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Pectinolytic yeasts from cold environments: novel findings of *Guehomyces pullulans*, *Cystofilobasidium infirmominiatum* and *Cryptococcus adeliensis* producing pectinases

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Abstract One hundred and three yeasts isolated from soil samples from King George Island and Tierra del Fuego province were screened in relation with their capability to produce pectinolytic enzymes. Although all the yeasts showed well-developed colonies at 20 °C, only eight showed a clear halo around the colony, indicative of pectin degradation. A secondary screening demonstrated that only four yeasts were capable to produce pectinases at low temperatures (8 °C). It could be seen that the selected yeasts were able to grow and produce high levels of polygalacturonase activity when submerged fermentations were performed using pectin-containing fruit wastes as substrates. None of the strains produced neither lyase nor rhamnogalacturonan hydrolase activities. Regarding pectin esterase activity, it was only produced in lower amounts by *G. pullulans* 8E (0.022 U ml^{-1}). A TLC analysis of the substrate cleavage pattern of the pectinolytic systems was consistent with an endo-type activity. The clarification of apple juice was only accomplished by *G. pullulans* pectinolytic system, with a clarification of 80% (%T_{650}) using 4 U/ml of enzyme at 20 °C. As far as we concern this work describes for the first time the production of pectinases by the cold-adapted yeasts species *Cystofilobasidium infirmominiatum*, *Cryptococcus adeliensis* and *G. pullulans.*

Keywords Cold-active enzymes · Polygalacturonases · Fruit juice clarification

Introduction

Cold-active enzymes are characterized by their high catalytic efficiency at low and moderate temperatures at which homologous mesophilic enzymes are less or not active, making them interesting for both, basic research and industrial applications. During the last years a wide variety of cold-adapted bacteria producing cold-active enzymes have been described. However, it has not been the same with cold-adapted yeasts, which represent a promising source for application in biodegradation processes and for enzyme production (Birgisson et al. 2003; Margesin et al. 2005).

Pectin is one of the main constituents of primary cell walls and the middle lamellae of higher plant cells together with cellulose and xyloglucan. This complex heteropolysaccharide is composed mainly of D-galacturonic acid residues joined through α-1,4-linkages forming homogalacturonan chains; some of the D-galacturonic acid residues in these chains could be methylated (Sakai et al. 1993). In food industry, pectic compounds can generate several problems during extraction, filtration, concentration and clarification of fruit juices. Therefore, pectinolytic enzymes are widely used for the treatment of pectic substances in the fruit and vegetable processing industries (Hoondal et al. 2002).

Pectinolytic enzymes or pectinases are depolymering enzymes that degrade pectin, and are divided into depolymerizing enzymes and de-esterifying enzymes. The first ones

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are polymethylgalacturonases, pectin lyases, polygalacturonases and pectate lyases, and the second are pectinesterases (Whitaker 1984). The production of these enzymes has been extensively reported in bacteria and filamentous fungi (Pedrolli et al. 2008; Sunnotel and Nigam 2002), *Aspergillus niger* being the main source of pectinases for industrial use since it produces high amounts of these enzymes and is a GRAS (generally recognized as safe) microorganism (Alkorta et al. 1998).

The possibility of using pectinases from cold-adapted yeasts for industrial applications has just started to be explored, with studies on isolation and characterization of pectinases from these microorganisms being scant.

This paper reports on three novel cold-adapted, pectinase-producing yeast species isolated from Tierra del Fuego province and King George Island, and presents the characterization of the pectinolytic systems produced by these microorganisms.

Materials and methods

Microorganisms

A collection of 103 yeast strains previously isolated and identified from soil and water samples from King George Island (Martinez et al. 2016) and Tierra del Fuego province (sampling points: Encerrada Bay, 54°48′44.6″S, 68°18′53.9″W and a large pristine area near Lago Escondido 54°39′0″S, 67°46′48″W) were used in this study. The culture collection is maintained by cryopreservation (−80 °C in 10% glycerol) at Research and Development Center for Industrial Fermentations (CINDEFI).

Screening and selection of pectinolytic yeasts

To detect pectinolytic enzyme-producing yeasts, a selective medium containing 10 g l^{-1} citrus pectin (Sigma-Aldrich), 1.4 g l^{-1} $(NH_4)_2SO_4$, 2.0 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4{\cdot}7H_2O$, 1 ml sol. A (5 mg l^{-1} $FeSO_4{\cdot}H_2O$, 1.6 mg l^{-1} $MnSO_4{\cdot}H_2O$, 2 mg l^{-1} $CoCl_2$) and 20 gl^{-1} agar were used. Isolates were point-inoculated and incubated at 8 and 20 °C for 72 h. Positive strains were selected by the formation of a clear halo around the colonies after flooding the solid media plates with Lugol's iodine solution (Buzzini and Martini 2002). Those yeasts which produce the largest halo diameter/colony diameter *n* (Dh/Dc) were selected to continue production studies.

Physiological characterization of yeasts

Pectinolytic yeasts were tested for the ability to grow at different temperatures (5, 15, 20, 24 and 28 °C) on PDA plates. The plates were inoculated with yeast cells previously grown for 24–48 h, and incubated at different temperatures. Growth was monitored daily for 5 days, except for the Petri dishes incubated at 5 °C, which were monitored for 1 week. Colony growth was assessed qualitatively.

To determine the effect of salt concentrations on growth of yeasts, PDA plates with different NaCl concentrations were prepared, and streaked with the isolates. The plates were incubated at 20 °C for 5–10 days and growth in different salinity ranges was recorded.

