

# INFLUENCE OF PECTIN, GLUCOSE, AND pH ON THE PRODUCTION OF ENDO- AND EXO-POLYGALACTURONASE BY *Aspergillus oryzae* IN LIQUID MEDIUM

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**Abstract** - Endo- and exo-polygalacturonase (PG) production by *Aspergillus oryzae* IPT 301 was evaluated in a batch bioreactor in media containing 0, 10, and 20 g.L<sup>-1</sup> pectin and 0 to 30 g.L<sup>-1</sup> glucose. For cultivations in which the pH was not controlled, the use of 20 g.L<sup>-1</sup> pectin and 10 g.L<sup>-1</sup> glucose resulted in superior enzyme activities compared to control media with 0 and 10 g.L<sup>-1</sup> of the inducer and the same glucose concentration. Maximum endo-PG and exo-PG activities were 125.0 and 76.3 U.mL<sup>-1</sup>, respectively. For cultivations in which the pH was controlled to a minimum value of 2.7, media containing 20 g.L<sup>-1</sup> pectin and 20 to 50 g.L<sup>-1</sup> glucose were tested and significant improvement of both activities was attained. Maximum enzyme activities (175.0 U.mL<sup>-1</sup> for endo-PG and 103.0 U.mL<sup>-1</sup> for exo-PG) were obtained in pH-controlled batch experiments with 30 g.L<sup>-1</sup> glucose.

**Keywords:** *Aspergillus oryzae*; Polygalacturonases; Glucose; Pectin; pH control.

## INTRODUCTION

Pectinases, or pectinolytic enzymes, are naturally produced by plants, filamentous fungi, bacteria and yeasts (Gainvors *et al.*, 1994; Schuster *et al.*, 2002). This group includes enzymes that act on pectin, leading to its de-polymerization by hydrolysis (exo-polygalacturonase and endo-polygalacturonase) or trans-elimination (pectin lyase) and to its de-esterification (pectin methylesterase) by breaking the ester bond between the carboxyl and methyl groups of the polysaccharide (Ceci and Lozano, 1998). The denominations exo- and endo-polygalacturonase are related to the pattern of action, random or terminal, of these enzymes (Kashyap *et al.*, 2001).

Because of their ability to degrade pectic substances, pectinases are widely used in the food

industry to assist in the extraction and clarification of fruit and vegetable juices (Koponen *et al.*, 2008; Rodriguez-Nogales *et al.*, 2008; Sandri *et al.*, 2011; Laaksonen *et al.*, 2012).

Pectinases can be produced by solid-state or submerged processes, and filamentous fungi belonging to the genus *Aspergillus* are most often used (Blandino *et al.*, 2001; Patil and Dayanand, 2006; Rodríguez-Fernández *et al.*, 2011). Generally, the fermentative production of enzymes requires special attention to the composition of the culture medium. Media can contain components involved in the induction, repression or inhibition of enzyme formation and secretion (in the case of extracellular enzymes) (Blandino *et al.*, 2001; Galiotou-Panayotou *et al.*, 1997). Besides the carbon source, other medium components must be carefully chosen to achieve

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good results (Thomas *et al.*, 1996). According to Roumbouts and Pilnik (1980), culture media with an adequate balance of simple carbohydrates and pectin lead to the best production of pectinases in a submerged process. Due to their role as pectinase inducers, pectin or agricultural residues with high pectin content are essential components of the culture medium (Dhillon *et al.*, 2004).

Pectinase production is regulated in different fungal species, depending on the carbon source (Olsson *et al.*, 2003). Aguilar and Huitrón (1987) described a stimulating effect of galacturonic acid and glucose on the production of pectinases by *Aspergillus* sp., although the enzyme production was strongly repressed by high concentrations of these compounds. Similar results were published by Teixeira *et al.* (2000), who described a repression of endo- and exo-PG production in *Aspergillus japonicus* when concentrations of glucose, pectin and sucrose were above certain levels. Solis-Pereyra *et al.* (1993) and Acuña-Arguelles *et al.* (1995) also reported that an excess of glucose in solid media reduced the formation of pectinases by *Aspergillus niger*. These authors claim that this result was due to a strong drop in the pH of the medium rather than catabolic repression or growth inhibition.

