Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Pectinolytic yeasts from viticultural and enological environments: novel finding of *Filobasidium capsuligenum* producing pectinases

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In this study indigenous yeasts associated with wineries, grapes and Malbec fermented must from San Rafael viticulture region (Argentina) were isolated to select pectinolytic strains for their potential use in enology. Pectinolytic yeasts were identified by physiological and molecular methods. Among 78 isolates, only nine were able to produce extracellular pectinases. Six isolated from berry surface were identified as *Aureobasidium pullulans* and the remaining isolates, recovered from wineries, belonged to *Saccharomyces cerevisiae* and *Filobasidium capsuligenum* species. Pectinase production was evaluated under vinification-related conditions: pH 3.5, 12 and 28 °C. *A. pullulans* U-12 produced the highest pectinolytic activity at low temperature (1.16 U ml^{-1}), while *F. capsuligenum* strains showed good activity at 12 and 28 °C (0.77 and 1.15 U ml^{-1}, respectively) being this study the first report on the capacity of this species to produce pectinases. The pectinolytic activity of *F. capsuligenum* B-13 showed an optimum at pH 4.5 and two peaks at 20 and 50 °C. The enzyme half-life was 2 h at 40 °C and retained 65% of its activity at 40 °C after 1 h of incubation. This pectinolytic system displayed remarkable activity at pH and temperatures found in vinification, suggesting a potential candidate for applying to wine-making.

Keywords: *Filobasidium capsuligenum* / Pectinolytic yeasts / Wine / San Rafael (Mendoza / Argentina) viticulture region

Received: September 6, 2012; accepted: February 17, 2013

DOI 10.1002/jobm.201200534

Introduction

Pectinolytic enzymes are a heterogeneous group of related enzymes that hydrolyze the pectic substances present in plant tissues. Pectinases have wide applications in winemaking. They improve grape must yield, clarification process of must and wine, extraction of polyphenols and pigments as well as the releasing of flavor compounds [1–3].

Commercial pectinases of fungal origin are usually a mixture of enzymes like polygalacturonases, pectato, or pectin lyases and also undesirable pectinesterase and side-activities [4, 5]. In the last few years different sources of pectinases have been explored, being yeasts a promising alternative [4]. Yeast pectinases are more preferable because they seem to produce only one type of pectinolytic enzymes mainly polygalacturonases [5–7]. Thus, the selection of pectinolytic yeasts from environments related to wine-making to be used as processing aids in vinification is of prime importance. This selection would allow to reduce the production costs by mean of secretion of pectinases "*in situ*."

"San Rafael" Designation of Origin (DO) represents an important wine region located in the Central-West part of Argentina. Malbec variety (*Vitis vinifera* L.) is one of the

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most characteristic red wine grapes of this region. However, very little is known about either the occurrence of pectinolytic microorganisms in local enological ecosystems or their abilities to contribute to winemaking.

In a previous work, yeasts producing cold-active pectinolytic activity from wine grapes of this viticulture region were selected [8]. In the present study, yeasts from grape berries, Malbec must fermentation and winery equipment surfaces were isolated to select and identify pectinolytic yeasts. Furthermore, the pectinase production under conditions associated with vinification (pH 3.5, 12 and 28 °C) and the characterization of its enzymatic activity were also performed.

Materials and methods

Studied area description and sampling

The study was performed during 2009/2010 vintage in San Rafael viticulture region (Mendoza, Argentina). Samples were collected from vineyards and wineries placed in different districts of San Rafael DO: Cuadro Benegas (lat. 34.62°S, long. 68.45°W), Las Paredes (lat. 34.59°S, long. 68.41°W), and Goudge (lat. 34.67°S, long. 68.12°W) districts.

Grapes. Five wine grape (*Vitis vinifera* L.) varieties (around 23–25° Brix maturity level) were harvested to isolate yeasts from berry surface. Sauvignon Blanc and Chardonnay were collected from Cuadro Benegas district whereas Semillon, Cabernet Sauvignon, and Malbec from Las Paredes district. For each grape variety around 2 kg of healthy grapes were randomly harvested, aseptically transported to the laboratory and kept cold until their study.

