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ORIGINAL ARTICLE

Highly cold-active pectinases under wine-like conditions from non-*Saccharomyces* yeasts for enzymatic production during winemaking

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Significance and Impact of the Study: Nowadays, there is increasing interest in low-temperature winemaking. Nevertheless, commercial oenological pectinases, produced by fungi, are rarely active at low temperatures. Cold-active pectinases that are stable under vinification conditions are needed. This study indicated that cold-active and acid-tolerant pectinases from non-*Saccharomcyes* yeasts were able to remain active at glucose, ethanol and SO_2 concentrations usually found in winemaking. Furthermore, not only are these yeasts a source of cold-active pectinases, but the yeasts themselves are also potential adjunct cultures for oenology to produce these enzymes during cold-winemaking.

Keywords
Aureobasidium pullulans, cold-active pectinases, enzyme inhibitors, low-temperature winemaking, oenological pectinases, pectinolytic yeasts.

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Abstract

The influence of oenological factors on cold-active pectinases from 15 preselected indigenous yeasts belonging to *Aureobasidium pullulans*, *Filobasidium capsuligenum*, *Rhodotorula dairenensis*, *Cryptococcus saitoi* and *Saccharomyces cerevisiae* was investigated. Pectinolytic enzymes were constitutive or partially constitutive; and high glucose concentration (200 g l^{-1}) did not affect or increased pectinase production at 12°C and pH 3·5 (up to 113·9 U mg^{-1}) only in *A. pullulans* strains. SO_2 (120 mg l^{-1}) slightly affected the growth of *A. pullulans* strains but did not affect pectinase production levels. Ethanol (15%) barely affected pectinase activity of *A. pullulans* strains but diminished relative activity to 12–79% of basidiomycetous yeasts. Moreover, non-*Saccharomyces* strains showed promising properties of oenological interest. This study demonstrates that cold-active pectinases from some *A. pullulans* strains were able to remain active at glucose, ethanol and SO_2 concentrations usually found in vinification, and suggests their potential use as processing aids for low-temperature winemaking.

Introduction

Pectinase is a generic name for a family of enzymes involved in the degradation of pectic substances, which are complex heteropolysaccharides present in plant cell walls (Alimardani-Theuil *et al.* 2011). In winemaking, these enzymes lead to key benefits like higher juice yield, easier pressing, more efficient clarification and filtration of wine (Fleet 2008), and enhanced extraction of chromatic and aromatic compounds from grape skins (Maturano *et al.* 2012; Sieiro *et al.* 2012).

Commercial pectinases are crude or partially purified fungal preparations, derived mainly from *Aspergillus*, which can contain impurities and undesired side-activities that can negatively impact the quality of wine (Alimardani-Theuil *et al.* 2011). An alternative to these pectinases could be indigenous yeasts that produce extracellular pectinolytic enzymes during wine making. This option would avoid the

use of costly exogenous pectinases and would improve the quality of wines, since these yeasts can produce substantial sensory complexity in wines (Fleet 2008).

Winemaking process is rather convenient to carry out at low temperature as it favours conservation of sensory characteristics of the end product (Adapa *et al.* 2014). Accordingly, oenological enzymes able to be active at low temperature are needed. In view of such interest, cold-active pectinases for winemaking have lately been reported (Merín *et al.* 2011, 2014; Sahay *et al.* 2013).

Most of exogenous pectinases are not notably inhibited at pHs of grape must (3·0–4·5) because their optimum pH usually varies between 2·0 and 5·5 (Alimardani-Theuil *et al.* 2011). However, their pectinolytic activity drops to negligible levels at low winemaking temperatures. Other factors that reduce the effectiveness of pectinases include ethanol and sulphur dioxide (SO_2) normally found in musts and wines.

Few studies of both pectinase production by yeasts under winemaking conditions (Radoi *et al.* 2005; Maturano *et al.* 2012) and pectinase activity of yeasts influenced by enzymatic inhibitors in vinification (Fernández-González *et al.* 2004) have been reported. Nevertheless, neither the production nor the activity has systematically been studied in cold-active pectinolytic enzymes from wine yeasts, affected by the presence of sugars or enzymatic inhibitors like ethanol and SO_2.

This study evaluates the effect of oenological factors that could affect yeast pectinase production (carbon sources and SO_2) and pectinase activity (ethanol and SO_2). Furthermore, it assesses the kinetic and metabolic properties of oenological interest of the pectinolytic yeasts to study preliminarily their potential application to cold-winemaking.