Previous published data suggest that the production of pectinases by *A. oryzae* is associated with the variation of the culture medium pH. Ueda *et al.* (1982) evaluated the influence of pH on the formation and the composition of the *A. oryzae* A-3 pectinolytic complex and they observed significant differences between cultivations with and without pH control. These authors also related their results to the stability profile of each enzyme in the pectinase complex. Malvessi and Silveira (2004) studied the production of PG by *A. oryzae* and observed that the microbial growth was favored by pH values close to 4, while pH values around 3 were needed for endo- and exo-PG production.

The aim of this study was to evaluate the production of endo- and exo-PG (the hydrolases of the pectinase group) by *A. oryzae*, using media containing different concentrations of glucose and pectin. Additionally, the effect of pH on the process was assessed.

## MATERIALS AND METHODS

### Microorganism

*A. oryzae* IPT301 from the Instituto de Pesquisas Tecnológicas de São Paulo (Brazil) culture collection

was used in the experiments. The strain was propagated in glycerine agar medium and incubated at 30 °C for 5 days. After incubation, the sporulated cultures were stored at 4 °C or were used for the inoculation of production media.

### Culture Media

The influence of culture medium composition on fungal growth and on the production of endo- and exo-PG was evaluated. Wheat bran extract medium (WBE) (Fontana *et al.* 2009), modified with respect to the concentrations of pectin and glucose, was used as the basis for the formulations tested and contained (per liter of distilled water): aqueous wheat bran extract, 500 mL; citric pectin (ESKISA S.A., Brazil), 0-20 g; glucose, 0-50 g; yeast extract, 0.05 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.0 g; MgSO<sub>4</sub>, 0.5 g; KH<sub>2</sub>PO<sub>4</sub>, 2.5 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 6.3 × 10<sup>-4</sup> g; ZnSO<sub>4</sub>, 6.2 × 10<sup>-4</sup> g; MnSO<sub>4</sub>, 1 × 10<sup>-5</sup> g. To prepare the wheat bran extract, 80 g of wheat bran (Moinho NORDESTE, Antônio Prado, Brazil) was finely milled (10 mesh), mixed with 500 mL of distilled water and autoclaved at 1 atm for 15 minutes. After cooling, the mixture was filtered and the extract was used in the preparation of WBE medium. The wheat bran extract was prepared before each experiment.

In order to evaluate the effect of pectin concentration on the main fermentation parameters, WBE medium formulated with 0, 10 and 20 g.L<sup>-1</sup> of pectin and 10 g.L<sup>-1</sup> of glucose was assayed. In additional runs, the effect of glucose concentration was investigated in processes performed with or without pH control. First, WBE medium with 0, 5, 10, 15, 20, 25 and 30 g.L<sup>-1</sup> of glucose, 20 g.L<sup>-1</sup> of citric pectin and no pH control was assessed (experiments G0, G5, G10, G15, G20, G25 and G30). Afterwards, the process was evaluated in WBE medium with 20, 30 and 50 g.L<sup>-1</sup> of glucose, 20 g.L<sup>-1</sup> of citric pectin and with pH control (experiments G20\*, G30\* and G50\*). All media were autoclaved at 1 atm for 15 minutes.

