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Screening of Pectinase-Producing Microorganisms with Polygalacturonase Activity

Jamile Zeni · Karine Cence · Camila Elis Grando · Lídia Tiggermann · Rosicler Colet · Lindomar A. Lerin · Rogério L. Cansian · Geciane Toniazzo · Débora de Oliveira · Eunice Valduga

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Abstract The aim of this work was to perform the screening of microorganisms, previously isolated from samples of agro-industrial waste and belonging to the culture collection of our laboratory, able to produce polygalacturonases (PG). A total of 107 microorganisms, 92 newly isolated and 15 pre-identified, were selected as potential producers of enzymes with PG activity. From these microorganisms, 20 strains were able to synthesize PG with activities above 3 UmL^{-1}. After the kinetic study, the enzyme activity was increased up to 13 times and the microorganism identified as *Aspergillus niger* ATCC 9642 and the newly isolated W23, W43, and D2 (*Penicillium* sp.) after 24 h of fermentation led to PG activities of 30, 41, 43, and 45 UmL^{-1}, respectively. The RAPD analysis demonstrated that the selected strains differs genetically, indicating that no duplication of strains among them in the experiments for polygalacturonases production was verified.

Keywords Screening · Microorganisms · Polygalacturonase · Kinetic study

Introduction

The commercial application of pectinases was firstly observed in 1930 for wines and fruit juices preparation. Only in the 1960s, when the chemical nature of plant tissues becomes apparent, the scientists began to use a greater range of enzymes more efficiently. As a result, pectinases are today one of the upcoming enzymes of the commercial sector. Primarily, these enzymes are responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant. Pectinases are now an integral part of fruit juice and textile industries as well as having several biotechnological applications [1].

J. Zeni · K. Cence · C. E. Grando · L. Tiggermann · R. Colet · L. A. Lerin · R. L. Cansian · G. Toniazzo · D. de Oliveira · E. Valduga (✉)
Department of Food Engineering, URI–Campus de Erechim,
Av. Sete de Setembro, 1621, Erechim, Rio Grande do Sul 99700-000, Brazil
e-mail: veunice@uricer.edu.br

Pectinases include depolymerizing and demethoxylating enzymes. Depolymerizing enzymes are called polygalacturonases (E.C. 3.2.1), which cleaves the α-1,4 glycosidic bonds between two galacturonic acid residues, and pectin-liase (E.C. 4.2.2), which catalyzes the β-elimination reaction between two methylated residues [3]. De-esterifying enzymes include pectinesterase (E.C. 3.1.1), which catalyzes the demethoxylation of methylated pectin, producing methanol and pectin [2].

Many plant-pathogenic bacteria and fungi are known to produce pectinolytic enzymes useful for invading host tissues. Moreover, these enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere [3]. Several species of microorganisms such as *Bacillus*, *Erwinia*, *Kluyveromyces*, *Aspergillus*, *Rhizopus*, *Trichoderma*, *Pseudomonas*, *Penicillium*, and *Fusarium* are known as good producers of pectinases.

Over the years, pectinases have been used in several conventional industrial processes, such as fruit juice extraction, textile processing and bioscouring of cotton fibers, retting and degumming of plant bast fiber, waste water treatment, coffee and tea fermentation, animal feed, purification of plant viruses, oil extraction, beverages, and wines [1, 4–6]. These several industrial applications justify the search for new microorganisms able to overproducing the desired enzyme.

Material and Methods

Isolation and Selection of Microorganisms

Samples were collected from soil, leaves (eucalyptus and orange), fruits (orange, papaya, persimmon, peach, apple, melon, guava, banana, grape, Kiwi, and black plum), teas, processed products, and agro-industrial wastes.

The samples were inoculated in Petri dishes containing plate count agar (PCA) and incubated at 25°C for approximately 72 h. The colonies were transferred to inclined test tubes with PCA and incubated at 25°C for 48 h. The selected cultures were stored after growth at 4°C. The purity of the strains was verified by microscopic examination.

