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Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems

MC Maldonado and AM Strasser de Saad

Departmento de Microbiologia Industrial y General, Facultad de Bioquimica, Quimica y Farmacia, Universidad Nacional de Tucuman, Ayacucho 491, 4000 Tucuman, Argentina

Production of pectinesterase and polygalacturonase by *Aspergillus niger* was studied in submerged and solid-state fermentation systems. With pectin as a sole carbon source, pectinesterase and polygalacturonase production were four and six times higher respectively in a solid state system than in a submerged fermentation system and required a shorter time for enzyme production. The addition of glucose increased pectinesterase and polygalacturonase production in the solid state system but in submerged fermentation the production was markedly inhibited. A comparison of enzyme productivities showed that those determined for pectinesterase and polygalacturonase with pectin as a carbon source were three and five times higher by using the solid state rather than the submerged fermentation system. The productivities of the two enzymes were affected by glucose in both fermentation systems. The membranes of cells from the solid state fermentation showed increased levels of C18:1, C16:0 and C18:0 fatty acids. Differences in the regulation of enzyme synthesis by *Aspergillus niger* depended on the fermentation system, favoring the solid state over the submerged fermentation for pectinase production.

Keywords: pectinase production; *Aspergillus niger*; solid state fermentation

Introduction

Pectinases are constitutive or inducible enzymes, produced by a variety of microorganisms that catalyze the breakdown of pectin. These enzymes are widely used in the industrial processing of fruit juices because of their capacity to degrade pectin and related substances, resulting in markedly enhanced yields [3,14], as the resultant decrease in the viscosity of fruit pulp facilitates extraction, maceration, liquefaction, filtration and clarification processes [10].

Pectinases can be produced either by submerged [4–6] or solid state [1,2,26–29] fermentation.

Submerged fermentation (SmF) systems have been used extensively for the study of the synthesis of microbial metabolites and for the production of high-priced materials [7,28]. Solid-state fermentation (SSF) has been used for the production of proteins [23], enzymes [8,24] and ethanol [12].

Studies concerning regulatory aspects of pectinase production by molds have been carried out in SmF systems. The enzymes are induced by pectin or by some of its derivatives [20,25] but production can be controlled by catabolite repression [11,15,21].

However, information about the regulatory mechanisms in pectinase production by SSF is scarce. The effect of carbon sources on endo- and exo-pectinase production by *A. niger* CH4 in both SmF and SSF systems was reported by Solis Pereira *et al* [28]. They concluded that high sugar concentration stimulated pectinase production in SSF, whereas in SmF this production was inhibited, probably by catabolite repression.

In our previous studies [19] it was shown that *Aspergillus niger* isolated from rotten lemons was able to produce extracellular pectinesterase (PE) and polygalacturonase (PG). The synthesis of both enzymes is induced by pectin, galacturonic and polygalacturonic acids at the transcription level [20]. Glucose represses the enzyme synthesis by a catabolite repression mechanism that occurs at the translation level [21]. In this current study, PE and PG production was studied in SmF and SSF systems with pectin as a carbon source. The effect of glucose addition on the production of enzymes by both systems was determined.

Materials and methods

Microorganism

The strain of *A. niger* used in this work was isolated from a rotten lemon [19]. It was maintained by monthly transfers to Czapek agar slant tubes, incubated at 30°C and stored at 4°C.

Submerged fermentation (SmF)

The fermentation medium contained in g L^{-1}: KH_2PO_4, 4; Na_2HPO_4, $FeSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.01; $(NH_4)_2SO_4$ 2; and (in mg L^{-1}): $MnSO_4 \cdot 7H_2O$, 70; H_3BO_3, 10. Pectin (15 g L^{-1}) and pectin supplemented with different concentrations of glucose (5, 10 and 20 g L^{-1}) were used as carbon source. Liquid media were adjusted to an initial pH 4.5 and sterilized for 15 min at 120°C. An inoculum of 2×10^6 spores ml^{-1} was used. Fermentation was carried out on a rotatory shaker at 300 rpm at 30°C. Samples were taken at 12-h intervals during 72 h, filtered through SS No. 595 filter paper and kept at 4°C for further assays.