### Operational Conditions

The experiments were carried out in batch mode using a 5-L BIOSTAT B bioreactor (B. BRAUN BIOTECH, Germany) with a working volume of 4 L and 3 flat-blade impellers. The temperature was controlled at 28 °C, and the initial pH was adjusted to 4.0 with 2 mol.L<sup>-1</sup> HCl or 3 mol.L<sup>-1</sup> NaOH. In experiments G0 through G30, no further pH control was done. In runs G20\*, G30\* and G50\* the pH was allowed to drop spontaneously from the initial value

of 4.0 to a minimum value of 2.7 and then controlled with automatic addition of 3 mol.L<sup>-1</sup> NaOH. The aim of the pH-controlled runs was to check whether the decreasing polygalacturonase activities achieved in experiments G15 though G30 could be related to the low pH values due to the increasing initial glucose concentrations. In an attempt to keep the dissolved oxygen concentration at non-limiting levels for the organism, the stirring speed and the specific air flow rate were varied from 300 to 750 rpm and 0.5 to 2.5 vvm (volume of air per volume of medium per minute), respectively.

Samples were collected periodically, centrifuged for 10 min at 10000 rpm, and stored at 4 °C for later analysis.

### Analytical Methods

The concentration of soluble reducing sugars was quantified by the dinitrosalicylic acid (DNS) method (Miller, 1959).

The endo-PG activity measurements were based on the viscosity reduction of a 0.63% (w/v) solution of citric pectin (ESKISA S.A., Brazil) prepared with 0.05 M acetate buffer (pH 4.0). The analysis mixture was 3.2 mL of sample conveniently diluted with 14.8 mL of the citric pectin solution. This reaction was carried out at 30 °C for 30 minutes and the viscosity of the reaction mixture was determined using a DV-II+ viscosimeter (BROOKFIELD ENGINEERING, USA). One endo-PG unit was defined as the quantity of enzyme that causes a 50% reduction in solution viscosity under the standardized conditions (Gainvors *et al.*, 2000). The exo-PG activity was estimated in a reaction mixture containing 50 µL of appropriately diluted sample and 2 mL of polygalacturonic acid (SIGMA, USA) in 0.1 M acetate buffer (pH 4). The exo-PG reaction was carried out at 35 °C for 30 minutes. The galacturonic acid released in the reaction was quantified by the Somogyi (1952) method. One unit of exo-PG was defined as the quantity of enzyme that leads to the release of 1 µmol of galacturonic acid per minute per milliliter of enzymatic extract (Couri and Farias, 1995). The activity of both enzymes was expressed in units per milliliter of medium (U.mL<sup>-1</sup>).

The spores used to inoculate the cultivation medium were enumerated by direct counting using a Neubauer chamber as previously described by Solis-Pereyra *et al.* (1993). The *A. oryzae* concentration, in grams of dry mass per liter, was gravimetrically determined by filtering a known

volume of culture medium through 0.45-µm-pore-size cellulose membranes (MILLIPORE, USA) and drying at 90 °C for 24 h.

The volumetric oxygen transfer coefficient ( $K_{La}$ ) in non-inoculated WBE medium was determined using the static method described by Moo-Young and Blanch (1989). For this determination, the stirring speed was 300 rpm, the specific air flow rate was 2.0 vvm, and the medium temperature was held at 28 °C.

### Statistical Analysis

Statistical tests were carried out by analysis of variance (one-way ANOVA) and the Tukey post-test using a probability level of less than 5% ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

### Endo- and Exo-Polygalacturonase Production in Medium Containing Different Pectin Concentrations

As already described in the present work, the addition of pectin to the cultivation medium is essential for inducing high titers of pectinase in *Aspergillus* fungi.

In WBE medium, the concentration of 20 g.L<sup>-1</sup> of citric pectin results in a viscosity of approximately 48 cP (at 30 °C), which is significantly higher than that verified in the medium without pectin (approximately 2.0 cP). When supplied with 10 g.L<sup>-1</sup> of pectin, WBE medium has a viscosity of approximately 18 cP.

