

Extraction and characterization of polygalacturonase of *Fomes sclerodermeus* produced by solid-state fermentation

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ABSTRACT

Polygalacturonase (PG) production by *Fomes sclerodermeus* using solid-state fermentation (SSF) was carried out. Maximal PG activity (26 U/gdw) was obtained between days 11 and 13 at the end of exponential growth. PG activity in the crude extract was more stable at pH 5-6 and 30 °C and had optimum activity at pH 5 and 50 °C. Optimal conditions for PG extraction were: one time extraction with Na₂SO₄ as solvent with 10 min. of agitation. In a scale-up system, PG activity per gram of dry substrate decreased about 60% compared with the activity obtained in an Erlenmeyer flask; however, high total PG activity was obtained.

Key words: polygalacturonase, solid state fermentation, *Fomes sclerodermeus*

RESUMEN

Extracción y caracterización de poligalacturonasa de *Fomes sclerodermeus* producida por fermentación en estado sólido. Se estudió la producción de poligalacturonasa (PG) por *Fomes sclerodermeus* usando técnicas de fermentación en estado sólido. La actividad PG máxima (26 U/g ps) fue observada entre los días 11 y 13. La actividad PG en los extractos crudos fue más estable a pH 5-6 y 30 °C, con una actividad óptima a pH 5 y a 50 °C. Las condiciones óptimas para la extracción de PG se lograron con una única extracción empleando Na₂SO₄ como solvente, con 10 minutos de agitación. En el escalado del sistema, la actividad PG por gramo de peso seco de sustrato disminuyó cerca de 60% comparada con la obtenida en frascos Erlenmeyer, pero la actividad total fue mayor.

Palabras clave: poligalacturonasa, fermentación en estado sólido, *Fomes sclerodermeus*

The pectic substances are present in plant cell walls, mainly localized in the middle lamella and the primary wall. Their amount can vary between 35% in dicot cell walls and 5% in monocot cell walls. These compounds are characterized by their capacity to form gels and their presence contributes to give firmness and structure to plant tissues (26).

D-galacturonic acid units in the pectic substances are joined by α -1, 4 linkages and can be classified as protopectin, pectin and pectic acid. In protopectin, the pectic substances are water-insoluble and they are in close relation with the other polymers present in the cell wall.

In pectin, the galacturonic acid chains contain significant amounts of methoxyl groups (15, 26).

Pectinases form a group of enzymes that degrade the pectic substances. They comprise enzymes that break the glycosidic bounds of the galacturonic acid chain and others that desesterify methoxyl groups of the galacturonic acid chains (6). The breakdown of polygalacturonic acid can occur through two enzymatic processes: Lyases split the α -1, 4 glycosidic bond by trans-elimination while polygalacturonase does so by hydrolytic cleavage (26).

Pectinases are industrially used for extraction and clarification of fruit juices (15), extraction of vegetable oils (8), extraction and clarification of wine (2) and treatment of vegetable fibers (27). Pectinases used in the food industry are commercially produced by *Aspergillus niger* (1, 14).

Solid state fermentation (SSF) is a process in which a microorganism is cultivated in solid substrate in the absence of free water. However, the culture medium must have necessary moisture to allow the growth of the organism (21). SSF presents some advantages with respect to submerged fermentation. In the first place, the culture conditions in SSF are more similar to those in the natural habitat of filamentous fungi, so that these are able to grow and excrete large quantities of enzymes (6, 11). Another advantage is the efficient rate of oxygen transfer through the substrate. Additionally, these processes are of special economic interest for countries with large amount of agroindustrial residues. Some of the substrates used are wheat, soy and rice bran, wheat, corn and rice straw and sugar cane bagasse (6, 10, 23).

The production of pectinases with the filamentous fungus *Aspergillus* sp. utilizing SSF methods has been studied at length (5, 28) while there are few works on pectinase production with white-rot fungi utilizing SSF methods.

The objective of this work was (i) to assess if *Fomes sclerodermeus* produces high polygalacturonase activity (PG) in SSF; (ii) to study the stability of PG activity and the optimum temperature and pH for the reaction; (iii) to optimize the extraction method and (iv) to assess the production of PG in a scale – up system.

