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Production of Cellulolytic and Hemicellulolytic Enzymes From *Aureobasidium pulluans* on Solid State Fermentation

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Abstract

This article investigates a strain of the yeast *Aureobasidium pullulans* for cellulase and hemicellulase production in solid state fermentation. Among the substrates analyzed, the wheat bran culture presented the highest enzymatic production (1.05 U/mL endoglucanase, 1.3 U/mL β-glucosidase, and 5.0 U/mL xylanase). Avicelase activity was not detected. The optimum pH and temperature for xylanase, endoglucanase and β-glucosidase were 5.0 and 50, 4.5 and 60, 4.0 and 75°C, respectively. These enzymes remained stable between a wide range of pH. The β-glucosidase was the most thermostable enzyme, remaining 100% active when incubated at 75°C for 1 h.

Index Entries: Cellulases; endoglucanase; hemicellulases; solid state fermentation; β-glucosidase.

Introduction

The hydrolytic action of cellulases and hemicellulases is of fundamental importance to obtain fermentable sugars from lignocellulosic biomass. These can be used as fermentation substrates to produce liquid fuels, food products, or other chemicals of interest *(1,2)*. The enzymatic hydrolysis of cellulose into glucose involves the synergistic action of at least three different enzymes: endoglucanase or endo-β-1,4-glucanase (EC 3.2.1.4), exoglucanase or exocellobiohydrolase (EC 3.2.1.91), and β-1,4-glucosidase or cellobiase (EC 3.2.1.21). Endoglucanase hydrolyze the polymers internally, resulting in a reduction of the degree of polymerization, whereas the exoglucanases act by removing units of cellobiose from either the reducing or the nonreducing

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ends of the molecule. β-glucosidase hydrolyzes cellobiose and other cellodextrins into glucose. β-glucosidase is responsible for the control of the entire speed of the reaction exerting a crucial effect on the enzymatic degradation of the cellulose, preventing the accumulation of cellobiose *(3,4)*. The β-glucosidase can also be used by the food industry to increase the bioavailability of the isoflavones in the human intestine, and by the beverage industry to stabilize the coloration of juices and wines *(5)*.

Because of xylan heterogeneity, the enzymatic hydrolysis of xylan requires different enzymatic activities. Two enzymes, β-1,4-endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37), are responsible for hydrolysis of the main chain, the former attacking the internal main-chain xylosidic linkages and the latter releasing xylosyl residues by means of endwise attack of xylooligosaccharides *(6)*. However, for complete hydrolysis of hemicellulose, side chain cleaving enzyme activities are also necessary, such as, α-L-arabinofuranosidases (EC 3.2.1.55), endomannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25), and α-galactosidases (EC 3.2.1.22) *(7)*. Solid state fermentation (SSF) is a well-known process for enzyme production and is defined as fermentation involving solids in absence (or near absence) of free water; however, the substrate must possess enough moisture to support growth and metabolism of microorganisms *(8)*. There is a current tendency to apply the SSF process in the development of bioprocesses to attain products with higher added values, such as antibiotics, alkaloids, organic acids, biopesticides, biofuel, aromatic compounds, and enzymes *(8,9)*.

Previous works described the production cellulases and hemicellulases using SSF and agricultural residues *(2,10–12)*. Brazil is an agroindustrial country known for its production of soy, corn, sugar cane, cassava, coffee, and so on, and for its high consumption of wheat, which generate large amounts of residues that have considerable potential for SSF applications *(13)*. On a previous study of screening of cellulolytic microorganisms, a strain of *A. pullulans* with high β-glucosidase activity was isolated. The objective of this work was the study of the production and the characterization of cellulolytic and hemicellulolytic enzymes secreted by *A. pullulans* using agroindustrial residues on SSF.

Material and Methods

Microorganism

The yeast *A. pullulans* ER-16 was isolated from orange juice residues in Catanduva, São Paulo State, Brazil. The stock culture was preserved in potato dextrose agar at 4°C.

Enzyme Production

The microorganism was cultivated using four different types of substrates: wheat bran, soy bran, soy peel, and corn cob. In order to evaluate

enzymatic production, samples were removed every 24 h throughout the period of 144 h.

Inoculation

The yeast was cultivated in 125-mL Erlenmeyer flasks containing 20 mL of potato dextrose agar medium for 48 h at a temperature of 28°C. A suspension was obtained by softly scraping the culture medium surface using 25 mL of mineral solution. Inoculation in the substrate was carried out by transferring 5 mL of the suspension into Erlenmeyer flasks containing the previously prepared production medium.