Such an increase in viscosity leads to a decrease in the oxygen transfer rate to the liquid phase, which can become a serious operational problem, especially in large-scale processing. To assess the effect that pectin has on oxygen transfer in the medium, the  $K_{La}$  was measured in WBE medium formulations containing 0 or 20 g.L<sup>-1</sup> pectin. For the medium containing pectin, a  $K_{La}$  of 16 h<sup>-1</sup> was determined, and the  $K_{La}$  of WBE medium without pectin was 26 h<sup>-1</sup>. In addition to reducing the cost of the medium, the use of lower pectin concentrations could therefore improve the process by improving the efficiency of oxygen transfer.

To examine this hypothesis, experiments were conducted with WBE medium containing 0 and 10 g.L<sup>-1</sup> pectin and the results were compared to those obtained with the original WBE medium (20 g.L<sup>-1</sup> pectin). Table 1 shows the general results achieved in these experimental conditions.

**Table 1: Results obtained for *Aspergillus oryzae* IPT301 cultivation in WBE medium containing 10 g.L<sup>-1</sup> glucose and different initial concentrations of citric pectin.**

Citric pectin (g.L <sup>-1</sup> )	0	10	20
Viscosity (cP)	2.0	18	48
S <sub>0</sub> (g.L <sup>-1</sup> )	11.6	12.1	11.9
S <sub>cons</sub> (g.L <sup>-1</sup> )	9.60	10.9	9.60
X <sub>max</sub> (g.L <sup>-1</sup> )	11.9 ± 0.001 <sup>c</sup>	12.1 ± 0.1 <sup>ab</sup>	12.3 ± 0.2 <sup>a</sup>
t <sub>Xmax</sub> (g.L <sup>-1</sup> )	24	36	36
pH <sub>min</sub>	2.33	2.45	2.48
pH <sub>final</sub>	7.76	7.60	5.55
Endo-PG <sub>max</sub> (U.mL <sup>-1</sup> )	9.4 ± 0.1 <sup>c</sup>	67.5 ± 0.3 <sup>b</sup>	125.0 ± 0.2 <sup>a</sup>
t <sub>endo-PG max</sub> (h)	96	96	96
Exo-PG <sub>max</sub> (U.mL <sup>-1</sup> )	6.0 ± 0.2 <sup>c</sup>	47.5 ± 0.2 <sup>b</sup>	76.3 ± 0.3 <sup>a</sup>
t <sub>exo-PG max</sub> (h)	60	96	96

Means followed by the same letter are not significantly different at a level of 5% ( $p < 0.05$ )

S<sub>0</sub>, initial reducing substance concentration (expressed as glucose); S<sub>cons</sub>, reducing substance concentration (expressed as glucose) consumed;

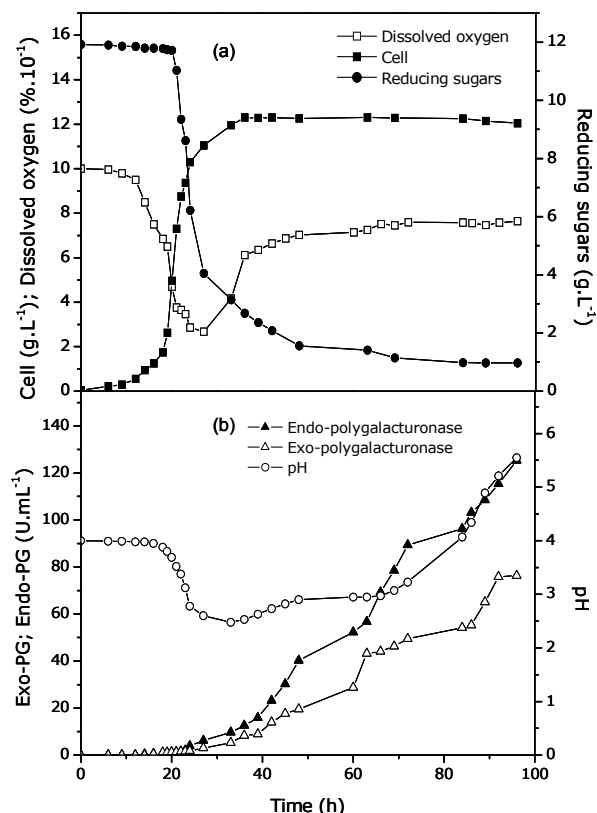
X<sub>max</sub>, maximum cell concentration; t<sub>Xmax</sub>, time for achieving X<sub>max</sub>; pH<sub>min</sub>, minimum pH value; pH<sub>final</sub>, pH value after 96 h of cultivation;