A total of 107 microorganisms, 92 filamentous fungi isolated in this work and 15 strains (*Penicillium camembertii* ATCC 4845, *Aspergillus niger* ATCC 16404, *A. niger* UFRJ collection, *A. niger* ATCC 1004, *A. niger* ATCC 9642, *Penicillium notatum* ATCC 9478, *Penicillium duclauxii* ATCC 9121, *Penicillium citrinum* ATCC 28752, *Penicillium digitatum* ATCC 26821, *Aspergillus oryzae* ATCC 1003, *Paecilomyces variotii* ATCC 22319, *Pseudomonas putida*, *Sporiodiobolus salmonicolor* CBS 2636, *Candida* sp. ATCC 34147 e *Hansenula* sp.) belonging to the culture collection of Laboratory of Biotechnology (URI–Campus de Erechim) were tested in this step.

Enzyme Activity Assays

The liquid medium for microbial growth and determination of polygalacturonase (PG) activity was proposed by Kashyap et al. [7], consisting of 10 gL^{-1} yeast extract, 10 gL^{-1} citrus pectin (Sigma), pH 5.5, autoclaved at 121°C/1 atm for 15 min.

The selected microorganisms were inoculated to 50 mL of medium and incubated on rotary shaker at 30°C, 100 rpm for up to 96 h. Every 24 h of fermentation cultures were centrifuged at 3,000×*g* for 10 min at 0°C to separate the supernatant free of cells and

subsequent determination of medium pH and polygalacturonase activity. The supernatant was stored in freezer at −18°C in glass vials with caps of 50 mL.

Polygalacturonase Activity

Initially, 1,000 μL of substrate (5 gL^{-1} solution of citrus pectin, Sigma) in acetate buffer (pH 4.5) was incubated and incubated at 40°C for 15 min. Then, 500 μL of enzyme extract was added and the mixture was incubated at 40°C for 40 min. The PG activity was determined by measuring the release of reducing groups using the method of dinitrosalicylic acid , initially proposed by Miller [8]. The absorbance was measured in spectrophotometer (Beckman Coutler, model DU640, 540 ηm). One PG unit was defined as the amount of enzyme that liberates 1 μmol of D-galacturonic acid per minute of reaction ($U=\mu mol min^{-1}$). The polygalacturonase activity was expressed in units of activity per milliliter (UmL^{-1}).

Kinetic Study of the Polygalacturonase Activity

The pectinolytic activities of the potential screened microorganisms were measured at 24, 48, 72, and 96 h of fermentation. The activity measurement was also carried out at different reaction times (5, 10, 15, 20, 25, 30, and 40 min).

Characterization and Identification of the Microorganisms

Microcultivation Technique

The microorganisms screened as promising for polygalacturonase production (activities higher than 3 UmL^{-1}) were identified by microcultivation technique. The fungi were inoculated on a slice of agar laid on a sterile glass slide and covered by a sterile coverslip. The slide was then placed in a Petri dish, and the setup was then incubated for 5 days at 25°C. The coverslip with the adhered hyphae was withdrawn and stained with Cotton Blue dye. The same procedure was adopted for examining spores and hyphae bound to the slide.

The identification of the fungi genus was based on the macroscopic morphology of the colonies and on the study of fructification structures of the strains, following the key of investigation of genera proposed by Barnett et al. [9].

Genetic Characteristics of the Microorganisms by RAPD Markers

The RAPD technique was used to correlate the results obtained in the fermentative screening with the genetic characteristics of the isolated microorganisms. Firstly, the extraction and quantification of the DNA was carried out, followed by amplification, electrophoresis in agarose gel, and the analysis of the data obtained.

The DNA was extracted from grown cells in liquid medium (potato dextrose—PD) for 72 h, according to the methodology proposed by Roeder and Broda [10]. Details about the experimental procedure used for genomic DNA extraction can be found in the work of Toniazzo et al. [11].

For DNA concentration, 980 μL of sterile ultrapure water (MilliQ) were added in a test tube with 20 μL of sample. DNA concentration was estimated by measuring the optical

density at 260 nm, using spectrophotometer (Agilent 8453), and checked by electrophoresis on a 0.8% agarose gel (Gibco BRL, Carlsbad, CA, USA) in 1×TBE (0.89 M Tris, 0.89 M of H_3BO_3, and 0.08 M EDTA).