Fermentation. One thousand five hundred kilograms of Malbec grape was picked up to conduct the spontaneous fermentation at pilot scale. Grapes were crushed in an automatic press and the must was transferred to a 1500-L steel tank and sulfured to give a final concentration of 50 mg/L SO_2. Alcoholic fermentation was conducted at 25 ± 1 °C and its progress was monitored daily by decline in total soluble solids measuring the density.

Winery equipments. Different equipment surfaces of two wineries that have never been inoculated with commercial yeasts were sampled to isolate autochthonous yeasts from San Rafael DO. Sampling was carried out at the beginning of vintage season when equipments were clean and without use since previous year. Crushers, presses and fermentation vats were sampled from the winery located in Goudge district, and filters, fermentation vats, and pipes from the winery located in Cuadro Benegas district. Samples were taken by streaking about 300 cm^2 with sterile cotton plugs. They were gently shaken in 5 ml of 0.1% (w/v) peptone-water for 15 min.

Yeast isolation

Aliquots of adequate dilutions in 0.1% (w/v) peptone-water from grapes, fermenting must, and cell suspensions from winery surfaces were plated onto Wallerstein Laboratory Nutrient Agar (WL). It allows presumptive discrimination between the yeast species by colony morphology and color [9]. Plates were incubated at 25 °C during 3–5 days. A proportional and representative number of each colony type was recovered. Isolates were purified by streak plating and subcultured onto YPD agar (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose, 20 g L^{-1} agar, pH 4.5) for subsequent identification.

Screening on plate for pectinolytic yeast selection

Isolates were screened respect to their capacity to hydrolyze pectin by the Petri plate method according to Merín *et al.* [8]. Briefly, isolates were point-inoculated onto a mineral medium containing citric pectin as a sole carbon source and incubated at 28 °C for 48–72 h. Enzyme activity was indicated by the formation of a clear halo around the colonies against a purple–brown background on pectin plate after lugol's solution addition. The ratio between clarification halo diameter and colony diameter (D_h/D_c) was determined as a semiquantitative assessment of extracellular pectinase production [10].

Yeast identification

Pheno- and genotypic identification of pectinase-secreting yeasts. All yeasts that showed pectinolytic activity were identified at species level following the taxonomic criteria described by Kurtzman *et al.* [11] as well as by PCR-RFLP analysis of the ITS1-5.8S-ITS2 region. PCR reaction was performed following protocols described by Esteve-Zarzoso *et al.* [12] using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR products were digested with *Cfo*I, *Hin*fI and *Hae*III restriction enzymes following the supplier's instructions. Amplified products and their restriction fragments were analyzed on 1.4 and 2.2% agarose gels, respectively, in 1× TAE buffer (Tris-acetic acid-EDTA, pH 8). Gels were stained with ethidium bromide. Fragment sizes were estimated comparing with a 100-bp ladder.

26S rRNA gene sequence analysis. Total DNA was extracted according to procedures described by Cocolin *et al.* [13] and amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGT

GTTTCAAGACGG-3′). Sequencing of the D1/D2 loop region of the 26S rDNA was performed directly from purified PCR products by the Sequencing Service from CERELA (Tucumán, Argentina). The resultant sequences were aligned in GenBank using the BLAST program for their identification. The 26S rRNA gene partial sequences were submitted to GenBank database available at NCBI under accession numbers: JN637173 and JN637174 (*Filobasidium capsuligenum*) and JN637175 (*Saccharomyces cerevisiae*).

Evaluation of pectinolytic activity under conditions associated to vinification

Production of extracellular enzymatic extracts. Yeasts that showed pectinolytic activity on plate were evaluated in liquid medium under conditions associated to vinification process. The selected yeasts were inoculated in a basal medium (containing per litre of 50 mM citric-citrate buffer: 20 g glucose, 10 g soy peptone, 10 g meat peptone, 10 g yeast extract, pH 3.5). The cultures were incubated with shaking (100 rpm) at 12 and 28 °C during 5 or 3 days, respectively. After removing cells by centrifugation (5000*g*, 15 min at 4 °C), the supernatants were filtered (0.22 μm) to assay the enzymatic activity.