Endo-PG<sub>max</sub>, maximum endo-PG activity; Exo-PG<sub>max</sub>, maximum exo-PG activity; t<sub>PGmax</sub>, time for achieving endo-PG<sub>max</sub> and exo-PG<sub>max</sub>.

Typical time courses of the main process variables in cultivation in WBE medium with 20 g.L<sup>-1</sup> pectin are shown in Figure 1. As can be seen, after a lag phase of 10 h, the cellular growth was intense up to approximately 36 h of cultivation, when culture reached the stationary phase. A maximum cellular concentration of 12.3 g.L<sup>-1</sup> was achieved. The substrate consumption rates were very high from about 18 h until 30 h, followed by a considerable reduction until about 45 h. From this point on the substrate consumption stopped. After the lag phase, dissolved oxygen concentrations decreased rapidly due to microbial metabolism and remained at approximately 30% of saturation between 24 and 30 h. After that, dissolved oxygen concentrations rose to about 60% and remained almost constant until the end. The endo- and exo-PG activities showed non-growth-associated production and their maximum values were attained after approximately 96 h of cultivation (125.0 and 76.3 U.mL<sup>-1</sup>, respectively). During the growth phase, the pH dropped to approximately 2.5. After this time, increasing pH values were measured in association with PG formation (Figure 1). Such a pH profile has been already reported for *A. oryzae* by Malvessi and Silveira (2004) and Fontana *et al.* (2009) and is probably associated with the metabolism of the fungus itself. According to Carlile and Watkinson (1997), the medium pH in fungus cultivation is affected by the formation and consumption of organic acids, absorption of the nitrogen source and release of H<sup>+</sup> ions.

As expected, the control of dissolved oxygen was notably easier in reduced-pectin or pectin-free cultivation media. Similar maximum cell concentrations (approximately 12 g.L<sup>-1</sup>) were measured in all three media conditions, indicating that pectin has little effect on growth. However, higher endo- and exo-PG

activities were found in media with increased pectin content, demonstrating the essential role of pectin as an enzyme inducer for this process (Table 1 and Figure 1).



**Figure 1:** Time course of *Aspergillus oryzae* IPT301 cultivation in WBE medium with 20 g.L<sup>-1</sup> citric pectin and 10 g.L<sup>-1</sup> glucose. (a) Cell, reducing sugar, and dissolved oxygen concentration. (b) Endo-polygalacturonase (endo-PG) activity, exo-polygalacturonase (exo-PG) activity, and pH.

### Endo- and Exo-Polygalacturonase Production in Media Containing Different Glucose Concentrations

In previous works (Fontana *et al.*, 2009; Ottoni *et al.*, 2012), important effects of simple carbohydrates on the growth and enzyme production by *A. oryzae* IPT 301 were observed. As such, the effect of glucose concentration on the batch process was tested in WBE medium containing initial glucose concentrations up to 50 g.L<sup>-1</sup> in runs without pH control (G0 to G30) or with pH controlled to a minimum value of 2.7 (G20\* to G50\*). The results are presented in Table 2.

Increasing maximum concentrations of *A. oryzae* were obtained as the initial glucose concentration in WBE medium was increased. This positive effect of glucose concentration on the growth of filamentous fungi has been reported by Gibson *et al.* (1994) and by Taragano *et al.* (1997). An exception to this behavior was observed in run G50\*, in which a cell concentration of 13.7 g.L<sup>-1</sup> was measured, suggesting

that the high initial glucose concentration (approximately 50 g.L<sup>-1</sup>) may lead to some inhibition of cell growth.

