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Studies on Productivity and Characterization of Polygalacturonase from *Aspergillus giganteus* Submerged Culture Using Citrus Pectin and Orange Waste

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Abstract Polygalacturonases are part of the group of enzymes involved in pectin degradation. The aim of this work was to investigate some of the factors affecting polygalacturonase production by an *Aspergillus giganteus* strain and to characterize this pectinolytic activity. Several carbon sources, both pure substances and natural substrates, were tested in standing cultures, and the best results were obtained with orange bagasse and purified citrus pectin. On citrus pectin as sole carbon source, the highest extracellular activity (9.5 U/ml and 40.6 U/mg protein) was obtained in 4.5-day-old cultures shaken at 120 rpm, pH 3.5 and 30°C, while on orange bagasse, the highest extracellular activity (48.5 U/ml and 78.3 U/mg protein) was obtained in 3.5-day-old cultures shaken at 120 rpm, pH 6.0 and 30°C. Optimal polygalacturonase activity was observed in assays conducted at pH 5.5–6.5 and 55–60°C. The activity showed good thermal stability, with half-lives of 90 and 30 min when incubated at 55 and 60°C, respectively. High stability was observed from pH 4.5 to 8.5; more than 90% of the activity remained after 24 h in this pH range.

Keywords Polygalacturonase · Pectinolytic enzyme · Orange waste · Citrus pectin · Enzyme characterization · *Aspergillus giganteus*

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Introduction

Many microorganisms are able to use pectic substances as carbon and energy sources, by releasing a variety of enzymes into widely differing environments [1, 2]. Microbial pectinases are important in plant diseases, in plant–microbe symbiosis, and in the decomposition of dead plant material, contributing to the natural carbon cycle [3, 4]. These enzymes are produced abundantly by saprophytic fungi, and decaying plant tissue represents the commonest substrate for pectinase-producing microorganisms [5].

Pectins are high molecular weight polysaccharides found in higher plants. They form the primary cell wall and are the main components of the middle lamella, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues [3, 6].

Filamentous fungi are widely used in industry for their capacity to produce efficiently a diverse range of enzymes [7]. In particular, commercial pectinase preparations employed in food processing are generally derived from species of *Aspergillus* and consist, traditionally, of mixtures of polygalacturonase, pectin lyase, and pectinesterase [5].

Polygalacturonases (PGs), the most researched of the family of pectinolytic enzymes, catalyze the hydrolytic cleavage of α-(1→4)-glycosidic bonds in polygalacturonic acid (pectic acid) [8, 9]. They may be either endopolygalacturonases [poly (1,4-α-D-galacturonide) glycanohydrolases], EC 3.2.1.15, which act randomly on internal 1,4-α-D-galactosiduronic linkages, or exopolygalacturonases [poly (1,4-α-D-galacturonide) galacturonohydrolases], EC 3.2.1.67, which remove D-galacturonide units in sequence from the nonreducing end of the pectin molecule [6, 10].

Acid pectinases, which are widely used in extraction, clarification, and removal of pectin in fruit juices, in maceration of vegetables to produce pastes and purées, and in wine-making, are often produced by fungi, especially *Aspergillus niger* [6, 9]. In the case of fruit juice, extraction by enzymatic maceration can lead to an increase in yield of more than 90% compared to conventional mechanical juicing, besides improving the organoleptic (color, flavor) and nutritional (vitamins) properties and technological efficiency (ease of filtering) [3].

The production of pectinases by microorganisms have been studied with several natural and purified carbon sources, and a great attempt have been done to the utilization of agroindustrial wastes like wheat bran, lemon peel, sugarbeet, apple, and orange bagasses [11].

A detailed knowledge of the physicochemical characteristics of these enzymes, with respect to their production and catalytic activity, is indispensable for their use in industrial processes [10]. Hence, the aim of this study was to investigate the influence of chemical and physical factors on the production of polygalacturonase by *Aspergillus giganteus* and to characterize this activity obtained under optimized conditions.

