CHAPTER 1.16 BIOREFINERY OF LEMON PEEL WASTE USING COLD ADAPTED YEASTS FROM ANTARCTIC AND SUB-ANTARCTIC REGIONS

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ABSTRACT

Cold adapted yeasts from soil samples from King George Island and Tierra del Fuego province were evaluated for their potential to produce extracellular pectinases. Pectinolytic yeasts were previously identified by 26S rDNA (D1/D2 domain) sequencing and phylogenetic analyses. Among 103 isolates, only eight showed pectinolytic activity at 20ºC, and only four -strains e9.2, 4.6, 5.9 and 8E- were capable to produce pectinolytic activity at 8ºC. Strain 8E identified as *Guehomyces pullulans* and the strains e9.2 and 5.9 identified as *Cystofilobasidium infirmominiatum* and *Cryptoccocus adeliensis* were selected for enzyme production under submerged fermentation.

All the strains were capable to grow in presence of lemon peel. *C. adeliensis* 5.9 produced the highest enzyme activity at 24 h (4.8 U/ml) while *C. infirmominiatum* e9.2 and *G. pullulans* 8E showed considerable activity at 45 h (3.9 U/ml and 2.83 U/ml, respectively). It could be seen that at 10ºC enzyme/s remained active. Besides polygalacturonase (PGase), presence of other pectin-degrading enzymes in the culture supernatants was investigated. None of the strains produce neither pectin or pectate lyase activity nor rhamnogalacturonan hydrolase activity. Regarding pectin esterase activity, it was only produced by *G. pullulans* (0.022 U/ml). All the strains produced enzymatic pools that showed higher activity against highly esterified pectin than against pectin with 63% methoxyl. This behavior could be attributed to the presence of polymethylgalacturonase activity (PMGase) in its supernatant. β-glucosidase activity was detected in all supernatants.

This is the first report on the capacity of these species to produce pectinases. Inulinase activity was detected in *G. pullulans* and *C. infirmominiatum* supernatants, while xylanase and cellulase activities were only detected in *G. pullulans* supernatants.

Keywords: agro-industrial wastes, cold adapted enzymes, lemon peel.

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INTRODUCTION

Twenty-two per cent of the global fruit market corresponds to citrus production. Within this group, oranges represent 66%, while tangerine and lemon represent 21% and 7% respectively, being 28% of citrus production industrialized. The most important producing countries are Brazil (orange), China (grapefruit and tangerine) and the US. Argentina is the eighth largest producer of citrus and world's largest producer of lemon and has been exporting fresh lemon fruits, juices and essential oils since 1970. The basic structure of the citrus activity in Argentina consists of 5,300 primary producers, which have a cultivable area of 147,466 ha with citrus, with a production of 2.7 million tons of fruit per year. There are 16 industries for juices and derivatives and 529 packaging plants that provides direct employment to about 120,000 people and generates a value of U$S 528 million annually, 64% of which are due the exportation of fresh fruit, juice concentrates and other derivatives. Production is mainly destined to industry (47%) and domestic consumption (32%), being the rest exported. Argentina's production covers a period of ten months from March to December and consist of lemons (49%), oranges (27%), tangerines (18%) and grapefruit (6%). 92% of fruit exports is conducted between May and September and the main destination is the European Union.

The main solid waste of the citrus industry is composed of peels, seeds, pulp and skins. The volumes that these industries handle makes this waste a true ecological problem, which is usually solved by disposing it in dumpsites or sometimes throwing it in river banks and margins and river courses, producing great contamination. Lemon peel is a by-product of the citrus industry that is mainly used as raw material for pectin extraction. Today the fall of this market makes necessary the study of other alternatives for the use of this waste. It is estimated that the lemon peel represents 19.8% of the dry weight of the fruit. Thus, an annual production of 1.5 million lemons generates approximately 148,000 tons of this waste.

Pectinolytic enzymes or pectinases are depolimerizing enzymes that degrade pectic susbtances present in cell walls of plant tissue[1,2]. This enzymes have wide-spread applications in the food industry for clarification of fruit juices, wines, coffee and tea fermentations[3]. Today, the main source of pectinases used in these industries is from fungi, mainly *Aspergillus niger*, since it produces high amounts of these enzymes and is a GRAS microorganism[4] . Yeasts declared as GRAS microorganisms, and which produce high levels of pectinases might be an interesting alternative source of pectinase for these industries.