MATERIAL AND METHODS

Microorganism and culture conditions

Fomes sclerodermeus (Léveillé) Cooke; BAFC 2752 was maintained in a malt extract-agar medium at 4 °C. The microorganism was cultivated in a 100 ml Erlenmeyer flask containing 3 g soy bran and 1 g wheat bran (dry weight). The mixture was hydrated with a 12 ml solution of malt extract 2% (w/v), $(\text{NH}_4)_2\text{SO}_4$ 0.4% (w/v) to 75% moisture content. The medium was autoclaved (20 min, 121 °C) and inoculated with 1 ml aliquots of mycelial suspension which was obtained from a 5 day- culture in 50 ml of malt extract 1.3% (w/v), wheat bran 4% (w/v) at 28 °C and 120 rpm. Cultivation was carried out at 28 °C for different times until the enzyme extraction.

Enzymatic extraction

The solid fermented material corresponding to one Erlenmeyer flask was stirred and 2 g were mixed with 10 ml distilled water. The mixture was stirred at 120 rpm for 20 min, filtered by compression with syringe through nylon filter of 0.23 mm pore diameter and centrifuged for 20 min at 2.000 rpm. The supernatant was stored at -20 °C until used for enzyme assays.

Enzyme assay

PG activity was measured by using 0.1% polygalacturonic acid in a 50 mM acetate buffer (pH 4.8) as a substrate and measuring the reducing sugars released by the Nelson (20) and Somogyi method (30) using mono-D-galacturonic acid as standard. Incubation was at 50 °C for 30 min. One enzyme unit was defined as 1.0 μmol of product formed per min. Enzyme activity was expressed as U/g dry weight of culture media.

Estimation of chitin and extracellular proteins

Total chitin in the solid fermented material was estimated by the content of N-acetylglucosamine after hydrolysis of 100 mg dry ground material in 6 N HCl for 4 h at 100 °C (22). Extracellular proteins of the crude extract were measured by the Bradford method (4).

Enzyme characterization

Temperature stability: The crude extract was incubated at different temperatures (30 – 60 °C) for 4 h. In the case of 30 °C, the period of incubation was extended to 120 h. The pH was maintained at 5 in all samples. Aliquots were taken at different times to determine the remaining PG activity. All the assays were done in triplicate.

pH stability: The crude extract was diluted (1:1) in 0.05 M buffer solutions pH 3-7 (citrate-phosphate) and pH 8 (boric acid-sodium borate) and maintained at 30 °C for 30 h. Aliquots were taken at different times to determine residual PG activity. All the assays were done in triplicate.

Optimum temperature and pH: The reaction mixture was incubated at temperatures in the range of 16-80 °C at pH 4.8. For the optimum pH, enzyme activity was assayed at different pH values using buffer citrate-phosphate (pH 3-7), boric acid-sodium borate (pH 8-9) and glycine-NaOH (pH 10). The reaction was incubated at 50 °C for 30 min. All the assays were done in triplicate.

Extraction analysis

Repeated extractions: To study the efficiency of the extracting method, five consecutive extractions were carried out with distilled water. Hundred per cent (100%) of PG activity was defined as the sum of the activities measured for each extract.

Extraction time: The effect of the extraction time on PG activity was studied. Different contact times (0-60 min) between the fermented bran and the solvent were allowed, with and without agitation. Aliquots were taken at different times to determine the optimum extraction time. All the other conditions were the same as described above.

Extraction efficiency with different solvents: Different solvents were used for PG extraction from the fermented medium. The solvents were distilled water; NaCl, 0.1 M; Na_2SO_4 , 0.1 M; acetate buffer 50 mM; glycerol, 50% and Tween 80, 0.1%. The pH of the solvents was 5 ± 0.2 . The extraction conditions were the same as described above. PG activity assays were carried out with blanks of all solvents to eliminate possible interferences.

