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Influence of pectin and glucose on growth and polygalacturonase production by *Aspergillus niger* in solid-state cultivation

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Abstract The solid-state production of endo- and exo-polygalacturonases (PG) by *Aspergillus niger* was studied in a media containing wheat bran, salts, and different citric pectin and/or glucose concentrations. Kinetic analysis of the process indicated that the formation of PG and the growth of *A. niger* are associated processes. By increasing citric pectin from 0 to 16% (w/w), the maximum *A. niger* concentration (X_m) was raised from 94 to 121 mg/g dry medium suggesting that pectin can be used by *A. niger* as a growth substrate besides its role as an inducer. With 16% (w/w) pectin, 281 U exo-PG/gdm and 152 U endo-PG/gdm were obtained. Otherwise, pectin concentrations from 20 to 30% (w/w) hindered both production and growth. *A. niger* concentrations of 108–113 mg/gdm were achieved in runs with glucose from 5 to 12% (w/w), whereas at 16 and 20% (w/w) glucose, lower X_m values (ca. 100 mg/gdm) were measured. The addition of glucose to the wheat bran medium, up to 10% (w/w) led to maximum endo-PG titers slightly lower than those found in the absence of glucose. Nevertheless, exo-PG formation in these media was strongly increased and activities over 370 U/gdm were achieved. The results suggest that in experiments with pectin concentrations until 16% (w/w), exo-PG production was repressed by pectin-degradation products although these same substances had favored biomass growth. When glucose concentrations over 10% (w/w) were added to the media, the maximum activities of both enzymes decreased drastically, suggesting that glucose at high concentrations also exerts a repressive effect on PG production.

Keywords Polygalacturonases · *Aspergillus niger* · Inducer · Production medium

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Introduction

Pectinases are a group of enzymes that attack and depolymerize pectin by hydrolysis and trans-elimination as well as by de-esterification reactions, which hydrolyze the ester bond between the carboxyl and methyl of pectin groups [4]. Among many different applications, especially in the food industry, these enzymes are widely used in the clarification of wines and fruit juices. Further applications include the maceration of vegetables to facilitate the extraction of oils and pigments [2, 15]. The filamentous fungus, *Aspergillus niger*, is most often used in the commercial production of pectinases because it is classified as 'generally regarded as safe' (GRAS) by the United States Food and Drugs Administration (US-FDA), which allows the use of its metabolites in the food industry [14].

Both submerged fermentation (SmF) and solid-state fermentation (SSF) can be used to produce pectinases. According to Solis-Pereyra et al. [17] and Maldonado and Strasser [11], much better results can be obtained in SSF as compared to SmF. Maldonado and Strasser [11] explain their results by the different responses observed in the regulation of pectinase synthesis by *A. niger*, depending on the fermentation technique used, that favor the SSF system.

Independent of the type of process, fermentation media normally contains carbohydrates, nitrogen sources, vitamins, mineral salts, and pectin or pectin-rich raw materials to induce the formation of pectinases [2]. In the present study, wheat bran was used as a support for SSF as well as a source of carbon, nitrogen, and other nutrients. Although wheat bran contains some pectin, a previous work showed that such an amount is not sufficient to obtain good PG levels [6].

Another important aspect to be considered in this comparison is the presence of simpler carbohydrates in the medium. In a study in which SmF and SSF production of pectinases by *A. niger* was compared, Solis-Pereyra et al. [16] reported that substrates such as

glucose, sucrose, and galacturonic acid strongly repressed the formation of these enzymes in submerged process, but did not have a major effect on the results when a solid-state reactor was used. In a further study, Solis-Pereyra et al. [17] examined the use of high concentrations of glucose in the SSF production of pectinases by *A. niger* and concluded that the presence of this carbohydrate in the medium had no repressive effect on pectinase formation, but glucose concentrations over 35% (w/w) led to a drastic drop in pH that affects the production. Dias-Godinez et al. [7] also compared solid-state and submerged processes and observed that in the SSF system the production of pectinases is enhanced due to a higher biomass level without catabolite repression and with low protease titers.