Fermentation

SSF was carried out in 500-mL Erlenmeyer flasks containing 5 g of moistened substrates (grounded to 2–3 mm size) with 10 mL of mineral solution aiming an initial humidity content of 75%. The mineral solution was made up of 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.1% NH_4NO_3 (w/v). After the inoculation of the microorganism, the fermentation was incubated at 28°C. Enzyme extraction was achieved by adding 50 mL of distilled water to each flask followed by 2 h on a rotary shaker at 80 rpm. Crude extracts were centrifuged (10,000*g*/20 min), and then the supernatant was used for enzyme activities assays.

Enzyme Assays

Xylanase, endoglucanase, and avicelase enzymes activities were measured by determining the release of reducing sugars by the 3,5-dinitrosalicylic acid method *(14)*. The 100 m*M* sodium acetate buffer pH 5.0 was used containing 0.5% of xylan (Birchwood-Sigma), 0.5% of carboxymethylcellulose (C5768 Sigma), and 0.5% of avicel (Co Sigma) as substrates for xylanase, endoglucanase, and avicelase enzymes, respectively. β-glucosidase activity was determined using 50 µL of the extract, 250 µL of 100 m*M* sodium acetate buffer pH 5.0, and 250 µL of 4 m*M* 4-nitrophenyl β-D-glucopyranoside (PNPG, Sigma). After 10 min, the reaction was stopped by the addition of 2 mL of 2 *M* sodium carbonate. The activities were measured at 410 nm and expressed in international units, defined as the amount of enzyme required to produce 1 µmole of nitrophenol (β-glucosidase), xylose (xylanase), and glucose (CMCase and avicelase) per minute, under assay conditions.

Enzyme Properties

The crude enzyme obtained in SSF using wheat bran as substrate was used for enzyme characterization. The activities were determined according to the standard conditions described above, except for the optimum temperature, which was determined in the range of 50–90°C and optimum pH between 3.0–8.0 in 100 m*M* McIlvaine buffer. For pH stability, enzymes were stored for 1 d at room temperature (about 25°C) diluted in 100 m*M*

McIlvaine buffer (pH 3.0–8.0), 100 m*M* Tris/HCI (8.0–9.0), and 100 m*M* glycine-NaOH (9.0–11.0). For temperature stability, enzymes were incubated at different temperatures (30–80°C) for 1 h. Both stabilities were determined by measuring the residual activities under standard conditions at optimum pH and temperature.

Results and Discussion

Enzyme Production

A. pullulans produced β-glucosidase in all substrates tested, but the production in wheat bran was found to be highest 1.3 U/mL after 120 h (Fig. 1A). The β-glucosidase production obtained in the present work is more than average results found in the literature. Iembo et al. *(10)* related a maximum production of 0.5 U/mL by *Aureobasidium* sp. in 168 h of culture in which wheat bran was used as substrate in SSF. Saha et al. *(15)* used wheat bran and corn bran as carbon source for *A. pullulans* cultivation in submerged fermentation (SMF), and obtained a maximum β-glucosidase production of 0.27 U/mL after 96 h. Wen et al. *(16)* related a β-glucosidase production of 0.0978 U/mL in *Trichoderma reesei* cultivation in SMF by using bovine manure as carbon source.

Wheat bran is a complex substrate rich in proteins (14%), carbohydrates (27%), minerals (5%), fat (6%), and B-vitamin *(17)*, this probably favored the growth and the production of enzymes for the microorganism. Previous works *(3,10–12,18)* report the production of cellulases and hemicelulases, using derived wheat (bran and straw) as substrate. The highest endoglucanase and xylanase production was obtained after 96 h of fermentation in wheat bran—1.05 and 5.0 U/mL, respectively. No endoglucanase production was detected in soy bran and corn-cob cultivation. In addition, no xylanase activity was detected (Figs. 1B,C) in soy bran cultivation. Avicelase activity was not detected from any of the substrates. Avicelase is the activity responsible for crystalline cellulose degradations and it is frequently found in low concentrations or absent on the cellulolytic system of many microorganisms *(19)*. This work confirms that the *A. pullulans* is among these. Previous studies *(1,11,12)*, with other species of fungi, relate endoglucanase and β-glucosidase productions with absence or low avicelase activity.

The xylanase and endoglucanase production for *A. pullulans* was not significant when compared with the production of fungi. Panagiotou et al. *(12)* reported 15.2 U/mL of endoglucanase and 92 U/mL of xylanase, in SSF, from the filamentous fungus *Fusarium oxysporum* using corn bran as substrate. Jecu *(3)* related an endoglucanase production of 14.8 U/mL in *Aspergillus niger* cultivation in a mixture of wheat straw and wheat bran in the ratio of 9 : 1, in SSF. Wen et al. *(16)* reported an endoglucanase production of 12.22 U/mL from *T. reesei*. Jorgensen et al. *(7)* obtained approx 38 U/mL of xylanase from *Penicillium brasilianum*, in SMF. Enzymes