Results and discussion

Influence of oenological parameters on pectinolytic enzymes

Effect of glucose and pectin on pectinase production

Figure 1 shows pectinase production in presence of glucose and pectin as carbon sources occurring in grape musts, and under oenological pH and low-temperature conditions (pH 3·5 and 12°C). When yeasts were grown on 20 g l^{-1} glucose as a sole carbon source, all non-*Saccharomyces* strains were able to produce pectinases, suggesting the constitutive expression of these extracellular enzymes as previously reported (Merín *et al.* 2011, 2014). *Saccharomyces cerevisiae* B-17 exhibited a negligible activity level. Similar results have been reported by Radoi *et al.* (2005) for *Saccharomyces* wine yeasts. Conversely, Fernández-González *et al.* (2004) have reported polygalacturonase production on glucose by wine *S. cerevisiae* strains.

When pectin (1 g l^{-1}) was supplemented to basal medium (Fig. 1), the synthesis of pectinases was significantly increased for *Aureobasidium pullulans* GM-Ma-16, -PN-22, -CS-123, -Cy-25, -Ch-24 and *S. cerevisiae* B-17 strains. The results suggest that these pectinases are inducible by pectin in agreement with results for *A. pullulans* NRRL Y-231 (Biely *et al.* 1996) and *Saccharomyces* wine yeasts (Radoi *et al.* 2005). Moreover, the fact that constitutive expression of pectinolytic enzymes was demonstrated when yeasts were grown on glucose, but a significant increase of pectinase activity was observed when pectin was added, indicated that the extracellular pectinase production was actually partially constitutive, similarly to pectinase synthesis in some fungi (Botella *et al.* 2007) and yeasts (Moyo *et al.* 2003) already reported.

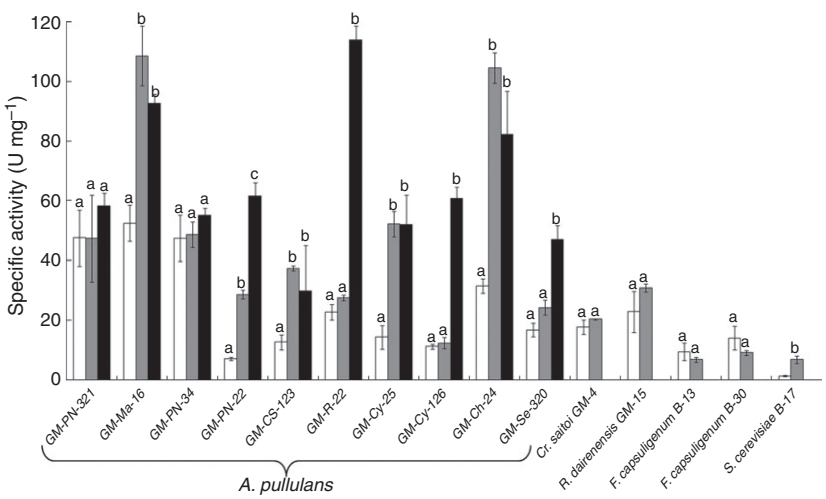

Figure 1 Effect of carbon sources on pectinase production. Pectinolytic yeast activity (U mg^{-1} total protein) produced on medium containing 20 g l^{-1} glucose as a sole carbon source (□), 20 g l^{-1} glucose and 1 g l^{-1} pectin (▒), and 200 g l^{-1} glucose and 1 g l^{-1} pectin (■), of pectinolytic yeasts. Data are mean values of two independent cultures. Vertical segments represent SD. Different letters above vertical bars indicate significant differences among treatments for each strain ($P < 0·05$).

Additionally, a significant increase in pectinase activity was observed when *A. pullulans* GM-PN-22, -R-22, -Cy-126 and -Se-320 strains were grown on 200 g l^{-1} glucose and 1 g l^{-1} pectin, usual concentrations at the beginning of fermentation, as compared to 20 g l^{-1} glucose and 1 g l^{-1} pectin (Fig. 1). *Aureobasidium pullulans* GM-R-22 showed the highest activity (113·9 U mg^{-1}). In accordance with our results, glucose addition to the production medium has been demonstrated to exert a positive effect on enzymatic synthesis at high glucose concentrations (Botella *et al.* 2007). On the contrary, no pectinase activity was detected in the *Saccharomyces* and basidiomycetous yeasts, suggesting the repression of pectinase synthesis by high glucose contents.