Enzyme production

Submerged fermentation was carried out in 250-ml Erlenmeyer flask containing 50 ml of culture medium (10 g l^{-1} citric pectin, 1.4 g l^{-1} $(NH_4)_2SO_4$, 2.0 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4{\cdot}7H_2O$, 5 mg l^{-1} $FeSO_4{\cdot}H_2O$, 1.6 mg l^{-1} $MnSO_4{\cdot}H_2O$, 2 mg l^{-1} $CoCl_2$, 5 g l^{-1} yeast extract and 2 g l^{-1} peptone, pH 5.0) and inoculated with 1 ml of yeast inoculums (DO 0.4). Flasks were incubated at room temperature (20 °C, 120 rpm) and samples were withdrawn at regular time intervals and assayed for polygalacturonase activity.

Substrates for pectinase enzyme production

Submerged fermentations were performed with selected yeasts using the medium presented earlier but replacing citrus pectin with different vegetables tissues. These substrates were prepared from different fruit sources like orange peels, Mexican lime, lemon and grape pomace, which were thoroughly washed with tap water to remove soluble sugars and dried in a hot air oven at 60 °C. Dried materials were milled (Mesh 35) and used as carbon and energy source. Enzyme production was carried out under conditions similar to those used when citrus pectin was used.

Culture was performed by duplicate.

Effect of glucose as carbon source on pectinase production

The effect of glucose on pectinase production was studied by inoculating the selected yeasts in the following media:

1. Basal medium supplemented with 10 g l^{-1} of citrus pectin.
2. Basal medium supplemented with 5 g l^{-1} of citrus pectin and 5 g l^{-1} of glucose.
3. Basal medium supplemented with 10 g l^{-1} of glucose.

Culture conditions were the same as those described earlier.

Enzymatic assays

Polygalacturonase activity (PGase) was determined using polygalacturonic acid (PGA, Sigma-Aldrich) as substrate. The reaction mixture containing 180 µl of 2 g l^{-1} polygalacturonic acid dissolved in CPB (12.5 mM; 6.25 mM, pH 5.0) and 20 µl of crude enzyme was incubated at 20 °C for 30 min. The release of reducing sugars was quantified by measuring the rate of increase of galacturonic acid concentrations using Nelson–Somogyi method. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of one micromole of galacturonic acid per minute under the given assay conditions. In addition, PGase activity was measured at 10 °C.

Pectinolytic activity was assayed by quantification of reducing sugars released using pectin 2 g l^{-1}in CPB (12.5 mM; 6.25 mM, pH 5.0) as substrate using the same protocol as PGase activity determination. High and low methylation pectins were used in this case, pectin esterified from citrus fruit (>86% methylation, Sigma) and pectin from citrus fruits (63% methylation degree, Sigma), respectively.

Pectinesterase activity was measured by titration method. To 10 ml of 5 g l^{-1} of citric pectin in 0.2 M NaCl, 250 µl of crude enzyme was added. The pH was adjusted to 5.50 with 0.1 M HCl and the admixture was incubated for 5 min at 20 °C. The carboxyl groups released were titrated with 0.002625 N NaOH. One unit of PE was defined as the amount of enzyme releasing one milliequivalent of carboxyl group per minute (Christensen et al. 1998).

Lyases

Pectin lyase and pectate lyase activity were determined by measuring the increased in absorbance at 235 nm, due to the formation of unsaturated products, as proposed by Albershein (1966). In case of pectate lyase, the assay was performed in the presence of 1.0 mM $CaCl_2$.

In case of pectin lyase, 180 µl of citric pectin (Sigma-Aldrich; 2 g l^{-1} in CPB (12.5 mM; 6.25 mM, pH 5.0)) and 20 µl of crude enzyme were incubated at 20 °C for 15 min. Absorbance at 235 nm were monitored during incubation time against a blank where heat-inactivated enzyme was used. One unit was defined as the increase in absorbance at 235 nm of 1.0 µl of the reaction mixture per minute under assay condition (Nakagawa et al. 2004).

For pectate lyase, 180 µl of polygalacturonic acid (Sigma-Aldrich; 2 g l^{-1} in citrate–phosphate buffer solution (25 mM citric acid and 12.5 Mm Na_2HPO_3, pH 5.0)) with 1 mM $CaCl_2$ and 20 µl of crude enzyme were incubated at 20 °C for 15 min. Absorbance at 235 nm were monitored during incubation time against a blank where heat-inactivated enzyme was used. One unit was defined as the increase in absorbance at 235 nm of 1.0 µl of the reaction mixture per minute under assay conditions.

Rhamnogalacturonan hydrolase was determined using rhamnogalacturonan (Sigma-Aldrich) as substrate. The reaction mixture, containing 180 µl of 2 g l^{-1} rhamnogalacturonan in CPB (12.5 mM; 6.25 mM, pH 5.0) and 20 µl of crude enzyme, was incubated at 20 °C for 30 min (Normand et al. 2010). The release of reducing sugars was quantified by measuring the rate of increase of rhamnose concentrations using Nelson–Somogyi method. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of one micromole of rhamnose per minute under the given assay conditions.

All enzyme assays were carried out in triplicate.