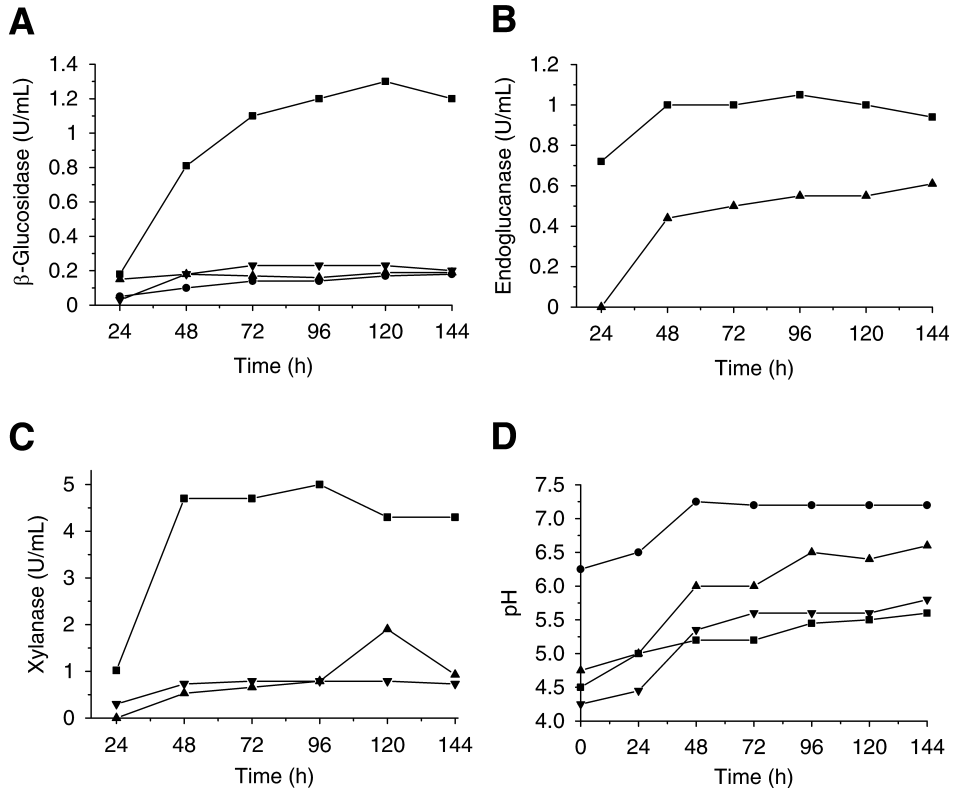

Fig. 1. Time-course of enzymatic production by *A. pullulans* in different cellulosic substrates. **(A)** β-glucosidase production, **(B)** endoglucanase production, **(C)** xylanase production, **(D)** pH variation during the fermentative process. (■) wheat bran, (●) soy bran, (▲) soy peel, (▼) corn cob.

production from yeasts is less than the production by filamentous fungi when cultivated on solid substrates. This may be explained because the growth conditions in SSF are very similar with their natural habitat, which favors the spreading of mycelium and consequently, results in a larger production of enzymes and better growth *(8,9)*. Diverse factors can influence enzyme production, such as pH, temperature, type of fermentation, carbon source used, and water availability (SSF). When cultivating *Thermoactinomyces thalophilus* by SSF, Kohli et al. *(20)* found an initial xylanase activities of 6.4 U/mL. After the attainment of the ideal conditions for the microorganism cultivation, the authors related a xylanase production of 42 U/mL in SMF.

A tendency for the pH to increase during the SSF was observed with all substrates tested, and was higher between 24 and 72 h of fermentation. No major alterations were observed after this initial increase, tending to stabilization (Fig. 1D). The pH variation fermentation was caused by the microorganism's metabolic activity, and may increase or decrease according

to what is secreted or consumed in the culture medium. The pH increase may be caused by organic acid consumption, such as citric, acetic, and lactic, or the release of ammonium salts resultant from the hydrolysis of protein and urea *(9,21)*. As proteolytic activity was observed in fermented extracts (data not shown), medium alkalinization was possibly caused by hydrolysis of protein as well as by the microbial metabolic action on the salts added for medium enrichment, such as ammonium sulfate and ammonium nitrate.