Material and Methods

Culture and Maintenance of Fungal Strain

The strain of *A. giganteus* CCT 3232 is in the culture collection of the "Fundação Tropical de Pesquisa e Tecnologia André Tosello" (Brazil), and it was previously isolated from soil of the Brazilian Atlantic Forest, at Peruíbe, São Paulo State. The fungus was maintained on Vogel medium [12] agar slopes. After inoculation, these cultures were incubated at 28°C

for 7 days and sterile distilled water was then poured on the mycelium, to prepare a suspension of 1.0×10^7 conidia/ml. In sterile Erlenmeyer flasks, 25 ml of liquid Vogel medium (containing ammonium sulphate as nitrogen source) and carbon source was inoculated with 1.0 ml of this conidial suspension. At intervals of 12 or 24 h, the mycelium was separated by vacuum filtration and the filtered medium used as extracellular polygalacturonase.

Extraction of Intracellular Proteins

The damp mycelium was cooled to 4°C and ground with three times its weight of washed sand. For each gram of mycelium, 15 ml 0.05 M sodium acetate buffer (pH 5.5) was then added, and the mixture was stirred and finally centrifuged at 4°C as previously described [13]. The supernatant (crude extract) was used to determine the quantity of mycelial protein.

Polygalacturonase Activity

Enzyme activity was assayed according to Carmona et al. [13] by incubation of the crude extract with polygalacturonic acid in buffer, at a suitable temperature (as indicated). The reaction was stopped by adding dinitrosalicylic reagent (DNS) at 10 and 20 min, and the unhydrolyzed substrate was removed by centrifugation. Reducing sugars were then measured in the supernatant by Miller's method [14], using galacturonic acid as standard. One unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing groups per minute under the experimental conditions. Specific activity was based on the concentration of extracellular protein in the filtrate.

Protein Determination

Protein concentrations were measured by the method of Lowry et al. [15], with bovine serum albumin (BSA) as standard.

Characterization of Enzyme Activity

The culture filtrate produced under optimized conditions for PG activity was used in assays designed to assess the effects of pH and temperature on that activity. To find the optimum pH for PG activity, the filtrate was incubated with the substrate at 50°C, at various pH values between 4 and 9, using 0.05 M sodium acetate buffer from 4.0 to 5.5, 0.05 M sodium imidazole buffer for 6.0 and 6.5, and 0.05 M Tris–HCl buffer from 7.0 to 9.0. Further buffers were also tested: 0.05 M sodium phosphate (pH 6.5–7.5), 0.05 M sodium maleate (5.5–7.0) and 0.05 M McIlvaine's buffer (5.0–7.0). The culture filtrate was then incubated with the substrate at pH 5.5, at various temperatures between 25 and 85°C, to find the optimal temperature for PG activity.

Thermal stability of PG activity was assayed as residual activity after incubating the culture filtrate with sodium imidazole buffer pH 6.0, at 55, 60, and 65°C, without substrate. The pH stability was determined as the residual PG activity after 24 h incubation of crude filtrate, at ambient temperature, without substrate, in the buffers 0.05 M glycine–HCl (pH 3.0–3.5), 0.05 M sodium acetate (4.0–5.5), 0.05 M sodium imidazole (6.0–6.5), 0.05 M Tris–HCI (7.0–9.0), and 0.05 M glycine–NaOH (9.5–10.0).

Results and Discussion

Enzyme Production and Fungal Growth

The time-course of polygalacturonase production by *A. giganteus* was followed by assaying the activity daily, during growth of the fungus in liquid Vogel medium containing 1% citrus pectin (*w*/*v*) as carbon source, at 28°C and pH 6.5, in standing and shaking (120 rpm) cultures. Under standing growth conditions (Fig. 1a), peak production of PG occurred at 6 days, when activity reached 3.09 U/ml and 7.52 U/mg of protein. With shaking (Fig. 1b), peak production was seen after 4.5 days of growth, the maximum activity being 4.03 U/ml and 12.97 U/mg of protein. The production of PG by *Sporotrichum thermophile* Apinis [2] also showed better results in shaking culture (200 rpm).