Evaluation of pectinolytic activity. Pectinolytic activity was assayed by quantification 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 reagent (DNS) [14]. Galacturonic acid was used as standard (Sigma, USA). The reaction mixtures containing 450 μl of substrate and 50 μl of enzymatic extract produced at 12 and 28 °C were incubated for 30 min at the same temperature of the respective enzyme production. The reaction was stopped by adding 500 μl DNS reagent and subjected to a boiling water bath for 15 min. After cooling, 1.5 ml of distilled water was added and the absorbance measured at 530 nm.

Characterization of pectinolytic system of *F. capsuligenum*

The temperature and pH effects on pectinolytic activity and thermal stability of pectinolytic system of *F. capsuligenum* B-13 were evaluated under the standard enzymatic assay in 50 mM citrate-phosphate buffer. The optimum temperature was evaluated at pH 4.5 in the temperature range from 5 to 60 °C. The optimum pH was determined at 20 °C in de pH range from 2.6 to 6.0. Thermostability of the pectinolytic system was determined by pre-incubating the enzymatic extract at temperatures of 30, 40 and 50 °C for different time intervals from 30 to 240 min, and residual activity was immediately measured at 20 °C and pH 4.5 as described above. Here, the pectinolytic activity of sample without pre-incubation was regarded as 100%.

Statistical analysis

Experimental data were subjected to ANOVA and Fisher LSD test ($p < 0.05$), 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.

Results

Sampling, isolation, and identification of pectinolytic yeasts

Seventy-eight yeasts were isolated from viticultural and enological environments belonging to San Rafael DO in order to select pectinolytic yeasts for their potential use in the winemaking process. For this purpose 24 yeast colonies from each source, berry surface and fermented Malbec must, and 30 colonies from winery equipment surfaces were collected. Among the 78 isolates, only nine showed pectinolytic activity on plate (Table 1). Six of them were isolated from berry surface while the remaining three were recovered from winery equipments. No pectinolytic activity was found among yeasts isolated from Malbec must during fermentation.

To identify pectinolytic species present in these ecosystems, conventional and molecular methods were performed. RFLP analyses of ITS1-5.8S-ITS2 region were highly correlated to the conventional identification and were able to correctly identify most of the isolates at the genus and species level [8, 12].

All pectinolytic yeasts isolated from the grape surface were identified as *Aureobasidium pullulans*, whereas the pectinolytic isolate recovered from the winery located in Goudge district was identified as *Saccharomyces cerevisiae* (Table 1). However, the two isolates collected from the winery located in Cuadro Benegas district could not be completely identified. These isolates were not identified by PCR-RFLP method since their *Hae*III restriction pattern was different from that available in yeast-id database for *Filobasidium capsuligenum*. In the database, the *Hae*III restriction pattern for this species is 550 + 100 bp, while in the two isolates only the 550 bp fragment was observed (Table 1). The 100 bp fragment was probably over-digested or this band was very faint on the gel (data not shown). In order to find their identities and to reconfirm the identity of the other winery pectinolytic yeast the D1–D2 loop region of 26S rRNA gene was sequenced and compared with the available DNA sequence database. Isolates B-13 and B-30 were

Table 1. Pectinolytic activity detection and identification of native pectinolytic yeasts isolated from grapes and wineries of San Rafael DO.

Isolation source	Number of isolates	D_h/D_c	AP	Restriction fragments (bp)			Assigned species[a]	Accession number[b]
				*Cfo*I	*Hae*III	*Hin*fI		
Grape surface								
(Sauvignon Blanc)	3 (U-15, U-17, U-21)	2.4–4.3	600	190 + 180 + 100	450 + 150	290 + 180 + 130	*Aureobasidium pullulans*	–
(Chardonnay)	2 (U-9, U-11)	3.1–4.2	600	190 + 180 + 100	450 + 150	290 + 180 + 130	*Aureobasidium pullulans*	–
(Semillon)	1 (U-12)	5.5	600	190 + 180 + 100	450 + 150	290 + 180 + 130	*Aureobasidium pullulans*	–
Winery equipments								
Goudge winery								
(Press)	1 (B-17)	3.9	880	385 + 365	320 + 220 + 180 + 145	365 + 155	*Saccharomyces cerevisiae*	JN637175
Cuadro Benegas winery								
(Fermentation vats)	2 (B-13, B-30)	8.6–9.3	650	340 + 300	550	360 + 280	*Filobasidium capsuligenum*	JN637173, JN637174

