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ORIGINAL PAPER

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Polygalacturonase production by *Aspergillus awamori* on wheat in solid-state fermentation

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Abstract The production of exo-polygalacturonase (exo-PG) and endo-PG by *Aspergillus awamori* grown on wheat in solid-state fermentation was studied. Endo- and exo-PG activities were detected after 24 h of inoculation. Glucose released from starch hydrolysis acted as a catabolite repressor for the exo-PG enzyme. In contrast, endo-PG production was not affected by glucose repression. When milled grains were used, the particle-size distribution and the chemical composition of the medium influenced the rate of micro-organism growth and therefore the trend followed by endo- and exo-PG production. However, these two parameters did not affect the maximum production of exo-PG and endo-PG. For one of the milled samples, three different moisture contents were used (50, 55, 60%). Moisture contents of 60% provide a higher yield of pectinases by *A. awamori*.

Introduction

Pectinases constitute a group of enzymes that catalyse the degradation of pectins, which are the structural polysaccharides present in vegetable cells and are responsible for maintaining the integrity of plant tissues (Alkorta et al. 1998). Pectinases are mainly used in the food industry to clarify fruit juices and wine, to improve oil extraction, to remove the peel from the citrus fruit, to increase the firmness of some fruits and to degum fibres (Uhlig 1990; Tanner et al. 1993; Chang et al. 1994; Baker and Wicker 1996). Commercial pectinase preparations are produced from fungal micro-organisms, mainly by *Aspergillus niger* strains cultured using two different fermentation techniques: submerged fermentation (SmF) or solid-state fermentation (SSF; Rambouts and Pilnik 1980).

In the past decade, SSF systems have generated increasing scientific interest, because they offer several economic and practical advantages over SmF: higher product concentration, improved product recovery, simpler fermentation technology, reduced waste-water output, lower capital investment and lower plant-operating costs (Pandey et al. 2000). These advantages have recently generated a number of studies on the production of enzymes by SSF; and several authors claim that the SSF technique can give greater enzyme yields than SmF. In particular, when pectinase yields are compared using both techniques, it is found that SSF is more productive than SmF (Nakadai and Nasuno 1988; Trejo-Hernandez et al. 1991;Solis-Pereira et al. 1993; Acuña-Argüelles et al. 1995). Another interesting finding is that pectinases produced by SSF have more stable properties: they have a higher stability to pH and temperature and they are less affected by catabolic repression than pectinases produced by SmF (Acuña-Argüelles et al. 1995).

The organic nature of agro-industrial crops and residues makes them good substrates for SSF processes; and they have already been tested for the production of enzymes (Pandey et al. 2001). SSF with complex media, such as wheat bran, wheat straw or wheat flour, can be used to enhance the production of pectinases and reduce the operational costs (Hours et al. 1994; Singh et al. 1999; Castilho et al. 2000; Kapoor et al. 2000; Blandino et al. 2001). The innovative use of crops for highly valuable chemical production can be especially interesting for those countries where there is sufficiency in cereal production. The use of cereal grains as the starting materials for the production of fine chemicals through fermentation offers potentially cleaner and more environmentally friendly processes (Webb and Wang 1997).

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This work studies the production of polygalacturonase (PG) enzymes [both endo-type (EC 3.2.1.15) and exo-type (EC 3.2.1.67)] from cereal raw materials, using SSF and a culture of *A. awamori*. Wheat grains and milled wheat were used as raw material and some of the factors influencing SSF, including particle size, chemical composition and moisture content, were evaluated.

Materials and methods

Inoculum preparation

A. awamori (NRRL 3312), classified by the Commonwealth Mycological Institute as *A. niger* complex, was propagated and stored on 5% hammer-milled whole-wheat flour and 2% agar slants at 4 °C. Preliminary work has shown this strain produces hydrolytic enzymes in SmF, using flour as substrate (Webb and Wang 1997; Blandino et al. 2001).