As can be seen in Table 2, the formation of endo- and exo-PG was not dependent on cell concentration. Among experimental runs performed without pH control, the highest activities for both endo- and exo-PG were obtained with 10 g.L<sup>-1</sup> glucose (G10), whereas the remaining runs (experiments G0, G5, G15, G20, G25 and G30) had significantly lower titers.

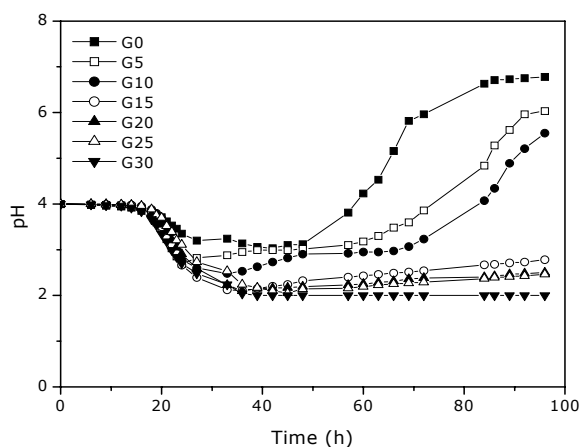
Figure 2 shows the variation of pH for experimental runs without pH control. With initial glucose concentrations up to 10 g.L<sup>-1</sup>, the characteristic kinetic behavior for pH was observed. However, as the initial glucose concentration was increased from 15 up to 30 g.L<sup>-1</sup>, the intense raise of the pH value was no longer noticed. In all cases of this group of experiments, lower and lower values of both minimum and final pH were measured as increasing amounts of glucose were added to the WBE medium (Table 2 and Figure 2).

**Table 2: Results obtained for *Aspergillus oryzae* cultivation in WBE medium containing 20 g.L<sup>-1</sup> citric pectin and different initial glucose concentrations. Cultivations were carried out with and without pH control.**

	G0	G5	G10	G15	G20	G25	G30	G20*	G30*	G50*
S <sub>0</sub> (g.L <sup>-1</sup> )	2.40	7.50	11.9	15.4	21.4	26.3	31.1	21.5	31.6	51.3
S <sub>cons</sub> (g.L <sup>-1</sup> )	-	6.90	10.9	14.2	20.1	25.0	29.7	19.0	30.5	47.2
X <sub>max</sub> (g.L <sup>-1</sup> )	11.0 <sup>l</sup>	11.9 <sup>l</sup>	12.4 <sup>lgh</sup>	12.6 <sup>fg</sup>	13.1 <sup>e</sup>	13.8 <sup>c</sup>	14.8 <sup>ab</sup>	12.8 <sup>fg</sup>	14.9 <sup>a</sup>	13.7 <sup>cd</sup>
t <sub>Xmax</sub> (g.L <sup>-1</sup> )	24	27	24	24	36	36	36	36	36	42
pH <sub>min</sub>	3.03	2.69	2.48	2.11	2.10	2.02	2.00	2.70	2.70	2.70
pH <sub>final</sub>	6.78	6.03	5.55	2.78	2.50	2.46	2.00	3.02	3.58	2.98
Endo-PG <sub>max</sub> (U.mL <sup>-1</sup> )	11.0 <sup>l</sup>	86.3 <sup>f</sup>	125 <sup>c</sup>	98.6 <sup>d</sup>	98.0 <sup>de</sup>	81.3 <sup>g</sup>	67.3 <sup>h</sup>	158 <sup>b</sup>	175 <sup>a</sup>	18.6 <sup>i</sup>
t <sub>endo-PG max</sub> (h)	72	96	96	96	96	96	96	96	96	96
Exo-PG <sub>max</sub> (U.mL <sup>-1</sup> )	4.86 <sup>l</sup>	62.7 <sup>c</sup>	76.3 <sup>c</sup>	68.8 <sup>d</sup>	61.5 <sup>ef</sup>	58.6 <sup>g</sup>	22.5 <sup>h</sup>	83.6 <sup>b</sup>	103 <sup>a</sup>	12.3 <sup>i</sup>
t <sub>exo-PG max</sub> (h)	92	96	96	96	96	96	89	96	96	96