Production of PG activity in scale – up system

193 g (dry weight) of medium (soy bran, 75%; wheat bran, 25%) were moistened with a solution of malt extract, 2% and $(\text{NH}_4)_2\text{SO}_4$ 0.4%, to 768 g of wet weight with 75% moisture content. The medium was autoclaved (20 min, 121 °C) and placed in plastic containers of 30 x 21 x 6 cm. The medium was inoculated with 1 ml aliquots of mycelial suspension/4 g of medium. Incubation was carried out at 28 °C. For the enzyme extraction, 20 g samples were taken from the bottom to the top of the container.

RESULTS AND DISCUSSION

Production of polygalacturonase using solid state fermentation

The white rot fungus *F. sclerodermeus* produced significant quantities of PG activity when grown in media containing soy and wheat bran and supplemented with malt and $(\text{NH}_4)_2\text{SO}_4$ (Figure 1A). The activity peak (26 U/g dw) was found between days 11 and 13 of cultivation. The N-acetylglucosamine content increased until day 13 too (Figure 1A). The pH decreased until day 6 and then increased until day 13 (Figure 1B). Finally, the dry weight loss of the cultures increased until day 11 (Figure 1B). The results show that PG activity increased during the primary metabolism and decreased when the secondary metabolism started. In *Botrytis cinerea* (17) and *Fusarium oxysporum* f. sp. *melonis* (18) the highest PG activities were obtained during the primary growth phase. In *Trametes troglia* (16), the PG peak was obtained when the biomass was at its highest level. The activity values were comparable to those reported for other fungi like *Aspergillus niger* (5) with 25 U/g d.w. and *Penicillium italicum* with 25 U/g d.w. (12).

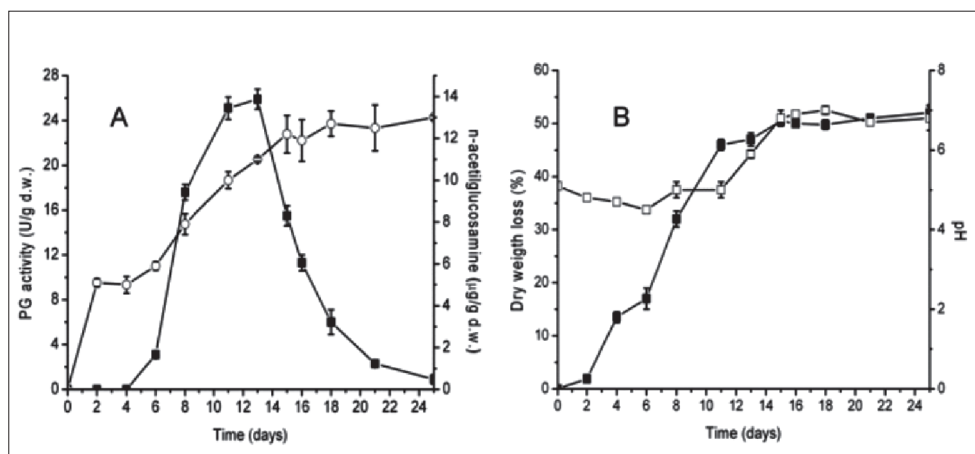


Figure 1. SSF of *F. sclerodermeus*. A; PG activity (■) and chitin levels (○). B; Dry weight loss (■) and pH (○). The error bars on the graphs represent three different fermentation runs.