Zymogram analysis of pectin-degrading enzymes

Zymograms were performed in conjunction with SDS-PAGE according to the method of García-Carreño et al. (1993) with slight modifications. SDS-PAGE was performed as described by Laemmli, using 5% (w/v) stacking gel and 12% (w/v) separating gel. For zymograms, after electrophoresis, the gel was submerged in CPB (12.5/6.25 mM, pH 5.0) containing 2.5% (v/v) Triton X-100 for 60 min, with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with CPB. The gel was later incubated with PGA solution 0.2% (w/v) at 20 °C for 20 min. Finally, gels were stained with Ruthenium red solution for zymography analysis. The development of clear zones on the pink background of the gels indicated the presence of pectinolytic activity. The molecular mass marker used was PageRuler™ prestained protein ladder, 10–180 kDa.

Thin-layer chromatography TLC

The endo- or exo-mode of action of the pectinolytic pools was studied by performing a TLC analysis of the final enzymatic degradation products.

For TLC analysis of PGA degradation products, heat-inactivated samples were spotted (2 µL) on aluminum sheets (silica gel 60 F254, Merck) and the chromatography performed using the ascending method with n-butanol:acetic acid:water (9:4:7, v/v/v) as the solvent system. Detection was accomplished by spraying the dried plate with 3% (w/v) phosphomolybdic acid dissolved in 10% (v/v) sulfuric acid in ethanol followed by heating at 105 °C for 5 min. Trigalacturonic acid (TGA, Sigma) and galacturonic acid (GA, Sigma) were used as standards.

An endo-polygalacturonase is characterized by the production mainly of oligomers, whereas an exo-polygalacturonase produces mostly monomers or dimmers.

Clarification of apple juice

Raw apple juice preparation

Apples (Golden delicious) were cut into cubes and pressed with a manual hydraulic press using double layer of cheesecloth to obtain raw unclarified juice. Aliquots of this juice were pasteurized (5 min at 90 ± 1 °C) and immediately cooled at room temperature.

The crude extracts—produced by each pectinolytic yeast in the optimum medium where maximum activity was achieved—were concentrated using a 10,000 MWCO laboratory crossflow cassette (Vivaflow 200, Satorious®).

The effect of enzyme concentration on apple juice clarification was determined by incubating, at 20 °C for 6 h, 15 ml of apple juice containing different amounts of enzyme (2.0, 4.0 and 6.0 U/ml of PGase). Every hour interval, a sample (1 ml) of each treatment was withdrawn and centrifuged at 1000 rpm for 10 min. The percent of transmittance was considered a measure of juice clarity and it was determined at 650 nm. Additionally, the color of the apple juice was measured at 440 nm (Sandri et al. 2011).

Results and discussion

Screening of pectinolytic activity for yeast selection

One hundred and three microorganisms isolated from soil and water samples from King George Island and Tierra del Fuego province were evaluated for their potential to produce extracellular pectinases. Among them, only eight isolates showed pectinolytic activity at 20 °C. These strains were selected and subjected to a new study to see their capability to produce pectinases at low temperature (8 °C). It could be seen that although all of them showed well-developed colonies, only the strains LP e9.2, LP 4.6, LP 5.9 and 8E were able to produce pectinolytic enzymes (Fig. 1). The relation Dh/Dc at 8 and 20 °C was used as the criterion to select the strains (Table 1).

All the strains were previously identified by 26S rDNA (D1/D2 domain) sequencing and phylogenetic analyses. The strain 8E from King George Island, identified as *Guehomyces pullulans*, and the strains LP e9.2 and LP 5.9 from Tierra del Fuego, identified as *Cystofilobasidium infirmominiatum* and *Cryptococcus adeliensis*, respectively, were selected for further studies. The strain LP 4.6 was not taken into account to continue with the production studies because it was identified as *G. pullulans* and, although it developed a great halo, the strain 8E displayed the largest Dh/Dc relation. The 26S rRNA gene partial sequences of the strains are deposited in Genbank database available at NCBI under the following accession numbers: *G. pullulans* 8E (KU 659491), *C.infirmominiatum* LP e9.2 (KU 659577) and *C. adelienses* LP 5.9 (KU 659556).

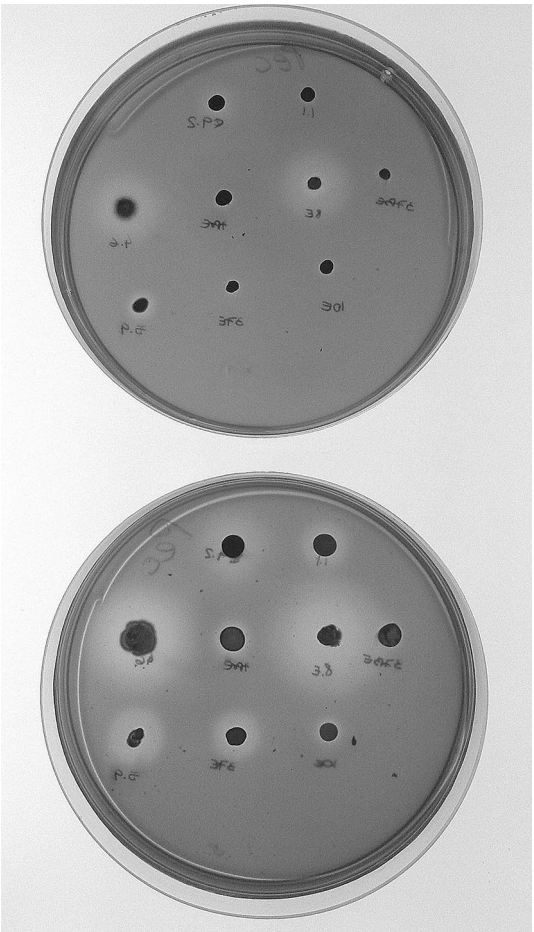

Fig. 1 Screening of cold-adapted yeasts in solid medium containing pectin after addition of Lugol's solution. **a** plates incubated at 8 °C and **b** plates incubated at 20 °C. A clear halo around the colonies indicates the production of pectinolytic enzymes