Amplification followed the procedure reported by Williams et al. [12], with some modifications. The reactions were carried out in 25 μL. The reaction mixture contained buffer (50 mM Tris–HCl pH 9.0, 50 mM KCl), dNTPs mix (200 mM of each nucleotide and 0.2 mM of primer, 3 mM $MgCl_2$ (Life Technologies, São Paulo, Brazil)), 0.25 mM, 1.5 U of Taq DNA polymerase recombinant (Invitrogen Life Technologies, São Paulo, Brazil, Triton-X-100 (Merck, Germany)), and 40 ng of DNA of each microorganism previously isolated.

The kits OPA, OPF, OPH, OPW, and OPY from the Operon Technologies (Nattermannallee, Cologne, Germany), with 20 primers of each one, were used to identify the primers that yielded the best results, estimating the number, intensity, size of each band, as well as reproducibility and the polymorphism.

The amplification was carried out in a Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA, model PTC100TM) as follows: one initial cycle of 3 min at 92°C, followed by 40 cycles comprising denaturation (1 min at 92°C), annealing (1 min at 35°C), and extension (2 min at 72°C) and then a final extension for 3 min at 72°C.

Amplification products were separated by electrophoresis in 1.4% agarose gels in buffer 1×TBE using a horizontal electrophoresis apparatus. The run was carried out at constant voltage of 90 V. DNA Lambda digested with *Eco*RI and *Hind* III of the Gibco BRL was included as molecular size marker. Gels were visualized by staining with ethidium bromide, and band patterns were photographed under UV light using a CCD camera (GEL-PRO, Media Cybernetics, Silver Spring, MD, USA).

The results obtained by determination of the presence or absence of band yielding a matrix that was analyzed with help of the software NTSYS version 1.7 (Numerical Taxonomy System of Multivariate Analysis System). The dendrogram was built by the algorithm UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) using the Jaccard coefficient of similarity.

Statistical Analysis

The results were treated by analysis of variance followed by Tukey's post hoc test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). All analyses were performed considering a level of 95% of confidence ($p<0.05$).

Results and Discussion

Isolation and Selection of Microorganisms

The selected microorganisms were isolated from samples of flour wheat (B), orange (E), mango (M), papaya (I), peach (H), melon (J), guava (L), banana (G), grape (O), soil of grape (R), kiwi (S), teas (D), samples of leaves of eucalyptus and orange (W), and processed product and agro-industrial waste (K). Most microorganisms were isolated from samples of eucalyptus, orange and orange sheets (48), guava (eight), peach (four), flour (three), and mango (three), respectively. In the screening for polygalacturonase-producing microorganisms, a total of 92 strains were isolated and screened.

Polygalacturonase Activity

Table 1 shows the results of the PG activity for the 20 strains selected as promising polygalacturonase producer. For better visualization, Fig. 1 presents the bioproduction profile (activities higher than 3 UmL^{-1}) for the five most promising microorganisms.

The strains *A. niger* ATCC 1004, *A. niger* ATCC 16404, D2 and W43 after 24 h of fermentation, and L1, H8, L4, W12, and T2 after 48 h of fermentation did not differ statistically ($p<0.05$) among them, with activities ranging from 3.5 to 4.2 UmL^{-1}, respectively. However, within 72 h of bioproduction, a decrease of activity of PG was observed for most microorganisms, except for strains W52 and W18 which showed a slight increase in their activities, with values close to 3 UmL^{-1}.

Possibly, the reduction in the polygalacturonase activity is related to the lack of substrate on the culture medium and/or variation of conditions of fermentation, such as pH. In this particular case, the highest enzyme production was correlated with lower pH values. In general, there is a slight decline on pH values in the first 24 h of fermentation (Fig. 2a, b). According to Uenojo and Pastore [13] and Cordeiro and Martins [14], the reason for this change in pH can be explained by the release of galacturonic acid in the medium by the action of pectinolytic enzymes produced by microorganisms during the first hours of bioproduction.