D_h/D_c: ratio between clarification halo diameter (D_h in mm) and yeast colony diameter (D_c in mm).
AP, amplified 5.8S-ITS product size (bp).
[a]According to Esteve-Zarzoso *et al.* [12], Merín *et al.* [8], and/or www.yeast-id.com database.
[b]Sequences information from the 26S rDNA PCR product obtained from the pectinolytic strains selected in this study.

conspecific with *F. capsuligenum*, while it was confirmed that strain B-17 was conspecific with *S. cerevisiae*.

Table 1 shows the ratio between clarification halo size and colony size (D_h/D_c), which was the first selection criterion of pectinolytic yeasts. According to these results, the isolate U-12 from Semillon grape, which showed the highest D_h/D_c ratio among the *A. pullulans* isolates, and the three pectinolytic yeasts isolated from winery surfaces (*S. cerevisiae* B-17, *F. capsuligenum* B-13, and B-30) were selected to continue the study of pectinase production.

Production of pectinases by selected yeasts under conditions associated to vinification

Figure 1 shows the pectinolytic activity of selected strains assayed on enzymatic extracts at pH 3.5 and 12 and 28 °C, conditions usually occurring in vinification process. The production of pectinases was also carried out under the same conditions, which are extreme conditions for normal production and activity of yeast pectinases.

The pectinolytic activity of secreted enzyme varied significantly ($p < 0.05$) between both tested temperatures for each yeast strain, and dissimilar results were obtained among isolates within each assayed temperature (Fig. 1). At 28 °C, *F. capsuligenum* B-13 and B-30 showed the highest pectinolytic activity (around 1.15 U ml^{-1}) without significant difference ($p < 0.05$) between them. *A. pullulans* U-12 (0.77 U ml^{-1}) and *S. cerevisiae* B-17 (0.51 U ml^{-1}) exhibited 70% and 44%, respectively, of the highest activity obtained at this temperature. At 12 °C, *A. pullulans* strain U-12 produced the highest enzyme activity (1.16 U ml^{-1}), around 34% higher than *F. capsuligenum* strains, while *S. cerevisiae* B-17 showed very low activity (0.13 U ml^{-1}).

Characterization of *F. capsuligenum* pectinolytic system properties

Considering that this is the first report about *F. capsuligenum* producing pectinases, we selected the strain B-13 in order to characterize some of the properties of its enzymatic activity. Even when both isolates of *F. capsuligenum* presented similar levels of pectinolytic activity, β-glucosidase (anthocyanase) activity was not detected in strain B-13, xylanase activity was similar to and cellulase activity was higher than those observed in strain B-30 (data not shown).

The pectinolytic activity of *F. capsuligenum* B-13 was evaluated at temperatures that range from 5 to 60 °C at pH 4.5. Figure 2A shows two peaks of activity at 20 °C (0.96 U ml^{-1}) and 50 °C (1.29 U ml^{-1}). The pH influence on pectinases produced by *F. capsuligenum* B-13 can be observed in Fig. 2B. The optimum pH of this enzymatic

