J Ind Microbiol Biotechnol
DOI 10.1007/s10295-016-1873-0

FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Pectinase production by *Aspergillus giganteus* in solid-state fermentation: optimization, scale-up, biochemical characterization and its application in olive-oil extraction

Gastón E. Ortiz[1] · María C. Ponce-Mora[1] · Diego G. Noseda[1] · Gabriela Cazabat[2] · Celina Saravalli[2] · María C. López[2] · Guillermo P. Gil[2] · Martín Blasco[3] · Edgardo O. Albertó[1]

Received: 12 July 2016 / Accepted: 12 November 2016

Abstract The application of pectinases in industrial olive-oil processes is restricted by its production cost. Consequently, new fungal strains able to produce higher pectinase titers are required. The aim of this work was to study the capability of *Aspergillus giganteus* NRRL10 to produce pectinolytic enzymes by SSF and evaluate the application of these in olive-oil extraction. *A. giganteus* was selected among 12 strains on the basis of high pectinolytic activity and stability. A mixture composed by wheat bran, orange, and lemon peels was selected as the best substrate for enzyme production. Statistical analyses of the experimental design indicated that pH, temperature, and $CaCl_2$ are the main factors that affect the production. Subsequently, different aeration flows were tested in a tray reactor; the highest activity was achieved at 20 L min^{-1} per kilogram of dry substrate (kgds). Finally, the pectinolytic enzymes from *A. giganteus* improved the oil yield and rheological characteristics without affecting oil chemical properties.

Keywords Solid-state fermentation · Pectinase production · *Aspergillus giganteus* · Agricultural waste · Design of experiment · Olive oil · Pectinolytic enzyme

Introduction

Pectinases constitute a large and complex group of hydrolytic enzymes that can be synthesized by a wide variety of plants and microorganisms [1]. They are involved in the degradation of pectin, a complex polysaccharide present in high amount in fruits' and vegetables' cell walls [2]. Due to the action exerted on vegetables cell walls, these enzymes are widely used in several industrial processes to clarify fruit juices and wines, improve cloud stability in fruit and vegetable nectars, and increase the juice and oil extraction yields [1]. It is noteworthy that pectinases constitute about 25% of the worldwide food enzymes market [3].

With respect to the oil extraction, the pectinases have been successfully employed to increase olive-oil extraction and improve the oil organoleptic properties, such as phenolic content. For this reason, the pectinolytic enzymes have a particular relevance in olive-oil industries [4, 5]. Likewise, the utilization of these enzymes in industrial processes is also important for the reduction of energy consumption and thereby to decrease environmental pollution [6]. This environmental role of pectinolytic enzymes is shown in a Life Cycle Assessment report which illustrates how the application of these enzymes reduces 18 times the CO_2 emission in the apple juice production [7]. In this sense, food and beverages industry generates as a result of its activity a large number of organic wastes as by-product

Electronic supplementary material The online version of this article (doi:10.1007/s10295-016-1873-0) contains supplementary material, which is available to authorized users.

✉ Gastón E. Ortiz
gas.ortiz@gmail.com

[1] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH), Universidad Nacional de San Martín (UNSAM)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Martín, Buenos Aires, Argentina

[2] Centro de Investigación y Desarrollo en Tecnologías de Industrialización de Alimentos, Instituto Nacional de Tecnología Industrial, Av. General Paz 5445, Edificio 40, San Martín, Buenos Aires, Argentina

[3] Centro de Investigación y Desarrollo en Biotecnología Industrial, Instituto Nacional de Tecnología Industrial, Av. General Paz 5445, Edificio 51, San Martín, Buenos Aires, Argentina

Published online: 22 November 2016

of manufacturing processes. Consequently, the accumulation of great quantities of organic residues represents a risk to health and environment. As an alternative, biotechnological processes for valorization of organic wastes generate products with high-added value resulting in economic and ecological advantages [8]. In this sense, production of industrial enzymes by solid-state fermentation using agricultural wastes as substrate represents a suitable alternative for re-valorization of such a type of residues reducing environmental pollution.

Numerous studies of pectinase production were carried out employing different wild type or recombinant strains of microorganisms as bacteria, fungi, and yeasts, using submerged culture fermentation (SmF) or solid-state fermentation (SSF) [9–11]. However, the SSF is most productive and ecofriendly than SmF, because such fermentation system requires lower quantities of water and several agricultural residues can be used as substrates for enzyme production [12]. Particularly, numerous species of *Aspergillus* genus grown on agricultural wastes with large amounts of pectin, such as orange, lemon, and banana peels or sugar beet pomace, has been successfully used for pectinase production by SSF [13, 14]. The state-of-the-art analysis allowed to determine that the most current scientific knowledge associated with pectinase production by *Aspergillus* fungus is related to the use of *A. niger* and *A. sojae* species (Supplementary Data S1). Taking into account this facts and the important impact that generates over the environment the use of enzymes in the industrial process, new strains suitable for pectinase production using agricultural residues are required. Likewise, an accordant optimization of strains' growth and enzymes' production conditions is necessary for an appropriate use of agricultural waste and achieves sustainability of the bioprocess. For this purpose, the design of experiment (DOE) is a widely used methodology [11, 15].

It should be mentioned that the pectinase production studies using *A. giganteus* are at an early stage and only few reports can be found in the current bibliography databases (Supplementary Data 1). However, in all these reports, the pectinase production was not studied in SSF and its applications have not been explored. Within this context, the main objectives of this work were to study the capability of *A. giganteus* to produce pectinolytic enzymes by SSF and evaluate the application of these enzymes in olive-oil extraction.

Materials and methods

Microbial strains

The *Aspergillus* strains used in this work were: (1) *A. terreus* ICFC 744/11; (2) *A. oryzae* NRRL 2217; (3) *A. awamori* NRRL 356; (4) *A. flavipes* NRRL 295; (5) *A. kawachii* IFO 4308; (6) *Aspergillus sp* ICFC 7/14; (7) *A. japonicus* NRRL 1782; (8) *A. oryzae* ICFC 8/12; (9) *A. giganteus* NRRL 10; (10) *A. rhizopodus* NRRL 6136; (11) *A. sojae* NRRL 5595; and (12) *A. sojae* ATCC 20235. ICFC strains are conserved in the IIB-INTECH Collection of Fungal Cultures (ICFC), reference in the WDCM database: WDCM 826. All the strains were periodically propagated and maintained in sugar cane molasses agar slants.

Inoculum preparation

The strains were grown on agar plates containing per liter of culture media: sugar cane molasses 45 g, glycerol 45 g, peptone 18 g, NaCl 5 g, KCl 0.5 g, $FeSO_4{\cdot}7H_2O$ 15 mg, KH_2PO_4 60 mg, $MgSO_4$ 50 mg, $CuSO_4{\cdot}5H_2O$ 15 mg, $MnSO_4$ 12 mg, and agar 20 g. Plates were incubated at 28 °C until sporulation. Conidia were harvested from the plates by the addition of 5 mL of 0.08% (w v^{-1}) Tween80. The concentration of conidia was determined using Neubauer cell-counting chamber.

Culture conditions for comparative study

Erlenmeyer flasks 100 mL containing 5 g of a dried mixture of wheat bran and orange peel in proportion 70:30, respectively, with a homogeneous particle size of ~2000 µm, were moistened with 110 mL of Hankin medium per each 100 g of dry substrate (gds) [16]. The composition of Hankin medium per liter is: yeast extract 1 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 4 g, Na_2HPO_4 6 g, $FeSO_4{\cdot}7H_2O$ 0.2 g, $CaCl_2$ 0.5 mg (this quantity per liter is 1X $CaCl_2$ solution), and traces compounds: H_3BO_3 0.01 mg, $MnSO_4{\cdot}H_2O$ 0.011 mg, $ZnSO_4{\cdot}7H_2O$ 0.118 mg, and $CuSO_4{\cdot}5H_2O$ 0.078 mg, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.012 mg (these quantities per liter is 1X trace solution). The flasks with the sterile culture substrate (sterilization conditions, 121 °C for 20 min) were inoculated with 10^6 conidia per gds and incubated at 28 °C in a moist chamber for 2, 4, and 6 days.

Enzyme recovery and total protein quantification

Enzymes produced were recovered by the addition of 10 mL gds^{-1} of distilled water into each culture flask and mixed in a shaker at 250 rpm, 28 °C, for 30 min. Then, the mixtures were clarified by filtration through cotton cloth followed by centrifugation at 2000×*g*, 4 °C, for 20 min. The clarified supernatants were used for the following analysis. The total protein was quantified by Bradford method using BIO-RAD™ protein assay reagent.

Semi-quantitative determination of pectinolytic activity by agar-plate assay

A semi-quantitative determination of pectinolytic activity was carried out at different values of pH using agar-plate diffusion assay according to the technique described by Hankin [16]. Agar plates were prepared with 1.5% (w v^{-1}) of agar and 1% (w v^{-1}) of citrus pectin as substrate. The agar and citrus pectin were dissolved in 100 mM citrate-sodium biphosphate buffer (BCP) to adjust and maintain the pH values between 3 and 6. Wells of 4 mm diameter were punched in the agarized substrate and loaded with 15 µL of crude enzymatic extracts normalized according to the total protein content. After 24 h incubation at 30 °C, the pectin-agar plates were flooded with 1% (w v^{-1}) of CTAB (cetyltrimethylammonium bromide) solution and maintained in agitation during 30 min. The diameter of the halos (*D*) corresponding to the zone of pectin degradation was converted to Log_{10} by Eq. 1 and reported as hydrolysis index (Log_{10} mm^2):

$$\text{Log}_{10}\text{ adjusted zone area } = \text{Log}_{10}\left[\left(\frac{D}{2}\right)^2\pi - \left(\frac{4.0}{2}\right)^2\pi\right] \quad (1)$$

Semi-quantitative determination was performed by means of two independent assays. Pectinolytic activity values from different crude extracts were evaluated by Multifactorial ANOVA and cluster analysis using the statistical software Statgraphic Centurion XVII trial version (Supplementary Data 2).