Table 1 Polygalacturonases activity (UmL^{-1}) during 96 h of bioproduction for the 20 potential microorganisms for polygalacturonase production.

Microorganism	Polygalacturonase activity (UmL^{-1})[a]			
	24 h	48 h	72 h	96 h
A. niger ATCC 16404	3.65 ± 0.09^{abA}	3.20 ± 0.07^{bAB}	2.99 ± 0.07^{aB}	2.91 ± 0.10^{aB}
A. niger ATCC 1004	4.16 ± 0.11^{aA}	2.43 ± 0.04^{dB}	1.70 ± 0.03^{dC}	1.69 ± 0.01^{cdC}
A. niger ATCC 9642	3.27 ± 0.23^{bA}	2.22 ± 0.04^{dB}	1.43 ± 0.02^{eC}	1.08 ± 0.08^{dCD}
Aspergillus sp. (L1)	1.93 ± 0.04^{cdC}	4.21 ± 0.04^{aA}	2.51 ± 0.01^{bcB}	2.34 ± 0.01^{bB}
Aspergillus sp. (L6)	3.00 ± 0.01^{bA}	1.63 ± 0.01^{eB}	1.73 ± 0.01^{dB}	0.64 ± 0.06^{eC}
Aspergillus sp. (H8)	2.25 ± 0.02^{cB}	4.11 ± 0.03^{aA}	1.45 ± 0.03^{eC}	1.36 ± 0.04^{dC}
Cephalosporium sp. (W18)	N.A.[b]	3.04 ± 0.01^{cA}	3.11 ± 0.01^{aA}	1.13 ± 0.01^{dB}
Penicillium SP. (W23)	0.68 ± 0.01^{eB}	3.12 ± 0.01^{bcA}	3.05 ± 0.01^{aA}	3.00 ± 0.01^{aA}
Penicillium sp.(W24)	3.15 ± 0.01^{bA}	3.13 ± 0.01^{bcA}	2.85 ± 0.01^{abAB}	1.17 ± 0.01^{dC}
Penicillium sp. (W12)	2.81 ± 0.01^{bcB}	3.85 ± 0.01^{aA}	1.97 ± 0.03^{dC}	2.00 ± 0.01^{cC}
Penicillium sp. (D2)	4.16 ± 0.08^{aA}	2.83 ± 0.04^{cB}	0.55 ± 0.02^{fC}	0.96 ± 0.01^{deC}
Penicillium sp. (W28)	3.00 ± 0.01^{bA}	2.19 ± 0.01^{dC}	2.47 ± 0.01^{bcB}	2.14 ± 0.01^{bcC}
Penicillium sp. (L4)	2.59 ± 0.01^{cB}	3.89 ± 0.02^{aA}	1.59 ± 0.01^{deC}	2.98 ± 0.01^{aB}
Penicillium sp.(T2)	2.75 ± 0.03^{bcB}	3.55 ± 0.04^{abA}	2.37 ± 0.02^{bcB}	0.25 ± 0.05^{fC}
Penicillium sp. (B9)	0.38 ± 0.01^{fC}	3.16 ± 0.01^{bA}	2.37 ± 0.01^{bcB}	0.13 ± 0.01^{fC}
Penicillium sp. (W35)	1.64 ± 0.01^{dC}	3.09 ± 0.01^{cA}	2.72 ± 0.01^{bAB}	1.73 ± 0.01^{cdC}
Penicillium sp. (W43)	3.55 ± 0.01^{abA}	2.76 ± 0.01^{cB}	1.97 ± 0.01^{dC}	1.93 ± 0.01^{cC}
Penicillium sp. (W52)	1.43 ± 0.01^{dC}	2.24 ± 0.01^{dB}	3.03 ± 0.01^{aA}	1.35 ± 0.01^{dC}
P. putida	0.22 ± 0.01^{fD}	3.17 ± 0.01^{bA}	2.29 ± 0.01^{cB}	1.69 ± 0.01^{cdC}
Rhizopus sp. (H9)	0.09 ± 0.01^{gC}	3.03 ± 0.01^{cA}	2.49 ± 0.02^{bcB}	NA