Aureobasidium pullulans strains produced pectinase activity in both media that mimic must-wine conditions regarding carbon source levels (pectin and glucose), besides pH and temperature, evidencing relevant features in these strains from a practical point of view.

Effect of ethanol and SO_2 on pectinase activity

The effects of two potential inhibitors (ethanol and SO_2) were assessed at 12°C and pH 3·5 on enzymatic activity of the selected strains to continue the study (Table 1). In presence of 15% (v/v) ethanol, pectinolytic activity of *Rhodotorula dairenensis* GM-15 and of most *A. pullulans* strains was not significantly affected or showed high relative activity (79–84%), except GM-PN-34 pectinase. Furthermore, the pectinase activity of *A. pullulans* GM-PN-22 and *S. cerevisiae* B-17 was increased by a 24 and 99% respectively. Nevertheless, it is remarkable that the net activity of the B-17 strain (2·39 U mg^{-1}) was notably lower than activity of the GM-PN-22 strain (6·20 U mg^{-1}). Other oenological enzymes, pectinases (Fernández-González *et al.* 2004), β-glucosidases (Rodríguez *et al.* 2007), a cold-active α-L-rhamnosidase (Orillo *et al.* 2007), have shown a decrease of around 50% at similar ethanol concentrations. Interestingly, cold-active pectinases from *R. dairenensis* and *A. pullulans* strains of this work showed higher relative activities, indicating that they could act efficiently in low-temperature vinifications.

Table 1 Relative activity (%) assayed in presence of ethanol and SO_2 with respect to reference activity†

	Potential pectinase inhibitor	
Pectinolytic strain	Ethanol (15%, v/v)	SO_2 (120 mg l^{-1})
Aureobasidium pullulans GM-PN-321	88^{ns}	190*
A. pullulans GM-Ma-16	106^{ns}	141*
A. pullulans GM-PN-34	53*	115^{ns}
A. pullulans GM-PN-22	124*	272*
A. pullulans GM-R-22	92^{ns}	90^{ns}
A. pullulans GM-Ch-24	84*	120*
Cryptococcus saitoi GM-4	12*	76*
Rhodotorula dairenensis GM-15	79*	88^{ns}
Filobasidium capsuligenum B-13	64*	126*
F. capsuligenum B-30	41*	76*
Saccharomyces cerevisiae B-17	199*	410*

Data are mean values of three determinations and two independent experiments.

The 100% of relative activity corresponds to activity values shown in Figure 1 in basal medium (20 g l^{-1} glucose). In all cases, SD < 10%.

†Reference activity: pectinolytic activity obtained by incubating the substrate with the enzymatic extract at pH 3·5 and 12°C in absence of inhibitor in the reaction mixture.

Significance: *, significant difference; ns, no significant difference, at which means differ from their respective reference activity ($P < 0·05$).

When 120 mg l^{-1} SO_2 was added to the reaction mixture, pectinase activity was not inhibited by the presence of this compound, except for pectinase of *Cryptococcus saitoi* GM-4 and *Filobasidium capsuligenum* B-30 that displayed 76% of relative activity (Table 1). Indeed, pectinase activity of some strains (*A. pullulans* GM-PN-321, -PN-22 and *S. cerevisiae* B-17) was significantly increased. These results are partially in agreement with those reported by Fernández-González *et al.* (2004), who observed that SO_2 had no significant inhibitory effect on polygalacturonase activity from a *S. cerevisiae* strain. Similarly, yeast β-glucosidase and β-xylosidase activities have not been inhibited by 150 mg l^{-1} SO_2 (Rodríguez *et al.* 2007). The fact that some pectinolytic yeasts slightly diminished their activity and others increased it in presence of SO_2 could be explained both by the intrinsic structural characteristics of the enzyme and its active site and by the unique ability of SO_2 to act as a reducing or an oxidizing agent that enables it either to inactivate enzyme systems by splitting their disulphide linkages (Cecil and Wake 1962) or activate certain hydrolytic enzyme systems, by bringing about conformational changes (Malhotra and Hocking 1976).