Interest in cold-active enzymes has been growing in recent years due to the possibility to use them in mild conditions. For example, cold-active pectinases are attractive for it usage in fruit juice industry, as colder temperatures hamper spoilage and favor milder conditions that avoid changes in organoleptic and nutritional properties[2].

This work reports on three cold-adapted, pectinase producing yeast species isolated in Antarctica and Tierra del Fuego province, and presents a preliminar characterization of polygalacturonase activities produced by them. Production of other enzymes such as inulinase, celullase, xylanase and β-glucosidase was also investigated.

MATERIALS AND METHODS

Microorganisms. A collection of 103 yeast strains previously isolated and identified from soil samples from King George Island and Tierra del Fuego province were used in this study. The culture collection is maintained by cryopreservation (-80ºC in 10% glycerol) at Research and Development Center for Industrial Fermentations (CINDEFI-Argentina).

Screening and selection of pectinolytic yeasts. In order to detect pectinolytic enzymes, a selective medium containing 10 g/l citrus pectin (Sigma-Aldrich), 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 1 ml sol. A (5 mg/l $FeSO_4.H_2O$, 1.6 mg/l $MnSO_4.H_20$, 2 mg/l $CoCl_2$) and 20 g/l agar was used. Isolates were point-inoculated and incubated at 8 ºC and 20ºC for 72 h. Yeasts producing pectinases were selected by the formation of a clear halo around the colonies after flooding the solid media plates with Lugol´s iodine solution[5]. Those yeasts which produced the largest halo diameter/colony diameter (Dh/Dc) were selected to continue with production studies.

Enzyme production. Submerged fermentation was carried out in 250 ml Erlenmeyer flask containing 50 ml of culture medium (10 g/l lemon peel, 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 5 mg/l $FeSO_4.H_2O$, 1.6 mg/l $MnSO_4.H_20$, 2 mg/l $CoCl_2$, 5 g/l yeast extract and 2 g/l peptone, pH 5.0) and inoculated with 1 ml of yeast inoculums (DO 0.4). Flasks were incubated at room temperature (20ºC) and samples were with draw at different intervals of time and assayed for polygalacturonase activity.

Lemon peels were thoroughly washed with tap water to remove soluble sugars and dried in a hot air oven at 60ºC. Then the peels were milled (Mesh 35) and used as source of pectin.

Enzymatic assays. Polygalacturonase activity (PGase) was determined using polygalacturonic acid (Sigma Aldrich) as substrate. The reaction mixture containing 180 μl of 0.20 % polygalacturonic acid dissolved in citrate - phosphate buffer solution (CPB, 12.5 mM; 6.25 mM, pH 5.0) and 20 μl of the crude extract was incubated at 20 ºC for 30 min (Haggag et al., 2006). The release of reducing sugars was quantified by the Nelson-Somogyi method, using galacturonic acid as standard. 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. PGase activity was additionally measured at 10º C.

Pectinolityc activity was assayed by quatifying the reducing sugars released from a pectin solution dissolved in CPB (12.5 mM; 6.25 mM, pH 5.0), after incubation with 20 μl of the crude extract at 20 ºC for 15 min. Reducing sugars were measured using the Nelson-Somogyi method; galacturonic acid was used as standard. One unit of pectinolytic activity was defined as the amount of enzyme required to release one micromole of reducing sugars per minute under the given assay conditions. High and low methoxyl pectin were used for this assay, i.e., pectin esterified from citrus fruits (> 86 % methoxyl, Sigma) and pectin from citrus fruits (63 % methoxyl, Sigma), respectively.

Pectin esterase (PE) activity was measured by a titration method. To 10 ml of 0.5% w/v of citric pectin in 0.2 M NaCl, 250 μl of crude enzyme was added. The pH was adjusted to 5.50 with 0.1M HCl and the admixture was incubated for 5 min at 20ºC. PE activity was measured by determining the carboxyl groups released by titration with 0.0026 N NaOH. One unit of PE was defined as the amount of enzyme releasing one milliequivalent of ester hydrolyzed (carboxyl group) per minute.

Lyases. Pectin-lyase and pectate-lyase activities were estimated by measuring the increased in absorbance, due to the formation of unsaturated products.