NA no detected PG activity

[a] Mean±standard deviation followed by equal letters, upper/lower cases, on columns does not differ statistically at a confidence level of 5% (Tukey's test)

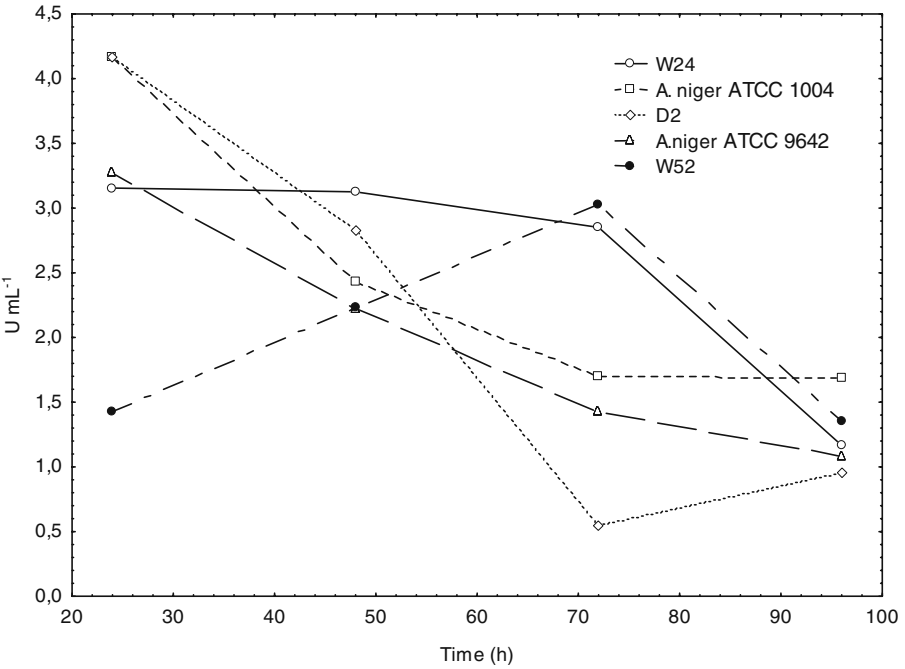

Fig. 1 Polygalacturonase activity (UmL^{-1}) during 96 h of for five potential microorganisms

After 48 h of fermentation, an increase in pH values and a reduction in enzyme activity were observed (Fig. 2a, b). Cordeiro and Martins [14] present that the enhancement on the pH can be due to the use of organic acids or production of alkaline compounds during the fermentation time. According to Ming-Chu et al. [15], the culture medium acidification or alkalinization reflects the substrate consumption. Due to this relation between the synthesis of polygalacturonases and the consumption of nitrogen compounds, the pH variation can be used to obtain important information about the enzyme production, as the beginning and ending of its synthesis.

Comparing the results presented in Table 1 with that reported in the literature, we can observe that similar pectinolytic activities were obtained. Galiotou-Panayotou and Kapantai [16] achieved 3.0 UmL^{-1} of PG by submerged fermentation of *A. niger* in medium composed by pectin of citrus. Soares et al. [17] selected six strains of *Bacillus* sp. as good PG producers. The activities varied from 0.3 to 4.0 UmL^{-1}. Martins et al. [18], using *Thermoascus aurantiacus*, reached a maximum activity of 5.0 UmL^{-1}. Kashyap et al. [6] obtained 15.7 UmL^{-1} after 30 h of fermentation by strains of *Bacillus* sp., *Aspergillus awamori* produced 0.05 UmL^{-1} of PG activity after 7 days of fermentation [19], and the use of *Lentinus edodes* led to an activity from 1.5 to 2.2 UmL^{-1} after 40 days [20].

Based on the results obtained in the screening step, a kinetic study for measuring activity of PG was carried out for the 18 most promising microorganisms (which presented PG activities above 3.0 UmL^{-1} in 24, 48, 72, and 96 h of bioproduction). The highest values for measurement of PG activity were found after 5 min of reaction, especially for the microorganisms *A. niger* ATCC 9642, W23, W43, and D2 (Fig. 3) after 24 h of fermentation with activities of 30, 41, 43, and 45 UmL^{-1}, respectively.