Total pectinolytic activity determination

Pectinolytic activities, polygalacturonase (PGase), and polymethylgalacturonase (PMGase) were quantitatively measured using the protocol described by Mora-Lugo et al. [17]. The reaction mixture contained 10 µL of enzyme extract diluted in 50 mM BCP buffer (pH 5) and 90 µL of 0.5% (w v^{-1}) polygalacturonic acid (PGA) or citrus pectin (CP) solution in BCP. The blank mixtures were performed by the addition of 90 µL of BCP plus 10 µL of diluted enzyme (enzyme blank) and 90 µL of PGA or CP plus 10 µL of BCP (substrate blank). The standard curve was performed using 100 µL of different concentrations of galacturonic acid (GA) solution (0, 0.125, 0.25, 0.375, and 0.5 µM of GA solution in BCP). All mixtures and the standard curve were performed in a 96-well thermocycler plate. After the addition of the reagents, the plate was centrifuged for 30 s at 2000×*g* and then placed into the thermocycler to conduce the reaction at 30 °C for 30 min. This reaction was stopped with the addition of 100 µL of dinitrosalicylic acid (DNS) prepared according to Miller [18]. The plate was centrifuged for 30 s at 2000×*g* and then placed into the thermocycler to conduce reaction at 95 °C for 5 min following with an additional step of 4 °C for 2 min. Finally, 150 µL of each reaction was added into an ELISA plate and the absorbance was read at 540 nm. An enzymatic unit is defined as the amount of enzyme required to generate 1 µmol GA per minute from its respective substrate (PGA or CP) in the conditions mentioned above. The activities were calculated using the following equation:

$$\frac{\text{U}}{\text{mL}} = \frac{\mu\text{mol } S - \mu\text{mol EB} - \mu\text{mol SB}}{30\text{ min} \times 0.01\text{mL}} \quad (2)$$

where *S*, EB, and SB are µmol of GA release by sample, enzymatic blank, and the substrate blank, respectively.

Endo-polygalacturonase (endo-PG) activity determination

Endo-PG activity was measured according to the microplate method reported by Ortiz et al. [19]. Briefly, the reaction was performed in a 96 microplate PCR plate by mixing 8 µl of diluted enzymatic sample in 100 mM BCP buffer pH 5.0 and 8 µL of 5 µg μL^{-1} PGA solution in a 100 mM BCP buffer pH 5.0. The mixture was incubated for 20 min of reaction at 40 °C in thermocycler. After incubation, the unhydrolyzed PGA was precipitated by the addition of 40 µL of 1.125 mg mL^{-1} Ruthenium Red (RR) solution and the remaining RR not involved in the precipitation reaction was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µg of PGA in small fragments unable to precipitate with RR per minute in the conditions mentioned above.

Enzyme characterization

The crude extract from *A. giganteus* NRRL 10 and the commercial enzyme-mix Pectinex® Ultra Olio enzyme from Novozyme were partially purified by diafiltration with 3 K ultrafiltration module (PALL, No. Part SIP-0013). The PMGase activity of diafiltered samples was adjusted to 1 IU mL^{-1} to carry out the enzyme characterization. Hence, the optimal pH was ascertained by assaying activities at different reaction pH values from 2.8 to 7.0 in 50 mM phosphate-citrate buffer and using the temperature described above for each enzymatic activity. For optimum temperature determination, pectinase activities were assayed at optimum pH for the three activities (pH 5.2) in a range of values from 20 to 50 °C. The PMGase, PGase, and endo-PGase thermal and pH stabilities were determined by incubating the samples under the optimal conditions for different times (0, 15, 45, 75, 105, and 135 min) before conducting the activity assays under optimal conditions.

Due to the complexity of the reactions occurring during inactivation by heat or pH, several equations have been proposed to model reaction kinetics. In this work, a first-order kinetic model was selected that fits the equation: $A/A_0 = e^{-kt}$, where A/A_0 represents the residual enzyme activity at time t (min) and k (min^{-1}) is the rate constant of the reaction at a given temperature.

Chemical composition of the agriculture waste

Protein and nitrogen content was determined by Kjeldhal method [20]. For this proposes, 0.250 g of dried agricultural waste was employed for each determination and the protein percentage in the samples was calculated using de followed conversion factors: 6.31 for wheat bran and 6.25 for apple pomace, orange peel, and lemon peel [21].

For total carbohydrate and glucose determination, 0.300 g of each dried agricultural waste was hydrolyzed by heating in 0.8 M HCl solution for 2 h in a reflux condenser device. The acid hydrolysates were neutralized by the addition of 10% NaOH solution, filtrated through paper filter No. 4 and made up to 200 mL with distiller water. Finally, total carbohydrates in the hydrolysates were quantified by measurement of reducing sugars according to Miller [18]; and total glucose content was measured by glucose oxidase kit from Wiener-Lab®.

For total pectin determination, 2 g of dried samples were transferred into a flask containing 30 mL of water and heating for 15 min in a reflux condenser device. After heating, each sample was immediately filtered by cheesecloth and the solids were washed twice with 20 mL boiling water. To prevent pectin degradation, the collected solutions were immediately cooled and two volumes of pre-chilled 96% (v v^{-1}) ethanol solution were added. Later, the samples were centrifuged at 7900×g for 30 min and the pellets were dried and restituted by the addition of 45 mL distilled water. The suspensions were clarified by centrifugation at 3000×g for 15 min, and supernatant were used to determine the pectin content by ruthenium red precipitation. The quantities of pectin content in the solutions were estimated by measuring absorbance of the non-precipitated ruthenium red at 535 nm. Standard curve was made using citric pectin 0.5% (w v^{-1}) solution in the proportions described by Ortiz et al. [19].

Substrate preparation and selection

Substrate screening was performed with wheat bran, citrus peel, lemon peel, and apple pomace. All substrates were dried at 80 °C and milled in a grain blender. For substrate selection, a mixture experiment design was carried out in a 100 mL Erlenmeyer flasks containing 5 g of each substrate mixture. All mixtures were hydrated at 110% with Hankin medium and sterilized by autoclaving. The flasks with the sterile culture mixture were inoculated and incubated in a moist chamber for 2 and 4 days with the conditions described above. The experimental designs and analysis of mixture experiments were carried out using Statgraphic Centurion XVII trial version. The compositions of mixtures are shown in Supplementary Data 3.

Plackett–Burman design (PBD)

A two-level fractional factorial design without interactions between factors (Plackett and Burman) was used for detection of significant parameters. The main effect of each parameter was calculated as the difference between the averages of the response values obtained for the high-level (+1) and low-level (−1) conditions.

The parameters screened for pectinase production were yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $FeSO_4{\cdot}7H_2O$, pH, $CaCl_2$, traces, pH, temperature, inoculum size, time, and moisture. The experiments were carried out by triplicate, and the PGase, PMGase, and endo-PGase activity values were taken as responses. The composition of the media, incubation conditions, and inoculum concentrations is shown in Supplementary Data 5. The experimental designs were formulated through the use of Statgraphics Centurion XVII trial version and the half-normal plot was used to assess which factors were significantly important to make the regression model.

Fractional factorial design (FFD)

A fractional factorial design with two levels was employed to study the major effects of the main parameters and their interactions. All experiments were performed in triplicate under the conditions shown in Supplementary Data 6. Statgraphics centurion XVII trial version was employed for the experimental design and ANOVA analysis. The pectinolytic activities PGase, PMGase, and endo-PGase were determined as described above and the second-order model represented by Eq. 3 was used to describe these responses. The half-normal plot was used to assess which factors are significantly important to make the regression model:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j \quad (3)$$

where Y is the estimated response, β_0 is the constant term, i and j have values from 1 to the number of variables (n), β_i is the linear coefficient, β_{ij} is the quadratic coefficient, and x_i and x_j are the coded independent variables. The coefficient of determination R^2 and the F value from analysis of variance (ANOVA) were used to confirm the quality of the model.

Bioreactor for SSF

A static tray-type bioreactor with a carbon-dioxide gas-analyzer module was used for SSF. The solid media consisted of 0.1 kg of wheat bran mixture in proportion 66:17:17 with orange and lemon peels, respectively. The mixture was hydrated with 130 mL of a saline solution composed by 150 µL of 1000X traces solution, 900 µL of 200X $CaCl_2$ solution, and 3.3 mL of 1 M NaOH solution to give the following initial condition: pH of 4.8, 1.5X gds^{-1} traces solution, 1.8X gds^{-1} $CaCl_2$, and moisture 130% (v gds^{-1}). The medium was sterilized and then inoculated with 1.5×10^6 conidia gds^{-1}. The culture was carried out at 28 °C and different aeration rates: 10, 15, and 20 L min^{-1} $kgds^{-1}$. The percentage of CO_2 in the exhausted gas was used for growth-phase monitoring and the cumulative amount of CO_2 produced was calculated using the area under the $\%CO_2$ vs. time curve. The biomass sampling for activities determination was done randomly from different zones of tray-type bioreactor.

Olive variety and olive-oil extraction

The experimental assays of olive-oil extraction were performed using *Frantoio* olive variety with a ripening degree of 1.4 determined according to Uceda and Frías [22]. Olive-oil extraction was carried out by the following steps: (1) the fruit samples were washed and dried and leaves were removed; (2) 300 g of fruit were milled two times for 30 s using a blade grinder Numak F400 to obtain a paste with ~5 mm particle size; (3) the paste was whipped using an Avencor-type system at 50 rpm for 45 min,; in this step, the active or heat inactive enzymes were added at 230 U_{PGase} per kg of olive paste; (4) after malaxation, the paste was centrifuged at $3000 \times g$ for 10 min; and (5) finally, the oil was separated by decantation in two phases: oil and waste water.

