity by 20%, while it enhanced GM-15 activity by 30% (Figure 3).

With respect to GM-4 pectinase activity, the combined effect of the two parameters produced a relative activity intermediate between the response to ethanol and SO_2 as single parameters at both temperatures. On the other hand, GM-15 pectinase behaved differently at the two tem-

Table 2 - Hydrolytic activities of enological interest of selected pectinolytic strains assessed at low temperature (12 °C) and grape pH (3.5).

Hydrolytic activities of enological interest	*Cr. saitoi* GM-4	*R. dairenensis* GM-15	*T. delbrueckii* BTd259[†]
Pectinase (U/mL)	0.858 ± 0.087^{b}	1.104 ± 0.034^{c}	0.423 ± 0.031^{a}
Cellulase (U/mL)	ND	0.549 ± 0.068	ND
Xylanase (U/mL)	ND	ND	0.243 ± 0.005
β-Glucosidase (U/mL)	ND	ND	0.0026 ± 0.0004
Protease (skim milk and gelatin)	-	-	+

[†]Control strain for enzymatic activities (Maturano *et al.*, 2012).

Different superscript letters within the same row indicate significant differences according to the LSD test ($p < 0.05$). Data are mean values ($n = 3$) ± SD. ND - not detected.

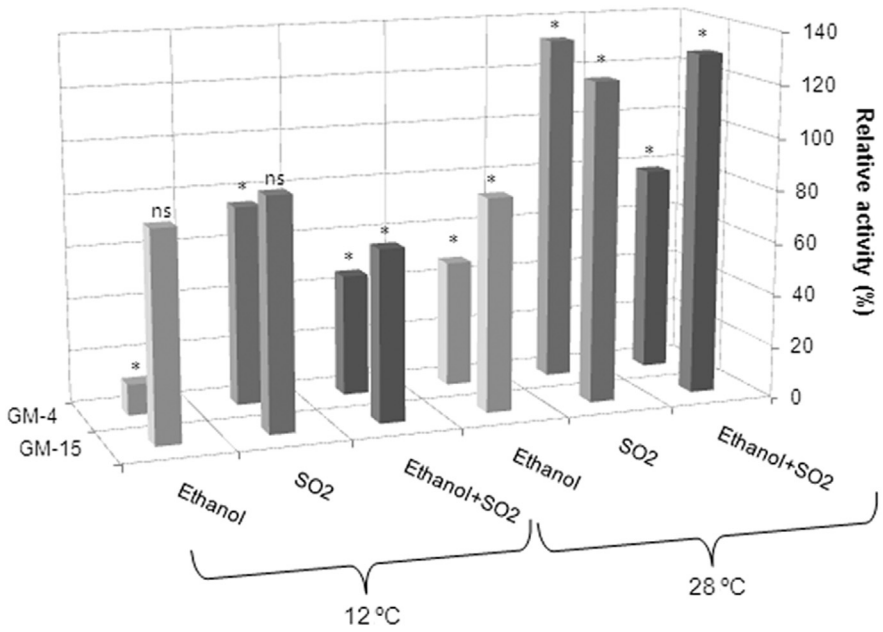

Figure 3 - Relative pectinolytic activity of *Cr. saitoi* GM-4 and *R. dairenensis* GM-15 assayed at 12 and 28 °C in presence of 15% (v/v) ethanol or 120 mg/L SO_2 or a combination of both compounds. Reference activity (100% of relative activity): 0.736 ± 0.071 U/mL at 12 °C and 1.349 ± 0.133 U/mL at 28 °C for *Cr. saitoi* GM-4, and 0.972 ± 0.083 U/mL at 12 °C and 1.483 ± 0.091 U/mL at 28 °C for *R. dairenensis* GM-15. (*): significant difference, (ns): not significant, according to the LSD test (n = 3, $p < 0.05$). In all cases SD was lower than 10%.

peratures assayed. Temperature seemed to affect the relative activity in presence of ethanol and SO_2, since at 12 °C the activity was negatively affected, but unexpectedly, at 28 °C the enzymatic activity was slightly increased. At the higher temperature, SO_2 probably counteracts the negative effect of ethanol on the relative activity. This could be explained by the unique ability of SO_2 to act as an oxidizing or a reducing agent that enables it either to inactivate enzyme systems by splitting their disulfide linkages (Cecil and Wake, 1962) or activate certain hydrolytic enzyme systems, probable by bringing about conformational changes (Malhotra and Hocking, 1976).

Different responses of related hydrolytic activities to these compounds have been reported. Ethanol concentrations of 12% (v/v) have been found to decrease polygalacturonase activity from *S. cerevisiae* (Fernández-González *et al.*, 2004), protease activity from *Ananas comosus* (Esti *et al.*, 2011) and α-L-rhamnosidase activity from *Aspergillus terreus* (Gallego *et al.*, 2001) to around 20-50%. Likewise, 15% (v/v) ethanol decreased the activity of β-glucosidases and β-xylosidases from wine yeasts to around 35% and 55%, respectively (Rodríguez *et al.*, 2007). Conversely, ethanol concentrations of 15 to 20% (v/v) produced significant increases (150-500% relative activity) in β-glucosidase (Barbagallo *et al.*, 2004) and β-xylosidase (Rodríguez *et al.*, 2007) activities from wine yeasts.

SO_2 has been reported to strongly inhibit protease activity from *Ananas comosus* (Esti *et al.*, 2011). Nevertheless, polygalacturonase (Fernández-González *et al.*, 2004) and α-L-rhamnosidase (Gallego *et al.*, 2001) activities were not affected by 50 mg/L SO_2. Rodríguez *et al.* (2007) observed that β-glucosidase activity from wine yeasts was not affected by 150 mg/L SO_2, which agrees with our results that showed a slight increase in pectinolytic activity in presence of 120 mg/L SO_2 compared with the reference activity. However, it is important to mention that the authors cited did not determine the effect of a combination of ethanol and SO_2 on the enzyme activities assayed.

These outcomes suggest that application of pectinase of *R. dairenensis* GM-15 in the production of red wine is preferably carried out at traditional temperatures (26-28 °C); although in vinifications at low temperature (12 °C) it would still retain high residual activity.

In conclusion, the current study has demonstrated that inoculation of pectinolytic yeasts or addition of pectinases in the vinification process is necessary since they do not naturally occur during wine fermentation. Our results also suggest that pectinolytic yeasts should be isolated from the grape surface. To our knowledge, this is the first report on pectinase production by *Cr. saitoi* and *R. dairenensis* species. The outcomes of the combined effect of ethanol and SO_2 at two possible fermentation temperatures on the pectinolytic activity indicated that the studied enzymes, particularly GM-15 pectinase, performed satisfactorily under wine-like conditions: at low pH (3.5), at low (12 °C) and traditional (28 °C) temperature in red winemaking, and in the presence of potential enzymatic inhibitors like ethanol

(15% [v/v]) and SO_2 (120 mg/L). *R. dairenensis* GM-15 also produced cellulase activity at low temperature and at grape pH, and did not produce β-glucosidase activity avoiding risks of color loss when it is used in red winemaking. Further studies regarding effects of these enzymes on wine processing and quality are needed in order to propose them in enology.

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