In the case of pectin-lyase, 180 μl of citric pectin (Sigma Aldrich) 0.2% w/v in CPB (12.5 mM citric acid and 6.25 Mm Na_2HPO_3, pH 5.0) and 20 μl of the crude extract were incubated at 20º C for 15 min. Absorbance at 235 nm was monitored during the incubation time against a blank, in which the heat inactivated enzyme extract was used. One unit was defined as the increase in absorbance at 235 nm of 1.0 in the reaction mixture per minute under the given assay conditions.

For pectate-lyase activity, determinations were performed as stated immediately above, except for the fact that polygalacturonic acid was used as substrate and 1mM $CaCl_2$ was added to the reaction mixture.

Rhamnogalacturonan hydrolase activity was determined using rhamnogalacturonan (Sigma Aldrich) as substrate. The reaction mixture containing 180 μl of 0.20 % rhamnogalacturonan dissolved in CPB (12.5 mM; 6.25 mM, pH 5.0) and 20 μl of the crude extract was incubated at 20º C for 30 min. The release of reducing sugars was quantified by using the Nelson-Somogyi method; rhamnose was used as standard. 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.

Inulinase activity was determined using inulin (Difco) as substrate. The reaction mixture containing 180 μl of 0.20 % inulin dissolved in CPB (12.5 mM; 6.25 mM, pH 5.0) and 20 μl of the crude extract was incubated at 20º C for 30 min. The release of reducing sugars was quantified by the Nelson-Somogyi method, using fructose as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of one micromole of fructose per minute under the given assay conditions. Activity against sacarose was additionally measured.

β-glucosidase activity was detected by zymogram using 4-methylumbelliferyl beta-glucoside (MUG).

All enzyme assays were carried out in triplicate.

Zymogram analysis of pectin degrading enzymes and β-glucosidase detection. Zymograms were performed in conjunction with SDS-PAGE according to the method of García-Carreño et al.[6]. 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% Triton X-100 for 60 min, with constant agitation in order to remove SDS. Triton X-100 was then removed by washing the gel three times with buffer. The gel was later incubated with PGA solution 0.2% (w/v) in buffer at 20ºC for 20 min. Finally, gels were stained with ruthenium red solution for zymographic analysis. The

development of clear zones on the pink background of the gels indicated the presence of pectinolytic activity. The development of clear zones on a pink background indicated the presence of pectinolytic activity. The molecular mass marker used was PageRuler™ prestained protein ladder, 10 to 180 kDa.

For β-glucosidase detection the gel was incubated with a 0.5mM MUG solution in sodium acetate buffer pH 4.5. After incubating the gels at 20° C for 15 min β-glucosidase activity was visualized as clear bands under UV light (λ=365 nm).

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 aluminium sheets (silica gel 60 F254, Merck) and the chromatography performed by 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. Tri galacturonic acid (TGA, Sigma) and galacturonic Acid (GA, Sigma) were used as standard.

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

RESULTS AND DISCUSSION

Screening of pectinolytic activity for yeast selection. One hundred and three yeasts isolated from soil 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 on plate at 20ºC. These strains were pre-selected and subjected to a secondary screening to study their capacity to grow and produce cold active pectinases (8 ºC). It could be seen that although all of them showed well developed colonies, only strains LP e9.2, LP 4.6, LP 5.9 and 8E were capable to produce pectinolytic enzymes (**Figure 1**).

Table 1 shows the ratio between clarification halo size and colony size (Dh/Dc) at 8 and 20º C. This relation was the first criteria used to select pectinolytic strains. According to these results, the isolate identified as *G*. *pullulans* 8E from King George Island, which showed the highest Dh/Dc ratio among the Antarctic isolates, and the two pectinolytic yeasts isolated from Tierra del Fuego province (*C*. *infirmominiatum* e9.2, *C*. *adeliensis* 5.9) were selected to continue with pectinase production studies. The strain 4.6 was not selected to continue with 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 all the three strains were deposited in Genbank database available at NCBI with the following accession numbers: KU 659491, KU 659577 and KU 659556 corresponding to *G. pullulans* 8E, *C. infirmominiatum* e9.2 and *C. adelienses* 5.9, respectively.

FIGURE 1. Screening of cold adapted yeasts in solid medium containing pectin after addition of lugol´s solution. a) plates incubated at 8 ºC and b) plates incubated at 20º C. A clear halo around the colonies indicates the production of pectinolytic enzymes.

TABLE 1. Halo diameter/colony diameter relation (Dh/Dc) for the pectinolytic strains at 8 and 20º C resulting from screening cultures in solid media containing pectin. Strains selected for production studies are indicated in bold.