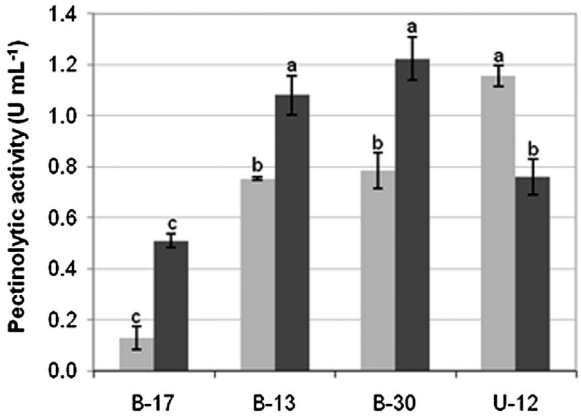

Figure 1. Pectinase activity at 12 (▒) and 28 °C (■) of selected yeast strains (*S. cerevisiae* B-17, *F. capsuligenum* B-13 and B-30, *A. pullulans* U-12) assayed on enzymatic extract produced in a basal medium at pH 3.5 (data are mean values of three experiments ± SD). Different lowercase letters on the bars mean significant differences among the activity of different strains at the same temperature, according to LSD test ($p < 0.05$). One unit of pectinase activity (U) was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar per min under assayed conditions.

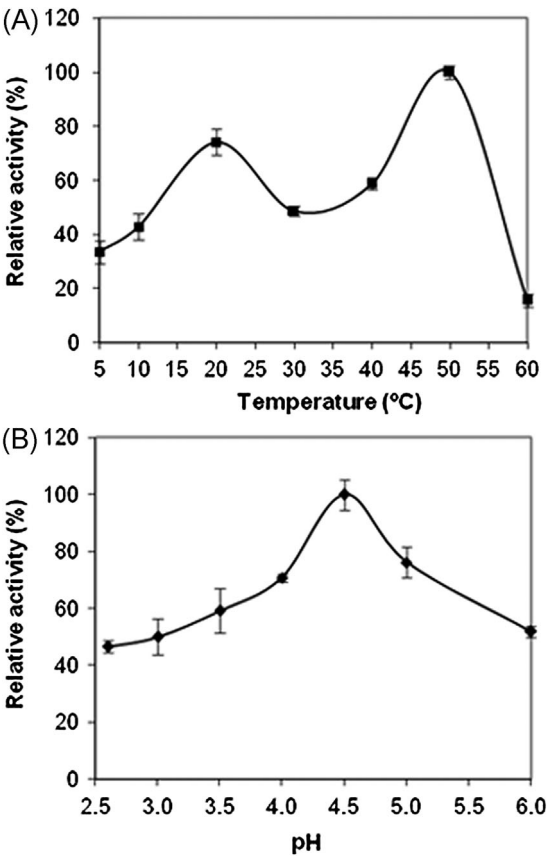

Figure 2. Effect of temperature (A) and pH (B) on activity of pectinolytic system of *F. capsuligenum* B-13. Data are given as mean ± SD, $n = 3$. The pectinase activity of 100% was equivalent to 1.29 and 1.03 U ml^{-1} for temperature and pH, respectively.

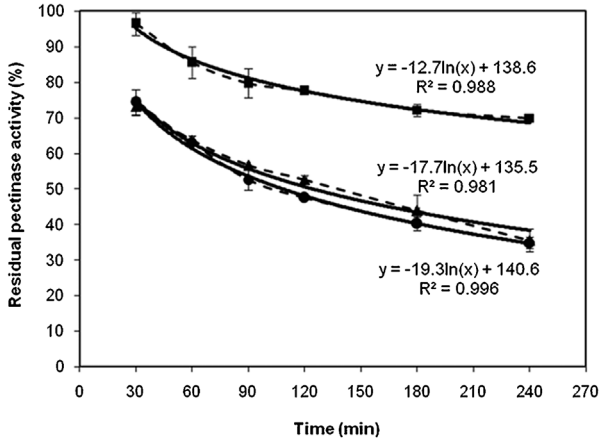

Figure 3. Thermostability profile of pectinolytic activity at low temperature (20 °C) of *F. capsuligenum* B-13. Thermostability was determined at temperatures of 30 (■), 40 (●), and 50 °C (▲), as a function of time. Data are given as mean ± SD, $n = 3$. Enzymatic reaction was carried out at 20 °C and pH 4.5 in citrate-phosphate buffer (50 mM). The pectinase activity of 100% was equivalent to 0.90 U ml^{-1}. Ecuations proximate to corresponding curves show that the thermostability adjusts to a logarithmic kinetics.

activity was 4.5. However, the pectinolytic system retained more than 50% of activity at pH values between 3.0 and 4.0. At pH 3.5, usually found in must and wine, the pectinolytic system maintained 60% of its maximum activity.