Guehomyces pullulans, basidiomycetous yeast belonging to the genus cystofilobasidiales, has diverse habitats. Sources include soil, glacial melt water rivers (Branda et al. 2010) and antarctic habitats (Connell et al. 2008). *G pullulans* has been studied for enzymatic activities that can be applied to the degradation of industrial waste products. This species have the ability to degrade lignin-containing wastes (Slavikova et al. 2002) by producing ligninase and Mn-peroxidase activities. In 2005, Nakagawa reported the production of cold-active β-galactosidase activity by *G. pullulans* (Nakagawa et al. 2005). Up to now there is no report on the production of pectinolytic activity by this yeast.

Cystofilobasidium infirmominiatum has been isolated from surface waters to a depth of 2000 m. This pigmented species, have been found in cold waters, at temperatures that ranged from 0 to 4 °C (Sampaoi 2011). In Argentina,

Table 1 Halo diameter/colony diameter relation (Dh/Dc) for the pectinolytic strains at 8 and 20 °C resulting from the screening cultures in solid media containing pectin

Strain	Dh (mm)	Dc (mm)	Dh/Dc
8 °C			
LPe 9.2	70	50	1.4
8E	130	35	3.7
LP 4.6	125	55	2.3
LP 5.9	50	30	1.7
20 °C			
LP 1.1	90	50	1.8
LPe 9.2	100	50	2.0
8E	200	50	4.0
7BE	70	60	1.2
LP 4.6	180	70	2.6
10E	50	40	1.3
37E	100	40	2.5
LP 5.9	100	30	3.3

Strains selected for production studies are indicated in bold

three strains of *C. infirmominiatum* have been isolated previously from freshwater ecosystems from lakes located in northwest Patagonia (Fonck, Mascardi and Nahuel Huapi lakes) (Brandão et al. 2011; Brizzio et al. 2007). Its bioprospection for the production of pectinases using pectin agar medium was positive but no further studies were performed.

Cryptococcus species are one of the most frequently found yeasts in a variety of Arctic and Antarctic environments, being able to utilize nutrients in oligotrophic systems, whereas most ascomycetes species cannot (Vaz et al. 2011). Two strains of *C. adeliensis* have been isolated from meltwater rivers from Nahuel Huapi National Park by de García et al. (2007) and Brizzio et al. (2007), which are non-pectinolytic yeasts. Among *C. adeliensis* strains isolated from Tierra del Fuego province (*n*: 5), only the LP 5.9 strain produced pectinolytic activity.

Physiological characterization

All pectinolytic yeasts were able to grow in PDA agar at 5, 15, 20 and 24 °C. Just *G. pullulans* (8E) *C. antarcticus* (LP 1.1) and *C. adeliensis* (LP 5.9) also grew at 28 °C. (Table 2).

These results confirm that these pectinolytic strains are adapted to cold environments. Lower growth temperature limits are difficult to determine since compounds required to prevent media from freezing are generally toxic (Vishniac 2005). Similar results were observed by Branda et al. (2010) for yeasts isolated from Calderone glacier (Appenines, Italy). These authors postulated that cold yeast populations probably evolved to replace obligate psychrophilic species with facultative ones. The prolonged exposure to suboptimal temperatures leads to acclimation, which implicates regulatory mechanisms resulting in the full adjustment of the genomic expression and the physiological state during growth under cold conditions (Morgan-Kiss et al. 2006).

The effect of NaCl concentration on the growth of the yeast isolates is given in Table 3. Except one isolate (7BE), the remaining seven yeasts grew well at 1.5 M salt concentration. None of the isolates were able to grow at 3.0 M NaCl and only *Metschnikowia australis* (37E) grew at 2.5 M, showing that the isolates are highly versatile in terms of their salt tolerance. As can be seen, all tested isolates can be considered moderately halotolerant because of their ability to tolerate up to 2.5 M NaCl. The yeasts from Antarctica could growth at higher NaCl concentrations than those isolated from Tierra del Fuego province. This higher halotolerance of Antarctic yeasts could be due to the permanent impact of high-salinity sea water during the formation of ground microcenoses in maritime Antarctica (Yukimura et al. 2009).

Table 2 Growth of yeast isolates at different temperatures

Strain	Identity	Isolation site	Temperature				
			5 °C	15 °C	20 °C	24 °C	28 °C
10 E	*Cryptococcus victoriae*	King George Island	+	+	+	+	–
37 E	*Metschnikowia australis*	King George Island	+	+	+	+	–
7 BE	*Candida davisiana*	King George Island	+	+	+	+	–
8 E	*Guehomyces pullulans*	King George Island	+	+	+	+	+
LP 1.1	*Cryptococcus antarticus*	Tierra del Fuego province	+	+	+	+	+
LP 4.6	*Guehomyces pullulans*	Tierra del Fuego province	+	+	+	+	–
LP e9.2	*Cystofilobasidium infirmominiatum*	Tierra del Fuego province	+	+	+	+	–
LP 5.9	*Cryptococcus adeliensis*	Tierra del Fuego province	+	+	+	+	+