Correspondence: Dr MC Maldonado, Departmento de Microbiologia Industrial y General, Facultad de Bioquimica, Quimica y Farmacia, Universidad Nacional de Tucuman, Ayacucho 491, 4000 Tucuman, Argentina
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Solid state fermentation (SSF)

The fermentation medium contained in g per 100 g: urea, 0.3; K_2HPO_4, 0.65; $(NH_4)_2SO_4$, 1.26; $MgSO_4$, 0.02; $FeSO_4$, 0.029; pectin, 1.5; sugar cane bagasse (SCB) pith as support, 23.1 and 70 ml distilled water. The pH was adjusted to 4.5. SCB was previously washed with hot water to eliminate sucrose. When glucose was used, the final concentrations were: 5, 10 and 20 g L^{-1}. The solution was sterilized at 120°C for 15 min; SCB was sterilized separately for 15 min at 120°C. After cooling, both fractions were mixed and inoculated with 2×10^7 spores g^{-1} dry matter; 10 g of the inoculated material was placed in a Petri dish and incubated at 30°C. Samples (10 g) of fermented material were taken at 12-h intervals during 72 h, mixed with 10 ml distilled water and pressed in a manual press. The liquid extract was kept at 4°C for enzymatic assays. It should be noted that differences in media composition for SmF and SSF were due to nutritional requirements optimized by previous work (unpublished results).

Separation of plasma membranes

Mycelium was washed and suspended in 0.02 M acetate buffer pH 4.5, disrupted (at 100 W for 5 min at 4°C) in an ultrasonic disintegrator (Megason, Branson Sonic Power Co, Danbury, CT, USA) and submitted to a differential centrifugation. Pellets from the last centrifugation were used for fatty acids extraction.

Analytical methods

Pectinesterase assay (PE): To 10 ml of 0.5% (w/v) pectin in 0.1 M NaCl, 2 ml of filtrate was added. The pH was adjusted to 4.5 with 0.1 M NaOH and the mixture was incubated for 60 min at 35°C. PE activity was measured by determining the carboxyl groups released by titration with 0.02 N NaOH [13]. One unit of PE was defined as the amount of enzyme releasing one milliequivalent of ester hydrolyzed (carboxyl group) per minute.

Polygalacturonase assay (PG): 1 ml of sample was added to a solution containing 1 ml of 0.9% polygalacturonic acid in 0.1 M acetate buffer, pH 4.5. After incubation at 45°C for 30 min, reducing sugars were determined by the dinitrosalicylic acid (DNS) method [22] using galacturonic acid as a reference. One unit of PG was defined as the amount of enzyme that liberates one micromole of galacturonic acid of enzymatic filtrate per minute.

For biomass determination in SmF the mycelium obtained after filtration was rinsed and dried at 90°C for 12 h until constant weight. In SSF, biomass could not be measured accurately by dry weight, so it was determined by measuring glucosamine concentration with Ehrlich's reagent [30]. Glucose was determined by using an enzymatic kit supplied by Boehringer Mannheim. Proteins were determined by the Lowry method [18].

Fatty acids were extracted by the Bligh and Dyer method [9] and were determined by HPLC using an analytical column C18. All fermentations and assays were carried out in triplicate. The results are presented as a mean value.

Results and discussion

Pectinesterase and polygalacturonase production in SSF

Enzyme production by *A. niger* in SSF was studied in the medium with pectin as the only carbon source and was also studied in the same medium supplemented with different glucose concentrations (5, 10 and 20 g L^{-1}).