Olive-oil phenolic compound determination

Total phenolic compounds were measured by means of an adapted protocol previously described by Hadj-Taieb et al. [4]. Briefly, phenolic compounds were extracted from 350 µL of olive oil using 250 µL of a solution 60:40 water–methanol mixture for each step of extraction (four times). After such extraction, 40 µL of each extract and standard curve solutions: 0, 50, 100, 150, 250, and 500 mg L^{-1} of galic acid were mixed with a 0.11% (v v^{-1}) of Folin–Ciocalteu (Merck) and incubated for 8 min in darkness. Subsequently, 300 µL of 20% (g gds^{-1}) Na_2CO_3 solution were added to the mixtures and then incubated in dark for 2 h. After incubation, the absorbance of each sample was measured at 727 nm. Total phenols values were expressed in milligrams of galic acid per kilogram of olive oil.

Fatty acid determination

For the determination of fatty acid composition, the methyl esters were obtained by vigorous shaking of a solution of oil in heptane (0.1 g in 2 ml) with 0.2 ml of methanolic potash 2 N. The samples were analyzed with a gas chromatograph (Perkin-Elmer Clarus 500) equipped with an FID detector. The analysis was carried out using a polysiloxane biscyanopropyl capillary column (100 mm length × 0.25 mm diameter) and hydrogen as carrier gas, operating at a flow of 20 cm s^{-1}. Temperatures of the injector and detector were 240 and 280 °C, respectively, and the volume injected was 1 µl.

Olive-oil physicochemical properties

The *K* values were determined by absorption at 232 and 270 nm, following the analytical method described in IOC/T.20/Doc no. 19 (International Olive Council) [23]. Iodine value was estimated assuming a stoichiometric addition of two atoms of iodine per double bond of carbon–carbon of the methyl esters determined by gas chromatography. The chlorophyll and carotenoid content was measured with an adapted protocol described by Hadj-Taieb et al. [4]. For this, the absorption spectrum of each oil sample (350 µL), dissolved in cyclohexane (650 µL), was evaluated at 670 and 470 nm for chlorophyll and carotenoid quantification, respectively. Turbidity evaluation was performed by measuring absorbance at 830 nm [24]. The draining time of olive oil was determined measuring the time required to elute 1.0 mL of oil in a 2.0 mL glass pipette at 25 °C [13]. All parameters were determined in triplicate for each sample.

Results and discussion

Comparative study of total pectinolytic activity

To evaluate the capability of *A. giganteus* to produce pectinolytic enzymes, a comparative analysis against different *Aspergillus* strains was carried out. For this purpose, the crude enzymatic extracts obtained from different days of fermentation were semi-quantitatively analyzed for total pectinolytic activity at different pH values by agar-plate diffusion assay. The diameters of pectinolytic halos were adjusted, as indicated in "Materials and methods" and reported as enzymatic activity index. Such activity values were employed to compare the strains through multifactorial analysis of variance (ANOVA) (Supplementary Data 2). A mixture composed by orange peel and wheat bran was chosen as substrate, due to the first one is a suitable pectinase inducer and the second provides an adequate

A

Escale Log_{10} mm^2

0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	2.4

	Day 2				Day 4				Day 6			
Strain	pH3	pH4	pH5	pH6	pH3	pH4	pH5	pH6	pH3	pH4	pH5	pH6
1	2.00	2.20	NA	NA	1.92	2.13	NA	NA	1.97	2.05	NA	NA
2	NA	2.05	1.20	1.20	NA	1.92	1.20	1.20	NA	1.58	1.05	1.05
3	NA	1.64	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	NA	1.97	1.71	NA	NA	1.71	1.41	NA	NA	1.41	NA	NA
5	1.49	1.71	1.41	NA	1.64	1.41	1.20	NA	1.28	NA	NA	NA
6	NA	1.71	1.41	NA	NA	1.41	1.20	NA	NA	NA	NA	NA
7	1.65	1.92	1.71	NA	2.06	1.82	1.64	NA	1.97	1.71	1.41	1.41
8	NA	1.20	1.58	1.41	NA	NA	0.85	1.41	NA	NA	0.85	0.85
9	NA	2.32	2.33	1.41	NA	2.26	2.32	1.71	NA	2.20	2.30	2.30
10	NA	1.71	1.71	NA	NA	1.41	1.58	NA	NA	1.41	NA	NA
11	1.55	2.08	1.82	1.58	1.55	2.20	1.41	NA	NA	2.13	1.20	1.20
12	1.82	2.42	2.15	1.92	1.82	2.28	2.05	2.05	NA	1.64	1.77	1.77

B

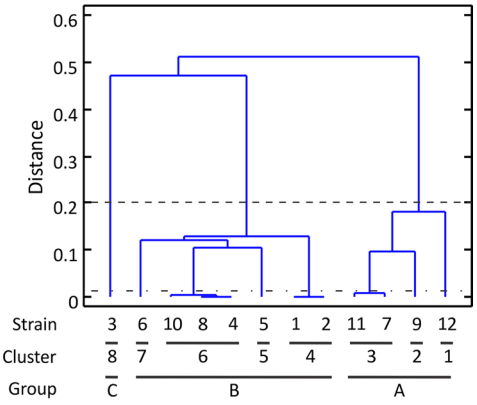

Fig. 1 Semi-quantitative analysis of total pectinolytic activity in different *Aspergillus* strains. **a** Total pectinolytic activity from crude extracts of *Aspergillus* cultures analyzed for different values of pH and time fermentation. The values correspond to the average of two independent studies and are expressed as hydrolysis index (log_{10} mm^2). *NA* no activity. **b** Dendrogram constructed from the pectinolytic activities using median method and squared Euclidean distance. Distance of 0.2 (*dash*) and 0.012 (*dash* and *dot*) indicates the cutline for the designation of groups and clusters, respectively. Strain reference numbers are indicated in "Materials and methods"

carbon-to-nitrogen ratio (C:N), necessary for an appropriate growth and pectinase production [11, 12, 25]. The *A. sojae* ATCC 20235 was included in the experiment as a control strain, because it has been reported to be an important pectinase producer [13, 26].

The analysis of variance revealed that most of the evaluated strains registered the highest pectinolytic activity around to pH 4.0 and with 2 days of fermentation (Fig. 1a; Supplementary Data 2). Furthermore, based on this enzymatic characteristic, the cluster analysis revealed the presence of two principal groups divided into seven clusters (Fig. 1b). One of these groups (Group B) is formed by clusters four, five, six, and seven. All the strains included in Group B showed intermediate activity values at pH 4.0 for days 2 and 4. In addition, we should mention the presence of acid-pectinases in the crude enzymatic extracts from *A. japonicus*, *A. terreus* and *A. kawachii*. The existence of acid-pectinases has been previously reported for *A. kawachii* by Contreras Esquivel and Voget [27], indicating that the results obtained through this screening methodology are consistent. The other group (Group A) is constituted by the clusters one, two, and three. The strains included in Group A presented high pectinase activity around pH 4.0–5.0, which remained stable throughout all period of fermentation. The control strain *A. sojae* ATCC 20235 showed a high pectinase activity throughout the tested pH range for days 2 and 4, while *A. giganteus* NRRL 10 was the second strain with the highest pectinolytic activity for pH 4.0–6.0 until day 6 (Fig. 1a). This result suggests that the pectinolytic complex of such strain possesses good stability. In this sense, Pedrolli and Carmona reported a pectin lyase with high stability produced by *A. giganteus* in SmF using orange waste as substrate [28]. Based on these results, it can be concluded that crude extracts from *A. giganteus* NRRL 10 are composed by stable pectinolytic enzymes with high-level activity.

To validate these results, a quantitative determination of the pectinolytic productivity was conducted. The results showed that *A. sojae* ATCC 20235 and *A. giganteus* NRRL 10 included in Group A (Fig. 1b), and presented the highest level of productivities with average values ranging around 55 U_{PGase} gds^{-1} day^{-1}, 35 U_{PMGase} gds^{-1} day^{-1}, and 10 $kU_{endo\text{-}PGase}$ gds^{-1} day^{-1} (Supplementary Data 3). These strains exhibited similar productivities patterns, where higher productivity was observed at the second day of fermentation, and then, it decreased constantly according to the progress of fermentation. This decline in the productivities could be due to the increase in pH values observed during fermentation (Supplementary Data 3). The dependence of productivity with the pH has been previously reported for *A. awamori* and *A. niger* strains [29, 30]. The pH is an important physicochemical factor that affects the growth and enzymatic productivities. In particular, for filamentous fungi, a pH value between 4.5 and 5.0 is considered as optimal for growth; likewise, an acid pH is appropriate for the production of pectinolytic enzymes [31, 32]. To verify that this decrease in pectinolytic productivity is not due to a cessation of metabolic activity, the extracellular protein content was determined (Supplementary Data 3). In addition, we made the determination of substrate weight loss as an additional indicator of metabolic activity; this weight loss is due to the CO_2 generated by respiration when the substrate is metabolized [33, 34]. For all the strains tested, the amount of extracellular protein increased or remained

constant throughout fermentation and the substrate weight loss increased during culture process, which indicated that the metabolism remained active all over fermentation (Supplementary Data 3). These results suggested that intermediate productivities exhibited by strains included in Group B were not due to a deprivation in its metabolic state.

Mixture designs for substrate selection

The mixture design approach was used to select the best combination of wheat bran with the inductors: apple pomace, orange peel, and lemon peel. In a mixture experiment, the independent factors were proportions of different components in a blend and the total proportions of the different factors had to be 100%. For this purpose, two mixture experiments were performed using a simplex-Lattice design with 17 experimental trials (Supplementary Data 4). The measured response of pectinolytic enzyme production was assumed as a dependent variable on the relative proportions of the mixture components. The activity values were fitted to curves for regression analysis to obtain the models for PGase, PMGase, and endo-PGase productivities (Supplementary Data 5). The adequacy of the models was significant when analyzed by ANOVA test, indicating that the models can be used for prediction of the responses (Supplementary Data 5).

