

Production and Characterization of Endo-Polygalacturonase from *Aspergillus niger* in Solid-state Fermentation in Double-Surface Bioreactor

Diogo Henrique Hendges*, Queli Montanari, Eloane Malvessi and Mauricio Moura da Silveira

Instituto de Biotecnologia; Universidade de Caxias do Sul; C. P.: 1352; 95001-970; Caxias do Sul – RS - Brasil

ABSTRACT

Endo-polygalacturonase (endo-PG) production by Aspergillus niger T0005/007-2 in solid medium with 170 mm of height was evaluated in a cylindrical double surface bioreactor in 96-h experiments. Cell concentration close to 92 mg.g⁻¹dm (mg per g of dry medium) in the standard condition (static) was achieved, whereas in tests under forced aeration of 1.4 and 2.8 L.min⁻¹.Kg⁻¹mm (L of air per minute per Kg of moist medium) and with the central shaft fungal biomass attained approximately 100 mg.g⁻¹dm. Superior endo-PG activity was obtained with the central-shaft system, 78 U.g⁻¹dm (units per g of dry medium). Forced aeration and pressure pulse showed no positive effect on the production of endo-PG, 45 U.g⁻¹dm and 28 U.g⁻¹dm, respectively. None of the conditions evaluated was efficient for medium temperature control. Endo-PG was stable up to 40°C. The activity decreased in 50% after 120 minutes at 50°C, which is a temperature normally found during this process.

Key words: *Aspergillus niger*, double-surface bioreactor, endo-polygalacturonase, solid-state fermentation, enzyme stability

INTRODUCTION

Pectinases are enzymes that break down pectic substances and include two groups – deesterification and depolymerization enzymes. Endo-polygalacturonase (endo-PG) is a depolymerizing enzyme, which is used in food, textile and paper industries (Kashyap et al., 2001; Kaur et al., 2004). In association with cellulases, endo-PG is used for essential oil extraction (Pedruzzi et al., 2001).

In solid-state fermentation (SSF), the microorganisms grow over a moist solid matrix, which simulates their natural conditions (Pandey,

2003; Hölker and Lenz, 2005; Singhania et al., 2009). SSF uses simple medium and low moist content; however, mass and heat transfer are limited by simple diffusion (Mitchell et al., 1992). Different configurations of SSF bioreactors have been described, such as rotating drum, column, and tray reactors. The last one is widely used at industrial scale (Mitchell et al., 1992; Mitchell et al., 2000; Durand, 2003). New designs have been tried to improve mass and heat transfer (Schmidell and Facciotti, 2001; Singhania et al., 2009; Mitchell et al., 2010). The efficient temperature control in *Bacillus thuringiensis* CM-1 cultivation in an aerated tray bioreactor with internal

*Author for correspondence: diogo@utfpr.edu.br

circulation of air was reported by Hongzhang et al. (2002), who observed temperature gradients of only 0.5°C during the process. Using a similar design, Fujian et al. (2002) reported that 0.20 MPa pressure coupled to internal air circulation favored heat and mass transfer in *Penicillium decumbens* JUA 10 culture. Tao et al. (1999) showed the positive influence of internal pressure over heat and mass transfer in tray reactor culture of *Trichoderma viride* SL-1. Pulsed bioreactor facilitated the heat dissipation during the cultivation of *Bacillus pumillus* 1.1625 for protease production (Aijun et al., 2005).

Aeration rate and gas composition into the reactor influenced *Aspergillus niger* growth and protease formation in SSF (Villegas et al., 1993). Mo et al. (2004) observed that air flow rate of 7 L.min⁻¹ facilitated the production of cellulase by *P. decumbens*, whereas a higher flow (10 L.min⁻¹) affected negatively the process, probably due to the drying of the medium.

This work aimed to study the production of endopolygalacturonase (endo-PG), a hydrolase of the pectinase group, by *Aspergillus niger* T0005/007-2 in solid-state fermentation in double-surface bioreactor (DSB). In this system, the use of different air flow rates, the application of pressure pulses coupled to forced aeration, and the adaptation of a central shaft to the medium bed were tested and the effects of these conditions on cell growth, enzyme production, and temperature control were assessed.

MATERIALS AND METHODS

Microorganism

Aspergillus niger T0005/007-2 was obtained from the Departamento de Bioquímica, Fundação Universidade Federal de Rio Grande (FURG-RS) and used in this work. For microorganism maintenance and spore production, a medium described by Maiorano (1982) was used.