PE activity (Figure 1a) was nearly the same in pectin (500 U L^{-1}) or in the presence of 5 g L^{-1} glucose (520 U L^{-1}) and it was maximum at 24 h of incubation. With the addition of 10 and 20 g L^{-1} glucose, there was a lag period in the enzyme synthesis that was proportional to glucose concentration. Under these conditions, enzyme production was maximum at 36 h (725 U L^{-1}) for 10 g L^{-1} and 72 h (600 U L^{-1}) for 20 g L^{-1} glucose. Enzyme production achieved with 10 g L^{-1} glucose was about 30% higher than that observed in the medium with pectin as the sole carbon source.

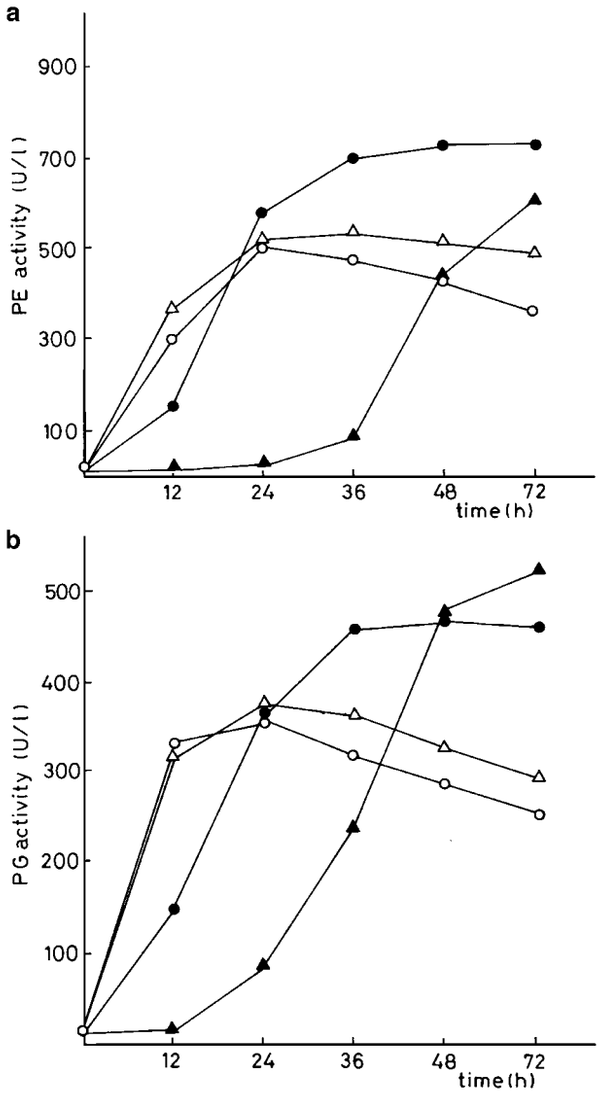

Figure 1 PE (a) and PG (b) yields by solid-state fermentation system (SSF) with pectin (○) as a substrate and with glucose as a supplement [5 g L^{-1} (△), 10 g L^{-1} (●) and 20 g L^{-1} (▲)].

Similar behavior was observed for PG production (Figure 1b) and the stimulation produced by glucose (10 and 20 g L^{-1}) increased enzyme levels about 23% (48 h) with respect to the medium without glucose.

Addition of glucose was associated with initial (12 h) high rates of sugar consumption, which increased in proportion to carbohydrate concentration (Figure 2a). Therefore, the glucose consumption rate may not be the limiting factor for pectinase production. The delay observed in enzyme production can be related to glucose concentration, which must be lower than 5 g L^{-1} when the production started.

Biomass production (Figure 2b) was faster and greater with 20 g L^{-1} glucose than that observed with solely pectin showing that glucose is more efficiently used by *A. niger* for growth than for pectin production.

Changes in pH were similar in pectin-containing medium with 5 g L^{-1} glucose; the final pH was 6.2 and 6.6 respectively. With 10 and 20 g L^{-1} glucose, the pH decreased in the first 24 h and remained at 2.3 and 2.9 respectively at the end of the experiment. The slight decrease of enzymatic activities observed after 24 h in the medium with pectin or with 5 g L^{-1} glucose addition (Figure 1a and b) could be related to pH level.