Spores stored on flour slants were washed in 10 ml of 0.01% Tween 80 solution. Then, 0.5 ml of the spore solution was spread on the surface of 100 ml of the solid flour medium in 500-ml Erlenmeyer flasks and the inoculated Erlenmeyer flasks were incubated at 30 °C for 5 days. After the incubation period, 100 ml of 0.01% Tween 80 solution was added to the flasks and the spores were suspended by gentle shaking. The number of spores was later counted in an improved Neubauer counting chamber (Assistent–Germany, BDH) and the inoculum concentration was adjusted to 1×10^5–5×10^6 spores/g solid by dilution with appropriate amounts of water.

Solid-state fermentation

Fermentation conditions

Wheat grains were used as the solid substrate for the production of PGs. The cereal grains were a Consort soft wheat variety from Yorkshire (UK) and were milled using the Satake STR-100 test mill at the Satake Centre for Grain Process Engineering, UMIST. The substrates were then sterilised at 121 °C for 30 min.

The required amount of spore suspension was poured onto disposable Petri dishes (15 cm diameter) and the solid fermentation was started by adding the sterilised solid substrate. This procedure minimised swelling and gelatinisation of the medium. pH was not controlled and the plates were incubated under static conditions at 30 °C for 7 days. Three whole plate samples were withdrawn for analysis after 24 h.

Effect of particle size, chemical composition and moisture content

The effect of the particle size of the substrate on the production of PGs was studied by growing the micro-organism on wheat grains and grains milled under two different conditions: milled stock 1 (MS1) and milled stock 2 (MS2). MS1: roll gap = 1.0 mm, speed roll A = 250 rpm, speed roll B = 100 rpm, corrugation direction = dull-to-dull. MS2: roll gap = 0.5 mm, speed roll A = 50 rpm, speed roll B = 100 rpm, corrugation direction = sharp-to-sharp. The initial moisture content of all substrates was adjusted to 60%. The particle-size distributions of MS1 and MS2 were determined by sieve analysis, using as Simon batch sifter.

The effect of chemical composition and particle size on the production of PGs was studied by growing the fungus in three different fractions of MS1 (MS1a, MS1b, MS1c, respectively). Fraction MS1a was made of particles from MS1 larger than 1.40 mm, fraction MS1b were particles of 0.85–1.40 mm and fraction MS1c particles were smaller that 0.85 mm. The initial moisture content of each substrate was also adjusted to 60% in all the media employed.

The effect of the initial moisture level of the substrate on the production of pectinases was studied by adjusting the moisture level of the MS1 to 50, 55 and 60%; and sterile distilled water was used as the moistening agent.

Peptinase extraction and analytical methods

All the fermented samples were treated with 100 ml of distilled water and mixed for 30 min on a rotary shaker at 200 rpm and 30 °C. The resulting solid suspensions were centrifuged at 5,000 rpm for 10 min and the supernatant liquor (the enzymatic extract) was used for the measurement of pH, exo-PG, endo-PG and reducing sugars (R.S.). The results shown represent the average of three sets of experiments and have a confidence interval of 95%.

Endo-PG was determined at 45 °C by viscometry and the exo-PG by the release of R.S., expressed as galacturonic acid. For endo-PG, 5 ml of enzyme extract were mixed with 15 ml of 1% (w/v) pectin in 0.1 M acetate buffer (pH 5.0). The reduction in viscosity was monitored with an Ostwald viscometer (Technico, BS/U, UK). A unit of endo-PG activity (U) was defined as the amount of enzyme that reduced the viscosity of the pectin solution by 50% per minute under the conditions described above. For exo-PG activity, 1 ml of enzyme extract was added to a solution containing 2 ml of 0.5% pectin in 0.1 M acetate buffer (pH 5.0). Samples were incubated at 45 °C for 10 min and the R.S. were determined by a modification of the dinitrosalicylic acid method (Miller 1959). A unit of exo-PG activity (U) was defined as the amount of enzyme that produced 1 µmol galacturonic acid/min under the conditions described above. All measurements were made in triplicate.

The reducing groups in the enzymatic extract were determined using a modification of the dinitrosalicylic acid method (Miller 1959). The results were expressed as galacturonic acid concentrations, using a calibration curve. The measurement of the pH in the enzymatic extract was performed using a standard pH meter.