Macroscopic morphology of the *A. giganteus* mycelium in standing and shaking cultures showed that in the former, the hyphae formed a homogeneous mass covering the air–medium interface, whereas in the latter, they grew in separate submerged pellets. Pirt [16] and Couri et al. [17] point out that the colonial morphology of fungi in submerged fermentation can affect enzyme production. According to these authors, the form taken by these colonies seems to be related to factors such as the kind of substrate, the presence of other nutrients in the medium, and growth conditions like agitation and pH. The floating mycelium formed in standing culture has a relatively small contact area with the medium and is nutritionally dependent on diffusion of the substrate through the liquid, while the pellets formed in shaking culture have a large contact surface with the substrate. This factor probably explains the greater production of PG activity in shaking than in standing culture, as it was the only difference between the cultures.

The peak of the fungal growth, measured as intracellular protein content, coincided exactly with the maximum PG production, both in the standing (maximum 1.095 mg protein) and shaking (maximum 1.887 mg) cultures (Fig. 1). The enhanced contact with nutrients in shaking culture could again explain this difference in growth.

The mycelium of *A. giganteus* was grown at 28°C for 6 days in standing Vogel liquid medium (pH 6.5), containing various carbon sources at 1% (*w*/*v*), both pure substances and natural substrates, to find out which substrates afforded better PG productivity. The pure substances were glucose, galacturonic acid, polygalacturonic acid, and 72% esterified citrus pectin, while the complex carbon sources were wheat bran, oat bran, solid orange bagasse, apple pomace and sugarcane bagasse.

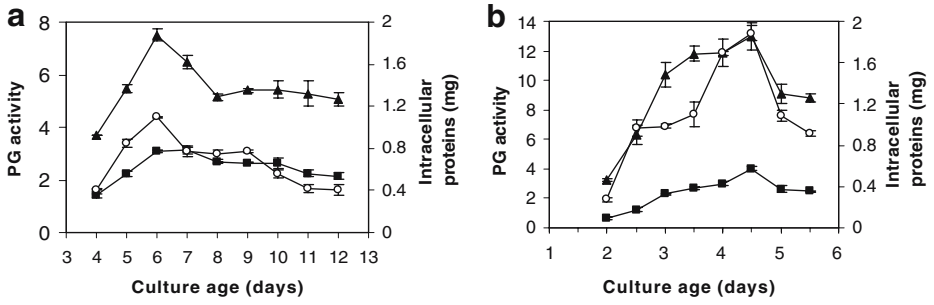

Fig. 1 Time-course of polygalactuonase production from *A. giganteus* in: **a** standing culture and **b** culture shaken at 120 rpm. *filled squares*: PG activity (U/ml), *filled triangles*: specific PG activity (U/mg of protein); *empty circles*: intracellular proteins (mg). The *bars* represent SD

As observed in Table 1, orange bagasse induced the highest production of PG activity per unit volume, approximately 11% higher than that induced by citrus pectin. Nevertheless, for specific activity, citrus pectin and polygalacturonic acid induced about 50% more PG per mg of protein than did orange bagasse. This is due to the low concentrations of extracellular proteins found in the filtrates of cultures grown on these substrates. The bacterium *Bacillus* sp. MG-cp 2 [18] and filamentous fungus *Thermoascus aurantiacus* [19] produced more PG activity when induced with citrus peel than with purified pectin.

A. giganteus produced PG in media containing galacturonic or polygalacturonic acid as the carbon source, but no activity was detected in glucose medium. This is consistent with the induction of PG by compounds whose structures are related to pectin [20, 21]. Galacturonic acid also induced PG activity production by *Sclerotinia sclerotiorum* [21] and by immobilized cells of *A. niger* [22].

The absence of PG activity in medium with glucose as sole carbon source was also reported by Runco et al. [23] and Fawole and Odunfa [24] in *Aspergillus terreus* and *A. niger*, respectively. It must be pointed out that PG synthesis by *A. giganteus* may be subject to catabolic repression, which has been proved in many other microorganisms [21, 23, 25].