Strain	Identity	Dh (mm)	Dc (mm)	Dh/Dc
8ºC				
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
20ºC				
LP 1.1	*Cryptococcus antarticus*	90	50	1.8
LPe 9.2	*Cystofilobasidium infirmominiatum*	100	50	2.0
8E	*Guehomyces pullulans*	200	50	4.0
7BE	*Candida davisiana*	70	60	1.2
LP 4.6	*Guehomyces pullulans*	180	70	2.6
10E	*Cryptococcus victoriae*	50	40	1.3
37E	*Metschnikowia australis*	100	40	2.5
LP 5.9	*Cryptococcus adeliensis*	100	30	3.3

Production of pectinases by selected yeasts under submerged fermentation. PGase activity was evaluated during the culture time course as is shown in **Figure 2**. *Cryptoccocus adeliensis* 5.9 showed the highest enzyme production with a maximum at 24 h of cultivation (4.8 U/ml), followed by *Cystofilobasidium infirmominiatum* (3.9 U/ml) and *Guehomyces pullulans* (2.9U/ml). Enzyme activity was evaluated at 10ºC and it could be seen that at this temperature PGAse activity remains active. *C. infirmominiatum* supernatant retained 46% of its activity and *G. pullulans* supernatant 44%, while the activity of *C. adeliensis* supernatant increased to 150% which could be attribute to the instability of the enzymes at 20ºC.

Pectinase production from cold adapted yeasts is a field that is still relatively unexplored and, to our knowledge there are no reports on pectinases from any *Guehomyces pullulans*, *Cystofilobasidium infirmominiatum* or *Cryptoccocus adeliensis.*

Furthermore, there are only two reports on polygalacturonases from cold-adapted *Cystofilobasidium* strains. Nakagawa et al. (2002, 2004) [2,8] and Birginson et al [7] isolated yeasts with pectinolytic activity belonging to the order Cystofilobasidiales which is a group of psychrophilic basidiomycetes[9]. *Cystofilobasidium capitatum* strains PPY-1, PPY-5 and PPY-6 were isolated from soil samples from Hokkaido, Japan[8] while *C. larimarini* S3B and *C. capitatum* S5 were isolated by Birginson et al (2003)[7] from frozen soils, leaves and branches from the south-west of Iceland.

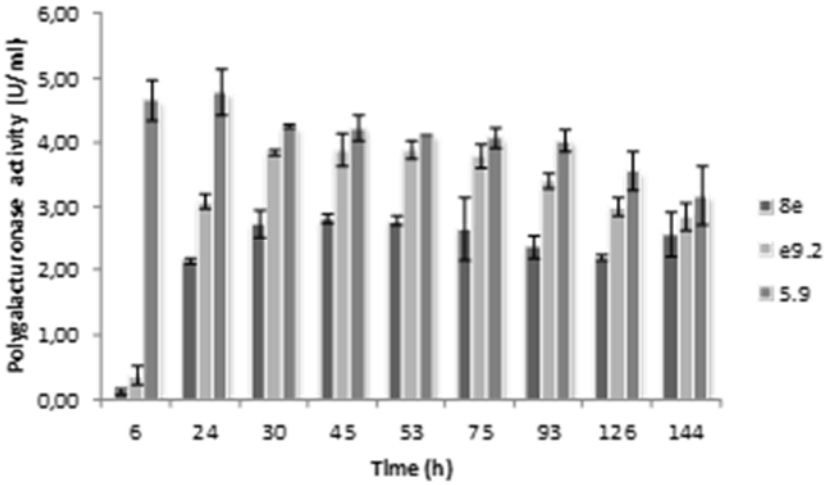

FIGURE 2. Time course of PGAse production by cold adapted yeast under submerged fermentation using cheap row material (lemon peels) as substrate.

Besides PGase, presence of other pectin-degrading enzymes in the culture supernatant of all three isolates was investigated. None of the strains produced neither pectin or pectate lyase activities nor 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 pectins were used. All strains produced enzymatic pools that showed higher activity against highly esterified pectin compared with that measured when pectin with 63% methoxyl degree was used. This behavior could be attributed to the presence of polymethylgalacturonase activity (PMGase) in their supernatants. β-glucosidase activity was detected in all the

supernatants (**Figure 3**). Inulinase activity was detected in *G*. *pullulans* and *C. infirmominiatum* supernatants, while xylanase and cellulase activities were only detected in *G*. *pullulans* supernatants.