The moisture content of the solid substrates was determined from the loss in weight after heating at 110 °C for 4 h. The starch was determined by measuring the glucose released after acid hydrolysis and the total nitrogen content was determined by the improved method of Kjeldahl (Wang 1999). The phosphorus concentration in the solid substrates was determined by the molybdovanadophosphoric acid colorimetric method, originally described by Kitson and Mellon (1944).

Results

Effect of the particle size of the milled wheat grains

The effect of the size of the solid substrate on the production of pectinases was tested using three different media: wheat grains and two fractions of milled wheat grains under two different conditions (MS1, MS2). The particle size distributions of the last two fractions are shown in Fig. 1. The mean diameter (the diameter below which 50% of the particles fall) of MS1 was 1.4 mm, while for MS2 was 1.8 mm.

Figure 2a–c shows the change in pH, endo-PG activity, exo-PG activity and R.S. concentration with time for MS1, MS2 and wheat grains, respectively. The exo-PG activity in MS1 reached a maximum value after 24 h of fermentation (7.7±2.4 U/g), after which a reduction in exo-PG activity was observed for 4 days. From day 6 an acute increase in exo-PG activity was noted. In contrast, endo-PG activity increased from the beginning of fermen-

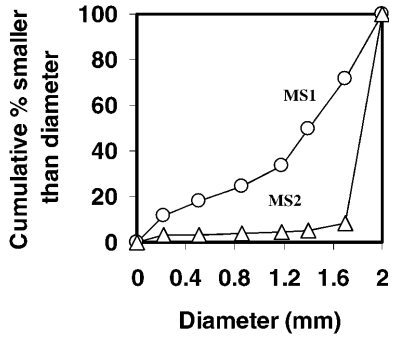

Fig. 1 Cumulative percentages in particle size distribution of milled grains. Milled stock 1 (*MS1*): roll gap = 1.0 mm, speed roll A = 250 rpm, speed roll B = 100 rpm, corrugation direction = dull-to-dull; Milled stock 2 (*MS2*): roll gap = 0.5 mm, speed roll A = 50 rpm, speed roll B = 100 rpm, corrugation direction = sharp-to-sharp

tation and reached a maximum (0.012±0.002 U/g) after 5 days, after which the enzyme titres slightly decreased.

In the evolution of the R.S., four trends were clearly observed (Fig. 2a). During day 1 after inoculation, a small increase in R.S. was observed, but after day 2, the concentration increased almost 50-fold. From then on, the concentration remained almost constant and, finally, over days 5–7, a significant decrease was measured. With regard to the evolution of pH, the value decreased during the first 5 days of fermentation, reaching a minimum of 3.9; and after that it increased.

The same tendencies were observed when MS2 (larger particle size) was used for fermentation (Fig. 2b). A maximum in the exo-PG activity (8.7±0.7 U/g) was measured 24 h after inoculation; and a subsequent reduction in exo-PG activity was also observed over days 1–5 of fermentation. Subsequently, a second maximum in the exo-PG activity (9.2±1.5 U/g) was observed on day 6. In relation to the kinetics of endo-PG activity, the major endo-PG activity (0.0130±0.0002 U/g) was found 24 h after inoculation and remained practically constant throughout fermentation.

There was no change in the concentration of R.S. from the day of inoculation to the first day of fermentation (Fig. 2b). A maximum concentration value was reached on day 3; and this remained almost constant until day 5. A minimum sugar concentration value was measured on day 6. The pH value did not change during the first 24 h of fermentation, but then it decreased steadily over days 1–5. A maximum in the pH curve was also observed on day 6 of fermentation.

Figure 2c shows the endo- and exo-PG activity profiles when wheat grains are used as solid substrate. Endo- and exo-PG activities were found after 24 h and then increased smoothly throughout fermentation. The pH values were slightly higher than those found when milled grains were used. There was practically no change in the concentration of R.S. until day 7 of fermentation.

The maximum values of pectinase production (endo-PG, exo-PG activities) on the different solid fermentation media are given in Table 1.