Culture medium and inoculum

The culture medium was formulated with wheat bran (35.6% w/w), citric pectin (Farmaquímica – Porto Alegre, RS, Brazil) (3.0% w/w), glucose (9.4% w/w), and nutrients salts solution (29.6% w/w), sterilized at 1 atm for 20 minutes.

Inoculation was done with suspensions of *A. niger* spores to provide a concentration of 10⁷ spores.g⁻¹

of moist medium and a moisture content of approximately 60%.

Experimental conditions

The double-surface bioreactor (DSB) used in this work was built with a PVC tube with 300mm height x 100mm diameter. Inside the tube, a nylon grid was installed at 65 mm from the bottom to hold the solid medium. In all experiments, the height of medium bed was 170 mm. Lateral holes allowed temperature measurement in different regions of the medium during the process.

As standard condition, both sides of DSB were covered with a thin layer of gauze, which allowed gas and heat changes with the external environment by simple diffusion.

Alternatively, DSB was operated under forced aeration through the medium, aiming to improve mass and heat transfer. In this case, both sides of DSB were closed with PVC covers and air inlet and gas outlet tubes were adapted to the extremities of the reactor. The air to be introduced in DSB was previously filtered and driven through a humidifier column to heat and saturate with water. Three flow rates were evaluated: 1.4, 2.8 and 8.5 L.min⁻¹.Kg⁻¹.mm (liters of air per minute per Kg of moist medium).

Another configuration tested for DSB was a pressure pulse system. For this purpose, the gas outlet duct was closed, causing the increase of the internal pressure up to 0.5 atm, and then opened to allow the instant decompression. The aim of this procedure was to expand the solid medium layer and improve heat and mass transfer through it. Similar designs have been described earlier in the literature (Tao et al., 1999; Zhao et al., 2001; Hongzhang et al., 2002; Zhang et al., 2003; Mo et al., 2004; Aijun et al., 2005).

Furthermore, the adaptation of a central shaft system to the bed medium, without aeration, was evaluated. The shaft was built with an 8 mesh screen, with 10 mm diameter and 170 mm height. In this experiment, the conditions were the same of the standard experiment.

All these processes were carried out in humidity saturated stove at 30°C for 96 h.

ANALYTICAL METHODS

Microorganism growth was indirectly estimated from oxygen balance data as described by

Zabriskie and Humprey (1978) and adapted to *A. niger* solid cultivation by Fontana (2005).

The moisture content of the medium was determined gravimetrically (A.O.A.C., 1998) with 1.0 g sample. To the dried sample, 10 mL of distilled water was added and the pH was measured in the liquid phase.

Endo-PG activity was estimated by the reduction of viscosity of a citric pectin solution (0.63% w/v) in acetate buffer (0.05M, pH 4.0) at 30°C for 30 min. One unit of endo-PG was defined as the amount of enzyme that reduced the solution viscosity by 50% under assay conditions (Maiorano, 1990).

Endo-PG activity and thermostability were evaluated by varying the temperature from 20 to 70°C and from 25 to 70°C, respectively. In thermostability tests, the maximum time of crude broth exposure to each temperature evaluated was 120 minutes, the samples being taken every 15 minutes during the first hour of test and then every 30 minutes.

The results regarding fungal growth and enzyme formation were compared statistically by one-way ANOVA and Tukey test with 5% level.

RESULTS AND DISCUSSION

With the operating conditions of the DSB, lower

colonization of the medium was achieved in the standard condition compared to the process carried out under forced aeration and with the central shaft and better than pressure pulse system.

The variation of biomass concentration of *A. niger* during the process in different experiments is presented in Figure 1. Maximum biomass concentration obtained under the standard condition was 92 mg.g⁻¹dm (mg per g of dry medium). With forced aeration system of 1.4 and 2.8 L.min⁻¹.Kg⁻¹mm and with the shaft system, an increase in the biomass concentration to approximately 100 mg.g⁻¹dm was observed. This increase could be associated due to the better colonization found in the deeper regions of the medium bed.

When a flow rate of 8.5 L.min⁻¹.Kg⁻¹mm was employed, the microorganism growth decreased (76 mg.g⁻¹dm), probably due to drying observed in both surfaces of the medium bed, indicating that the air humidifier system was not efficient for this high flow rate. Mo et al. (2004) reported that high air flow rates affected *P. decumbens* growth and cellulase production.