The mixture composed by wheat bran, orange peel, and apple pomace showed a quadratic dependence for PGase productivity with R^2 higher than 0.95 at 2 days of fermentation, which explains 95% of variability of the response (Supplementary Data 5). The contour plot for this response showed that apple pomace did not exert a positive effect on the induction of PGase productivity. This fact is evidenced by the higher response close to the triangle edge, where the percentage of apple pomace in the blend was cero (Supplementary Data 6A, day 2). When we analyzed the PGase productivity at day 4 of fermentation, the response presented a cubic behavior and it was possible to evidence that the highest productivity was near to the vertex of the triangle corresponding to 50% of orange peel (Supplementary Data 6A, day 4). This suggests that the major contribution in the PGase productivity was given by orange peel. When PGase productivity was compared between both days, the maximum value (50 U_{PG} gds^{-1} day^{-1}) was obtained at day 2 of fermentation (Supplementary Data 6A). It is important to mention that this maximum value was obtained for a mixture similar to the screening mixture (wheat bran and orange peel 70:30). The PMGase productivity displayed a similar behavior, where the apple pomace had a little contribution on PMGase productivity and the major contribution was given by orange peel (Supplementary Data 6B). In addition, the highest PMGase productivity (12 U_{PMG} gds^{-1} day^{-1}) was registered on the second day of fermentation. Likewise to what was observed for PGase and PMGase productivity, the apple pomace did not exercise an inductive effect on endo-PGase productivity (Supplementary Data 6C). These results are in accordance with the study conducted by Zheng and Shetty that reported the effect of apple pomace on pectinase production by *Lentinus edodes* [35]. In this study, the authors concluded that, the apple pomace did not contain a sufficient amount of pectin to be a good inducer. Furthermore, Hours et al. studied the factors that affect the pectinolytic enzyme production by *A. foetidus* using apple pomace as carbon and energy source [35]. In this report, they demonstrated that availability of organic nitrogen in apple pomace is the main factor that affected the productivity of these enzymes. Taking into account these studies, the apple pomace could be acting in our experiments as a weak inductor due to a low pectin or nitrogen content (Table 1).

Table 1 Chemical composition of agriculture waste

Values in % (w/w)	Wheat bran	Orange peel	Lemon peel	Apple pomace
Nitrogen	2.48	0.86	1.18	0.31
Protein	15.65	5.37	7.37	1.95
Carbohydrates	72.00	55.44	42.46	65.89
Glucose	22.01	12.00	3.24	21.63
Pectin	ND[a]	4.40	2.07	0.85

[a] Not detected

Considering that orange peel is a suitable inductor and that apple pomace is not an appropriate inductor, we decided to evaluate another citric-origin inductor. For this purpose, lemon peel was used in combination with wheat bran and orange peel. A cubic model was used to describe the dependence of the PGase productivity with the component of mixture. In Fig. 2a, it can be note that at day 2 of fermentation, the maximum zone of PGase productivity is near the center of the triangle, the contour suggesting a strong interaction between the three components. Likewise, lemon peel did not act as inducer at day 2 of fermentation, which can be seen in the triangle vertex corresponding to 50% of lemon peel (Fig. 2a, day 2). However, lemon peel showed higher induction at day 4, which can be observed by a maximum PGase productivity zone near the vertex of 50% lemon peel (Fig. 2a, day 4). When PMGase was evaluated, we found a similar behavior to that observed for PGase, with a strong interaction between the three components at day 2 and a major influence of the lemon peel on day 4 (Fig. 2b). For endo-PG, the highest productivity was obtained with a high proportion of wheat bran and a smaller proportion of orange and lemon peel close to zero (Fig. 2c, day 2). The cubic model indicates that the interaction

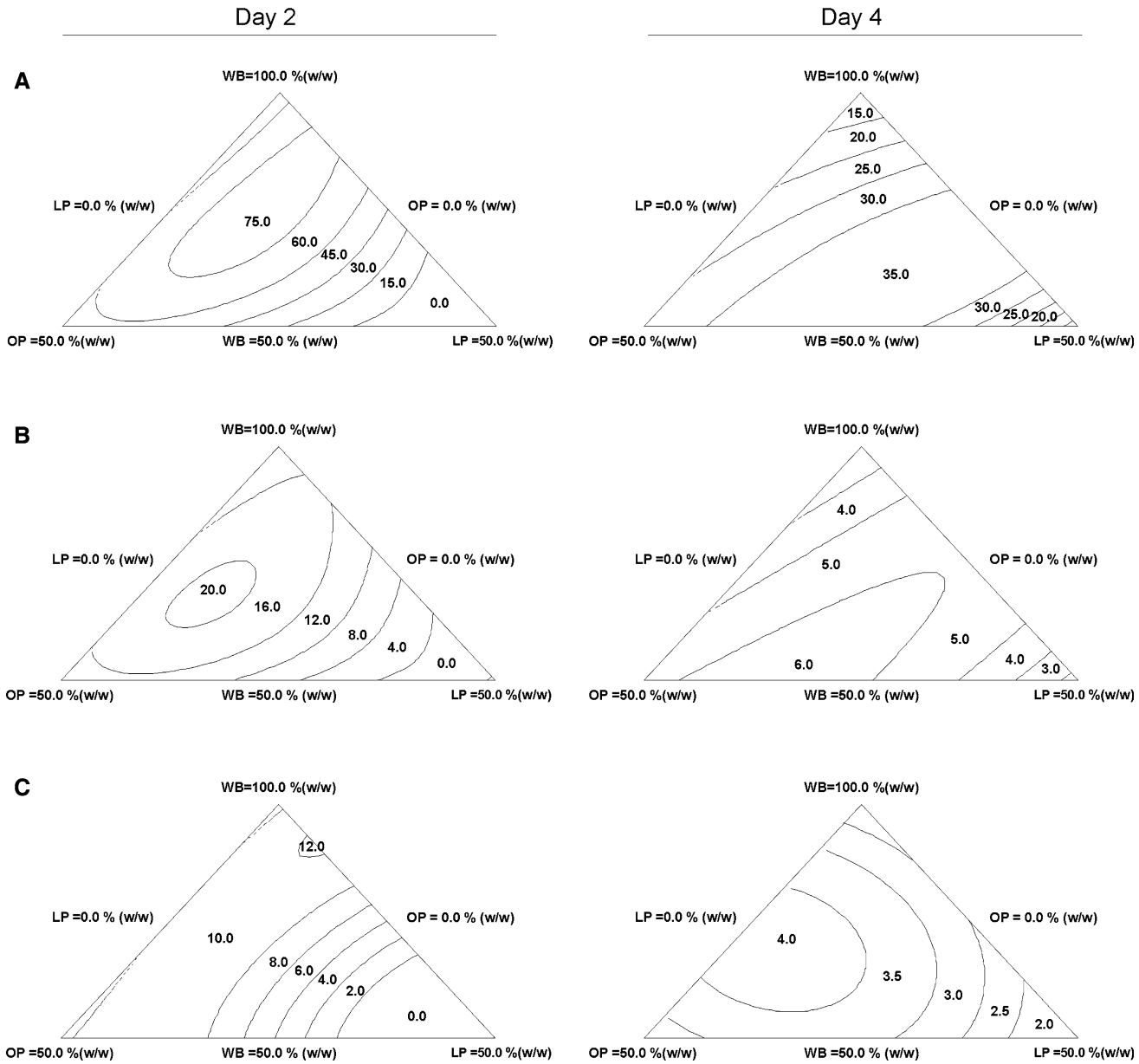

Fig. 2 Mixture contours plots for pectinase production by *A. giganteus* as a function of wheat bran (WB), orange peel (OP), and lemon peel (LP). Each *contour line* represents combinations of WB, OP, and LP; which shows a selected value for **a** PGase productivity (U_{PG} gds^{-1} day^{-1}), **b** PMGase productivity (U_{PMG} gds^{-1} day^{-1}), and **c** endo-PGase productivity ($kU_{endo\text{-}PG}$ gds^{-1} day^{-1})

between the three components was positive for endo-PG productivity and the effect of wheat bran and orange peel positively contributed on productivity, while the effect of lemon peel was not significant (Supplementary Data 5). On day 4, it is possible to note that orange peel begun to exert an inductive effect on endo-PG productivity (Fig. 2c, day 4). Although the model did not fit significantly with confidence interval of 95% (p value 0.05), it is possible to approach these statements considering the behavior previously observed for PGase and PMGase. The maximum PGase, PMGase, and endo-PG productivity was achieved at day 2. To obtain the optimal mixture, the desirability function was used for the simultaneous optimization of the three pectinolytic activities. The optimal values obtained for the maximal response correspond to trial eight, composed by 66.6% wheat bran, 16.7% orange peel, and 16.7% lemon peel (Supplementary Data 4B). Finally, to confirm the model prediction, the optimal mixture predicted by model, optimal experimental mixture, and screening mixture was tested (Supplementary Data 7). In this sense, Mrudula and Anitharaj reported that the orange peel was a good inducer for pectinase production [30]. Likewise, Heerd et al. and

Silva et al. described that the use of wheat bran in combination with orange peel increased the inductive effect of these substrates on the pectinolytic enzymes production [13, 36]. Furthermore, lemon peel has been reported by Ruiz et al. as a suitable carbon source and inducer for pectinase production [14]. Moreover, a comparative study of pectinase production carried out by Martínez-Trujillo et al. indicated that lemon peel was better than apple pomace for the production of such enzymes [37]. According to the analysis of mixtures design, it is important to mention that pectinolytic activity could be detected at day 2 of fermentation in the control trial (wheat bran 100%) suggesting that this strain expresses constitutively pectinolytic enzymes (Fig. 2). However, these activity values were lower in comparison with those obtained when the inducers were included in an optimal ternary mixture.

Finally, when the metabolism of *A. giganteus* was evaluated, the wheat bran was the most important factor that affected the metabolic state (Supplementary Data 8). In other hand, we did not detect a significant effect of inductors on the metabolic state at day 2. This suggests that the differences between productivity for both mixtures were due to the biochemical composition of the evaluated inductors and not due to a metabolic state deprivation.