In this context, the aim of the present work was to evaluate the SSF production of endo- and exo-PG, two enzymes of the pectinases group, by *A. niger* in experiments carried out with different pectin and glucose concentrations. In the interpretation of results, PG synthesis, carbohydrate consumption, and biomass growth, indirectly estimated from respiratory parameters, were considered. Furthermore, a kinetic analysis of the process was evaluated.

Materials and methods

Microorganism

Aspergillus niger T0005007-2 (Universidad de Salta, Argentina) was used in all the experiments. The strain was plated onto glycerin agar [10] and incubated at 30°C for 5 days, plates being stored at 4°C until needed. Replicate cultures were made monthly.

Production media

Wheat bran was used as the support and main carbon source for all solid media. After inoculation, each 100 g of the reference medium had the following composition [6]: 36.6 g of wheat bran (Moinho Nordeste, Antonio Prado, Brazil), 6.0 g of citric pectin (Delaware, Brazil), 30.6 ml of concentrated salt solution, and a volume of suspension of *A. niger* spores in distilled water to complete 100 g of medium. The concentrated salt solution contained (g/l): $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 1.0; $FeSO_4.H_2O$, 6.3×10^{-5}; $ZnSO_4$, 6.2×10^{-5}; $MnSO_4$, 1.0×10^{-6}. Variations of this composition were tested by adding different amounts of citric pectin (2, 4, 12, 16, 20, 25, and 30 g) and/or glucose (5, 8, 10, 12, 16, and 20 g) to the medium as mentioned in the discussion of results.

The initial moisture of the media was adjusted to 63% by the addition of distilled water and the flasks were autoclaved at 121°C for 20 min.

Experimental conditions

The experiments were carried out in 800 ml Becher flasks, each containing 56 g of medium. The flasks were inoculated with suspensions of *A. niger* spores to provide an initial concentration of 7.10^7 spores/100 g and statically incubated at 30°C in a humidified atmosphere.

For each sampling point two flasks were removed and analyzed. In a preliminary experiment with the reference medium containing 6% (w/w) citric pectin and no glucose, samples were taken at short intervals and analyzed according to the techniques described below. In further experiments, samples were taken after 48 and 72 h of fermentation to quantify enzyme production and carbohydrate consumption, and also to determine pH and moisture level in the media, these process times being identified as those in which the maximum titers in exo- and endo-PG occurred, respectively. Additionally, total carbohydrate (TC) concentration was quantified also after 40 h of process when the culture reached the stationary phase. In all runs, oxygen uptake rates (OURs) values were measured every 2–4 h from 16 to 60 h of process and biomass growth curves could be made from these values for all conditions.

Analytical methods

The moisture of culture media was determined according to AOAC [3] and pH by drying samples to constant weight and suspending them in 10 ml of deionized water and measuring the pH of the resultant suspension in a pHmeter.

To quantify the TC content, 10 ml of 1.5 M H_2SO_4 were added to 0.7 g of sample and this suspension was hydrolyzed at 100°C for 30 min, neutralized with 4 M NaOH, and treated with potassium ferricyanide and cupric sulfate solutions to remove proteins. The concentration of soluble carbohydrate was estimated by the 3,5-dinitrosalicylic acid (DNS) method [12] as total reducing sugars. TC concentration is expressed as mg of reducing sugars per g of dry medium (g/gdm).

Endo- and exo-PG were extracted from the media by suspending 2.7 g of sample in 15 ml of water (pH 4) contained in 125 ml flasks shaken at 200 rpm in a reciprocal shaker, and the enzyme solutions were centrifuged to remove suspended solids.

Endo-PG activity was determined by measuring the reduction of viscosity of a standard citric pectin solution. For this estimation, 3.2 ml of diluted sample were mixed with 14.8 ml of a 1% (w/v) solution of citric pectin (Delaware, Brazil) in 0.05 M acetic acid-acetate buffer (pH 4) and the reaction mixture incubated at 30°C for 30 min, after which the viscosity was measured in a DV-II$^+$ viscometer (Brookfield Engineering, USA). One unit of endo-PG activity was defined as the quantity of enzyme that caused a 50% reduction in the viscosity of the reaction mixture after 30-min incubation at 30°C

[9]. Endo-PG activity in this study is expressed as units per gram of dry medium (U/gdm).