With the pressure pulse system, the fungal growth decreased (28 U.g⁻¹dm) possibly as a consequence of mycelium disruption when the pressure shock was applied, as also observed by Hongzhang et al. (2005), reaching 73 mg.g⁻¹dm. Table 1 resumes the results of fungal growth at 96h of process.

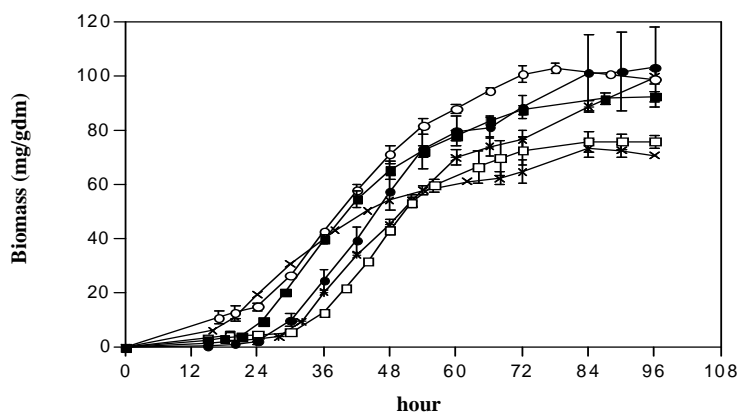


Figure 1 - Biomass variation during the process in solid-state culture of *Aspergillus niger* under the different conditions evaluated. (■) standard condition; air flow rates: (○) 1.4, (●) 2.8 and (□) 8.5 L.min⁻¹.Kg⁻¹mm; (×) pressure pulses coupled to the forced aeration (1.4 L.min⁻¹.Kg⁻¹mm); (*) central shaft.

Table 1 - Maximum biomass concentration reached in the different experiments.

Condition	Biomass (mg.g ⁻¹ dm)
Standard Condition	92.3 ^b ±2.54
1.4 L.min ⁻¹ .Kg ⁻¹ mu	103 ^a ±2.54
2.8 L.min ⁻¹ .Kg ⁻¹ mu	103 ^a ±20.9
8.5 L.min ⁻¹ .Kg ⁻¹ mu	75.8 ^c ±3.57
Pressure pulse system	73.3 ^c ±4.74
Central shaft	99.7 ^a ±3.74

Biomass^{a,b,c} – different letters are statistically different at 5% level.

As shown in Table 2, maximum enzyme activity was obtained with the central shaft system adapted to the medium bed after 96 h of process (78 U.g⁻¹dm, which was about 25% higher than that achieved under the standard condition (63 U.g⁻¹dm). The last one was similar to the obtained with

forced aeration with flow rate of 1.4 L.min⁻¹.Kg⁻¹mm. With pressure pulse system, a significant decrease in enzyme production was found (28 U.g⁻¹dm), following the inferior cell growth attained in this experiment.

Table 2 - Maximum endo-PG activity in the different experiments.

Condition	Endo-PG (U.g ⁻¹ dm)	Time (h)
Standard	63.2 ^b ±4.03	96
1.4 L.min ⁻¹ .Kg ⁻¹ mu	62.6 ^b ±1.58	96
2.8 L.min ⁻¹ .Kg ⁻¹ mu	54.8 ^c ±14.8	96
8.5 L.min ⁻¹ .Kg ⁻¹ mu	44.9 ^d ±1.70	96
Pressure pulse system	28.0 ^e ±6.78	72
Central shaft	78.5 ^a ±3.39	96

Endo-polygalacturonase^{a,b,c,d,e} – different letters are statistically different at 5% level.

In this work, in all the experiments, no evident effect on the heat dissipation from medium bed of *A. niger* culture was found as described in the literature (Tao et al., 1999; Zhang et al., 2003; Mo et al., 2004; Aijun et al., 2005), since temperatures around 50°C were measured, between 36 and 41h of process, remaining close to 46-47°C until the end of the cultivations. As such, one can suggest that the higher endo-PG activity obtained with a central shaft adapted to the bed medium was associated with the larger fungal growth, probably due to better mass transfer – oxygen supply and carbon dioxide release – to deeper settings of the bed. However, further studies using central shaft are needed to understand completely this system.