Properties of oenological interest

Table 2 shows the characteristics of oenological interest of the selected yeasts. *S. cerevisiae* strains showed good growth properties in grape must, like final biomass and resistance to SO_2, but produced H_2S, as expected. Non-*Saccharomyces* strains did not produce H_2S and some of them were resistant to SO_2. The latter trait is an important criterion for selection of yeasts to be used as adjunct cultures for vinification, given that SO_2 is commonly added to musts for its antimicrobial and antioxidant properties (Fleet 2008).

Considering the enzymatic activities, *A. pullulans* and basidiomycetous strains showed better enzymatic profiles

Table 2 Properties of oenological interest of selected pectinolytic strains

Pectinolytic strains	Growth properties in grape must			Killer phenotype‡	Activity of oenological interest (U ml^{-1})		
	Final biomass (log CFU ml^{-1})	Resistance to SO_2*	Production of H_2S†		Cellulase	Xylanase	β-Glucosidase
Aureobasidium pullulans GM-PN-321	$6{\cdot}92 \pm 5{\cdot}57^{g}$	++	–	ND	$0{\cdot}191 \pm 0{\cdot}016^{b}$	$0{\cdot}216 \pm 0{\cdot}038^{cd}$	$0{\cdot}096 \pm 0{\cdot}015^{c}$
A. pullulans GM-Ma-16	$6{\cdot}62 \pm 5{\cdot}44^{e}$	+	–	ND	$0{\cdot}477 \pm 0{\cdot}027^{d}$	$0{\cdot}104 \pm 0{\cdot}023^{b}$	$0{\cdot}157 \pm 0{\cdot}020^{d}$
A. pullulans GM-PN-22	$6{\cdot}93 \pm 5{\cdot}74^{g}$	++	–	ND	$0{\cdot}103 \pm 0{\cdot}009^{a}$	$0{\cdot}122 \pm 0{\cdot}041^{bc}$	nd
A. pullulans GM-R-22	$6{\cdot}90 \pm 5{\cdot}55^{g}$	+++	–	$K^- R^+$	$0{\cdot}481 \pm 0{\cdot}093^{d}$	$0{\cdot}552 \pm 0{\cdot}061^{f}$	nd
A. pullulans GM-Ch-24	$6{\cdot}81 \pm 5{\cdot}65^{f}$	++	–	ND	$0{\cdot}371 \pm 0{\cdot}026^{c}$	$0{\cdot}238 \pm 0{\cdot}058^{d}$	$0{\cdot}124 \pm 0{\cdot}035^{cd}$
Cryptococcus saitoi GM-4	$4{\cdot}53 \pm 3{\cdot}38^{a}$	+	–	$K^- R^+$	nd	nd	nd
Rhodotorula dairenensis GM-15	$5{\cdot}18 \pm 4{\cdot}04^{b}$	+	–	$K^- R^+$	$0{\cdot}549 \pm 0{\cdot}068^{d}$	nd	nd
Filobasidium capsuligenum B-13	$6{\cdot}08 \pm 4{\cdot}92^{d}$	++	–	$K^- R^+$	$0{\cdot}368 \pm 0{\cdot}097^{c}$	$0{\cdot}278 \pm 0{\cdot}037^{de}$	nd
F. capsuligenum B-30	$5{\cdot}92 \pm 4{\cdot}62^{c}$	++	–	$K^- R^-$	$0{\cdot}153 \pm 0{\cdot}019^{b}$	$0{\cdot}334 \pm 0{\cdot}081^{e}$	$0{\cdot}108 \pm 0{\cdot}030^{c}$
Saccharomyces cerevisiae B-17	$7{\cdot}71 \pm 6{\cdot}61^{h}$	+++	+	$K^- R^-$	nd	$0{\cdot}062 \pm 0{\cdot}005^{a}$	$0{\cdot}010 \pm 0{\cdot}001^{b}$
S. cerevisiae IOC 18-2007§	$8{\cdot}52 \pm 6{\cdot}91^{i}$	+++	+	$K^+ R^-$	ND	ND	nd
Torulaspora delbrueckii BTd259¶	ND	ND	ND	ND	nd	$0{\cdot}243 \pm 0{\cdot}005^{de}$	$0{\cdot}003 \pm 0{\cdot}0004^{a}$