For the evaluation of exo-PG, 50 µl of diluted sample of culture media were added to 2 ml of a 0.25% (w/v) of polygalacturonic acid (SIGMA) in 0.05 M acetic acid-acetate buffer (pH 4) and the reaction mixture incubated at 35°C for 30 min, after which the quantity of reducing sugars liberated was measured by the Somogyi [18] method, using D-galacturonic acid (SIGMA) as a standard. One unit of exo-PG activity was defined as the quantity of enzyme which released 1 µmole of reducing sugar, measured as galacturonic acid, from polygalacturonic acid per milliliter of culture media per minute [5]. Exo-PG activity is expressed as units per gram of dry medium (U/gdm).

Biomass concentration was indirectly estimated from the values of OUR measured during the experiments as described for the submerged process by Zabriskie and Humphrey [19] and adapted by Maiorano [10] for SSF. This calculation is based on the relationship between OUR and the biomass growth rate (dX/dt) [13],

$$\mathrm{OUR} = \frac{1}{Y_{GO}}\frac{dX}{dt} + m_O X \quad (1)$$

where: X, biomass concentration; Y_{GO}, true cell yield from oxygen consumption; m_O, maintenance coefficient for oxygen.

The result of the integration of Eq. 1 is

$$X_n = \frac{Y_{GO}\cdot(\Delta t/2)\cdot[(\mathrm{OUR})_n + (\mathrm{OUR})_{n-1}] + [1 - m_O\cdot Y_{GO}\cdot(\Delta t/2)]\cdot X_{n-1}}{1 + m_O\cdot Y_{GO}\cdot(\Delta t/2)} \quad (2)$$

where: X_n and $X_{n\text{-}1}$, biomass concentration at cultivation times t_n and $t_{n\text{-}1}$; Δt, time interval from $t_{n\text{-}1}$ to t_n; OUR_n and $\mathrm{OUR}_{n\text{-}1}$, OURs at cultivation times t_n and $t_{n\text{-}1}$.

Equation 2 provides cumulative cell concentrations with time. The first value of $X_{n\text{-}1}$ must be estimated from the biomass concentration in inocula. The values for Y_{GO} and m_O were previously assessed by Fontana [8] in solid media as 0.014 g biomass/mmol O_2 and 2.75 mmol O_2/g biomass/h.

Results and discussion

Description of a solid-state fermentation run

A preliminary solid-state run was carried out to assess the main kinetic characteristics of either *A. niger* growth or the production of endo- and exo-PG.

As shown in Fig. 1a, after a lag-phase of 5–10 h, the biomass growth curve assumed an exponential profile for approximately 15 h, followed by decreasing growth rates up to the stationary phase after 48–50 h of process. During the exponential phase, the maximum specific growth rate was calculated as 0.17/h. As can be observed from the same figure, the variation of TCs with time followed the growth curve, with approximately 70% of sugars being consumed up to 30 h of process.

In Fig. 1b, one can observe the time course for the production of endo- and exo-PG. It was observed that the formation of exo- and endo-PG was associated with both *A. niger* growth and carbohydrate consumption. However, during the stationary phase, the exo-PG activity decreased whereas the titer in endo-PG presented a slight increase. The maximum values for enzymatic activities were 232 U/gdm in 48 h for exo-PG, and 135 U/gdm in 72 h for endo-PG.

The variation of pH with time, depicted in Fig. 2, showed the typical profile previously described in the literature [17] although higher values have been measured in our work probably due to the differences in medium composition. During the exponential growth phase, decreasing pH values, from 5.0 to 4.1, were measured. After the exponential phase, pH increased to values of about 5.2. In the same figure, it can be seen that the moisture content of the medium remained nearly constant and therefore this parameter did not affect the results. Such behavior for pH and moisture can be considered a pattern for this process since it was similar in all other experiments discussed in this study.