Optimization of culture conditions

Evaluation of main parameters in pectinase productivity

The effects of the medium composition and culture conditions were evaluated by means of the Plackett–Burman design in 12 experimental trials involving 12 parameters at two levels. The conditions tested in the 12 trial resulted in following maximal activities values: 90.16 U_{PG} gds^{-1} day^{-1}, 26.81 U_{PMG} gds^{-1} day^{-1}, and 11.09 $kU_{endo\text{-}PG}$ gds^{-1} day^{-1}. In contrast, the conditions selected in the fifth trial resulted in the minimal values (Supplementary Data 9).

To obtain a linear model for PGase, PMGase, and endo-PGase productivities, the activity values were fitted by regression analysis (Supplementary Data 10). The adequacy of these models was analyzed by ANOVA test. p values <0.05 were obtained for the three models, indicating that these models can be used to predict the responses (Supplementary Data 10).

Accordingly, the main effects of the individual parameters on enzyme production were calculated, as described in Materials and methods. The significance levels (p values) identified by ANOVA test obtained for each variable are shown in the (Supplementary Data 10). The estimated effect obtained for each variable is illustrated in the Pareto chart (Fig. 3). As shown in Fig. 3a, an increase in $(NH_4)_2SO_4$ and KH_2PO_4 factors exerted a positive effect on enzyme productivity; while an increase in time, pH, and temperature factors exercised a negative effect on enzyme productivity. Figure 3b shows that an increase in pH, temperature, and time presented a negative effect on PMGase productivity; meanwhile, an increase in $(NH_4)_2SO_4$, $CaCl_2$ and KH_2PO_4 enhanced this productivity. With respect to the effects on endo-PG productivity, we note that an increase in time and temperature produced a negative effect in the response; however, in contrast to PGase and PMGase productivity, the traces solutions exerted a positive effect on endo-PG productivity (Fig. 3c).

Study of pectinolytic production by fractional factorial design

To model the pectinolytic production in *A. giganteus*, the main parameters that affect the enzymes production, such as temperature, pH, $CaCl_2$, $(NH_4)_2SO_4$, and KH_2PO_4, were optimized by means of a fractional factorial design involving 26 experimental runs (Supplementary Data 11). The experiments were carried out with 2 days of fermentation, because maximum productivity was observed with this period of time. The other critical parameters, such as moisture, traces solution, and inoculum, were fixed in the quantities that gave the maximal activities values in the Plackett–Burman experiment: moisture 130% (v gds^{-1}), 1.5X gds^{-1} traces solution, and 1.5×10^6 conidia gds^{-1}. To obtain the dependency of the pectinolytic activities (responses) with the parameters tested, the responses were fitted to a second-order polynomial equation (Supplementary Data 12). The ANOVA test showed not significant lack of fit for PGase, PMGase, and endo-PGase models, suggesting that all models are suitable to represent each response (Supplementary Data 12).

The polynomial equations were used to find the optimized response values for each model; and the contour plot or 3D mesh plots were used to visualize the effect of interaction between the parameters (Fig. 3). The Pareto charts showed that the factors pH, KH_2PO_4, and $CaCl_2$ had a significant effect (p value <0.05) in PGase activity, demonstrating that an increase in KH_2PO_4 and $CaCl_2$ factors exerts a positive effect on enzyme productivity (Supplementary Data 13A). According to the profile observed in the PGase 3D mesh plot, it can be noted that the maximum production (217.6 U_{PG} gds^{-1}) was reached in the region established by pH 4.4–4.8, 10–12 mg gds^{-1} KH_2PO_4, and 1.8–1.9X gds^{-1} $CaCl_2$ solution, with a strong interaction between factors (Fig. 3d). Likewise, considering that pH, temperature, and $CaCl_2$ are the main factors affecting PMGase activity (Supplementary Data 13B). A 3D mesh plot including these factors was conducted to find the region with maximal activity, and a region of (58.00 U_{PMG} gds^{-1}) was found in the range to pH 4.4–4.8, 24–26 °C and

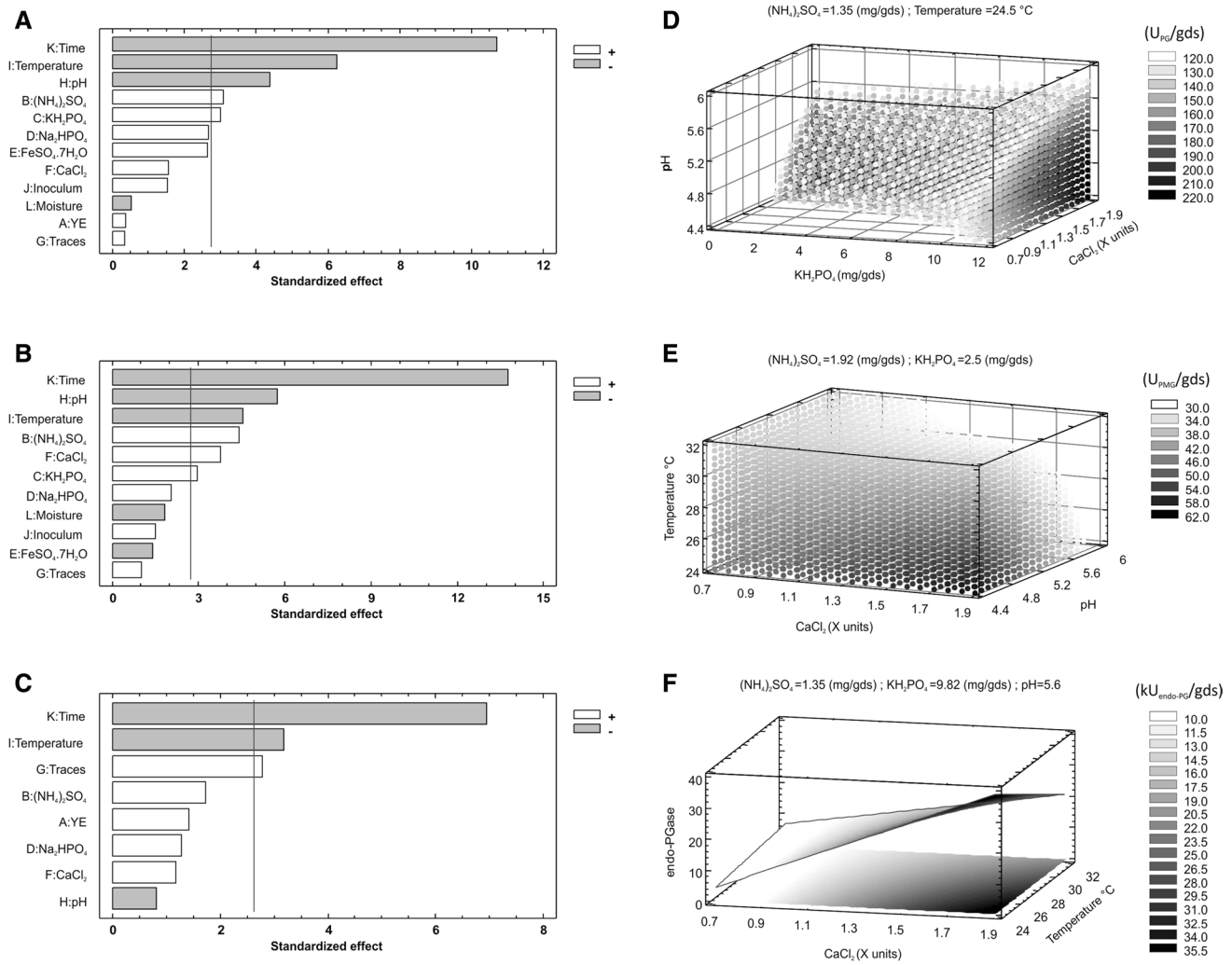

Fig. 3 Optimization of culture condition by PDB and FFD. Pareto chart for the effect of each parameter on pectinase productivity evaluated by PBD. **a** PGase productivity; **b** PMGase productivity; and **c** endo-PGase productivity. Cutline ($p < 0.02$) obtained by ANOVA test for each effect. *YE* yeast extract. Estimated mesh and surface plots obtained by FFD. **d** PGAase activity; **e** PMGase activity; and **f** endo-PGase activity. The constant factors, which are showed in the *top* of each graph, were fixed in the optimal values predicted by desirability function

1.72–2.00X gds^{-1} $CaCl_2$ (Fig. 3e). When the endo-PGase activity was analyzed, we found that only temperature and $CaCl_2$ factors affected this activity (Supplementary Data 13C). Figure 3f shows a zone of a maximal endo-PGase activity (34–35 $kU_{endo\text{-}PG}$ gds^{-1}) in the range to 24–26 °C and 1.7–1.9X gds^{-1} $CaCl_2$.

In addition, the desirability function was used for the simultaneous optimization of the three pectinolytic activities, and we note that optimal values of variables for the maximal response corresponded to the 16th trial (Supplementary Data 14). Finally, based on the Pareto charts (Supplementary Data 13), it is possible to conclude that pH, temperature, and $CaCl_2$ are the most significant factors that affect the three activities. Supplementary Data 15 shows the optimal range for the three activities, and it is comprised within the values pH 3.75–4.50, 24–26 °C, and 1.7–1.9X gds^{-1} $CaCl_2$.