Pectinesterase and polygalacturonase production in SmF

Enzyme production by *A. niger* was studied by submerged fermentation in the medium with pectin as a sole carbon source and in the same medium supplemented with different glucose concentrations (5, 10 and 20 g L^{-1}). In the absence of glucose, maximum production of PE (127 U L^{-1}) and PG (55 U L^{-1}) occurred at 48 h of incubation (Figure 3a and b); the further increases observed at 72 h were not significant. When glucose was added to the medium, production of both enzymes was lower and slower compared to pectin alone. The inhibitory effect increased as glucose concentration increased and there was an inhibition of about 74 and 90% for PE and PG activities respectively by 20 g L^{-1}.

Reduction and delay of the production of both enzymes is related to the sugar concentration in the medium since enzyme production begins when glucose is exhausted (Figure 4a). These results are in accordance with the catabolic repression by glucose reported for *A. niger* growing in liquid medium [21].

Maximum biomass concentration was observed at 48 h in all studies. When glucose was present, the biomass con-

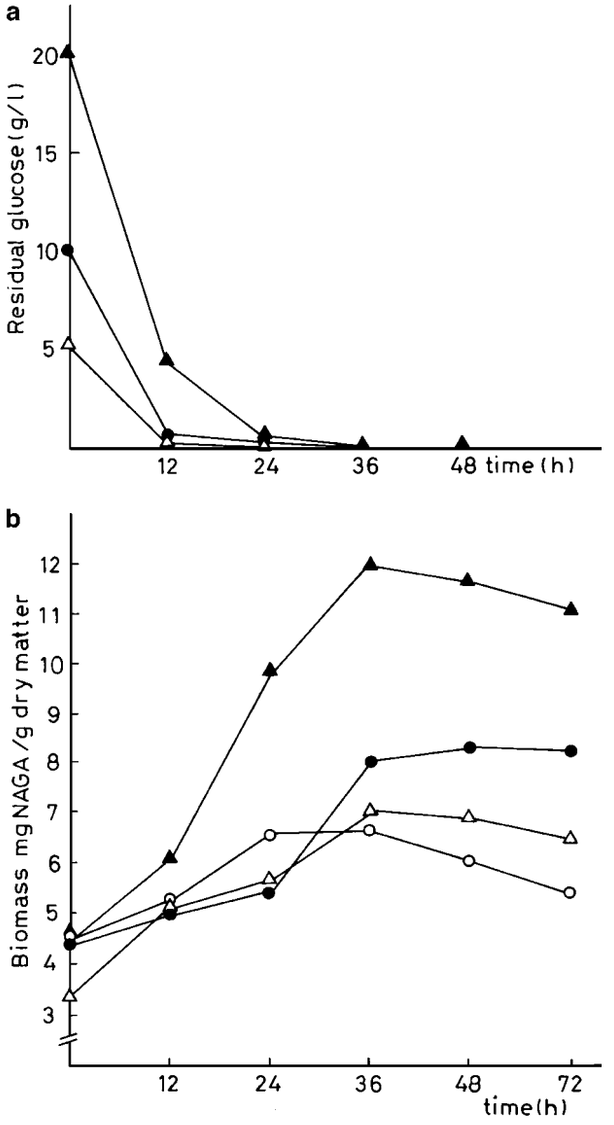

Figure 2 Glucose consumption (a) and biomass production (b) during PE and PG production in SSF; symbols as in Figure 1.