Note these values, obtained from a simple kinetic evaluation of the enzyme activity, after 5 min of reaction, are 5 to 13 times higher compared to the PG activity obtained in the

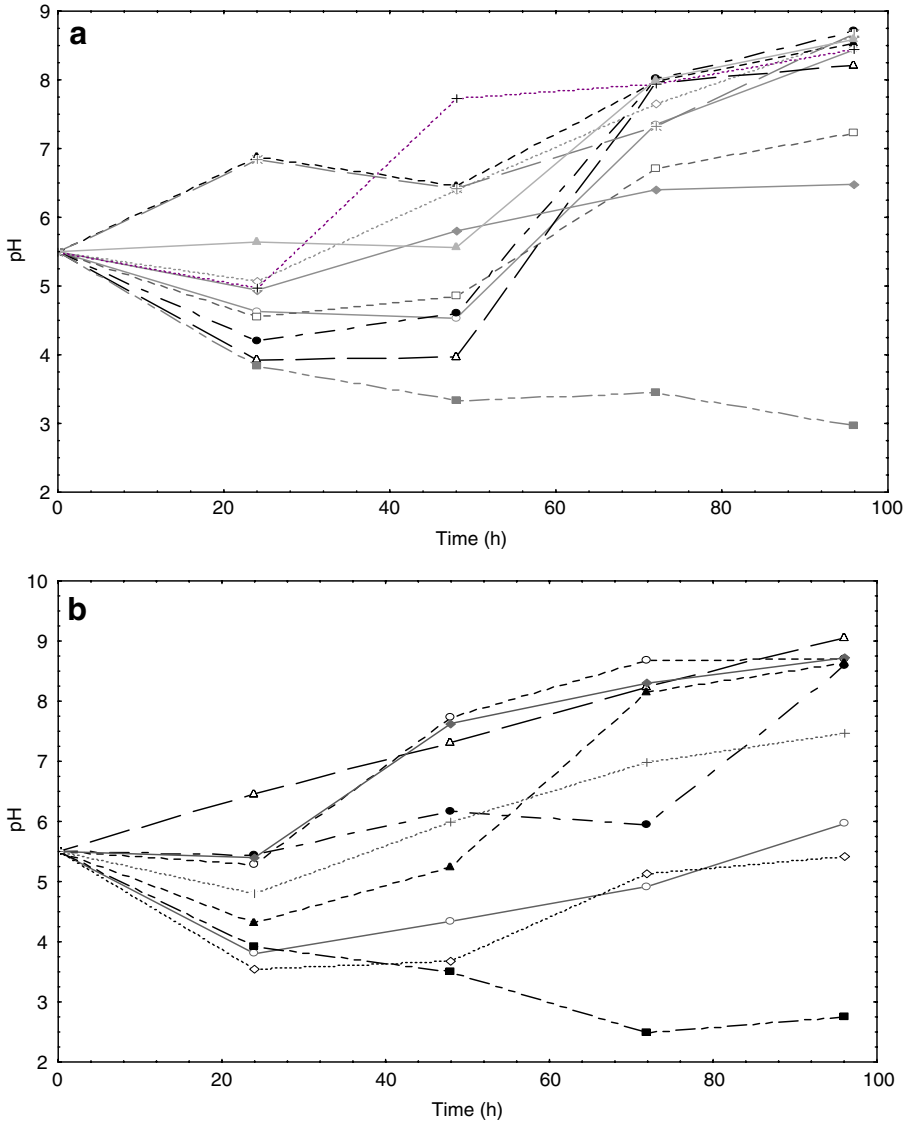

Fig. 2 pH values during 96 h of bioproduction for the 20 potential microorganisms for polygalacturonase production: (**a**) *Penicillium*: *Penicillium* sp. (W23), *Penicillium* sp. (W24), *Penicillium* sp. (W12), *Penicillium* sp. (D2), *Penicillium* sp. (L4), *Penicillium* sp. (T2), *Penicillium* sp. (B9), *Penicillium* sp. (W35), *Penicillium* sp. (W43), *Penicillium* sp. (W52), and *Penicillium* sp. (W28). (**b**) *A. niger* ATCC 9642, *A. niger* ATCC 16404, *A. niger* ATCC 1004, *P. putida*, *Aspergillus* sp. (L1), *Aspergillus* sp. (L6), *Rhizopus* sp. (H9), *Cephalosporium* sp. (W18), and *Aspergillus* sp. (H8)