In addition, the thermostability of the pectinolytic system was studied (Fig. 3). The enzymatic system was thermostable and retained 86, 63, and 64% of its activity at 30, 40, and 50 °C, respectively, after 1 h of incubation. The half-life of pectinase was 2 h at 40 and 50 °C. At the highest tested temperature the residual activity was still 35% after 4 h of incubation.

Discussion

The study of presence of pectinolytic yeasts to obtain more information about their occurrence and biodiversity on grape berries, wines, and enological surfaces as well as their influence on the winemaking process is a major challenge in wine microbiology [6, 15, 16].

This work focused on the isolation of yeasts from viticultural and enological environments belonging to San Rafael DO in order to select pectinolytic yeasts for their potential use in the vinification process. It was observed low frequency of pectinolytic yeasts in these ecosystems, only 9 among 78 isolates showed activity (Table 1). These results are in agreement with those reported by other authors [15, 16] who indicated low occurrence or absence of pectinolytic enzymes in wine

yeasts. A 25 and 10% of total yeast colonies isolated from grape and winery surfaces, respectively, corresponded to pectinolytic yeasts; whereas no pectinolytic isolates were recovered from Malbec must during fermentation. It has been previously detected low incidence of pectinolytic yeasts in other ecosystems. Among yeasts isolated from tropical fruits only a 7% was positive for polygalacturonase activity [5]. Buzzini and Martini [17] also reported a low frequency of pectinolytic yeasts in natural environments of Brazilian rain forest (1.5% of ascomycetes, 18.7% of basidiomycetes, and 21.7% of yeast-like organisms).

Pectinolytic yeasts were identified by physiological and molecular methods. Only three species were recovered from these ecosystems. *A. pullulans* was the only species found on grape berry surfaces (Table 1). Previous studies carried out in our laboratory have evidenced the presence of pectinolytic isolates of *A. pullulans* on wine grapes and the ability of this species to produce extracellular pectinases with high level of activity at low temperature [8].

Regarding winery pectinolytic yeasts, the strain *S. cerevisiae* B-17 was recovered from a wooden press of the winery located in Goudge district (Table 1). Although many studies have confirmed the dominant presence of *S. cerevisiae* species in winery surfaces [1, 18], only a few works have reported the pectinolytic capacity of winery *S. cerevisiae* strains [6, 19]. This is a relevant finding since this *Saccharomyces* strain could conduct the fermentation and positively influence the wine-making by releasing pectinases into the medium.

The isolation of *F. capsuligenum* strains from fermentation vats of the winery located in Cuadro Benegas district (Table 1) is in agreement with previous results. This species was isolated first in a South-African winery [20] but additional strains had been isolated from different locations around the world and from diverse sources (brewery, soil, and fruits) [21]. Hence, this discovery represents the first finding of this species in Argentinean wineries. On the other hand, some strains were found to secrete extracellular hydrolytic enzymes, such as amylases [22] and proteases [23]. However, as far as we know, this is the first study that reports regarding strains of *F. capsuligenum* that produce pectinolytic enzymes.

According to the results obtained in the present work and those from the literature, the occurrence of extracellular pectinases in yeasts appears to be a strain-related character [17, 24]. Ahansal *et al.* [24] informed that several strains of *F. capsuligenum* isolated from indigenous forest in Morocco were not able to produce pectinases, while in the present work the two winery strains showed pectinolytic activity.

In order to select and characterize those yeasts with optimum capacity to produce active pectinases under vinification conditions, different criteria were applied. The first selection was based on the ratio between clarification halo diameter and colony diameter (D_h/D_c) at 28 °C as a measure of the relative production of pectinases (Table 1). De García *et al.* [10] reported similar or lower ratios for yeasts isolated from glacial meltwater rivers in Patagonia (Argentina).