Data are mean values of three determinations and two independent cultures ± SD.
nd: not detected; ND: not determined.
Different superscript letters within the same column indicate significant differences ($P < 0{\cdot}05$).
*Growth in red grape must supplemented with 120 mg l^{-1} SO_2 (as sodium metabisulphite) compared with its control (+++) without the additive.
†High (++) and low (+) sulphide producers or (–) no sulphide production.
‡Killer phenotype: $K^+ R^-$, killer/sensitive; $K^- R^+$, neutral; $K^- R^-$, sensitive.
§Control strain for growth tests (Institut Œnologique de Champagne, France).
¶Control strain for enzymatic activities (Maturano *et al.* 2012).

of oenological interest than ascomycetous strains, as can be seen in Table 2. All *A. pullulans* strains were also able to produce cellulase and xylanase activities at 12°C and pH 3·5 as previously reported (Chi *et al.* 2009), displaying *A. pullulans* GM-R-22 the highest levels; but this is the first time that *F. capsuligenum* species is reported to produce cellulase and xylanase activities. Half of the analysed *A. pullulans* and *F. capsuligenum* strains were able to produce β-glucosidase activity, suggesting a strain-related character. Cellulase and xylanase enzymes are positive for contributing to the increase in juice yield and colour extraction, and improve the clarification properties of wines by degrading cellulose and hemicelluloses present in grape cell walls (Sieiro *et al.* 2012). However, although β-glucosidases hydrolyse glucosilated complexes from grapes by releasing volatile compounds that contribute to wine aroma (Rodríguez *et al.* 2007; Sieiro *et al.* 2012), some commercial β-glucosidases can affect the colour of red wines since they are able to degrade anthocyanins. Thus, in some cases the presence of β-glucosidases in winemaking may be negative.

The principal component analysis (PCA) illustrates these results (Fig. 2). The horizontal component (PC1) explained 52·7% of the total variance and was positively associated with the production of cold-active hydrolytic enzymes (pectinase, cellulase and β-glucosidase) while production of H_2S, final biomass and resistance to SO_2 presented a negative correlation; and PC2 (23·1%) was mainly positively associated with the xylanase production. It can be seen that ascomycetous yeasts were clearly separated from basidiomycetous and *A. pullulans* strains, the latter group being related to cold-active hydrolytic activities, particularly *A. pullulans* GM-R-22, which was distinguished by high levels of cold-active xylanase and cellulase activities.

Regarding killer phenotype, most of the pectinolytic strains evidenced a neutral phenotype ($K^- R^+$), which is particular relevant when *Saccharomyces* fermentation starters used in the vinification have a killer/sensitive phenotype ($K^+ R^-$), like the commercial strain used in this study (Table 2). *Filobasidium capsuligenum* B-30 and *S. cerevisiae* B-17 presented a sensitive phenotype ($K^- R^-$). Production of killer toxins by *F. capsuligenum* strains has been reported earlier (Keszthelyi *et al.* 2008) indicating that this characteristic would be strain-dependent.

Growth kinetics and pectinase production in presence of SO_2

Figure 3 shows the pectinolytic activity evolution at 12°C and pH 3·5 during yeast growth in absence and presence of 120 mg l^{-1} SO_2 of the four *A. pullulans* strains selected on the basis of previous results. In the absence of SO_2, the strains showed a similar growth profile, with slight differences in their specific growth rate (0·14–0·19 h^{-1})