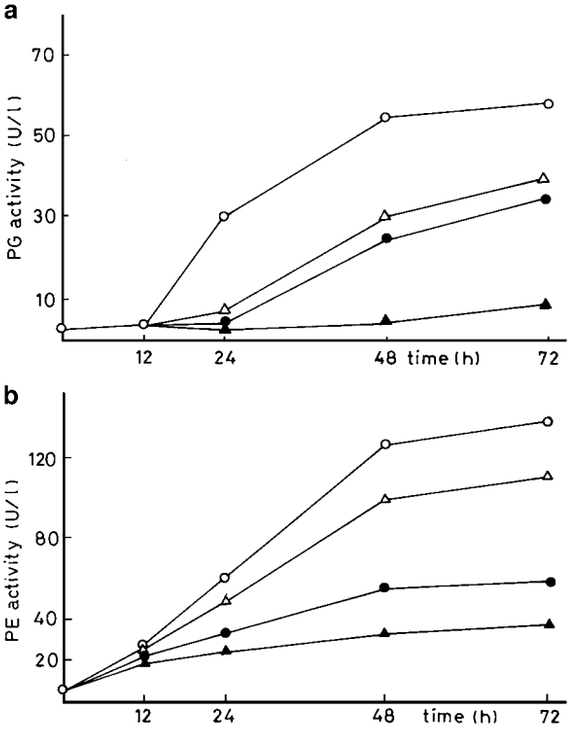

Figure 3 PE (a) and PG (b) yields by submerged fermentation system (SmF) with pectin (○) as a substrate and with glucose as a supplement [5 g L^{-1} (△), 10 g L^{-1} (●) and 20 g L^{-1} (▲)].

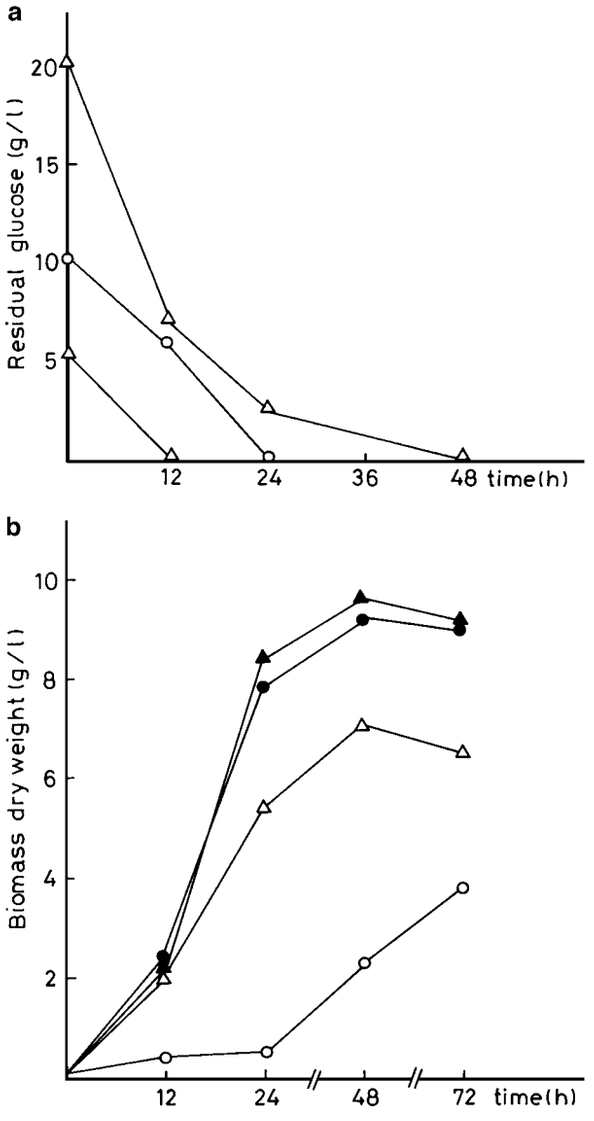

Figure 4 Glucose consumption (a) and biomass production (b) during PE and PG production in SmF; symbols as in Figure 2.

centration was increased, higher values were obtained with 20 g L^{-1} (Figure 4b).