The natural carbon sources oat bran and wheat bran were not able to induce PG production by *A. giganteus* under these experimental conditions. Sugarcane bagasse did not even support growth of this fungus, most probably because of the relatively short period of culture (6 days), once the growth of this microorganism on this substrate could be observed after 11 days of culture (data not shown). In solid state fermentation, *Aspergillus carbonarius* [26], *A. niger* [27], and *Termoascus aurantiacus* [28] produced polygalacturonase with wheat bran as sole carbon source.

The influence of the initial pH of the medium was evaluated in shaking cultures (120 rpm) of 4.5 days, with liquid Vogel medium containing 1% pectin (*w*/*v*) as the carbon source, at 28°C. The pH medium was adjusted before inoculation to values from 3.0 to 10.0. The fungal growth and PG production were detected over the whole pH range tested (Fig. 2a); the acid media showed the best results. The highest PG production, expressed either as activity unit per volume or activity per milligram of protein, was obtained in growth media initially adjusted to pH 3.5, the best mean values being 4.05 U/ml and 26.0 U/mg of protein. PGs from filamentous fungi are commonly produced under acid conditions [24, 29, 30].

Table 1 Influence of carbon source on polygalacturonase production by *A. giganteus*.

Carbon Source	PG activity (U/ml)	Specific PG activity (U/mg)	Intracellular proteins (mg)
Orange waste	3.33±0.05	4.81±0.34	0.825±0.004
Apple pomace	0.36±0.02	1.28±0.01	0.868±0.004
Oat bran	ND	ND	0.674±0.020
Wheat bran	ND	ND	0.678±0.008
Sugarcane bagasse	ND	ND	ND
Citrus pectin	3.00±0.15	7.35±0.13	0.961±0.100
Polygalacturonic acid	2.15±0.04	7.24±0.06	0.708±0.007
Galacturonic acid	0.92±0.09	3.01±0.08	0.171±0.030
Glucose	ND	ND	2.014±0.100

ND Not detected

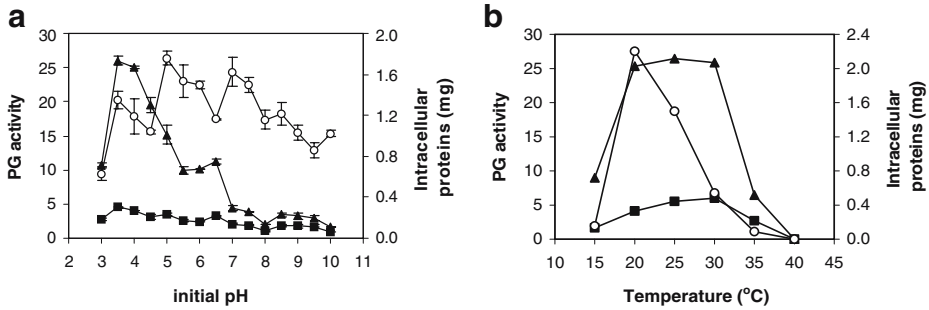

Fig. 2 Influence of **a** initial pH and **b** temperature on polygalacturonase production from *A. giganteus*. *filled squares*: PG activity (U/ml), *filled triangles*: specific PG activity (U/mg of protein); *empty circles*: intracellular proteins. The *bars* represent SD

During growth, the pH of the culture medium was seen to change (data not shown): in cultures whose initial pH was between 3.0 and 7.0, the pH increased and became the media less acid, and in those starting at 8.0 to 10.0, the pH decreased and became the media less alkaline. Cultures with an initial pH between 4.5 and 10.0 exhibited a final pH between 6.2 and 8.2, showing the buffering effect of some metabolite(s) released by the fungus.

The highest intracellular protein content (1.760 mg) in 4.5-day shaking cultures was seen at initial pH 5.0. Furthermore, the intracellular protein content was high in cultures whose initial pH was between 3.5 and 8.5, and lower but considerable contents were found up to pH 10.0. This indicated that fungal development also occurred at neutral and alkaline pH, in agreement with observations made by Rapper and Fennel [31].

The effect of temperature on PG production was tested in medium with initial pH 3.5. The highest PG activity per unit volume, 6.01 U/ml (Fig. 2b), was produced at 30°C, while the maximum values of specific activity were obtained in the range 20–30°C, the mean value being 25.9 U/mg of protein. It has been reported that 30°C is also the best culture temperature for PG production by *Aspergillus foetidus* [32].

