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Effect of initial moisture content on two Amazon rainforest *Aspergillus* strains cultivated on agro-industrial residues: Biomass-degrading enzymes production and characterization

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ABSTRACT

Production of biomass-degrading enzymes using inexpensive and readily available agricultural residues as substrates for solid-state fermentation (SSF) can contribute to a broader application of enzymes for the conversion of biomass into biofuels and chemicals. Among the operational parameters that affect SSF process efficiency, moisture content is one of the most important. This work evaluates the effect of initial moisture content on two *Aspergillus* strains (*Aspergillus niger* P47C3 and *Aspergillus fumigatus* P40M2), isolated from the Amazon rainforest and grown under SSF. Analyses were made of the biomass-degrading enzymes produced using different agro-industrial residues as carbon sources (wheat bran, sugar cane bagasse, soybean bran, and orange bagasse). The enzymatic complex produced by a selected strain of *A. fumigatus* was characterized in terms of optimum pH and temperature, and thermal stability. The most effective carbon sources for multienzyme production during *Aspergillus* cultivation were wheat and soybean bran, as well as a 1:1 mixture of sugar cane bagasse and wheat bran. Much higher activity values were achieved for β-glucosidase (105.8 IU/g) and xylanase (1055.6 IU/g) when wheat bran with 50% initial moisture content was used as substrate. Under this condition, endoglucanase and total cellulase activity values were 56.6 IU/g and 5.0 FPU/g, respectively. Characterization of the crude enzymatic complex showed that the *A. fumigatus* P40M2 enzymes were active in the acidic pH range, with maximal activities at the range of 50–65 °C, demonstrating the potential of the organism for the production of acidophilic and thermophilic biomass-degrading enzymes.

1. Introduction

The conversion of lignocellulosic materials into biofuels and other chemicals can be achieved using a multienzyme system composed predominantly of cellulases and xylanases, acting in synergy. However, the major bottleneck for a broader application of enzymes in the process of biomass conversion is their high cost. Cellulolytic enzyme production under solid-state fermentation (SSF), using inexpensive and easily available agricultural residues as substrates, can contribute to cost reduction (Sukumaran et al., 2009). In addition, growth of filamentous fungi using SSF is advantageous since the solid medium simulates the natural habitat of these organisms (Singhania et al., 2009). This benefit is extended to the production of enzymes, with SSF yielding greater productivity when compared to submerged fermentation (SmF) (Raghavarao et al., 2003). In SSF processes, the water content is one of the most important operational parameters affecting process efficiency. If the moisture content is too high, the void spaces in the solids are filled with water, resulting in oxygen limitation. At the other extreme, if the moisture content is too low, microorganism growth will be hindered (Raghavarao et al., 2003). Consequently, identification of the optimal moisture content for each solid substrate is crucial to promote favorable growing conditions and maximize the production of metabolites.

The effect of the initial moisture content of the substrate on the production of biomass-degrading enzymes by SSF has been described in the literature. Singhania et al. (2007) found that higher initial moisture levels had a negative effect on cellulase production by *Trichoderma reesei* grown on wheat bran under SSF. Mamma et al. (2008) evaluated enzyme production in SSF using orange peel as substrate and the fungus *Aspergillus niger*, and were able to significantly increase enzyme activities after optimizing

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the initial moisture content of the solid medium. Gao et al. (2008) found that increased initial moisture content enhanced enzyme activity during SSF cultivation of the thermoacidophilic fungus *Aspergillus terreus* M11 using corn stover. Similarly, Brijwani et al. (2010) and Farinas et al. (2011) found a positive effect of initial moisture content on cellulase production under SSF.

The optimal moisture content depends on both the solid substrate and the microorganism used. The choice of fungal strains with high expression capacity and a diversity of cellulolytic enzymes can contribute to obtaining the enzymatic complex required to hydrolyze plant biomass. In this context, Brazil's high level of biodiversity is suggestive of an environment that could potentially be explored in the search for microorganisms possessing the desired characteristics for this application (Delabona et al., 2012). In addition to identifying the conditions required for higher enzyme productivity, another important issue is that the enzymes need to fulfill special requirements in terms of optimum pH and temperature, and be thermo stable.

This work investigates the effect of initial moisture content on enzyme production by two *Aspergillus* strains isolated from the Amazon rainforest (*A. niger* P47C3 and *Aspergillus fumigatus* P40M2). Analyses were made of the biomass-degrading enzymes produced during cultivation under SSF, using different agro-industrial residues as carbon sources. The enzymatic complex produced by a selected strain of *A. fumigatus* was characterized in terms of its optimum pH and temperature, and thermal stability.

2. Materials and methods

2.1. Microorganisms

The wild-type *Aspergillus* strains used in this study were *A. niger* P47C3 and *A. fumigatus* P40M2. These strains were isolated by screening soil and decomposed wood samples from the Amazon forest reserve of EMBRAPA Eastern Amazon (Brazilian Company for Agricultural Research), located in Belém, Brazil (Delabona et al., 2012). Stock cultures were maintained on potato dextrose agar (PDA) plates at 4 °C.