Means followed by the same letter are not significantly different at a level of 5% ( $p < 0.05$ )

<sup>l</sup>pH controlled at a minimum value of 2.7 with 3 M NaOH



**Figure 2:** Time course of pH in batch cultivation of *Aspergillus oryzae* IPT301 without pH control. Cultivations were performed using WBE medium with initial glucose concentrations ranging from 0 to 30 g.L<sup>-1</sup> (G0 to G30).

One potential reason for the low activity titers in runs G15 to G30 (Table 2 and Figure 2) is that the pH of the cultures was too low for *A. oryzae* metabolism, hindering the enzyme production. Another possibility, according to Ueda *et al.* (1982), is that the low pH has a negative effect on the stability of enzymes. In this work, the effect of pH on the production of endo- and exo-PG was examined in the experiments G20\*, G30\* and G50. Evidence for pH-dependent expression of pectinolytic genes has also been pointed out for some *Aspergillus* strains (De Vries and Visser, 2001; Peñalva and Arst, 2002). As shown in Table 2, the activities of both endo- and exo-PG were dramatically increased in experimental runs with initial glucose concentrations of 20 to 30 g.L<sup>-1</sup> and minimum pH of 2.7 (experiments G20\* and G30\*). On the other hand, in WBE medium containing 50 g.L<sup>-1</sup> glucose (experiment G50\*), negligible PG activities were achieved, despite the control of pH and the considerable biomass production. As reported by other authors (Acuña-Arguelles *et al.* 1995; Solis-Pereyra *et al.* 1996), this result may be due to the catabolic repression exerted by the high glucose concentration.

## CONCLUSIONS

The results presented here confirm, as previously observed for different *Aspergillus* species, that the presence of high glucose concentrations in the cultivation medium can hinder the production of fungal pectinases. This hindrance is likely due to excessively low pH values as well as catabolic repression. The results indicate that the production of PG by *A. oryzae* is especially dependent on the composition of the cultivation media and the variation of the pH during the process.

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## NOMENCLATURE

DNS dinitrosalicylic acid  
Endo-PG endo-polygalacturonase

Endo-PG <sub>max</sub>	maximum endo-PG activity	
Exo-PG	exo-polygalacturonase	
Exo-PG <sub>max</sub>	maximum exo-PG activity	
G0, G5, G10, G15, G20, G25 and G30	experiments in WBE medium with initial glucose concentrations of 0, 5, 10, 15, 20, 25 and 30 g.L <sup>-1</sup> without pH control	
G20*, G30* and G50*	experiments in WBE medium with initial glucose concentrations of 20, 30 and 50 g.L <sup>-1</sup> and pH controlled to a minimum value of 2.7	
K <sub>L</sub> a	volumetric oxygen transfer coefficient	h <sup>-1</sup>
pH <sub>final</sub>	pH value at the end of a cultivation	
pH <sub>min</sub>	minimum pH value in a cultivation	
S <sub>0</sub>	initial reducing substance concentration (expressed as glucose)	g.L <sup>-1</sup>
S <sub>cons</sub>	reducing substance concentration (expressed as glucose) consumed	g.L <sup>-1</sup>
t <sub>PGmax</sub>	time for achieving endo-PG <sub>max</sub> and exo-PG <sub>max</sub>	
t <sub>Xmax</sub> , WBE medium	time for achieving X <sub>max</sub> wheat bran extract medium	h
X <sub>max</sub>	maximum cell concentration	g.L <sup>-1</sup>

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