Pectinase production in a tray bioreactor

The scaling-up of pectinase production was carried out using a tray bioreactor under the optimal conditions: pH 4.5, 1.8X gds^{-1} $CaCl_2$ and moisture 130% (g gds^{-1}), previously determined by FFD analysis of PMGase, PGase and endo-PGases enzymes. The impact of the aeration rate on pectinase production was evaluated at 10, 15, and 20 L min^{-1} kgds^{-1}. At the lower aeration rate, the maximum pectinolytic enzymes production (72 $kU_{endo\text{-}PG}$ gds^{-1}, 130 U_{PG} gds^{-1}, and 70 U_{PMG} gds^{-1}) was reached at 48 h of fermentation (Fig. 4a). For an air flow

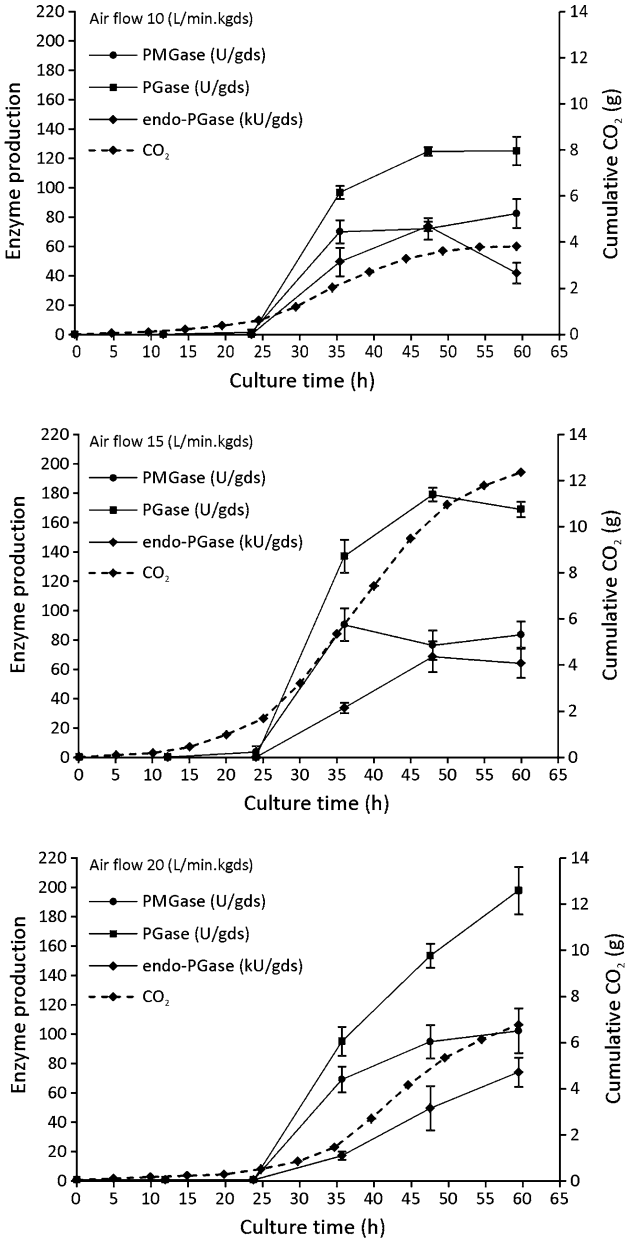

Fig. 4 Bioreactor enzyme production. Evaluation of pectinase production at three airflow rates: 10, 15, and 20 L min^{-1} $kgds^{-1}$ (**a**, **b**, and **c**, respectively). The PMGase (*dots*), PGase (*squares*), and endo-PGase (*diamonds*) activity values are the average of five samples withdrawn from different sites of the culture. The growth phase was monitored online by the analysis of cumulative carbon-dioxide production (*dashed line*). The *error bars* represent the standard deviation of each experimental point ($n = 5$)

of 15 L min^{-1} $kgds^{-1}$, the PGase activity increased up to 180 U_{PG} gds^{-1} at 48 h and the other activities (PMGase and endo-PGase) remained around 70 U gds^{-1} (Fig. 4b). It should be mentioned that these activities were similar to those predicted by the FFD analysis. Finally, when the air flow was increased up to 20 L min^{-1} $kgds^{-1}$, the maximum activity values (73 $kU_{endo\text{-}PG}$ gds^{-1}, 197 U_{PG} gds^{-1}, and 101 U_{PMG} gds^{-1}) were achieved at 60 h of culture (Fig. 4c). These results suggest a strong dependence of the pectinolytic activities with the aeration rate. A similar dependence of pectinase production with aeration rate was reported for *A. niger* F3 growing on citrus peel by Rodríguez-Fernández et al. [38]. In addition, the analysis of the carbon-dioxide accumulation was possible to determinate that the maximum values of activities were obtained at the end of the exponential phase for each evaluated flow rate; suggesting that this is an appropriate time to finish the fermentation procedure (Fig. 4).

Biochemical characterization of the crude enzyme extract

To establish whether crude extracts from *A. giganteus* can be useful as an alternative in processes, where pectinolytic activity is required, some biochemical properties of the extracts were determined and then compared with those of a well-known commercial pectinase extract (Pectinex® from Novozyme). When the optimal temperature profiles were evaluated on both extracts, a similar dependence of the activity with temperature was found for PMGase and PGase with maximum values around 37 and 20 °C, respectively (Fig. 5a, b). Likewise, endo-PGase profile of pectinolytic extracts from *A. giganteus* exhibited in contrast to Pectinex®, two peaks with maximal activity at lower temperature (23 and 37 °C) (Fig. 5c). This biochemical property for endo-PGase activity is advantageous, because the majority of industrial processes, where these enzymes, are used to require high endo-pectinolytic activity at room or low temperature [39]. When pectinolytic activities were analyzed in function of different pH values, both extracts displayed an optimal activity around pH 5.2 for the three enzymatic activities analyzed (Fig. 5d–f). However, *A. giganteus* extract showed a higher residual activity for PGase, PMGase, and endo-PGase enzymes in the range from pH 5.8–7.0 with respect to Pectinex® blend (Fig. 5d–f). As it was mentioned above, this behavior was previously observed in the biochemical characterization of a unique pectinlyase produced by *A. giganteus* [40]. This differential tolerance of pH confers an advantage to the *A. giganteus* extract over Pectinex®, because allowed extend the pH range, where pectinases are useful.

The stability of PMGase enzymes is an important and critical aspect to conduct industrial application. Therefore, thermal and pH stability of pectinolytic extracts were evaluated at short time under the optimal PMGase conditions previously determined (pH 5.2, 37 °C). The PMGase activity of Pectinex® blend presented an inactivation constant of 0.0005 min^{-1}, a one order less than the *A. giganteus* extract (0.0022 min^{-1}) indicating that Pectinex® exhibited

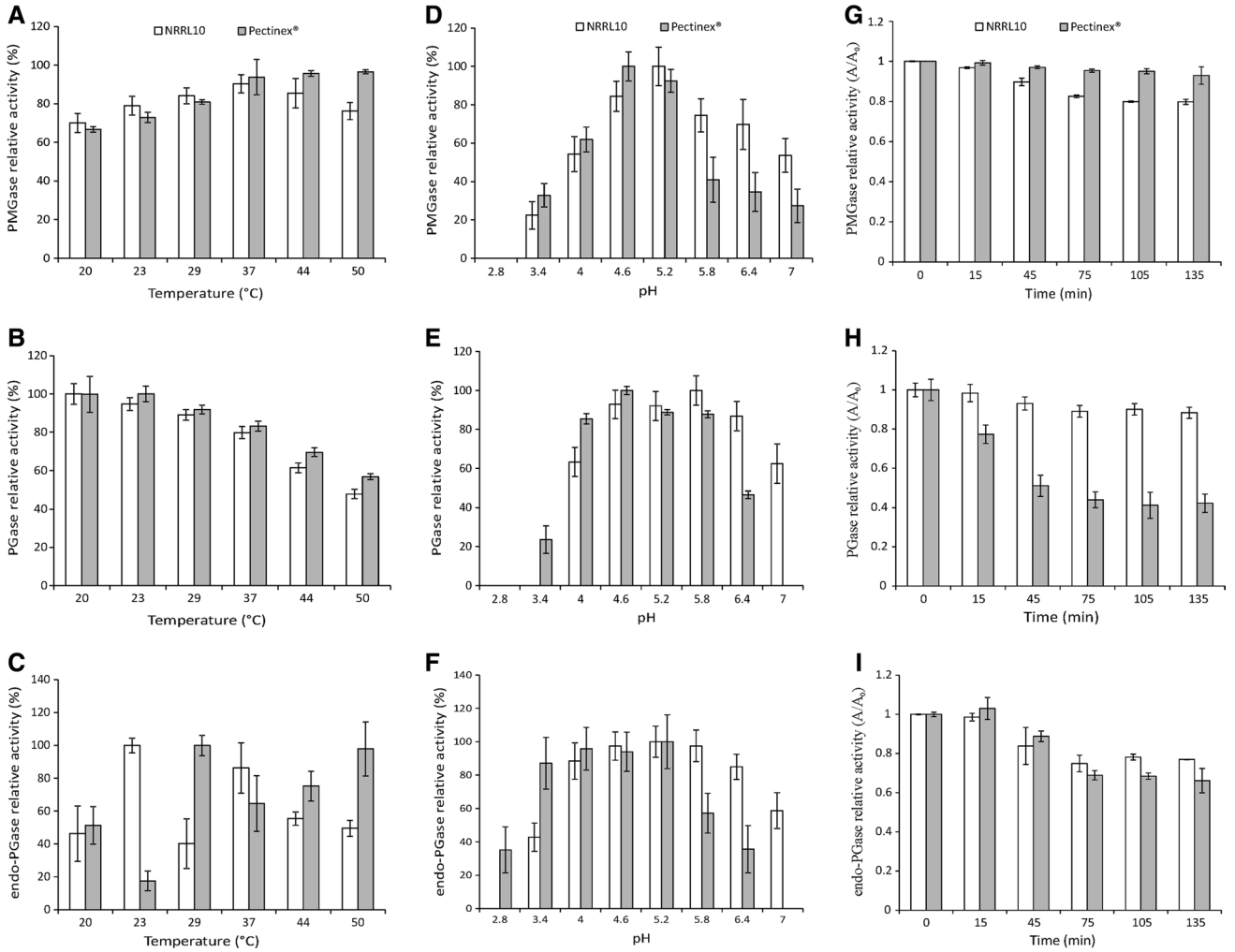

Fig. 5 Biochemical characterization of pectinolytic extract. Effect of temperature on **a** PMGase, **b** PGase, and **c** endo-PGase. Residual pectinolytic activities are expressed as a percent of the original activity. Effect of pH on **d** PMGase, **e** PGase, and **f** endo-PGase. Residual activities are expressed as a percent of the original activity. Stability at pH 5.2 and 37 °C of **g** PMGase, **h** PGase, and **i** endo-PGase. The *white bars* represent the enzymatic extract from *A. giganteus* NRRL 10 and the *gray bars* the commercial blend Pectinex®. The *error bars* represent the standard deviation ($n = 3$)

an appropriate stability for PMGase activity (Supplementary Data 16A). It should be mentioned that PMGase activity is the result of the synergistic action of exo-PGase, endo-PGase, pectin Lyase PL, and accessories enzymes, such as ramnhogalacturonase; therefore, a good stability of PMGase is required to achieve a complete degradation of pectin [41]. However, is important to highlight that *A. giganteus* enzymatic extracts retain 80% of PMGase activity at 135 min of incubation (Fig. 5f). Therefore, this residual enzyme activity is acceptable for using the extract in processes that do not require long times of incubation. When PGase stability was analyzed (Fig. 5g), *A. giganteus* extract exhibited a higher resilience to thermal inactivation than Pectinex® with inactivation constants of 0.0016 and 0.011 min^{-1}, respectively (Supplementary Data 16B). Finally, Fig. 5H shows that endo-PGase stability exhibited a similar behavior for thermal inactivation with constant values of 0.0046 and 0.0067 min^{-1} for *A. giganteus* and Pectinex® extracts, respectively (Supplementary Data 16C).