Production of polygalacturonases in media with different exogenous pectin concentrations

As previously mentioned, PG are inductive enzymes and therefore for their production by *A. niger* the presence of pectin as an inducer in the medium is needed. Then, this set of experiments was carried out to evaluate the effect of the addition of different exogenous citric pectin concentrations (0, 6, 12, 16, 20, 25, and 30% w/w) on the production of PG by the SSF process.

In Fig. 3, the growth curves for these runs are presented. As can be seen, the maximum biomass concentrations (X_m) for all experiments were attained in ca. 40 h. At this process time, significant amounts of TC were still present in the medium, varying from 62 to 110 mg TC/gdm as the initial citric pectin concentration was increased. By increasing citric pectin concentration from 0 to 16% (w/w), X_m was raised from 94 to 121 mg/gdm suggesting that the products of hydrolysis of pectin can be used by *A. niger* as an additional growth substrate to the carbohydrates contained in wheat bran besides its role as an enzyme inducer. On the other hand, citric pectin concentrations from 20 to 30% (w/w) resulted in lower X_m values. The maximum specific growth rates calculated from the curves presented in Fig. 3 for all conditions varied from 0.20 to 0.23/h, with the smaller values being related to media with 25 and 30%

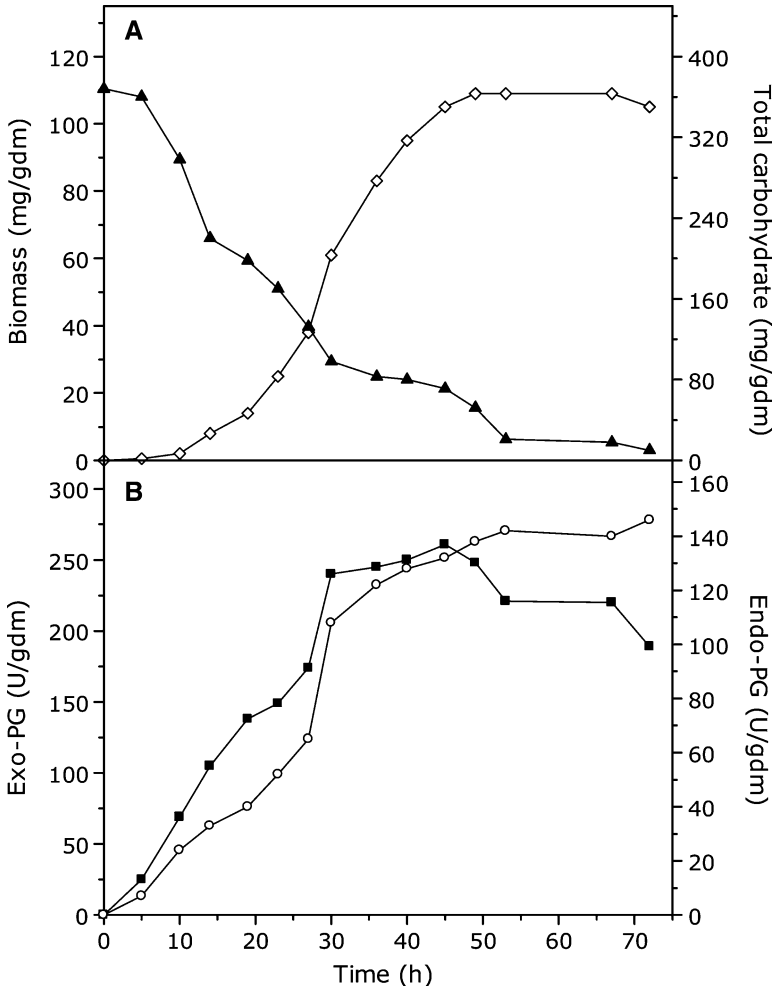

Fig. 1 Time course of *Aspergillus niger* cultivation in wheat-bran solid medium containing 6% (w/w) citric pectin. Biomass concentration (*open diamond*); total carbohydrate concentration (*filled triangle*); exo-polygalacturonase (PG) activity (*filled square*); endo-PG activity (*open circle*)

(w/w) pectin. This means that the poorer growth and carbohydrate consumption observed with high citric pectin concentrations cannot be attributed to an inhibition by the substrate but to other factors such as the limitation of some essential nutrient, due to the replacement of part of the wheat bran by pectin, or to the reduction of oxygen diffusion as a consequence of the hardening of the medium caused by the excess of pectin.