Table 3 Growth of yeast isolates at different NaCl concentrations

Strain	Identity	NaCl concentration					
		0.5 M	1.0 M	1.5 M	2.0 M	2.5 M	3.0 M
10E	*Cryptococcus victoriae*	+	+	+	+	–	–
37E	*Metschnikowia australis*	+	+	+	+	+	–
7BE	*Candida davisiana*	+	+	–	–	–	–
8E	*Guehomyces pullulans*	+	+	+	–	–	–
LP 1.1	*Cryptococcus antarticus*	+	+	+	–	–	–
LP 4.6	*Guehomyces pullulans*	+	+	+	–	–	–
LP e9.2	*Cystofilobasidium infirmominiatum*	+	+	+	–	–	–
LP 5.9	*Cryptococcus adeliensis*	+	+	+	–	–	–

Metschnikowia species have been recovered from water samples of the Atlantic and Pacific oceans, lakes and ponds, algae and corals, invertebrates (brine shrimp and copepods) and fish (Kutty and Philip 2008). *M. bicuspidata* has been isolated from saline environments with 10% NaCl (Gunde-Cimerman et al. 2005). *M. australis* is endemic to Antarctica and is the ascomycete with major prevalence in all Antarctic marine environments. It is the only yeast recovered from intravascular fluid of the macroalgae *Adenocystis utricularis,* so it is considered as a representing habitat for this yeast (Fell and Hunter 1968).

Production of pectinases by selected yeasts

Figure 2a–c shows the pectinolytic activity of selected strains assayed on enzymatic extracts at pH 5.0 and 20 °C when different raw pectins and citrus pectin (control) were used as substrate for the submerged cultures. All the strains were capable to grow in the presence of cheap substrates, and pectinolytic activity started to be detected at 6 h of cultivation. Figure 2a shows the polygalacturonase production profile for *G. pullulans* 8E. Maximum enzyme production was observed at 30 h of cultivation with grape pomace (3.68 U/ml) followed by lemon peel, orange peel and Mexican lime peel. Similar titles of enzyme activity were reached by *C. infirmominiatum* LP e9.2 using lemon peel as substrate (3.8 U/ml, Fig. 2b), this activity being even higher than the one detected when citrus pectin was used. In this case, maximum enzyme production was detected at 30 h and continued up to 75 h of cultivation. Compared to *G. pullulans* and *C. infirmominiatum*, *C. adeliensis* LP 5.9 produced higher amounts of pectinolytic enzymes when it grows on Mexican lime peel (4.7 U/ml), lemon peel (4.8 U/ml) and citrus pectin (7.5 U/ml). Except for citrus pectin, the pectinolytic activity detected in *C. adeliensis* cultures remained at constant levels at least up to 75 h of cultivation. It could be seen that at 10 °C enzyme activity remains active. *C. infirmominiatum* and *G. pullulans* supernatants retained 46 and 44% of their activities, respectively, while the activity of *C. adeliensis* supernatant increased to 150%; the latter could be attributed to the instability of the enzymes at 20 °C.

Pectinase-producing yeasts may be discerned as two types according to Blanco et al. (1999): those that cannot use pectic substances or their hydrolysis products as carbon sources, and those that can. *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Geotrichum klebahnii* represent the first type of pectinase-producing yeasts (Cavalitto et al. 2000) while the yeasts studied here belong to the latter type. Pectinases from these yeasts are still relatively unexplored and to our knowledge there are no reports on pectinases from any *G. pullulans*, *Cystofilobasidium infirmominiatum* or *Cryptococcus adeliensis.* Furthermore, there are only two reports on polygalacturonase from cold-adapted *Cystofilobasidium* strains.

Nakagawa et al. (2002) and Birgisson et al. (2003) isolated yeasts with pectinolytic activity belonging to the order Cystofilobasidiales, which is a group of psychrophilic basidiomycetes (DePriest et al. 2002). *Cystofilobasidium capitatum* strains PPY-1, PPY-5 and PPY-6 were isolated from soil samples from Hokkaido, Japan, while *C. larimarini* S3B and *C. capitatum* S5 were isolated by Birgisson et al. (2003), from frozen soils, leaves and branches from southwest of Iceland.

Besides polygalacturonase (PGase), presence of other pectin-degrading activities in the culture supernatant of the three isolates was investigated. None of the strains produced pectin or pectate lyase activity or rhamnogalacturonan hydrolase activity. Regarding pectin esterase activity, it was only produced in lower amounts by *G. pullulans* (0.022 U/ml). Differences were observed among the isolates when different soluble pectic substrates were used (Supplementary Fig. 1). All the strains produced enzymatic pools that showed more activity against highly esterified pectin (>86% methylation) compared to the activity measured when pectin with 63% methylation was used. Due to the absence of pectin esterase activity, this behavior could be attributed in all cases to the presence not only of polygalacturonase enzymes but also to the presence of polymethylgalacturonase activity (PMGase) in their supernatants.