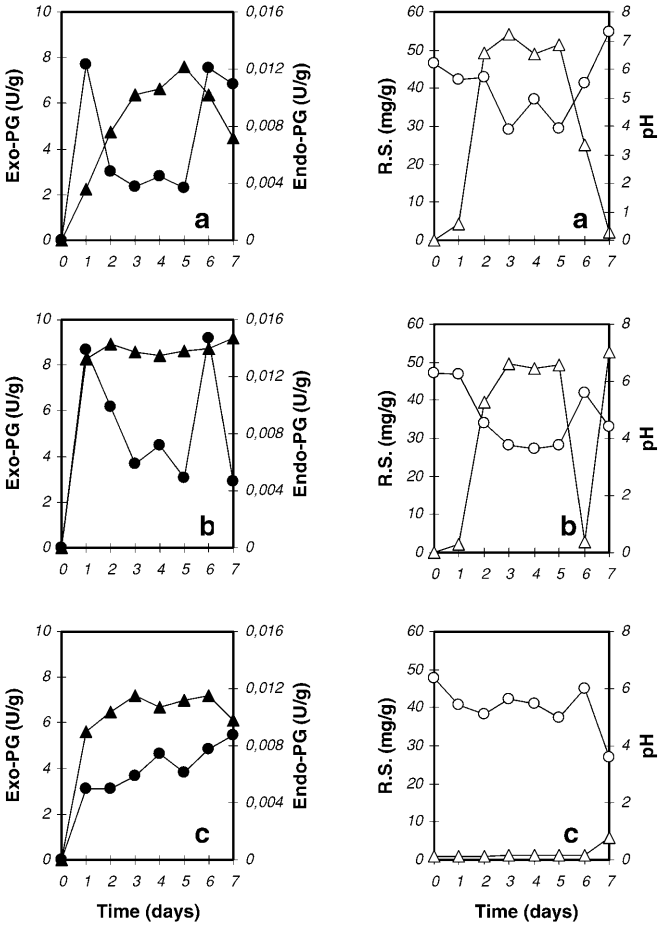

Fig. 2 Kinetics of reducing sugar concentration (*R.S.*; ■), pH (○), exo-polygalacturonase activity (*Exo-PG*; ●) and endo-polygalacturonase activity (*Endo-PG*; ▲) during solid-state fermentation (SSF) of *Aspergillus awamori* on: **a** MS1 medium, **b** MS2 medium and **c** whole wheat grain. *U* Units of enzymatic activity

Table 1 Highest endo- and exo-polygalacturonase (*PG*) activities measured on the different media employed. *U* Units of enzymatic activity

	Highest enzymatic activities (U/g)		
	MS1	MS2	Wheat grain
Exo-PG	7.7±2.4	9.2±1.5	4.9±0.1
Endo-PG	0.012±0.001	0.015±0.002	0.011±0.001
	MS1a	MS1b	MS1c
Exo-PG	9.6±4.2	8.5±1.9	9.2±1.6
Endo-PG	0.014±0.0005	0.014±0.0002	0.013±0.002
	MS1, 50% humidity	MS1, 55% humidity	
Exo-PG	3.8±1.0	3.4±0.4	
Endo-PG	0.008±0.003	0.010±0.0002	

Effect of chemical composition and particle size of the medium

The effect of the chemical composition and the particle size of the medium on the production of pectinases were

Table 2 Chemical composition and particle size of media MS1, MS1a, MS1b and MS1c used as solid substrates

Medium	Particle size (mm)	Chemical composition (% dry weight)		
		Starch	Total nitrogen	Phosphorus
MS1	<0.212–2.0	70.91	10.88	0.40
MS1a	>1.4	65.42	12.05	0.38
MS1b	0.85–1.4	68.52	11.28	0.46
MS1c	<0.85	78.76	8.46	0.19