The influence of the temperature on endo-PG activity and thermostability was evaluated. As shown in Figure 2, among the different conditions tested, the maximum activity of endo-PG was found at 50°C, which was approximately the double of the activity obtained under the standard analytical condition (30°C). Malvessi and Silveira (2004) reported that temperatures between 35-

40°C were ideal for the activity of endo-PG produced by *Aspergillus oryzae* in submerged process.

Nevertheless, when this temperature (50°C) was applied to estimate the thermostability of endo-PG, an enzyme inactivation of approximately 50% was observed after 120 minutes of treatment (Figure 3). On the other hand, after the same time of treatment at 25, 30 and 40°C, almost all enzyme activity was preserved. The total inactivation of endo-PG was quickly noticed after about 15 minutes at 60 and 70°C, as reported by Naidu and Panda (2003) for polygalacturonase produced by *A. niger* in submerged process. This study corroborated the conclusions reported by Daniel (1996), who suggested the low stability of enzymes at temperatures higher than the ideal for the microbial growth.

By comparing the results found in these tests, particularly those performed at 40 and 50°C, one can confirm the importance of fermentation temperature control to avoid, or to reduce, enzyme denaturation during the own production process.

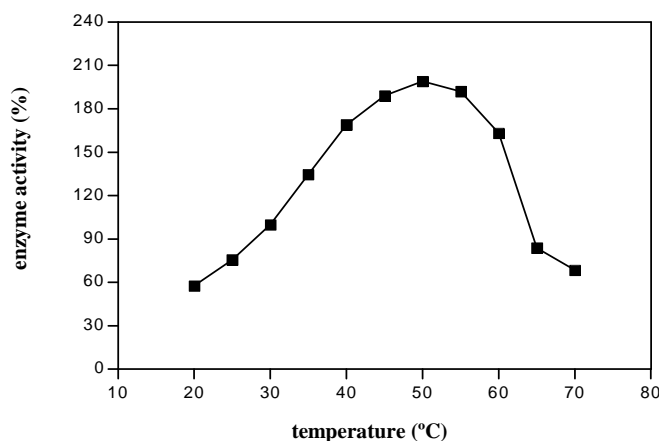


Figure 2 - Variation of endo-PG activity produced by *Aspergillus niger* in solid-state fermentation with the temperature. The value of 100% presents the standard condition of enzyme reaction (30°C).

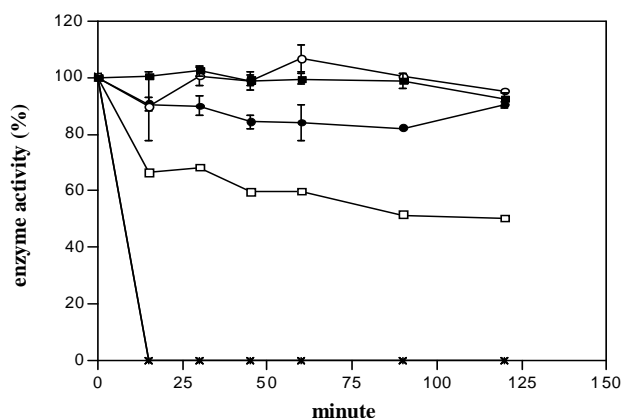


Figure 3 - Thermostability of endo-PG produced by *Aspergillus niger* in solid-state fermentation after previous treatment at different temperatures. (■) 20°C, (○) 30°C, (●) 40°C, (□) 50°C, (×) 60°C; (*) 70°C.

CONCLUSIONS

The results of the present work showed that the use of strategies such as forced aeration or air pressure pulses had no positive effect on the production of endo-polygalacturonase by the strain T0005/007-2 of *A. niger* in comparison to static conditions.

From the data obtained, it is possible to suggest that the use of one or more perforated shafts along a double-surface bioreactor is a simple and more efficient solution to the task of improving mass transfer in solid-state fermentations.

For temperature control, however, further efforts are needed to develop a simple bioreactor design that allows an efficient heat release from the

medium. In this case, it is interesting to highlight the remarkable difference in endo-PG stability at 40 and 50°C indicating that even a small improvement in temperature control could lead to substantial gains in production by avoiding enzyme denaturation.

ACKNOWLEDGMENTS

The authors acknowledge the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Universidade de Caxias do Sul (UCS) for the financial support of this work.

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Received: August 28, 2009;

Revised: February 02, 2010;

Accepted: September 14, 2010.