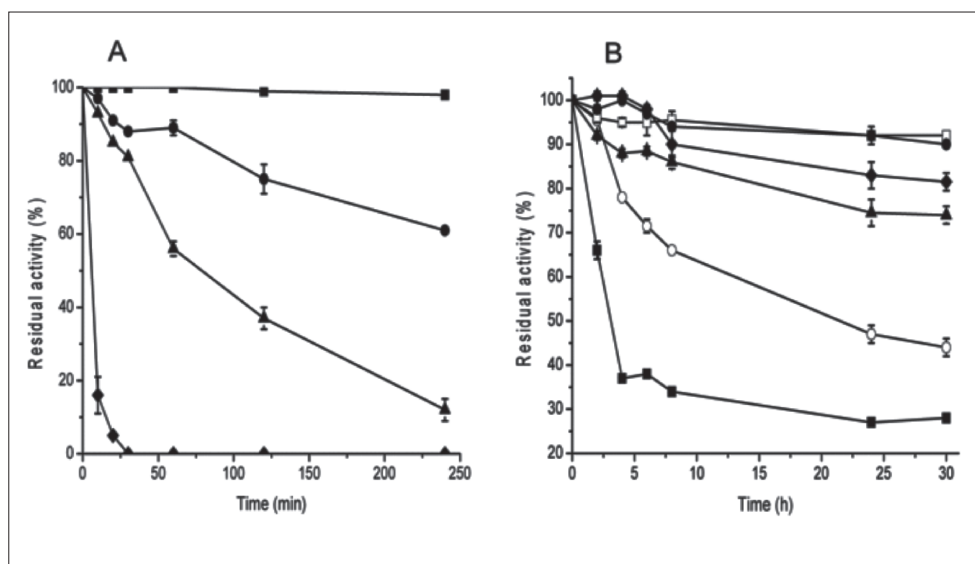


Figure 2. Stability of the PG activity produced by *F. sclerodermeus*. A; Stability at different temperatures: 30 °C (■), 40 °C (●), 50 °C (▲), 60 °C (◆). The incubation pH was 5.0. The 100% of PG activity was 0.7 ± 0.02 U/ml. B; Stability at different pH: 3 (■), 4 (●), 5 (□), 6 (●), 7 (◆), 8 (▲). The temperature of incubation was 30 °C. The 100% of PG activity was 0.9 ± 0.04 U/ml. The error bars on the graphs represent three different experiments.

Enzyme Characterization

The stability of PG activity in the crude extract was studied. When the extract was maintained at 60 °C, no PG activity was measured after 30 minutes (Figure 2A). The retained activity was 55% when maintained at 50 °C for 60 min while at 40 °C for 240 min still retained 60%. At 30 °C after 120 h, 73% of the original activity was retained (data not shown). Singh and Rao, (29) reported for *Aspergillus niger* the irreversible inactivation of the PG with 10 min of incubation at 48 °C. These results would support the observation that fungal PGs are not stable at temperatures close to 60 °C (7).

As regards to pH (Figure 2B), the highest stabilities were obtained in the range of 5-6.

The optimum activity was found at 50 °C and pH 5 (Figure 3). When pH increased to 6, the activity fell abruptly. This result shows that the PG of *F. sclerodermeus* works at slightly acid pH. Huang and Mahoney (13) found an optimal temperature and pH of 55 °C and 7 respectively for the PG of *Verticillium alboatum*. In *Aspergillus niger* (29), the optimum temperature for the PGVI was found between 45-51 °C, and the optimum pH was 4.6. Riou *et al.* (24), found the optimum temperature at 45 °C in *Sclerotinia sclerotiorum* and at 50 °C. in *Saccharomyces cerevisiae* (7).