Comparison of enzyme production between SmF and SSF systems

In SSF with pectin as a carbon source, PE and PG productions were achieved in 24 h and were about four and six times higher respectively than those observed in the SmF system. The addition of glucose in the SSF produced a stimulation of activities when compared to the same system with pectin alone. This behavior was in contrast with the antagonistic effect of sugar addition observed in SmF. The effect can be related to the higher rate of glucose consumption in SSF. In this system at 12 h, more than 95% of the initial sugar was consumed when the initial concentration was 5 or 10 g L^{-1} and 80% glucose was consumed in the medium with 20 g L^{-1} initial glucose.

In SmF, 95% of the sugar consumption was attained after 24 h when the initial glucose concentration was 10 or 20 g L^{-1}.

The enzyme production and the effect of glucose are true if the comparison is made using the results expressed as units L^{-1}. However, if the more useful values of enzyme productivity (Units g^{-1} dry cell per hour in SmF or Units g^{-1} NAGA per hour in SSF) are used in the comparison, the following conclusions can be established.

In SmF with the pectin medium, maximal productivities were obtained at 48 h, whereas in the SSF system, higher values were achieved in a shorter time (24 h). PE productivity in SSF was about three times higher than in SmF (3.16 and 1.14 respectively). For PG, the productivity values in SSF were about five times higher than that attained in SmF (2.28 and 0.48 respectively). The differences in productivity and the shorter times required for enzyme production favour SSF over SmF techniques.

With respect to the effect of glucose addition, differences were also observed in productivities. There is about 90% decrease of productivity for both enzymes (1.14 to 0.12 for PE and 0.47 to 0.054 for PG) in SmF when 10 g L^{-1} glucose was added. In SSF, the addition of the same glucose concentration produced about 45% inhibition of PE and PG productivities (3.16 to 1.81 for PE and 2.28 to 1.18 for PG) in 48 h, whereas at 24 h in the pectin medium, there was an increase of productivity of PE (41%) and PG (21%).

These results show that the enzyme production is affected in both fermentation systems but the sensitivity of enzyme synthesis to glucose is different. The existence of physiological differences between SSF and SmF is possible and it may be related to different patterns of pectinase production for each fermentation technique. Similar behavior was observed by Solis Pereira *et al* [28,29] in *A. niger* CH4 but enzyme yields reported in this strain were higher than those obtained by us. However, our results are at levels similar to those reported by Acuna *et al* [1] in the same strain of *A. niger* CH4.

Studies have indicated that under stress conditions, microorganisms show changes in plasma membrane composition [16]. The fatty acid composition of the cell membranes of *A. niger* was studied in both fermentation systems.

Results in Table 1 show that in the membranes of the *A. niger* cultivated in SSF there is an increase of unsaturated fatty acids C18:1 (oleic) and long chain saturated fatty acids C16:0 (palmitic) and C18:0 (stearic). There is not a significant difference in other fatty acids concentrations (C16:1 palmitoleic; C14:0 miristic). These changes may be related to the difference in glucose consumption and enzyme production.

Table 1 Fatty acid variations in plasma membrane of *Aspergillus niger*

Fermentation system	Palmitic acid	Estearic acid	Oleic acid
Submerged	17.3[a]	7.4	19.8
Solid state	31.6	16.4	29.4

[a]Results are expressed as % of total fatty acid concentration in plasma membrane of *Aspergillus niger*.

We can conclude that the production of PE and PG by *A. niger* is affected by the fermentation system used. Even in the presence of glucose, higher production was obtained in SSF than in SmF, suggesting a different level of sensitivity to catabolic repression. Changes observed in the concentration of unsaturated fatty acids of the plasma membrane could be related to stress conditions as reported by Lesage *et al* [17] who observed that a decrease in water activity of growing cultures of fungi leads to modifications in the phospholipid fatty acid saturation and consequently to reduction in the membrane fluidity and permeability. The inherent differences in mixing and nutrient diffusion between SSF and SmF could also be related to the differences observed.

We have established that the SSF system furnishes an important tool to obtain higher productivity of pectinases by *A. niger*, on the basis of greater enzyme yields and shorter fermentation times.

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