The pectinolytic activity of selected strains was assayed under conditions usually occurring in the vinification process.

Pectinolytic yeasts belonging to *F. capsuligenum* (B-13 and B-30) and *A. pullulans* (U-12) produced good levels of pectinolytic activity in liquid medium at 12 and 28 °C (Fig. 1). These values are comparable to some pectinolytic activities reported for other yeasts such as *S. cerevisiae* [25] or higher than those produced by *Zygoascus hellenicus* [24]; whereas *S. cerevisiae* B-17 produced lower levels of activity. Nevertheless, it is important to highlight that conditions of enzymatic tests for strains cited from the literature were less extreme (30–50 °C and pH 5.0–5.5) than those applied in this study.

The other selection criterion was the production of good levels of pectinase activity at low temperature (12 °C). *F. capsuligenum* strains were able to produce high level of pectinase activity at red wine fermentation temperature (28 °C) and also showed a good level of activity at low temperature (12 °C). Despite the statistically similar values of pectinase activity of both strains of *F. capsuligenum*, the strain B-13 was selected based on the production of other hydrolytic activities involved in the breakdown of the cell wall (cellulase activity = 0.37 U ml^{-1}, xylanase activity = 0.28 U ml^{-1}). This strain secreted cellulase and xylanase activities desirables in winemaking for their contribution to the extraction phenomenon of color and tannins from grape berry to must [2, 26]. In addition, strain B-13 did not produce undesirable β-glucosidase (anthocyanase) activity. Anthocyanins are phenolic molecules composed of a glycosilated flavylium ion mainly responsible for red wine color. Some β-glucosidases from fungi and yeasts break the linkage between the glucose and the anthocyanidin moieties inducing loss of wine color [27].

The pectinases produced by *F. capsuligenum* B-13 showed maximum activity at pH and temperatures that are typical for these enzymes secreted by yeasts (Fig. 2A and B) [4, 5, 28]. The literature has reported that yeast and fungal species produced pectinases showing the highest activity at 50 °C [29, 30]. Nevertheless, good levels of relative pectinolytic activity (60–75%) were obtained at low and medium temperatures (15–30 °C), and also

significant levels of relative activity (60–100%) were observed at pH of wines. This pectinolytic system had high activity over a broad range of temperatures, particularly towards low temperature region, consequently was more complete than usual pectinases from yeasts. The two peaks of activity at 20 and 50 °C (Fig. 2A) indicated that it is likely to exist more than one pectinolytic activity in the enzymatic system in concordance with results reported by van Dyk *et al*. [31].

The good level of activity observed at pH 3.5 and 20 °C as well as the high enzyme thermostability at 30 °C evidenced that *F. capsuligenum* or its pectinases could be used in the maceration stage of wine production. Besides, the stability against higher tested temperatures was high during long intervals. Other authors have found that cold-active pectinases from yeasts were thermostable at 20 and 30 °C, but they lost a high percentage of their maximum activities at 40 °C and did practically not have activity at 50 °C [32].

In summary, *A. pullulans* was the only pectinolytic species found on grapes, which was able to produce high activity levels, while a winery strain of *S. cerevisiae* (B-17) was able to produce pectinases under winemaking conditions, but with lower levels of activity. *F. capsuligenum*, found on winery surfaces, was reported as a pectinolytic species for the first time; and particularly *F. capsuligenum* B-13 was able to produce a pectinolytic system constituted by active and thermostable pectinases under winemaking conditions. More detailed studies on enzymatic production by these yeasts during winemaking process along with its characterization are required to provide further information on their potential contribution to winemaking.

Acknowledgments

This work was supported by UNCuyo N° 06/L096 project and I+D (UNCuyo) and ANPCyT (PICT 2010 N° 0847) program grants. The authors thank Ing Raúl Carrión and Dra Silvina Cabeza for carefully supervising the spontaneous fermentation, as well as Raúl Raya (PhD) for his advice in yeast identification. We are grateful to UVEG and CSIC for kindly supplying online yeast database access (http://www.yeast-id.com).

Conflict of interest statement

All authors declare that there is no financial/commercial conflict of interest.

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