Application of pectinolytic enzymes in olive-oil extraction

During the last years, several pectinolytic enzymes have been used to improve the olive-oil yield and quality properties [4, 42, 43]. In this context and considering that *A. giganteus* achieved the highest pectinase productivity and exhibited similar pectinolytic biochemical characteristics in comparison with Pectinex® Ultra Olio enzyme, we decided to explore the potentiality of *A. giganteus* pectinolytic extract to improve the olive-oil extraction. As shown in Fig. 6, the addition of *A. giganteus* extract at the

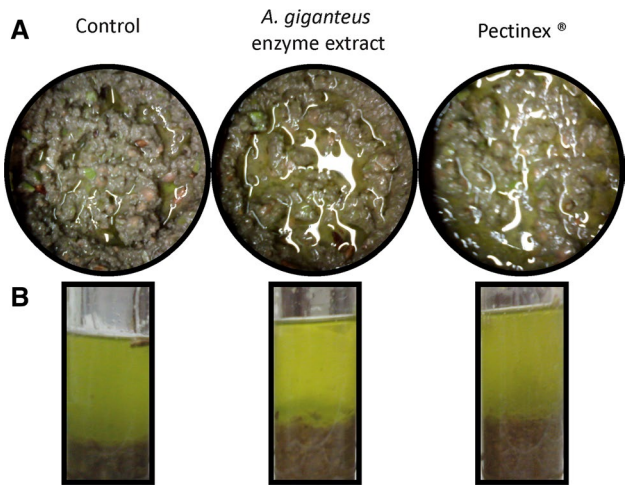

Fig. 6 Effect of pectinolytic enzymes in olive-oil extraction. **a** Pectinase effect during malaxation. **b** Pectinase effect on oil decanting step

Table 2 Olive-oil physicochemical properties obtained with or without the addition of pectinolytic enzymes during malaxation

Determination	Samples		
	Control	Pectinase	Pectinex®
Iodine index	85.55 ± 0.64	[b]85.6 ± 0.57	[b]85.3 ± 0.28
K_{232}	1.61 ± 0.04	[b]1.58 ± 0.01	[b]1.54 ± 0.16
K_{270}	0.15 ± 0.02	[b]0.15 ± 0.07	[b]0.15 ± 0.06
ΔK	0	0	0
Cholophylls (ppm)	6.4 ± 0.3	[b]5.4 ± 0.3	[b]5.7 ± 0.1
Carotene (ppm)	3.0 ± 0.1	[b]2.4 ± 0.7	[b]2.8 ± 0.2
Relative oil turbidity %	100	[a]30 ± 25	[b]90 ± 18
Total polyphenols (ppm)	77.5 ± 1.98	[b]77.5 ± 7.26	[b]89.64 ± 7.92
Oil yield %(w w^{-1})	8.17 ± 0.57	[a]9.42 ± 0.62	[a]8.95 ± 0.65
Oil draining time (s)	14.36 ± 0.51	[a]6.17 ± 0.16	[a]9.38 ± 0.18

[a] Significant differences with control p value <0.05 (one-tailed t test); $n = 3$

[b] No significant differences with control p value >0.05 (one-tailed t test); $n = 3$

beginning of the malaxation produced an increase in oil droplet content indicating an improved in the oil release and malaxation properties. The malaxation step is considered the main bottleneck in the olive-oil process [44]. As shown in Table 2, the oil yield achieved using *A. giganteus* extract was similar to that obtained with Pectinex®; however, both were higher comparing to the control. The increase in oil yield using *A. giganteus* extract achieved 15.30%; similar values were obtained by Hadj-Taieb et al. and Najafian et al. [4, 43]. Furthermore, Fig. 6 and Table 2 show that pectinolytic extract from *A. giganteus* exerted a 70% decrease in relative turbidity of olive oil with respect to control, demonstrating a remarkable effect on the clarification of olive oil after the decanting step.

The viscosity is an important factor that affects the settling velocity, which is essential in olive-oil production for removing solid and liquid particles suspended in the oil that contribute to turbidity [45]. Thereby, to analyze the change in the viscosity of olive oil after enzymatic treatment, the draining time was evaluated. As shown in Table 2, the treatment with pectinolytic extract from *A. giganteus* decreased the oil-draining time with respect to control and Pectinex® treatment, indicating a viscosity reduction in olive oil.

Several studies reported that the action of pectinolytic enzymes reduced the complexation of phenolics with the pectic polysaccharides of the fruit, increasing the phenolic compounds in olive oil [4, 43, 46]. As shown in Table 2, a significant increase in the polyphenol content was not observed when *A. giganteus* extract was used in oil extraction. Our results are in accordance with the results previously published by Peres et al. [5]. Furthermore, chlorophylls and carotenoids are an important pigment that contributes to the appearance of olive oil [45]. In our experiments, the pectinolytic enzyme treatment did not affect the pigments composition in the olive oil (Table 2). To investigate the effect of pectinolytic treatment on the oil chemical composition, the iodine index (unsaturated fatty acids estimation), K_{270} (secondary oxidation), K_{232} (conjugate trienes and carbonylic compounds), and fatty acid profile were evaluated. No significant changes in K values and iodine index were detected between the different treatments (Table 2). Likewise, the enzyme did not exert variation on the fatty acid composition (Table 3). These results are consistent with previously data reported for chemical composition of oil from green olives (*Chemlali* variety) by Hadj-Taieb et al. [4]. Finally, the chemical composition results (pigments, K values, polyphonic, and fatty acid) suggested that enzymatic treatment is innocuous for the final product.

Conclusions

In the present work, *A. giganteus* NRRL 10 exhibited higher productivity and stability of pectinases produced, in comparison with other strains tested. The mixture analysis allowed identifying a blend of wheat bran (66.7%), orange (16.7%), and lemon (16.7%) peel as the optimal substrate for *A. giganteus* pectinase production. The subsequent PBD and FFD analysis showed that pH, temperature, and $CaCl_2$ affect enzyme production. The tray bioreactor experiments indicated that an increase in aeration rate is necessary to enhance the pectinolytic enzyme production. The comparative biochemical analysis showed that *A. giganteus* pectinases exhibit a higher residual activity at pH 5.8–7.0 with respect to Pectinex®. Finally, *A. giganteus* pectinases

Table 3 Olive-oil fatty acid profile obtained with or without the addition of enzymes during malaxation

Fatty acids composition	g of fatty acid per 100 g of methyl ester		
	Control	Extract	Pectinex®
C12:0 Lauric	0.04[a]	ND	ND
C14:0 Miristic	0.05[a]	ND	ND
C16:0 Palmitic	15.06 ± 0.1	15.05 ± 0.04	15.35 ± 0.3
C16:1 Isomer + palmitoleic	1.1 ± 0.11	1.06 ± 0.02	1.06 ± 0.02
C17:0 Margaric	0.05[a]	0.05[a]	0.05[a]
C17:1 Heptadecenoic	0.09[a]	0.1[a]	0.09[a]
C18:0 Estearic	1.83 ± 0.04	1.78 ± 0.09	1.83 ± 0.04
C18:1 Isomers + Oleic	69.62 ± 0.4	69.78 ± 0.12	69.43 ± 0.44
C18:2 Linoleic + isomers	10.51 ± 0.65	10.51 ± 0.56	39.63 ± 41.71
C18:3 Linolenic + isomers	5.98 ± 7.06	5.95 ± 7	5.92 ± 6.97
C20:0 Araquidonic	0.65 ± 0.34	0.64 ± 0.32	0.66 ± 0.35
C20:1 Gondoic	0.35 ± 0.03	0.35 ± 0.02	0.34 ± 0.01
C22:0 Behenic	0.13[a]	0.13[a]	0.13[a]
C24:0 Lignoceric	0.08[a]	0.08[a]	0.08[a]
C18:1 Trans	ND	ND	ND
C18:2 Trans + C18:3 trans	ND	ND	ND

ND no detected

[a] Only detected in a one trail; $n = 3$

improved oil yield and rheological features without altering chemical composition of olive oil. Therefore, pectinolytic extract from *A. giganteus* NRRL 10 is suitable for its application in the olive-oil extraction process.

Acknowledgements This work was supported by the PICT Start Up 2010-1312 Grant from ANPCyT and MINCyT Argentina (issued to Dr. E. Albertó). We would like to acknowledge Dr. Fernandez-Lahore (Jacobs University Bremen, Germany) for providing *A. sojae* ATCC 20235 strain. The NRRL strains employed in this work were kindly provided by ARS Culture Collection.