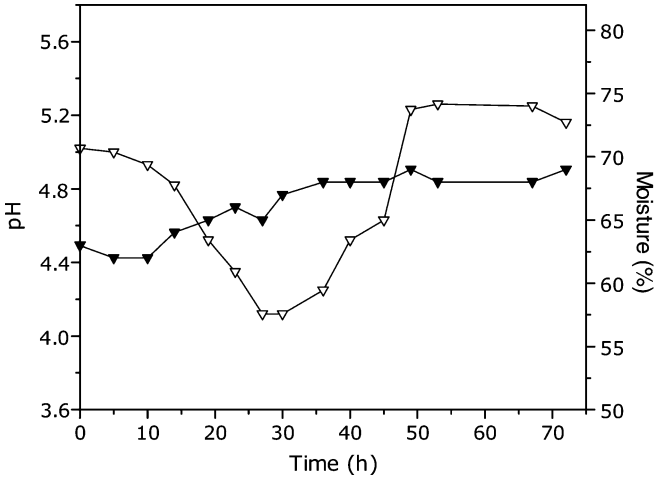

Fig. 2 Variation of pH (*inverted open triangle*) and moisture content (*inverted filled triangle*) with time during the cultivation of *A. niger* in wheat-bran solid medium containing 6% (w/w) citric pectin

In Fig. 4, the variation of exo- and endo-PG activities with the citric pectin content of each medium is shown for 48 and 72 h of process, respectively, with the best results in terms of enzyme production being achieved under the same conditions that favored biomass growth. Citric pectin concentrations from 0 to 16% (w/w) led to increasing activities of both enzymes but when the concentration of inducer was raised to 20–30% (w/w) the peaks of activities were reduced following the biomass growth. As shown in the figures, the maximum activities of exo-PG (281 U/gdm) and endo-PG (152 U/gdm) were obtained in 48 and 72 h of process, respectively.

The yields for biomass ($Y_{Xm/TC}$) and enzymes ($Y_{Exo\text{-}PG/TC}$ and $Y_{Endo\text{-}PG/TC}$) as compared to the total carbohydrates consumed are presented in Table 1. The highest $Y_{Xm/TC}$, $Y_{Exo\text{-}PG/TC}$, and $Y_{Endo\text{-}PG/TC}$ were found with the medium containing 16% (w/w) pectin, con-

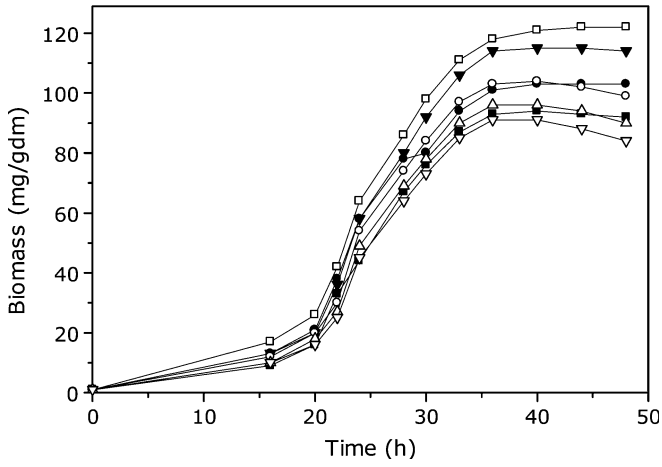

Fig. 3 Growth of *A. niger* in wheat-bran solid medium containing different concentrations of citric pectin (% w/w): 0 (*filled square*); 6 (*filled circle*); 12 (*filled inverted triangle*); 16 (*open square*); 20 (*open circle*); 25 (*open triangle*); 30 (*open inverted triangle*)

firming that biomass growth and formation of enzymes were similarly affected by the process conditions. Although experiments have clearly demonstrated that polygalacturonase formation and *A. niger* growth are associated processes, these results do not provide information on the possible occurrence of catabolic repression of exo- and endo-PG formation.