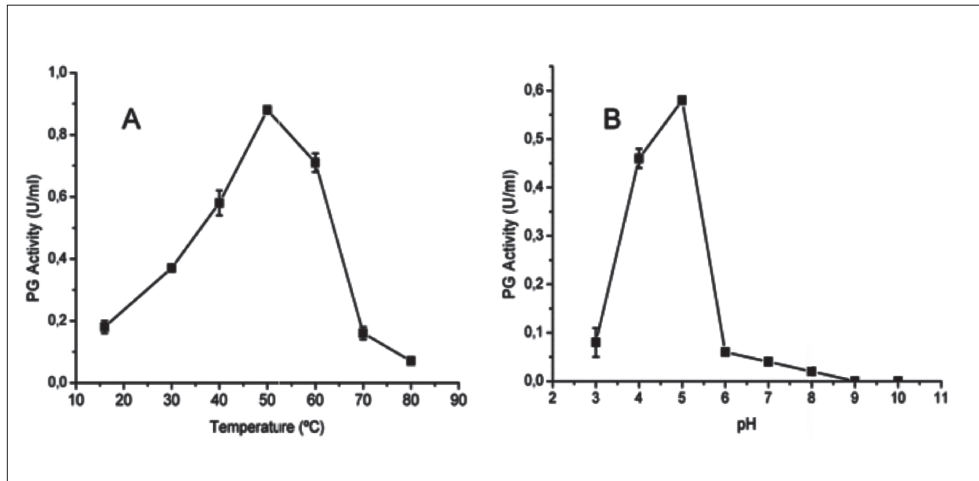


Figure 3. Effect of temperature and pH on PG activity in *F. sclerodermeus*. A; PG activity vs. temperature of reaction. The pH was fixed at 4.8. B; PG activity vs. pH of reaction. The temperature was fixed at 50 °C. The error bars on the graphs represent three different experiments.

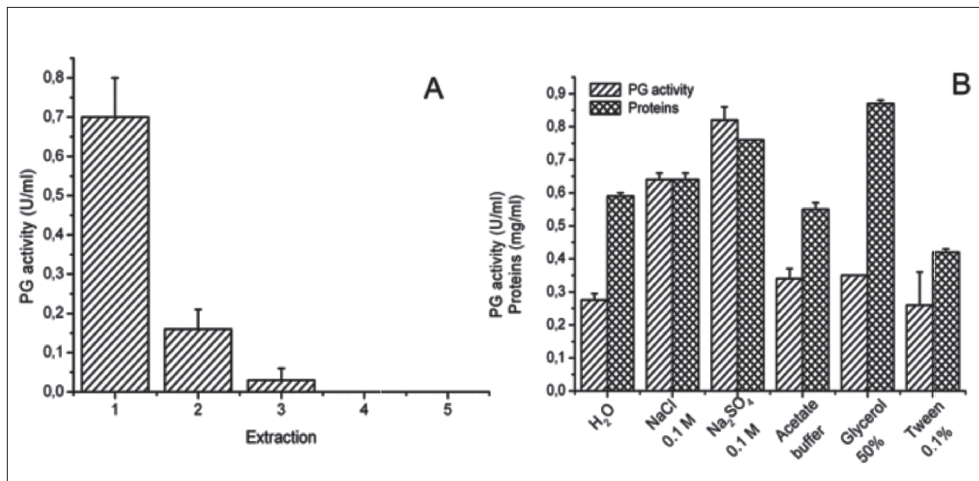


Figure 4. The efficiency of repeated extractions and the effect of different solvents on PG extraction from *F. sclerodermeus* grown in SSF. A; PG activity recovery in the repeated extractions. B; PG activities and extracellular proteins obtained with different extraction solvents. The error bars on the graphs represent three different experiments.

Optimization of downstream process

Repeated extractions. The efficiency of extraction of PG activity from the fermented bran with distilled water was carried out in five serial extractions. Fresh solvent was added in each extraction to the same fermented bran. The first extraction (Figure 4A) recovered 79% of PG activity (taking as 100% the addition of the activities obtained in all extractions). In the second and third extractions 18% and 3% were recovered respectively. Subsequent extractions did not have PG activity. Singh *et al.* (28) carried out repeated extractions on *Aspergillus carbonarius* cultures obtaining more than 80% of PG activity in the first extract and 15% in the second.

Effect of different solvents on recovery of PG activity. The effect of different solvents on PG extraction was studied. Distilled water, NaCl, Na₂SO₄, acetate bu-

ffer, glycerol and Tween 80 were tested. The best solvent to obtain PG activity was Na₂SO₄ followed by NaCl (Figure 4B). The use of Tween 80 or glycerol produced similar values to those of distilled water. The extracellular proteins were measured for each solvent (Figure 4B). The maximum amount of proteins was obtained with Na₂SO₄ and glycerol. Then, glycerol is effective to extract other proteins but not PG. Castilho *et al.* (6) assayed PG extraction using different solvents, obtaining the highest activities with acetate buffer. Singh *et al.* (28) obtained the highest activities with Na₂SO₄ 0.1M. According to these authors, the action of this salt could help break the bonds between carbohydrates and proteins.