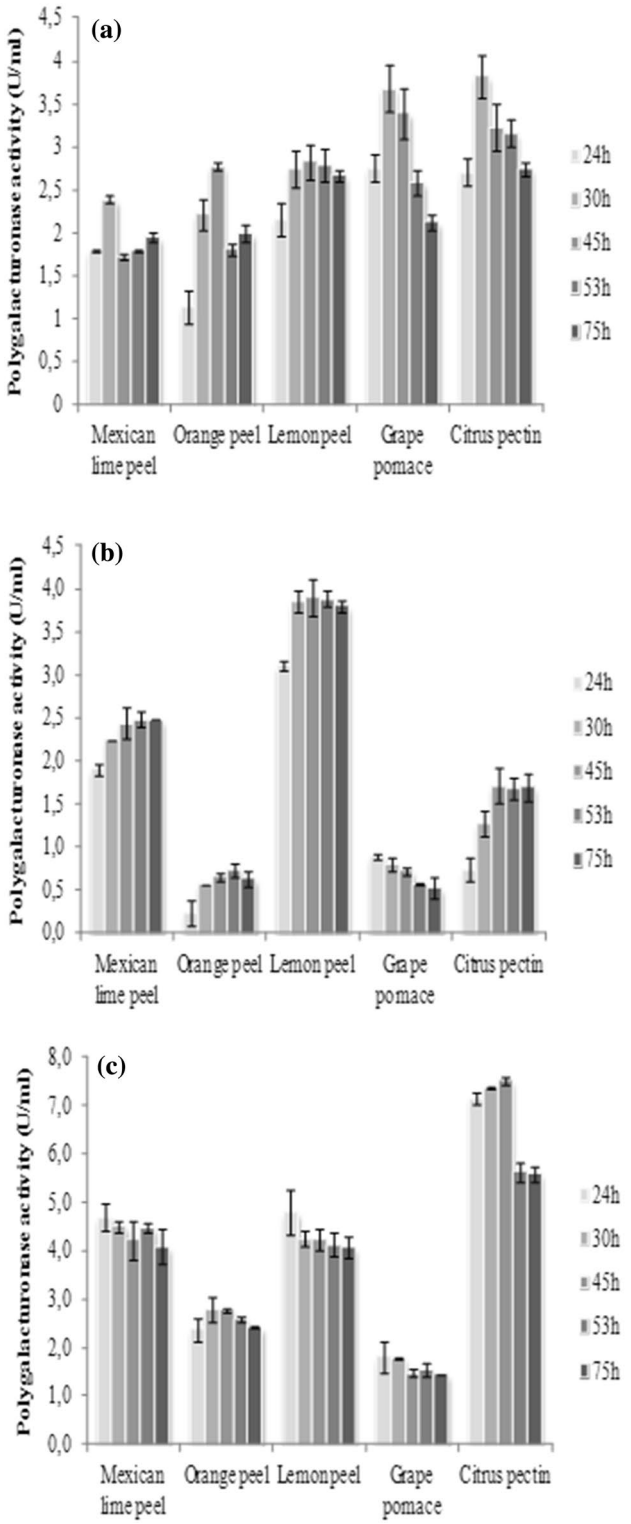

Fig. 2 Polygalacturonase production by cold-adapted yeasts using different low cost pectic substrates. **a** *G. pullulans 8E*, **b** *C. infirmominiatum e9.2* and **c** *C. adeliensis 5.9*

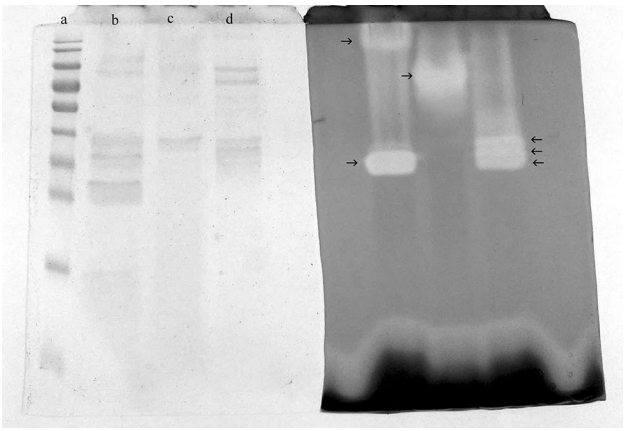

Fig. 3 SDS page (*left*) and zymogram analyses (*right*) of pectinolytic enzymes in culture supernatants of cold-adapted yeasts. The supernatants were run as follows: *lane b C. adeliensis* 5.9; *lane c C. infirmominiatum* e9.2 and *lane d G. pullulans* 8E. *Lane a* protein molecular weight marker (10–180 kDa)

Comparing cold-adapted yeasts with pectinolytic activity belonging to the order Cystofilobasidiales, *C. infirmominiatum* pectinolytic profile differs to the one reported for the yeasts *C. capitatum* reported by Nakagawa et al. (2005) and Birgisson et al. (2003); the latter produced pectin lyase activity while the first ones not only produced pectin lyase but also pectin esterase activity.

Effect of glucose and pectin on PGase production

When yeasts were grown on 10 g l^{-1} glucose as the sole carbon source, it could be seen that, even though *C. infirmominiatum* e9.2 consumed this carbon source, enzyme production was not detected. As for *G. pullulans* and *C. adeliensis*, the strains were able to produce pectinases, suggesting the constitutive expression of these enzymes (Supplementary Fig. 2 shows PGase production in the presence of glucose and citrus pectin as carbon sources).

When citrus pectin (5 g l^{-1}) was supplemented to a basal medium containing 5 g l^{-1} of glucose, PGase production by *G. pullulans* and *C. adeliensis* significantly increased, which suggests PGase production was partially constitutive and partially inducible. However, in the case of *C. infirmominiatum,* as no PGase production was attained with glucose, PGase production appears to be exclusively inducible. Moreover, PGase production decreased when glucose was increased from 5 to 10 g l^{-1}, suggesting the repression of polygalacturonase synthesis by high glucose concentrations. Adding glucose along with pectin to the culture

medium is known to act as a repressor to the production of this kind of enzymes in *Cystofilobasium* species reported by Birgisson et al. (2003). This behavior has already been reported also for *Saccharomyces* and basidiomycetous yeasts by Merín and Morata de Ambrosini (2015).