References

1. Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. Bioresour Technol 77:215–227
2. Thakur BR, Singh RK, Handa AK (1997) Chemistry and uses of pectin—a review. Crit Rev Food Sci Nutr 37:47–73
3. Mercimek Takcı HA, Ucan Turkmen F (2016) Extracellular pectinase production and purification from a newly isolated *Bacillus subtilis* strain. Int J Food Prop 2912(10942912):1123270. doi:10.1080/10942912.2015.1123270
4. Hadj-Taieb N, Grati N, Ayadi M et al (2012) Optimisation of olive oil extraction and minor compounds content of Tunisian olive oil using enzymatic formulations during malaxation. Biochem Eng J 62:79–85. doi:10.1016/j.bej.2011.04.003
5. Peres F, Martins LL, Branco C et al (2014) Laboratory-scale optimization of olive oil extraction using enzymes and microtalc. Eur J Lipid Sci Technol. doi:10.1002/ejlt.201400060
6. Arunachalam C, Saritha K (2009) Protease enzyme: an eco-friendly alternative for leather industry. Indian J Sci Technol 2:29–32
7. Jegannathan KR, Nielsen PH (2013) Environmental assessment of enzyme use in industrial production—a literature review. J Clean Prod 42:228–240. doi:10.1016/j.jclepro.2012.11.005
8. Liguori R, Amore A, Faraco V (2013) Waste valorization by biotechnological conversion into added value products. Appl Microbiol Biotechnol 97:6129–6147. doi:10.1007/s00253-013-5014-7
9. Patil SR, Dayanand A (2006) Optimization of process for the production of fungal pectinases from deseeded sunflower head in submerged and solid-state conditions. Bioresour Technol 97:2340–2344. doi:10.1016/j.biortech.2005.10.025
10. Rojas NL, Ortiz GE, Baruque DJ et al (2010) Production of heterologous *polygalacturonase* I from *Aspergillus kawachii* in *Saccharomyces cerevisiae* in batch and fed-batch cultures. J Ind Microbiol Biotechnol. doi:10.1007/s10295-010-0929-9
11. Demir H, Göğüş N, Tari C et al (2012) Optimization of the process parameters for the utilization of orange peel to produce *polygalacturonase* by solid-state fermentation from an *Aspergillus sojae* mutant strain. Turk J Biol 36:394–404. doi:10.3906/biy-1104-23
12. Ortiz GE, Noseda DG, Ponce Mora MC et al (2016) A comparative study of new *Aspergillus* strains for proteolytic enzymes production by solid state fermentation. Enzyme Res 2016:3016149. doi:10.1155/2016/3016149
13. Heerd D, Yegin S, Tari C, Fernandez-Lahore M (2012) Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: a comparative study. Food Bioprod Process 90:102–110. doi:10.1016/j.fbp.2011.08.003
14. Ruiz HA, Rodríguez-Jasso RM, Rodríguez R et al (2012) Pectinase production from lemon peel pomace as support and carbon source in solid-state fermentation column-tray bioreactor. Biochem Eng J 65:90–95. doi:10.1016/j.bej.2012.03.007
15. Ortiz GE, Guitart ME, Cavalitto SF et al (2015) Characterization, optimization, and scale-up of cellulases production by *Trichoderma reesei* cbs 836.91 in solid-state fermentation using agro-industrial products. Bioprocess Biosyst Eng 38:2117–2128. doi:10.1007/s00449-015-1451-2
16. Hankin L, Anagnostakis SL (1975) The use of solid media for detection of enzyme production by fungi. Mycol 67:597–607. doi:10.2307/3758395
17. Mora-Lugo R, Madrigal M, Yelemane V, Fernandez-Lahore M (2015) Improved biomass and protein production in solid-state cultures of an *Aspergillus sojae* strain harboring the *Vitreoscilla* hemoglobin. Appl Microbiol Biotechnol 99:9699–9708. doi:10.1007/s00253-015-6851-3
18. Miller GL (1959) Use of dinitrosalicyclic reagent for determination of reducing sugar. Anal Chem 31:426–438
19. Ortiz GE, Guitart ME, Albertó E et al (2014) Microplate assay for endo-polygalacturonase activity determination based on ruthenium red method. Anal Biochem 454C:33–35. doi:10.1016/j.ab.2014.02.028
20. Lynch JM, Barbano DM (1999) Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. J AOAC Int 82:1389–1398
21. Aldermann GA, Morgan DE, Harvard A, Edwards RE, Todd JR (1975) Energy allowances and feeding systems for ruminants. Minist Agric Fish Food Tech Bull 33:34–36
22. Uceda M, Frías L (1975) Epocas de recolección. Evolución del contenido graso del fruto y de la composición y calidad del aceite (Seasons of harvest. Changes on fruit oil content, oil composition and oil quality). Proceeding II Semin. Oleícola Int

23. International Olive Council (2015) Spectrophotometric investigation in the ultraviolet. COI/T.20/D:1–10
24. Mignani AG, Ciaccheri L, Mencaglia AA, Cimato A (2012) Optical absorption spectroscopy for quality assessment of extra virgin olive oil. Olive Oil-Const Qual Heal Prop Bioconvers. doi:10.5772/1378
25. Nighojkar S, Phanse Y, Sinha D et al (2006) Production of polygalacturonase by immobilized cells of *Aspergillus* niger using orange peel as inducer. Process Biochem 41:1136–1140. doi:10.1016/j.procbio.2005.12.009
26. Ustok FI, Tari C, Gogus N (2007) Solid-state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. J Biotechnol 127:322–334. doi:10.1016/j.jbiotec.2006.07.010
27. Contreras Esquivel JC, Voget CE (2004) Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. J Biotechnol 110:21–28. doi:10.1016/j.jbiotec.2004.01.010
28. Pedrolli DB, Carmona EC (2009) Pectin lyase from *Aspergillus giganteus*: comparative study of productivity of submerged fermentation on citrus pectin and orange waste. Appl Biochem Microbiol 45:610–616. doi:10.1134/S0003683809060064
29. Baladhandayutham S, Thangavelu V (2011) Optimization and kinetics of solid-state fermentative production of pectinase by *Aspergillus awamori*. Int J ChemTech Res 3:1758–1764
30. Mrudula S, Anitharaj R (2011) Pectinase production in solid state fermentation by *Aspergillus niger* using orange peel as substrate. Glob J Biotechnol Biochem 6:64–71
31. Alcântara SR, da Silva FL, Leite NJ (2013) Scale up of polygalacturonase production by solid state fermentation process. doi:10.5772/53152. http://www.intechopen.com/books/food-industry/scale-up-of-polygalacturonase-production-by-solid-state-fermentation-process
32. Galiotou-Panayotou M, Kapantai M, Kalantzi O (1997) Growth conditions of *Aspergillus* sp. ATHUM-3482 for polygalacturonase production. Appl Microbiol Biotechnol 47:425–429. doi:10.1007/s002530050951
33. Hours RA, Voget CE, Ertola RJ (1988) Some factors affecting pectinase production from apple pomace in solid-state cultures. Biol Wastes 24:147–157. doi:10.1016/0269-7483(88)90057-2
34. Larroche C, Moksia J, Gros J et al (1998) A convenient method for initial dry weight determination in samples from solid state cultivations. Process Biochem 33:447–451
35. Zheng Z, Shetty K (2000) Solid state production of polygalacturonase by *Lentinus edodes* using fruit processing wastes. Process Biochem 35:825–830. doi:10.1016/S0032-9592(99)00143-0
36. Silva D, Martins ES, Leite RSR et al (2007) Purification and characterization of an exo-polygalacturonase produced by *Penicillium viridicatum* RFC3 in solid-state fermentation. Process Biochem 42:1237–1243. doi:10.1016/j.procbio.2007.05.025
37. Martínez-Trujillo A, Arreguín-Rangel L, García-Rivero M, Aguilar-Osorio G (2011) Use of fruit residues for pectinase production by *Aspergillus flavipes* FP-500 and *Aspergillus terreus* FP-370. Lett Appl Microbiol 53:202–209. doi:10.1111/j.1472-765X.2011.03096.x
38. Rodríguez-Fernández DE, Rodríguez-León JA, de Carvalho JC et al (2011) The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation. Bioresour Technol 102:10657–10662. doi:10.1016/j.biortech.2011.08.106
39. Cabeza MS, Baca FL, Puntes EM et al (2011) Selection of psychrotolerant microorganisms producing cold-active pectinases for biotechnological processes at low temperature. Food Technol Biotechnol 49:187–195
40. Pedrolli DB, Gomes E, Monti R, Carmona EC (2008) Studies on productivity and characterization of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. Appl Biochem Biotechnol 144:191–200
41. Schols HA, Geraeds CCJM, Searle-van Leeuwen MF et al (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105–115. doi:10.1016/0008-6215(90)84010-R
42. Aliakbarian B, De Faveri D, Converti A, Perego P (2008) Optimisation of olive oil extraction by means of enzyme processing aids using response surface methodology. Biochem Eng J 42:34–40. doi:10.1016/j.bej.2008.05.006
43. Najafian L, Ghodsvali A, Haddad Khodaparast MH, Diosady LL (2009) Aqueous extraction of virgin olive oil using industrial enzymes. Food Res Int 42:171–175. doi:10.1016/j.foodres.2008.10.002
44. Clodoveo ML, Dipalmo T, Schiano C et al (2014) What's now, what's new and what's next in virgin olive oil elaboration systems? A perspective on current knowledge and future trends. J Agric Eng 45:49–59. doi:10.4081/jae.2014.193
45. Mateos RG-MJA (2006) Rapid and quantitative extraction method for the determination of chlorophylls and carotenoids in olive oil by high-performance liquid chromatography. Anal Bioanal Chem 385:1247–1254
46. Vierhuis E, Korver M, Schols HA, Voragen AG (2003) Structural characteristics of pectic polysaccharides from olive fruit (*Olea europaea* cv moraiolo) in relation to processing for oil extraction. Carbohydr Polym 51:135–148. doi:10.1016/S0144-8617(02)00158-3