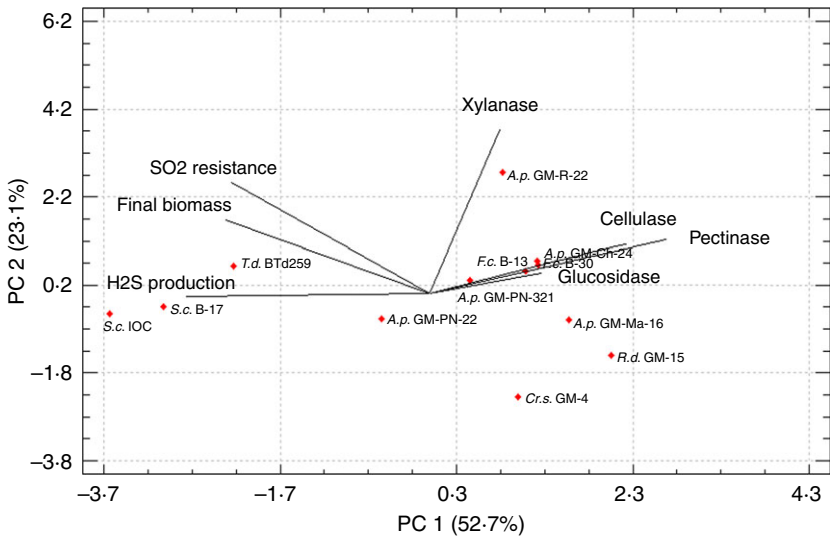

Figure 2 Biplot graph of the first two principal components for the characteristics of oenological interest of the studied yeasts. Cellulase, xylanase and β-glucosidase activities of *Saccharomyces cerevisiae* IOC 18-2007 were negligible at 12°C and pH 3·5. Growth properties in grape must of *Torulaspora delbrueckii* BTd259 were taken from Maturano *et al.* (2012) and Comitini *et al.* (2011).

and in the time to reach the stationary phase (48–72 h). As expected, the four strains diminished their growth after addition of SO_2 to the basal medium until the beginning of the exponential phase. However, they attained a high cell density at the end of the incubation (around 7·0 log CFU ml^{-1}), except GM-Ma-16 strain.

Pectinase activity level was not significantly influenced by SO_2 in any of the *A. pullulans* strains although enzyme production kinetics was affected along with the growth curve (Fig. 3). Two profiles were observed: GM-PN-321 and -Ma-16 changed their maximal enzymatic production time from 48 to 96 h, while GM-PN-22 and -R-22 changed slightly their maximal production time and their activity levels in presence of SO_2 were higher than those in absence of this compound from 24 h of cultivation.

In conclusion, the pectinases of non-*Saccharomyces* strains seem to be interesting systems for low-temperature winemaking, such as the enzymatic system of *A. pullulans* GM-R-22. This strain was able to produce constitutive cold-active pectinases that were non-repressible by high glucose concentration and tolerant to major vinification factors, in addition to its promising oenological properties. Our results also suggest that these novel yeasts have potential to be used as vinification adjunct cultures for enzyme production during cold-winemaking, as recently proposed (Merín *et al.* 2011; Sahay *et al.* 2013). In this regard, further studies on co-cultures of pectinolytic strains and *S. cerevisiae* starter cultures, as well as the evaluation of the effect of pectinolytic strains on aroma, colour, and clarification properties of red wines are being performed.

Likewise, the application of these cold-active enzymatic systems could be extended to the hydrolysis of cellulose, hemicellulose and pectin in vegetable waste treatment as well as the production of biofuel, as the conversion of lignocellulosic biomass into fuels and other chemicals can be achieved using a multi-enzyme system, containing cellulases, pectinases, endo-β-xylanase, arabinofuranosidase, among other related enzymes, acting in synergy (Delabona *et al.* 2014). Moreover, on-site production of enzymes, this is the use of the same lignocellulosic biomass for enzyme production and hydrolysis, could reduce production costs of enzymes and bioethanol, as both processes could be co-located and share infrastructure and utilities (Delabona *et al.* 2012). The mentioned enzymatic processes could be carried out at low temperatures (12–18°C) with high catalytic efficiency, contributing to reduce processing costs.

Materials and methods

Micro-organisms and culture conditions

Aureobasidium pullulans strains GM-PN-321, -Ma-16, -PN-34, -PN-22, -CS-123, -R-22, -Cy-25, -Cy-126, -Ch-24 and -Se-320, isolated from wine grapes (Merín *et al.* 2011), *Filobasidium capsuligenum* B-13 and B-30, and *Saccharomyces cerevisiae* B-17, isolated from wineries (Merín *et al.* 2014), and *Cryptococcus saitoi* GM-4 and *Rhodotorula dairenensis* GM-15, indigenous yeasts from wine grape ecosystem (Merín *et al.* in press), were used in this study. All of them were isolated from the viticulture region DOC San Rafael (Mendoza, Argentina). These yeasts were preselected for their ability to produce pectinolytic activity at some oenological conditions (12°C and pH 3·5).