Zymogram analysis of pectin-degrading enzymes

SDS-PAGE was conducted for all strains growing on the substrate which gave for each one the highest PGase concentration, followed by zymogram analysis for pectinase activity using PGA as substrate. SDS-PAGE and zymograms are shown in Fig. 3. As can be seen, when *C. adeliensis 5.9* grew on citrus pectin, it produced at least two active pectinases against PGA (180 and 40 kDa). The supernatant of *C. infimominiatum e9.2,* growing on lemon peel, revealed a PGase whose molecular weight is close to 70 kDa, whereas *G. pullulans 8E* produced at least 3 active bands at approximately 55 kDa with grape pomace as substrate. Comparing with previous reports on pectinases characterization, PGases produced by these new pectinolytic yeasts have higher molecular weight; *Kluyveromyces marxianus* NCYC 578 produces four isoenzymes whose molecular weight ranges between 46 and 49 kDa (Barnby et al. 1990), while *Saccharomyces pastorianus* produces a 43 kDa PGase and *Cryptococcus albidus* produces a polygalacturonase whose molecular weight is 41 kDa. Nakagawa et al. (2005) reported the production of five active pectinases for *Cystofilobasidium capitatum* PPY-6 and a single activity band for Makria frigid PPY-4 grown on pectin.

TLC analysis

Figure 4 shows the reaction products of PGA hydrolysis by yeast supernatants. Monogalacturonic acid (AGA) and trigalacturonic acid (Tri-gal) were used as standards. After 20 h of hydrolysis, the pectinolytic systems of all of the strains produced Tri-gal and Di-gal whereas AGA was produced in smaller amounts. These results indicate that the polygalacturonases present in the supernatants can be classified as endo-polygalacturonases (EC 3.2.1.15), since exo-polygalacturonases, if present in the culture supernatants, would have catalyzed the degradation of PGA to monomers mainly or exclusively. In this endo-mechanism, the enzyme complex might catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex with another polymer chain, resulting in the liberation of oligogalacturonates (Contreras Esquivel and Voget 2004). Similar results were reported by Nakagawa et al. (2005) for an endo-polygalacturonase produced by the psychrophilic yeast *Cystofilobasidium capitatum* strain PPY-1. On the other hand, *Cryptoccocus liquefaciens* N6 isolated from the Japan Trench at a depth of 6,500 m produced two

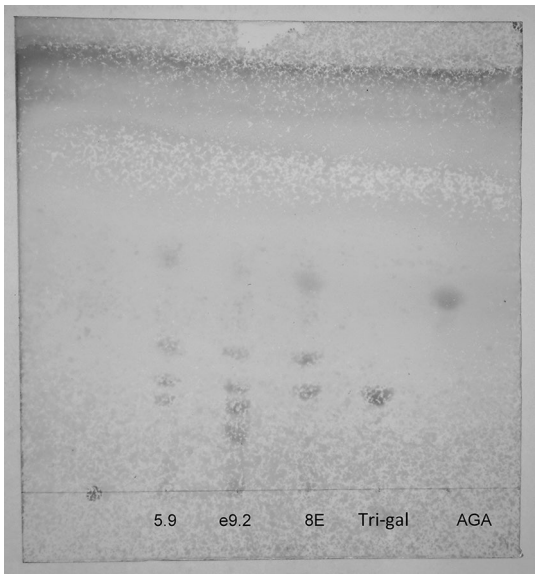

Fig. 4 PGA degradation pattern produced by the pectinolytic systems from the cold-adapted yeasts. Tri-gal and Aga are trigalacturonic acid and monogalacturonic acid (standard), respectively

endo-PGases that retained 40–45% of their activities at 10 °C (Abe et al. 2006).

Only a few cold-adapted yeast species able to produce PGases have been reported, but to our knowledge no cold-active endo-PGases from *G. pullulans*, *C. adeliensis* and *C. infirmominiatum* have been reported yet, being this the first one.

Clarification of apple juice

Pectin substances are responsible for the consistency, turbidity and appearance of fruit juices. In fact, the presence of these substances in fruit juices causes an increase in their viscosity, prolonging the processes of filtration and concentration.

Pectin enzymes have been used for a long time to increase juice yield and to clarify juices, and they account for about one-quarter of the world's food enzyme production.

All supernatants were applied for the clarification of fresh apple juice to determine whether these enzyme preparations were able to clarify the juice. As shown in Fig. 5i, *C. adeliensis* 5.9 (Fig. 5b) and *C. infimominiatum* e9.2 (Fig. 5c) could not clarify apple juice, while *G. pullulans* 8E (Fig. 5a) clarified apple juice at every enzyme concentration tested. It could be seen that, in general, apple juice clarification increased together with the holding time, but not with an increase in enzyme amounts, being the optimum enzyme concentration 4 U/ml. In general, enzyme concentration is the most important factor influencing enzymatic fruit juice clarification. Higher PGase