The highest intracellular protein contents were produced at 20°C (Fig. 2b), although temperatures from 24 to 26°C are recommended for growing this species [31].

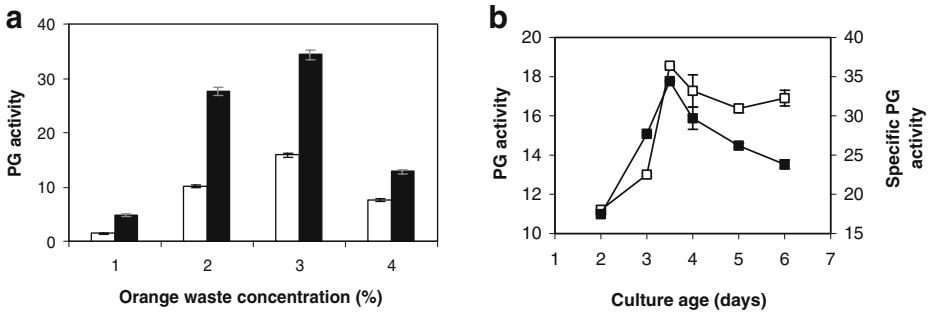

Fig. 3 **a** Variation with orange waste concentration and **b** time-course of activity of polygalacturonase produced by *A. giganteus* in liquid shake culture. *empty squares*: PG activity (U/ml), *filled squares*: specific PG activity (U/mg of protein). The *bars* represent SD

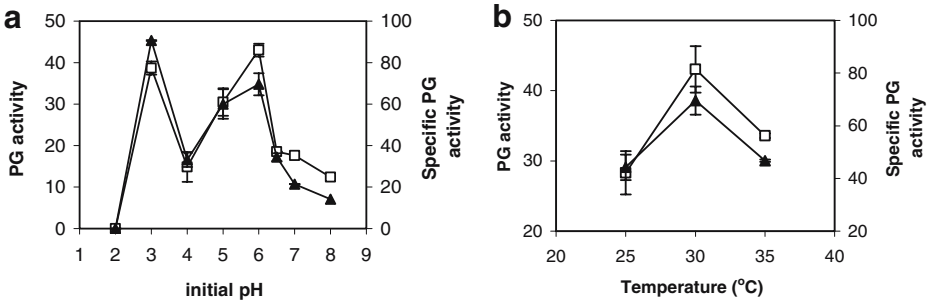

Fig. 4 Influence of **a** initial pH and **b** temperature on polygalacturonase production from *A. giganteus*. *empty squares*: PG activity, (U/ml), *filled triangles*: specific PG activity (U/mg of protein). The *bars* represent SD

Optimizing Production on Orange Waste

As orange waste is an agroindustrial residue abundantly produced in Brazil by the citrus juice industry, and *A. giganteus* is able to produce good amounts of polygalacturonase in liquid medium with this material as carbon-source, the culture conditions were optimized.

As observed in Fig. 3a, *A. giganteus* produced PG activity in medium containing a high concentration of orange waste, with peak production at 3% (*w*/*v*). It is uncommon in submerged cultures, and according to Naidu & Panda [11], high concentrations of carbon sources inhibit enzyme synthesis.

The time-course of PG was followed in liquid Vogel medium, at 28°C and pH 6.5, in shake cultures (120 rpm), using 3% (*w*/*v*) orange waste as the sole carbon source. The peak production of PG, seen after 3.5 days of growth (Fig. 3b), occurred 1 day earlier than in medium with 1% (*w*/*v*) citrus pectin as carbon source.

The influence of the initial medium pH was evaluated in 3.5-day shake cultures (120 rpm) in liquid Vogel medium containing 3% (*w*/*v*) orange waste as the carbon source at 28°C. The pH was adjusted before inoculation to values from 2.0 to 8.0. The acid media gave the best results (Fig. 4a). The highest levels of PG production were obtained in media initially adjusted to pH 6.0 and 3.0, with mean values of 43.03 U/ml and 26.0 U/mg of protein, and 38.70 U/ml and 90.61 U/mg of protein, respectively.