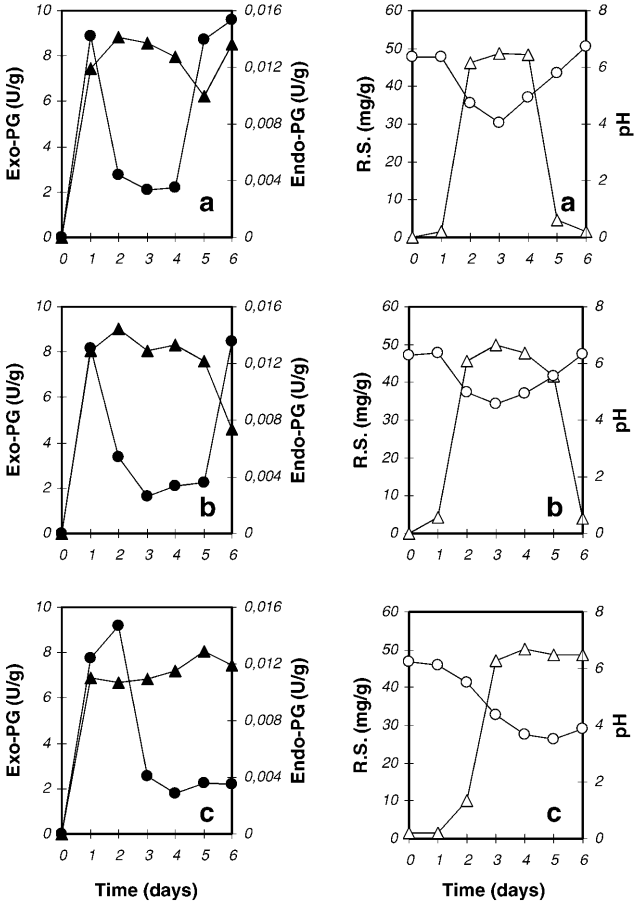

Fig. 3 Kinetics of reducing sugar concentration (■), pH (○), exo-polygalacturonase activity (●) and endo-polygalacturonase activity (▲) during SSF of *A. awamori* on: **a** MS1a, **b** MS1b and **c** MS1c

studied by cultivating the fungus on three different media, referred to as MS1a, MS1b and MS1c. As described previously, these media were obtained by dividing MS1 into three different fractions: MS1a contained particles larger than 1.4 mm, MS1b was particles that fell between 0.85–1.4 mm and MS1c was particles smaller than 0.85 mm. The chemical compositions of these three fractions in terms of starch, total nitrogen and phosphorus content are given in Table 2. MS1a and MS1b did not differ significantly in their starch and total nitrogen concentration, although the particle size of MS1a was larger than that MS1b. MS1c differed substantially from the other two: it contained the highest levels of starchy material and the lowest levels of total nitrogen and phosphorus; and it also had the smallest particle size.

Figure 3a, b shows the evolution of R.S., pH and the endo-PG and exo-PG activities on media MS1a and MS1b. It can be seen that the trends for each of the parameters studied were similar to those found in the medium, MS1. However, starch hydrolysis was delayed in MS1c (particles smaller than 0.85 mm, richer in starch), as indicated by the trend of soluble R.S. (Fig. 3c). In this medium, a sharp increase in the concentration of R.S. was observed on day 3 of fermentation.

Effect of moisture content of the solid substrate

The purpose of this experiment was to determine the optimum moisture content of milled wheat grains for the production of PG enzymes. Two different moisture levels were tested (50%, 55%) and the results were compared with those previously obtained for a 60% moisture content. No higher moisture contents were used, to avoid the presence of free water and possible contamination. In all cases, MS1 was used as solid substrate. The results are also shown in Table 1.

The trends observed at 50% and 55% humidity were the same as those previously reported for MS1 at 60% moisture (data not shown). However, the highest values of exo-PG activity were achieved at a moisture level of 60% (Table 1). A decrease in the moisture content from 60% to 55% resulted in a 56% decrease in the enzymatic activity; but approximately the same activity was measured at 55% and 50% moisture. Similarly, the best results for endo-PG activity were obtained at 60% moisture content.