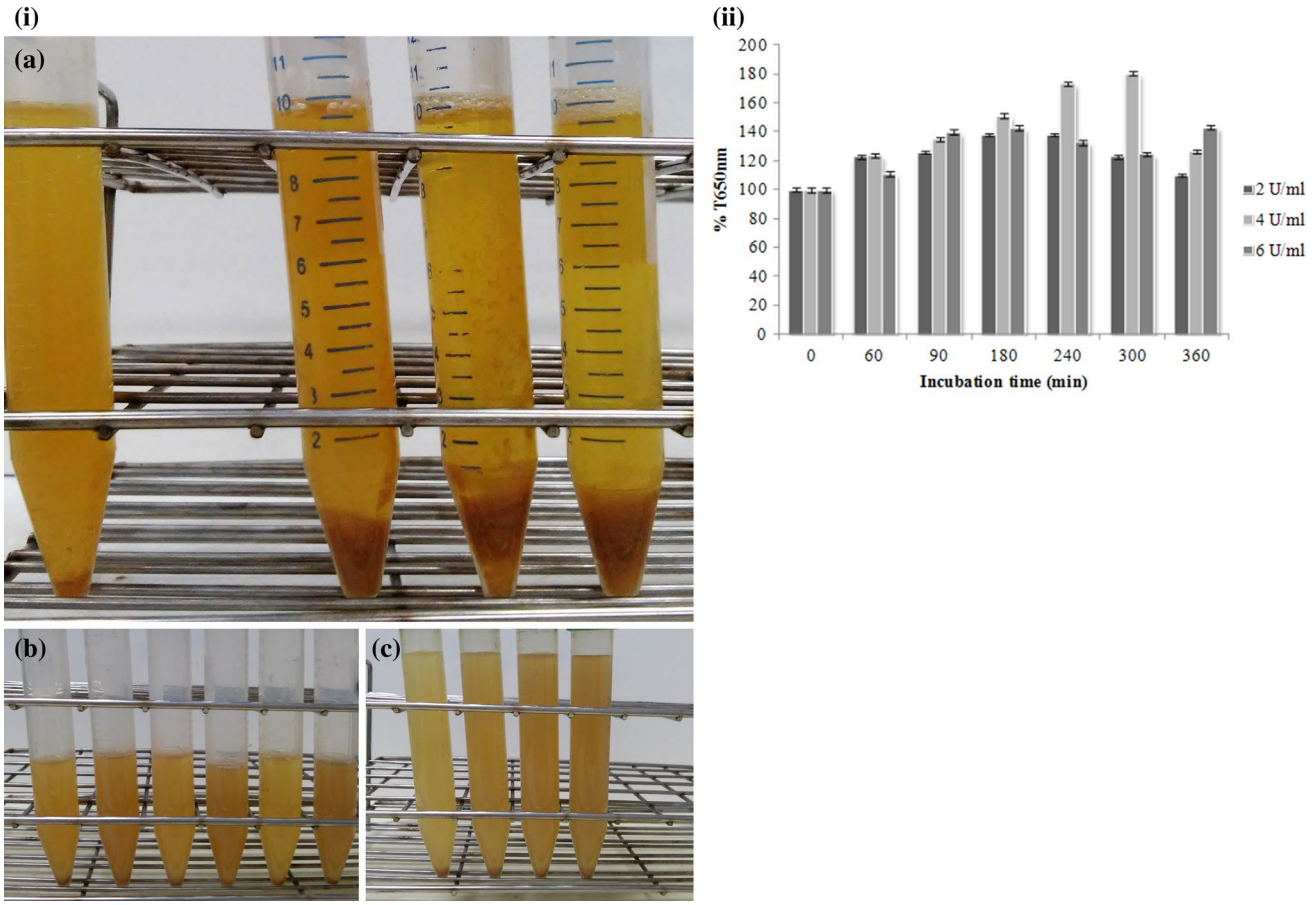

Fig. 5 **i** Clarification of apple juice by the pectinolytic systems of *G. pullulans* 8E (**a**), C. *adeliensis* 5.9 (**b**) and *C. infirmominiatum* e9.2 after 18 h of incubation at 20 °C. Clarification controls were performed using heat-inactivated enzymes (*left*-hand side of each illustration). **ii** Effect of holding time and enzyme concentration on apple juice clarification ($\%T_{650nm}$) by *G. pullulans*'8E supernatant. Values are mean ± SD of three replicates

concentrations are expected to increase $\%T_{650nm}$ by exposing part of the positively charged protein regions, reducing electrostatic repulsion between clouds and, therefore, causing the aggregation and settling out of the flocs. Here, enzyme concentrations higher than 6 U/ml did not increase juice clarification. Similar results were found by Nakkeeran et al. (2011) and Dey and Banerjee (2014), where an increment in PGase activity did not result in an improvement of the clarification process.

In the present study, pectinolytic enzymes of *G. pullulans* with 4 U/ml and 5 h of incubation at 20 °C clarified the juice efficiently with %T650 nm of 80%. The value of A420 nm in clarified juice was 0.56, while in raw juice it was around 0.874. The formation of pectin flocs facilitated the production of a clear supernatant with the removal of the colloidal part of the juice (Fig. 5ii).

Conclusions

Cold-adapted pectinolytic yeasts were selected by screening a culture collection isolated and identified from soil samples from King George Island and Tierra del Fuego province. The yeasts showed adequate growth and PGase production at 20 °C using cheap pectin-containing fruit wastes. PGases have an *endo* mode of action and are active at 10 °C. This is the first report of these species as pectinolytic yeasts, and based on the results presented here, it can be concluded that they have a great potential for its use in the food industry.

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Compliance with ethical standards

Conflict of interest The authors declare no commercial or financial conflict of interest.

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