For enzyme production, yeasts were inoculated with 10^5 CFU ml^{-1} of 24–36 h pre-cultures in basal medium (containing per litre of 50 mmol l^{-1} citric-citrate buffer, pH 3·5: 2 g yeast extract, 20 g peptone, 20 g glucose, 1 g K_2HPO_4) and were incubated under shaking conditions (100 rev min^{-1}) at 12°C for 5 days. Cells were removed

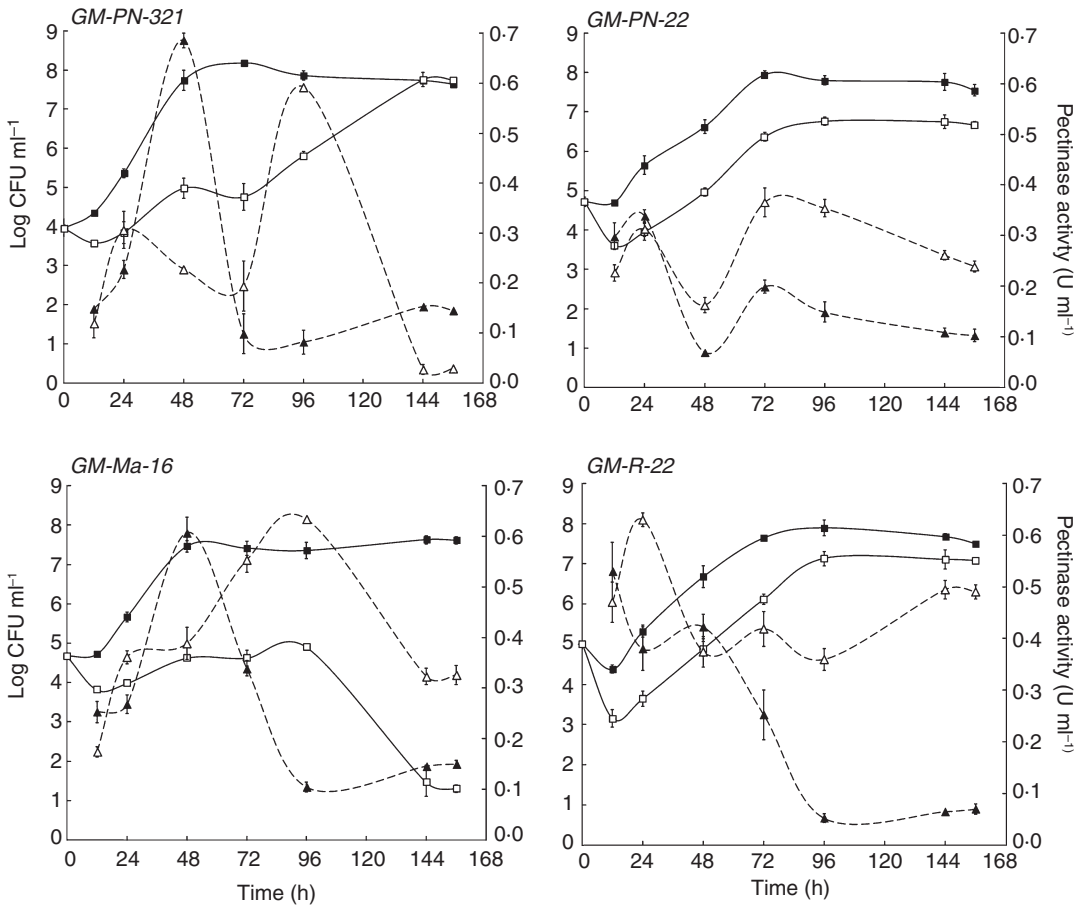

Figure 3 Growth and activity kinetics of selected *Aureobasidium pullulans* strains. Cellular growth and pectinase activity produced in absence (■, ▲) or presence (□, △) of 120 mg l^{-1} SO_2 respectively. Data are mean values of two independent cultures. Vertical segments represent SD.

by centrifugation (5000 ***g***, 10 min at 4°C) and supernatants were filtered (0·22 μm) to obtain cell-free supernatants (enzymatic extracts).

Enzyme and total protein determinations

Pectinase activity was assayed by measuring the amount of reducing sugars released from a pectin dispersion (0·25%, w/v, in 50 mmol l^{-1} citric-citrate buffer, pH 3·5) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959). Galacturonic acid was used as standard. The reaction mixtures contained 0·45 ml of substrate and 0·05 ml of enzymatic extract and were incubated at 12°C for 30 min. The reaction was stopped by adding 0·5 ml DNS reagent and the mixture was placed in a boiling water bath for 15 min. After cooling down, 1·5 ml of distilled water was added, and the absorbance was measured at 530 nm against a blank for each tube. One pectinase unit (U) is defined as the enzymatic activity that releases 1 μmol of reducing sugar per min under assay conditions.

Total protein determination was performed according to Bradford (1976), using bovine serum albumin as standard.