screening step (when 40 min was used for activity measurement). At this point, it is worth to emphasize that the pectinolytic activity can be maximized if other variables are optimized, such as culture medium (synthetic substrate and agro-industrial waste) and type of fermentation process (submerged and/or solid state) as well as operating conditions (temperature, initial pH, agitation, and moisture).

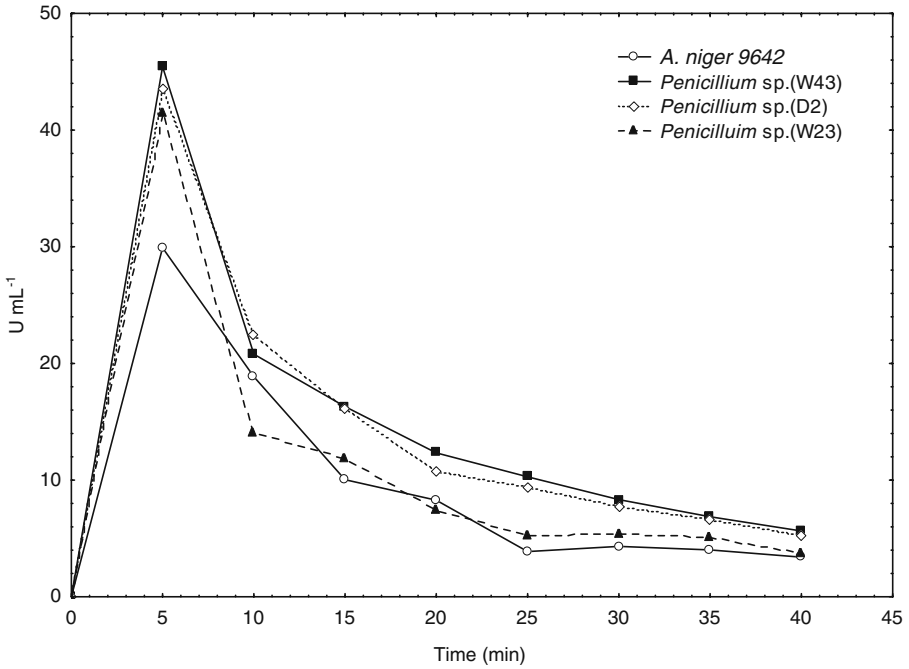

Fig. 3 Measurement of polygalacturonase activity after 24 h of fermentation at different reaction times

Rare are the information that relate kinetic study for measuring polygalacturonase activity. Soares et al. [17] performed a study of measurement of PG activity after incubation at 40°C for 10 min of reaction, resulting in activities from 0.3 to 0.8 $U mL^{-1}$ for *Bacillus* sp. in submerged fermentation.