Enzyme Properties

The optimum pH and temperature of β-glucosidase produced by *A. pullulans* in this study was determined to be 4.0–4.5 and 75°C, respectively. This enzyme remained stable within a broad range of pH 4.5–10.0 and its original activity was constant after 1 h at 75°C (Fig. 2). When compared with other microbial β-glucosidases found in literature *(4,22–24)*, this enzyme presented a very high level of thermal stability. Previous works *(10,15,25)* described the production of β-glucosidase from *Aureobasidium* sp. and *A. pullulans* as having noticeable stable levels of both pH and temperature. Other research conducted with stable enzymes disclosed very little difference between stable and unstable enzymes regarding amino acid's sequence. Slight alterations in the molecule, such as an increase in hydrophobicity, ionic interaction, and disulfide bridge may lead to major changes in the stability of a molecule *(26)*. Thermostable enzymes are commonly produced by thermophilic microorganisms *(27)*. *A. pullulans* has an optimum growth at around 28°C typical of mesophilic microorganisms. Future studies of β-glucosidase produced by *A. pullulans* may contribute to the understanding of evolutionary interactions between thermophilia and mesophilia, besides presenting characteristics that allow its use in biotechnological processes.

The optimum pH and temperature obtained for endoglucanase were 4.0–4.5 and 60°C, respectively. Xylanase had a maximum activity at pH 5.0 and with temperature at 50°C and remained stable within a wide pH range. Xylanase remained stable after 24 h between pH 3.0–8.0 and maintained 53% of its original activity in pH 11.0. Endoglucanase remained stable within pH 3.5–7.5, maintaining only 54% of its catalytic activity when incubated at pH 8.0. Both enzymes had a considerable reduction in their activity after 1 h of incubation at temperatures more than 50°C (Fig. 2). Thermal inactivation is commonly observed in xylanases and endoglucanases produced by mesophilic microorganisms *(18,28–30)*.

The application of microbial xylanases for biobleaching of pulp by the paper industry is intrinsically related to the absence of cellulases in the fermented medium *(18)*. The hydrolytic action of such enzymes on cellulose fibers may decrease the quality of pulp resulting in inferior paper *(30)*. Hence, the presence of cellulolytic enzymes in the enzymatic extract

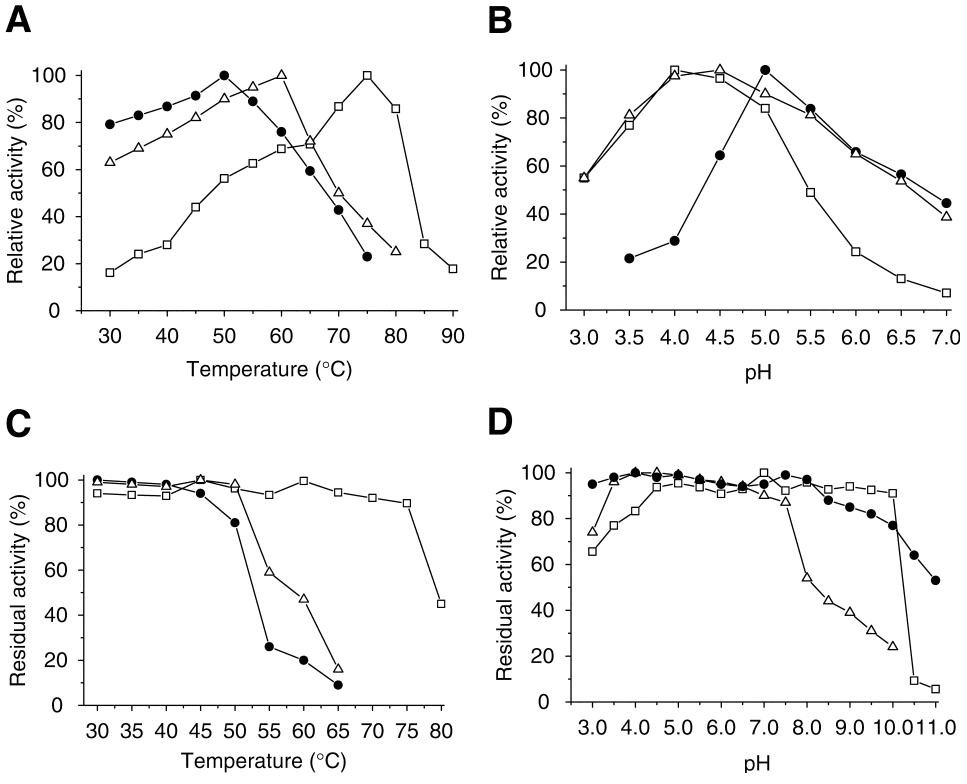

Fig. 2. Enzyme activities and stability relative to pH and temperature: **(A)** effect of temperature on the enzyme activities, **(B)** effect of pH on the enzyme activities, **(C)** temperature-stability curve, **(D)** pH-stability curve. (□) β-glucosidase, (●) xylanase, (△) endoglucanase.

produced by *A. pullulans* in SSF along with the low level of activity of xylanase in alkaline pH, impairs its use by the paper industry. On the other hand, the synergistic action of such enzymes benefits the attainment of fermentable sugars from agroindustrial residues that can be used to develop alternative fuels. This fact prompts us to continue with studies of these enzymes in order to contribute to the development of new sources of energy.

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