2.2. Agro-industrial residues

The sugar cane bagasse used as solid substrate was kindly provided by Edra Eco Sistemas (Ipeúna, SP, Brazil). It was used without any pretreatment and a particle size between 1 and 2 mm was selected. Exploded sugar cane bagasse was kindly provided by a local sugar cane mill (Usina Nardini, Vista Alegre do Alto, SP, Brazil). Soybean and wheat bran where purchased from a local store. Orange bagasse was kindly provided by a local orange processing industry (Citrosuco, Matão, SP, Brazil). These materials were milled with a knife mill and classified to particle size between 1 and 2 mm. Characterization of materials in terms of C:N ratio was done using an elemental analyzer CHNS/O (2400 series II, PerkinElmer). All processed material was stored in sealed plastic bags and maintained refrigerated at 4 °C until experimentation.

2.3. Enzyme production

Solid-state fermentation was used for fungal cultivation and evaluation of the enzymatic complexes produced using different carbon sources. For preparation of fungal inoculum for fermentation, 2 mL of sterile distilled water containing 0.1% Tween 80 was added into sporulated slants containing each fungus, and the spores were dislodged into the liquid by gentle pipetting. The fungi were cultured in 250 mL Erlenmeyer flasks containing 5 g of substrate and an appropriate volume of mineral salt medium (Mandels and Sternberg, 1976) required to achieve the desired initial moisture content (50, 60, 70, or 80%, w/w). The substrate bed height was approximately 1.5 cm. Each flask was sterilized by autoclaving at 121 °C for 20 min, and then inoculated with 107 spores per g of raw material. Dried and ground lignocellulosic materials (wheat bran, exploded sugar cane bagasse, sugar cane bagasse, sugar cane bagasse and wheat bran (1:1), soybean bran, orange bagasse, and orange bagasse and wheat bran (1:1) were used as substrates. The flasks were incubated at 37 °C for 5 days. Samples (whole flasks) were withdrawn at 24-h intervals and the enzymes were extracted by adding 50 mL of citrate buffer (pH 5.0, 50 mmol/L) and stirring at 120 rpm for 30 min. The solids were separated by centrifugation at 10,000 × *g* (at 4 °C) for 15 min, and the enzymatic activity of the supernatant was assayed.

2.4. Enzyme assays

Filter paper cellulase (FPase), endoglucanase, xylanase, and β-glucosidase activities were determined. All enzymes were analyzed according to the standard procedure recommended by the IUPAC Commission on Biotechnology (Wood and Bhat, 1988), with some modifications. FPase activity was assayed by incubating the properly diluted enzyme extract (0.1 mL) with 0.9 mL of citrate buffer (50 mmol/L, pH 5.0) containing Whatman No. 1 filter paper (50 mg, 1 cm × 6 cm). The reaction mixture was incubated at 50 °C for 60 min. Endoglucanase activity was measured using 0.1 mL of properly diluted enzyme and 0.9 ml of a 4% (w/v) solution of carboxymethylcellulose (CMC) (Sigma, St. Louis, USA) in citrate buffer (50 mmol/L, pH 5.0). This mixture was incubated at 50 °C for 15 min. Xylanase activity was determined under conditions similar to those described above, except that a 1% solution of birchwood xylan (Sigma) was used as substrate. Finally, the β-glucosidase activity was determined using cellobiose (Sigma) as substrate, and quantifying the sugars released with an enzymatic glucose measurement kit (Laborlab, São Paulo, Brazil). All experiments were performed in duplicate. One unit of FPase or endoglucanase activity corresponds to 1 μmol of glucose released per minute. One unit of xylanase activity corresponds to 1 μmol of xylose released per minute. The quantification of reducing groups was performed using the dinitrosalicylic acid (DNS) method (Miller, 1959). All enzymatic analyses were carried out in duplicate. Results were expressed as activity units per mass of initial dry solid substrate (IU/g).

2.5. Partial crude enzyme characterization: influence of pH and temperature, and thermal stability

The temperature profiles for the activities of endoglucanase, FPase, xylanase, and β-glucosidase (using supernatants produced under the optimal SSF production conditions) were obtained by assaying the activities at different reaction temperatures (30, 35, 40, 45, 50, 55, 60, 65, and 70 °C) in 50 mmol/L sodium citrate buffer (pH 5.0). The effect of pH on enzyme activities (at 50 °C) was determined using different reaction buffers: 50 mmol/L glycine–HCl (pH 3.0); 50 mmol/L sodium citrate (pH 3.5–6.0); 50 mmol/L citrate phosphate (pH 6.5–7.0); 50 mmol/L phosphate (pH 7.5–8.0); and 50 mmol/L Tris–HCl (pH 8.5–10.0). For thermal stability determination, the crude supernatant obtained under the optimal production conditions was incubated at 60 °C for 1 h, in the absence of substrate. The residual enzyme activity was measured after different time intervals. At the end of the incubations, the test tubes containing the enzymes were immediately cooled by placing them on ice, and were then kept at 4 °C overnight. Measurement of enzyme activity was performed under standard pH and temperature conditions.