Production of polygalacturonases in media with citric pectin and different glucose concentrations

In the sequence, SSF runs with media containing 6% (w/w) citric pectin and glucose concentrations of 0, 5, 8, 10, 12, 16, and 20% (w/w). The aim of these experiments was to test the replacement of a part of the pectin by a simpler and cheaper carbon source for the growth of *A. niger* as well as to obtain more information on the induction/repression process of polygalacturonase production by this fungus.

Table 1 Yields for biomass and polygalacturonases (PG) in solid-state cultivation of *Aspergillus niger* in media with different exogenous citric pectin concentrations

Pectin (% w/w)	$Y^{a}_{Xm/TC}$ (mg/mg)	$Y^{b}_{Exo\text{-}PG/TC}$ (U/mg)	$Y^{c}_{Endo\text{-}PG/TC}$ (U/mg)
0	0.305	0.344	0.257
6	0.343	0.773	0.391
12	0.382	0.849	0.421
16	0.406	0.912	0.461
20	0.351	0.792	0.380
25	0.325	0.649	0.316
30	0.319	0.508	0.267

Biomass concentrations of 108–113 mg/gdm were achieved in 40 h in runs with initial glucose from 5 to 12% (w/w), whereas in media containing 16 and 20% (w/w) lower values (ca. 100 mg/gdm) were measured (Fig. 5). The maximum specific growth rates were almost identical for SSF experiments with 5 to 16% (w/w) glucose (ca. of 0.17/h) and slightly lower with the highest sugar concentration (0.16/h). Thus, as observed in tests with different citric pectin concentrations, the variation of X_m cannot be attributed to inhibition by the substrate.

As shown in Fig. 6, with the addition of glucose to the wheat bran medium in concentrations up to 10% (w/w), the maximum titers in endo-PG were slightly lower than those found in the media without glucose. Nevertheless, exo-PG formation in these media was strongly increased and activities over 370 U/gdm were achieved. These results suggest that in experiments with citric pectin concentrations, up to 16% (w/w) exo-PG production was repressed by pectin-degradation products although these same substances had favored biomass growth. Our results agree with those reported by Aguilar

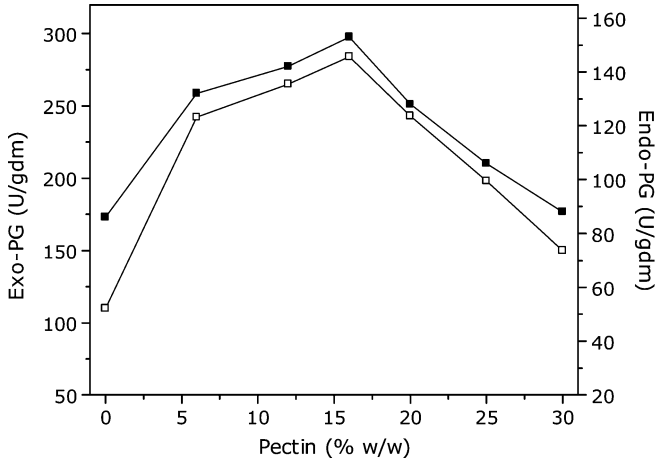

Fig. 4 Variation of exo-PG (*open square*) and endo-PG (*filled square*) activities with the initial concentration of citric pectin in wheat-bran solid medium (48 and 72 h of process, respectively)

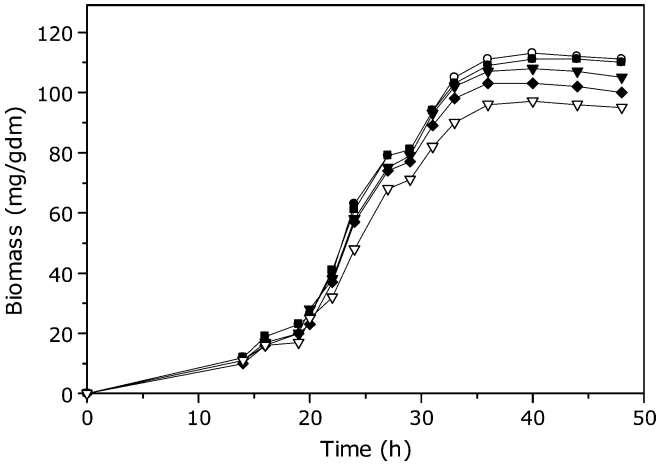

Fig. 5 Growth of *A. niger* in wheat-bran solid medium containing 6% (w/w) citric pectin and different concentrations of glucose (% w/w): 5 (*filled square*); 8 (*open circle*); 10 (*filled circle*); 12 (*inverted filled triangle*); 16 (*filled diamond*); 20 (*open inverted triangle*)