Discussion

In all media employed, the trend followed by the R.S. concentration was the result of a balance between the rate of secretion of starch-degrading enzymes and the rate of glucose consumption by *A. awamori* cells. In previous studies, it was found that, when *A. awamori* grows on wheat, it secretes amyloglucosidase enzymes, which hydrolyse the carbon source (starch) and release glucose (Webb and Wang 1997). Therefore, the sharp increase in the concentration of R.S. observed in the early stages of the solid state fermentation was the result of a high amylolytic activity, while the drop in concentration observed

in the later stages was probably the result of the exhaustion of free starch in the medium.

The differences observed in the R.S. concentrations in MS1, MS2 and wheat grains (Fig. 2) can be explained in terms of their particle sizes or, in other words, the availability of their starch. For MS2 and wheat grains, with larger particles than MS1, the reduced surface area/volume ratio provided a smaller surface for *A. awamori* growth and may have made it difficult for the micro-organisms to penetrate the aleurone layer. As a result, given that starch was less available in MS2 than in MS1, glucose was almost exhausted by the sixth day of fermentation. The subsequent increase in R.S. was probably the result of the induction of amylolytic enzymes to assimilate more starch. Similarly, the R.S. concentration remained constant during the first 6 days of fermentation on wheat grains, demonstrating that the micro-organism growth was hindered. In the same way, starch hydrolysis was delayed in MS1c, probably due to the fact that the particles were too small and substrate agglomeration may have interfered with microbial aeration.

In contrast, endo- and exo-PG activities were detected after 24 h of fermentation, when the concentration of R.S. in the different media was extremely low. This result provides evidence that pectinases are synthesised earlier than amylolytic enzymes. Similar patterns were observed by Sarrete et al. (1992), who found that, with *Rhizopus oligosporus*, the production of polysaccharidases occurred in a sequential manner and the first enzyme to be detected was PG (Sarrete et al. 1992).

It seems clear there is a relationship between the R.S. concentration and the evolution of exo-PG activity. The highest values of exo-PG activity were obtained when the concentrations of R.S. in the medium were low. These results suggest that the glucose released by starch hydrolysis acts as a repressor of exo-PG activity. A glucose concentration above 40 mg/g completely suppressed the production of exo-PG activity. In contrast, the fluctuations observed in the R.S. concentration did not seem to affect the trends followed by endo-PG activity, suggesting that endo-PG enzyme production was not influenced by glucose repression. These differences in endo- and exo-PG evolution indicate that these enzymes are independently controlled. Catabolic repression of pectic enzymes in the presence of glucose and other sugars in SmF have also been reported by other authors (Solis-Pereira et al 1993; Taragano et al. 1997). Nevertheless, other works point out that SSF is more suitable than SmF for the growth of filamentous fungi, especially under conditions where catabolic repression applies (e.g. at high glucose concentration; Solís-Pereira et al. 1996; Favela-Torres et al. 1998).

With regard to the temporal evolution of pH, the value decreased during the first days of fermentation, possibly due to microbial production of organic acids. After that, when the concentration of soluble R.S. decreased, the pH increased, probably owing to microbial assimilation of organic acids previously released into the medium. The same pH trend was reported for *A. awamori* grown on wheat bran in SSF (Roque and Takuo 1994).

In general, it seems that the particle size of the solid substrate and its chemical composition affect the rate of micro-organism growth and therefore the trend followed by endo- and exo-PG production. However, these two parameters did not influence the maximum production of exo-PG and endo-PG enzymes when milled stocks were used (Table 1). Practically the same enzymatic activities were registered in MS1 and MS2, despite the fact that they had different particle size distributions (Fig. 1). For MS1 (with a higher percentage of smaller particles) the surface area for growth was greater, but the inter-particle porosity was lower. In contrast, with MS2 the porosity was greater, but the saturated surface area was lower. A combination of these two factors was probably responsible for the measured values of enzyme production.

A moisture content of 60% seems to give a good compromise between water availability, substrate swelling and oxygen diffusion effects, thus favouring the formation of pectinases by *A. awamori*. Similar results have been reported suggesting that, in media with low water-availability, fungi suffer modifications to their cell membrane that affect microbial metabolism (Acuña-Arguelles et al. 1994). As a result, suboptimal product formation occurs, due to reduced mass transfer processes, such as diffusion of solutes and gas to cells during fermentation (Ngadi and Correia 1992).

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