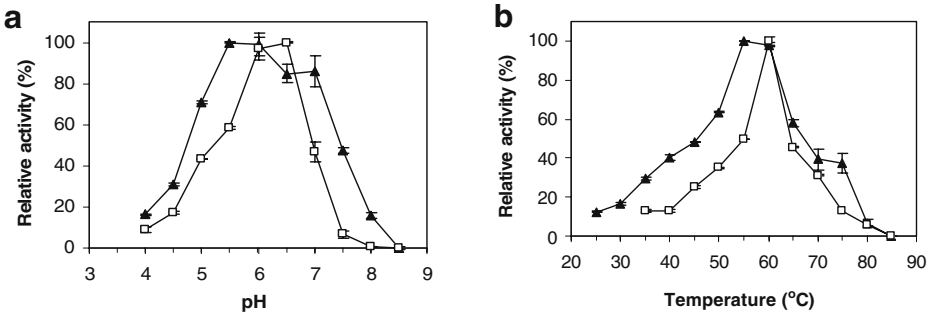

Fig. 5 Influence of **a** pH and **b** temperature on polygalacturonase activity from *A. giganteus*. *filled triangles*: PG-cp; *empty squares*: PG-ow. The *bars* represent SD

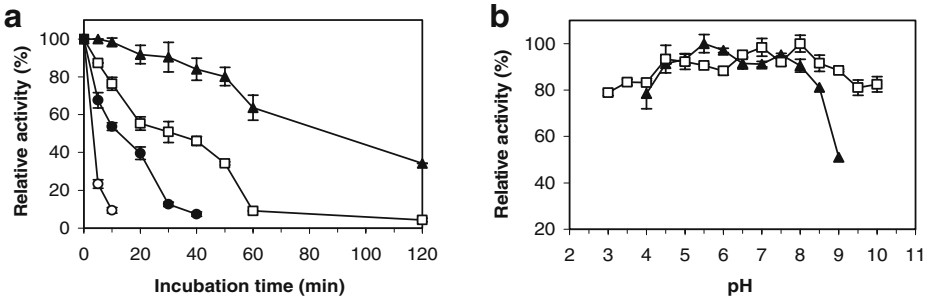

Fig. 6 **a** Thermal stability and **b** pH stability of polygalacturonase activity from *A. giganteus*. **a** PG-cp 55°C (*filled triangles*), PG-cp 60°C (*empty squares*), PG-cp 65°C (*empty circles*), PG-ow 55°C (*filled circles*); **b** PG-cp (*filled triangles*);PG-ow (*empty squares*).The *bars* represent SD

The effect of temperature on PG production was tested in medium with initial pH 6.0. The highest PG production, expressed either as activity per unit volume or activity per milligram of protein, was obtained at 30°C, with mean values of 43.0 U/ml and 69.5 U/mg protein (Fig. 4b).

Enzyme Characterization

The optimal pH for PG activity was found to be 5.5–6.0 (Fig. 5a), for filtrate from *A. giganteus* cultivated in medium with 1% (*w*/*v*) citrus pectin (PG-cp), and pH 6.0–6.5 for filtrate from *A. giganteus* cultivated in medium with 3% (*w*/*v*) orange waste (PG-ow). These are higher pH optima than those usually observed for PGs produced by filamentous fungi, between 3.0 and 5.0 [28, 30, 33–37].

McIlvaine, sodium phosphate and sodium maleate buffers were tested in the pH range 5.0 to 7.0, but they gave results markedly lower than those obtained with the other buffer solutions at the same pH, indicating their strong interference in PG activity. As some pectinases depend on cations, especially Ca^{++} [2, 18, 35, 38], it is possible that complexation had occurred between these anions and this cation or any other required for the enzyme activity.