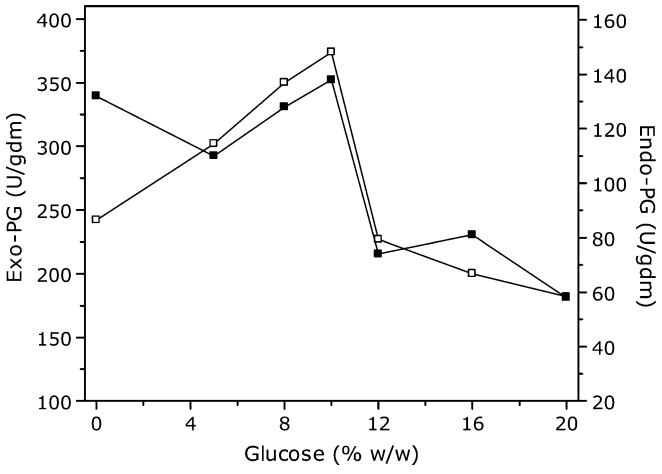

Fig. 6 Variation of exo-PG (*open square*) and endo-PG (*filled square*) activities with the initial concentration of glucose in wheat-bran solid medium containing 6% (w/w) citric pectin (48 and 72 h of process, respectively)

and Huitron [1], who analyzed the catabolic repression of pectinase formation by *Aspergillus* sp. in liquid medium, as measured by viscosimetry (endo-PG) and by reducing groups (exo-PG), and concluded that the first type of enzyme could be repressed either by galacturonic acid or glucose, whereas the second group was not affected by glucose. When glucose concentrations over 10% (w/w) were used in the solid media, the maximum activities of both enzymes decreased drastically. For the medium with 16% (w/w) glucose, for instance, the reduction of exo- and endo-PG activities was close to 40% as compared to the 10% (w/w) glucose medium. Since the reduction of X_m for the same runs was less than 4%, that effect cannot be directly related to this factor, suggesting that the presence of glucose in high concentrations also exerts a repressive effect on the production of PG. The previously mentioned conclusion of Solis-Pereyra et al. [17] that the decreasing activities of PG in SSF production with *A. niger* are due to the drop of pH, cannot be considered in the present work because, as a consequence of medium composition, the pH was never lower than 4.2 even when 20% (w/w) glucose was used.

In Table 2, the yields calculated for biomass and PG are shown. It can be seen that the conversion of TC into biomass and especially into exo-PG was increased in media containing glucose, with no remarkable effect of glucose on the formation of endo-PG.

From the previous experiments, we concluded that the addition of a limited amount of citric pectin, up to 16% (w/w), to the wheat bran medium has a positive effect on *A. niger* growth although the formation of exo-PG was repressed. In addition, we observed that such repressive effect could be avoided or strongly reduced by adding 6% (w/w) citric pectin and 10% (w/w) glucose to the medium. Thus, a further set of experiments was performed with media containing 10% (w/w) glucose

Table 2 Yields for biomass and PG in solid-state cultivation of *A. niger* in media with 6% (w/w) citric pectin and different glucose concentrations

Glucose (% w/w)	$Y^{a}_{Xm/TC}$ (mg/mg)	$Y^{b}_{Exo\text{-}PG/TC}$ (U/mg)	$Y^{c}_{Endo\text{-}PG/TC}$ (U/mg)
0	0.343	0.773	0.391
5	0.458	1.208	0.423
8	0.505	1.522	0.457
10	0.419	1.336	0.445
12	0.491	0.987	0.274
16	0.624	0.952	0.352
20	0.693	1.213	0.232

[a]Cell yield at 40 h of process (mg biomass/mg TC consumed)
[b]Exo-PG yield at 48 h of process (units/mg TC consumed)
[c]Endo-PG yield at 72 h of process (units/mg TC consumed)

and citric pectin at 0, 2, 4, and 6% (w/w) to check the possibility of achieving higher exo-PG titers with lower inducer concentrations.