Influence of oenological parameters on production and activity of pectinases

Effect of carbon sources on pectinase production

To study the effect of pectin and high glucose concentration, naturally present in grape must, on pectinase production, yeasts were inoculated in the following four media: (i) basal medium (20 g l^{-1} glucose) (ii) basal medium supplemented with 1 g l^{-1} citrus pectin; (iii) medium 'ii' supplemented with 200 g l^{-1} glucose instead of 20 g l^{-1}; (iv) medium 'i' supplemented with 200 g l^{-1} glucose instead of 20 g l^{-1} (control). Culture conditions and enzymatic extract preparation were the same as described above.

Effect of ethanol and SO_2 on pectinase activity

The effects of ethanol and sulphur dioxide on pectinase activity were evaluated in enzymatic extracts, obtained on

basal medium, 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^{-1} respectively.

Effect of SO_2 on growth kinetics and pectinase production of selected strains

Yeast growth evolution and pectinase production in presence of SO_2, usually found in grape musts, were evaluated at low temperature (12°C). Pre-cultures 36–48 h old grown in basal medium were inoculated at a concentration of 10^5 CFU ml^{-1} in the same medium supplemented with 120 mg l^{-1} SO_2 (as sodium metabisulphite). Control cultures were conducted in absence of SO_2. Cultures were incubated at 12°C with agitation (100 rev min^{-1}) and samples were taken periodically during cultivation. Yeast growth was monitored by successive dilutions and pectinase production was assayed in enzymatic extracts as previously described.

Oenological properties of pectinolytic yeasts

Determination of growth properties

Selected strains were inoculated at a concentration of 10^5 cells ml^{-1} in sterilized red grape juice (cv. Malbec) and incubated at 28°C for 7 days. Yeast growth was determined by viable cell counting on plates containing YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, 20 g l^{-1} agar) medium. Resistance to SO_2 was evaluated by adding 120 mg l^{-1} SO_2 (as sodium metabisulphite) to sterilized grape juice and assessing yeast viability. Hydrogen sulphide production was assessed by the lead acetate method (Fernández de Ullivarri *et al.* 2011).

Characterization of killer phenotype

Killer activity of pectinolytic strains was assayed against a sensitive reference strain, *Candida glabrata* NCYC 388 obtained from North Patagonian Culture Collection (NPCC, Neuquén, Argentina). To test killer sensitive character, five reference killer strains were used: *Saccharomyces cerevisiae* NPCC 145 (K1 type), *C. glabrata* NCYC 388 (K4 type), *Wickerhamomyces anomala* NRRL Y-366 (K5 type) and two indigenous killer strains from NPCC, *Torulaspora delbrueckii* NPCC 1033 and *W. anomala* NPCC 1027 (Lopes and Sangorrín 2010), following the protocol proposed by Fernández de Ullivarri *et al.* (2011).

Determination of hydrolytic activities of oenological interest

Cellulase and xylanase activities were assayed in enzymatic extracts by measuring reducing sugars according to Miller (1959). Cellulase was measured using microgranular cellulose and xylanase using birchwood xylan as substrate at a concentration of 0·25% (w/v) in 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 assay conditions. β-Glucosidase activity was assayed by incubating 0·1 ml of 15 mmol l^{-1} D-(+)-cellobiose solution in citric-citrate buffer (pH 3·5) with 0·1 ml of enzymatic extract at 12°C for 30 min. Glucose production was quantified using the enzymatic-colorimetric test (GOD-POD). One β-glucosidase unit is defined as the enzymatic activity that releases 2 μmol of glucose from cellobiose per min under assay conditions.

The commercial fermentation starter strain *S. cerevisiae* IOC 18-2007 (Institut Œnologique de Champagne, France) was employed as control strain for yeast growth experiments. *Torulaspora delbrueckii* BTd259 (Maturano *et al.* 2012) was used as positive control for hydrolytic activities. *S. cerevisiae* IOC 18-2007, not possessing pectinolytic activity, served as negative control.

Statistical analysis

Analysis of variance (ANOVA) and Fisher's LSD test ($\alpha = 0{\cdot}05$) were applied to the experimental data. Principal components analysis (PCA) was used to simplify the interpretation of the results for oenological properties. All statistical analyses were performed with STATGRAPHICS PLUS ver. 5.1 (Manugistics, Rockville, MD).

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Conflict of Interest

No conflict of interest declared.

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