Zymogram analysis of pectin degrading enzymes and β-glucosidase detection. Zymogram analyses of pectinolytic enzymes in culture supernatants of the selected strains were performed. As can be seen in **Figure 3** *C*. *adeliensis 5.9* produces at least two active pectinases against PGA (180 kD and 40 kDa). *C*. *infimominiatum e9.2* produces a pectinase whose molecular weight is close to 70 KDa, whereas *G*. *pullulans 8E* produces at least 3 pectinases whose molecular weights are close to 55 KDa. Comparing with previously reports on pectinases characterization, the ones presented here have higher molecular weight; *Kluyveromyces marxianus* NCYC 578 produces four isoenzymes whose molecular weight ranges between 46 to 49 KDa[10] while *Saccharomyces pastorianus* produces a 43 KDa PGase and *Cryptococcus albidus* produces a polygalacturonase whose molecular weight is 41 KDa[11]. Nakagawa et al. (2005)[2] reported the production of five active pectinases for *Cystofilobasidium capitatum* PPY-6.

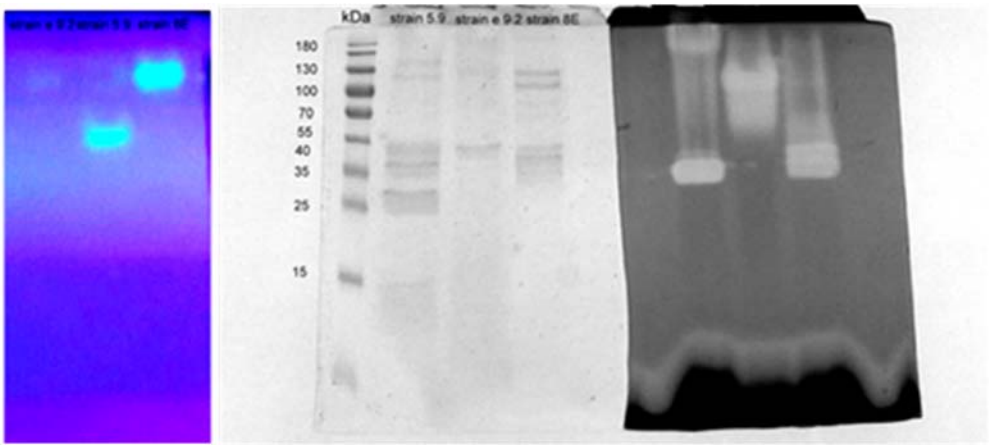

FIGURE 3. Zymogram analyses of pectinolytic enzymes and detection of b-glucosidase enzymes in culture supernatants of cold adapted yeast.

Thin-Layer chromatography TLC. TLC analysis of the products of PGA hydrolysis indicates that di-, and tri-galacturonates,as well as higher oligosaccharides were produced from the initial stages of the hydrolysis, and accumulated throughout the incubation period. PGases present in cold adapted yeasts supernatants did not seem to be able to attack dimers and trimers as these products were accumulated throughout the incubation period (**Figure 4**). From these results, it can be deduced that they act by an endo-splitting mechanism, and so they are endo-PGases (EC 3.2.1.15).

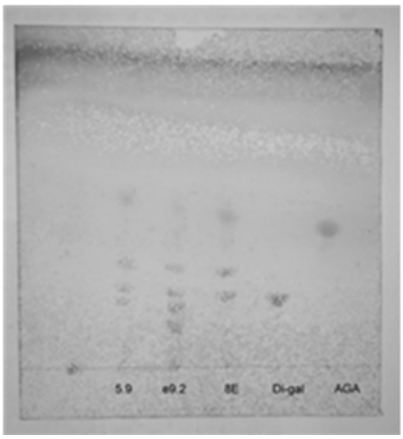

FIGURE 4. Thin-layer chromatography of the degradation products during enzymatic digestion of PGA with culture supernatants of pectinolytic yeasts.

CONCLUSION

The present study emphasized on cold active enzyme production and the capability of the yeasts to use lemon peels as a low cost raw pectin source for pectinolytic production at room temperature. This is commercially very important as the use of cheaper raw materials reduces the production costs significantly. Since the pectinolytic systems remain active at 10ºC they can be applied in fruit juice clarification. Fruit juices are cold stored to prevent spoilage and to increase its shelf life. Clarification at low temperature by these enzymes is cost effective since the temperature of the juice does not need to be raised so as to allow mesophilic enzymes work.

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