In Fig. 7, it can be seen that with 2–4% (w/w) citric pectin, comparable X_m values (110–113 mg/gdm) were achieved, whereas with the medium with no added pectin, X_m was smaller (102 mg/gdm), that effect possibly being related to the lower TC content of this medium as confirmed by the similar $Y_{Xm/TC}$ calculated for these runs (ca. of 0.40 mg/mg).

As presented in Fig. 8, no significant difference of maximum titers in endo-PG was measured with citric pectin concentration from 2 to 6% (w/w), whereas in the absence of added inducer endo-PG activity decreased. Exo-PG seems to be more dependent on the concentration of inducer since the highest activities were obtained only with 4 and 6% (w/w) citric pectin.

The results of this study enabled us to show that the formation of PG and the growth of *A. niger* in SSF are associated processes. As such, to some extent, alterations in SSF medium could affect both enzyme production or biomass growth. It was confirmed that the

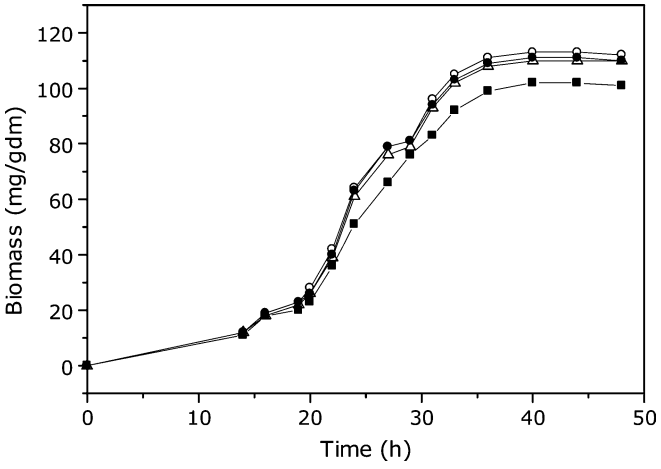

Fig. 7 Growth of *A. niger* in wheat-bran solid medium containing 10% (w/w) glucose and different concentrations of citric pectin (% w/w): 0 (*filled square*); 2 (*open triangle*); 4 (*open circle*); 6 (*filled circle*)

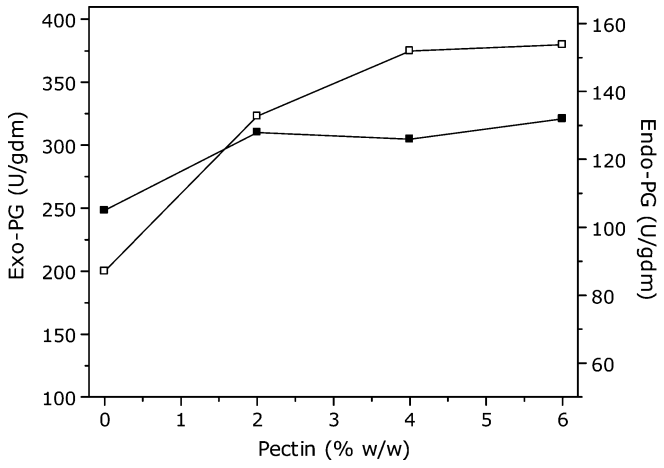

Fig. 8 Variation of exo-PG (*open square*) and endo-PG (*filled square*) activities with the initial concentration of citric pectin in wheat-bran solid medium containing 10% (w/w) glucose (48 and 72 h of process, respectively)

addition of well-defined concentrations of exogenous pectin and glucose to the wheat bran medium is indispensable to attain high titers in exo- and endo-PG. However, considering the cost of the purified pectin, other formulations, including pectin-rich low-cost residues such as apple or citrus bagasse, must be evaluated.

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