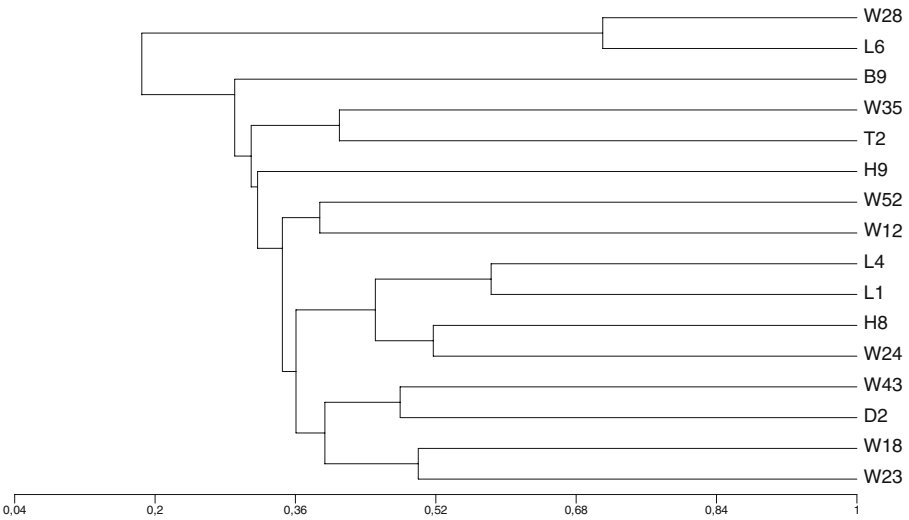

Fig. 4 Dendrogram based on analysis of grouping (UPGMA) for estimation of genetic similarity (Jaccard coefficient) by RAPD of pectinolytic (PG) fungi

Pre-identification of the Microorganisms Polygalacturonase Producers and RAPD Analysis

The identification of the microorganisms PG producers was carried out by the evaluation of morphological and reproduction characteristics and the genetic differentiation by molecular markers. The analysis of the results indicated that the isolated fungi presented higher potential for pectinases production than bacteria. The best pectinase-producing strains were identified by microcultivation technique, based on the shape of fructification body. By this procedure and by comparison of macro and microscopic aspects, of a total of 16 identified microorganisms, 68.75% of the fungi were identified as belonging to the *Penicillium* genera, coded as B9, D2, L4, T2, W12, W23, W24, W28, W35, W43, and W52 (presence of paintbrush-like conidia). From all the isolated fungi, 18.75% (H8, L1, and L6) were classified as *Aspergillus* genera, based on the presence of hyaline and dark septated mycelium [21]. A strain belonging to *Rhizopus* genera (H9) and other to *Cephalosporium* genera (W18) were also pre-identified.

Soares et al. [17] isolated 168 strains of bacteria, six of them identified as *Bacillus* sp. Kashyap et al. [7] isolated strains of *Bacillus* sp. from soils and fruit residues. Martin et al. [22] screened 16 strains, obtaining good results using *Moniliella* sp. SB9, *Penicillium* sp. EGC5, *Thermoascus* sp-179.5, and *Phanerochaetes* sp-291, besides others.

Species of *Aspergillus* are considered initiators of deterioration of seeds and grains, since they could grow at low water content. In a second step, the contamination by *Penicillium* follows, at higher water content, as a result of metabolic activity of the first microorganisms [23]. *Penicillium* and *Aspergillus* are common contaminants of tropical and subtropical regions, predominating among other fungi [24].

For the genetic evaluation, ten primers (OPY 18, OPA 17, OPH 03, OPA 12, OPW 18, OPW 20, OPY 15, OPY 09, OPY 01, and OPW 16) were used in RAPD analysis and the relation between the total number of fragments and polymorphic fragments generated by RAPD markers. A total of 102 and 95 fragments polymorphic for fungi were observed. With the average number of fragments per primer of 9.5 we can observe a high polymorphism (93.1%). Similarity indices (Jaccard coefficient) among the pectinolytic fungi and bacteria evaluated in this study ranged from 0.18 to 0.70. This result may be better observed by the dendrogram presented in Fig. 4.

Although no groups could be identified, these results are important, since they prove that all the screened microorganisms are different, avoiding the possibility of selection of the same strains. These results indicate a high variability among the strains, related to distinct species or genera of fungi and bacteria.

Conclusions

In the screening step, 92 newly isolated microorganisms and 15 pre-identified strains were potentially selected as polygalacturonase producers. From these, 16 strains of isolated filamentous fungi pre-identified and four from microorganisms belonging to culture collection were able to synthesize PG with activities above 3 $U mL^{-1}$. After the kinetic study, the enzyme activity was increased up to 13 times and the microorganism identified as *A. niger* ATCC 9642 and the newly isolated W23, W43, and D2 (*Penicillium* sp.), after 24 h of fermentation, conducted to PG activities of 30, 41, 43, and 45 $U mL^{-1}$, respectively. The analysis of RAPD demonstrated that the strains differ genetically among them.

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