Effect of contact time on recovery of PG activity. Different contact times with and without agitation were assayed to obtain the highest PG activity. Shaken samples

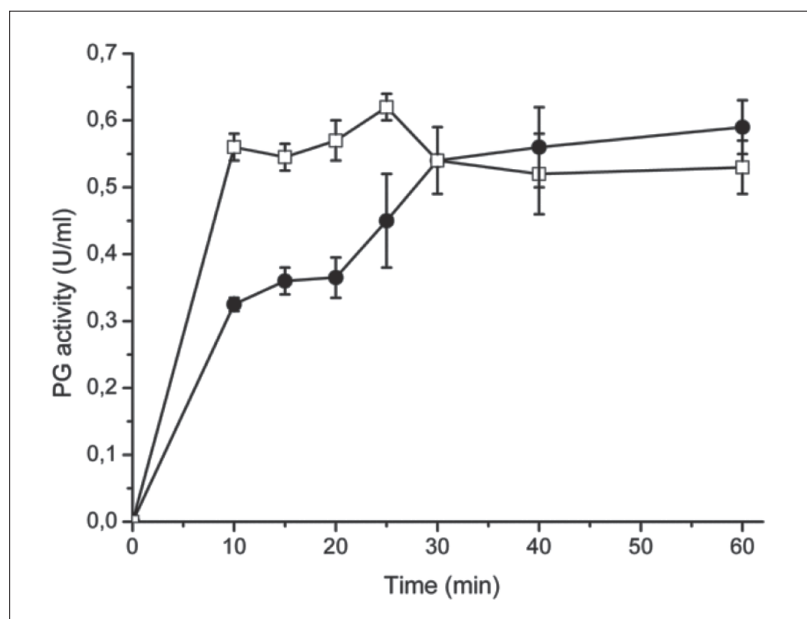


Figure 5. Influence of contact time on recovery of PG activity in *F. sclerodermeus* grown in SSF. With agitation (□), without agitation (●). The error bars on the graphs represent three different experiments.

Table 1. PG activity and extracellular proteins production in the scale up system. U = $\mu\text{mol}/\text{min}$; g d.w. = gram of dry weight

	Culture day		
	7	10	14
PG activity/g d.w. (U/g d.w.)	8.3 ± 0.54	9.6 ± 0.69	9.1 ± 0.52
Total PG activity (U)	1303 ± 85	1488 ± 102	1406 ± 93
Extracellular proteins (mg/g d.w.)	4.2 ± 0.27	4.5 ± 0.34	9.9 ± 0.79

reached the highest activity after 10 min of contact with the solvent (distilled water) while, the samples without agitation reached the highest activity in 30 min (Figure 5). Hence, the more convenient conditions for extraction would be 10 min of contact time with agitation. In cultures of *Aspergillus niger* (6), the optimum time of agitation for PG extraction was 30 min while in *A. carbonarius* (28) the optimum time was 15 min. Moreover, it was observed that when shaken for more than 25 min, PG activity decreased. According to Ghildyal *et al.* (9) this loss of activity could be explained by the adsorption of the enzyme on the substrate or by the liberation of denaturing agents as proteases.

Scale-up of PG activity production

The production of PG activity in a scale-up system was studied. For this, 768 g of culture medium was placed in a 3780 cm³ plastic container. The PG activity/g (dry weight) medium in the scale-up system decreased about 60%

compared with the highest activity obtained in the laboratory – scale experience (Table 1, Figure 1). This indicates the existence of other variables such as aeration rate, medium temperature and inoculum distribution that could influence PG activity production.

The total PG activity was similar on the three sample days and the extracellular protein level was highest on day 14 (Table 1), indicating that this day is not suitable for PG purification. Mitchell *et al.* (19) commented that in scale-up of the SSF process, the substratum bed can reach high temperatures that could denature the enzymes that are being produced. In this case, the temperature of the medium throughout the culture time did not increase beyond 30 °C and at this temperature, PG activity of *F. sclerodermeus* is quite stable.

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