PG activity was assayed at various temperatures by incubating the culture filtrate with the substrate at pH 6.0. Greatest activity of 9.4 U/ml was observed at 55–60°C for filtrate from *A. giganteus* cultivated in medium with 1% (*w*/*v*) citrus pectin, while a maximum activity of 48.5 U/ml was observed at 60°C (Fig. 5b), for filtrate from *A. giganteus*

Table 2 PG activities of filtrate from *A. giganteus* grown in medium with 1% (*w*/*v*) citrus pectin, at pH 3.5 (PG-cp), and in medium with 3% (*w*/*v*) orange waste, at pH 6.0 (PG-ow).

Substrates	PG-cp (U/ml)	PG-cp (%)	PG-ow (U/ml)	PG-ow (%)
Polygalacturonic acid	17.80±0.94	100±5.3	43.03±3.31	100±7.7
Citrus pectin (MD 34%)	16.05±0.99	90.3±5.6	29.82±1.46	69.3±3.4
Citrus pectin (MD 72%)	3.96±0.24	22.2±1.3	13.85±0.99	32.2±2.3
Apple pectin (MD 75%)	2.07±0.13	11.6±0.7	2.25±0.33	5.2±0.8
Citrus pectin (MD 90%)	1.64±0.03	9.2±0.2	4.13±0.24	9.6±0.6

MD Methylation degree of the carboxyl groups

cultivated in medium with 3% (*w*/*v*) orange waste. Temperatures in the range 50–60°C are often reported as optimal for PG activity in other filamentous fungi [2, 18, 33, 34, 36, 37].

Considering that this fungus is mesophilic, the PG-cp produced by it can be described as having good thermal stability (Fig. 6a). After 50 min of incubation at 55°C, it maintained 80% of its activity, and the half-life at this temperature was found to be 90 min. At 60°C, the half-life was 30 min, while at 65°C, the enzyme denaturated quickly, only about 9.5% of its activity remaining after 10 min. The PGs of *Aspergillus japonicus* [35] and *A. niger* [39], for example, proved much less thermostable, with half-lives of 5 and 20 min, respectively, at 50°C. After 1 h of incubation at 55°C, the PG produced by *Penicillium viridicatum* RFC3 had its activity reduced to approximately 10% of its initial activity [34]. More prolonged stability, at temperatures higher than those cited, has been found in PGs produced by thermophilic fungi [2, 28].

PG-ow had a half-life of 10 min at 55°C (Fig. 6a), shorter than that of PG-cp at the same temperature. The PGs studied here proved to be very stable over the entire range of pH tested, and the PG-ow gave better results than PG-cp at extreme pHs (Fig. 6b). In both cases, more than 90% of PG activity remained after 24 h at any pH between 4.5 and 8.0. A similar result was obtained with the PG from *P. viridicatum* mentioned above, which maintained 90% of its activity after incubation in pHs from 5.0 to 8.5 [34]. In contrast, the PG from *Thermoascus aurantiacus* [28] only retained more than 90% of its activity when incubated in pH between 7.0 and 8.0.

The polygalacturonases produced in optimized culture conditions with citrus pectin and with orange waste differ in their characteristics, as observed in Figs. 5 and 6. This indicates a differential expression of the enzymatic complex on these carbon sources, as reported by Ribon et al. [40] in *Penicillium griseoroseum*, besides the different composition of these filtrates.

Table 2 compares the activities of the two PGs, towards pectic substrates of various methylation degrees on the carboxyl groups (MD), with the activities on unmethylated polygalacturonic acid. Both enzymes exhibited higher activity on substrates with a low MD and were thus classified as polygalacturonases.

A. giganteus can be considered a good producer of polygalacturonase in submerged fermentation, compared to other filamentous fungi. This enzyme has a considerable potential for commercial application, primarily in the food and animal feedstock industries, due to features such as its optimum activity in acid medium, which remains at a high level at neutral pH, and good stability in general from pH 4.5 to 8.0 and up to 60°C.

The utilization of orange waste in PG production leads to an increase in yield with a reduction in process cost; moreover, it adds value to the waste from the orange juice industry. Some processes have been developed for PG production with orange waste in solid state fermentation [28, 34, 37], but submerged fermentation offers some advantages for enzyme production, such as easy extraction, pH control, diffusion control by agitation, and a shorter period of culture.

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