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Understanding the cellulolytic system of *Trichoderma harzianum* P49P11 and enhancing saccharification of pretreated sugarcane bagasse by supplementation with pectinase and α-L-arabinofuranosidase

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HIGHLIGHTS

- The Amazon rainforest *Trichoderma harzianum* is a potential candidate for saccharification of plant biomass.
- Important roles for pectinase and α-L-arabinofuranosidase in the enzymatic synergies.
- Sugarcane bagasse hydrolysis is more effective and competitive for second generation ethanol.

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ABSTRACT

Supplementation of cellulase cocktails with accessory enzymes can contribute to a higher hydrolytic capacity in releasing fermentable sugars from plant biomass. This study investigated which enzymes were complementary to the enzyme set of *Trichoderma harzianum* in the degradation of sugarcane bagasse. Specific activities of *T. harzianum* extract on different substrates were compared with the extracts of *Penicillium echinulatum* and *Trichoderma reesei*, and two commercial cellulase preparations. Complementary analysis of the secretome of *T. harzianum* was also used to identify which enzymes were produced during growth on pretreated sugarcane bagasse. These analyses enabled the selection of the enzymes pectinase and α-L-arabinofuranosidase (AF) to be further investigated as supplements to the *T. harzianum* extract. The effect of enzyme supplementation on the efficiency of sugarcane bagasse saccharification was evaluated using response surface methodology. The supplementation of *T. harzianum* enzymatic extract with pectinase and AF increased the efficiency of hydrolysis by up to 116%.

1. Introduction

The conversion of lignocellulosic biomass into fuels and other chemicals can be achieved using a multi-enzyme system acting in synergy. An enzymatic cocktail containing different enzymes involved in the hydrolysis of each part of the lignocellulosic structure is crucial to increase enzymatic hydrolysis (EH) yields (Alvira et al., 2011; Berlin et al., 2007; Gao et al., 2011). In addition, the viable bioconversion of biomass requires not only that the cost of the enzymes be reduced, but also that improvements be made in the effectiveness of the enzymatic extracts used in the hydrolysis mixture. These aspects are highly dependent on both the raw material and the pretreatment employed (Delabona et al., 2012a; Jorgensen and Olsson, 2006; Sorensen et al., 2011; Sukumaran et al., 2009). On-site production of enzymes can help to reduce enzyme costs since there is less need to stabilize the enzyme preparations, avoiding costs associated with transport and storage for long periods. Furthermore, the use of the same lignocellulosic biomass for enzyme production and hydrolysis could reduce the production costs of bioethanol, since both process could be co-located and share infrastructure and utilities (Delabona et al., 2012a). The on-site enzyme production using a split stream from the bioethanol process as part of the fermentation medium could be an attractive alternative (Kovacs et al., 2009; Sorensen et al., 2011). Moreover, the production of enzymes using as carbon source the same lignocellulosic material that will be used in the hydrolysis has shown that these enzyme preparations can present better performance (Delabona et al., 2012a; Jorgensen and Olsson, 2006).

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Efficient degradation of cellulose requires the cooperation of at least three types of enzymes: cellobiohydrolases, endoglucanases, and β-glucosidases. The organism *Trichoderma reesei* has typically been used for industrial cellulase production, due to its ability to produce extracellular protein in high amounts. However, the amount of β-glucosidase secreted by *T. reesei* is insufficient for an efficient saccharification (Berlin et al., 2007; Jorgensen and Olsson, 2006). A strain of the fungus *Trichoderma harzianum* P49P11, recently isolated from the Amazon rainforest, has shown relatively high β-glucosidase and xylanase activities when cultivated in a culture medium consisting of pretreated sugarcane bagasse and sucrose, using submerged fermentation in a bioreactor (Delabona et al., 2012a). Previous studies demonstrated that supplementation of cellulase preparations with endoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase and other accessory enzymes can positively affect EH of different lignocellulosic biomasses such as corn stover, wheat straw and soft wood (Alvira et al., 2011; Berlin et al., 2007; Gao et al., 2011; Kovacs et al., 2009; Kumar and Wyman, 2009). The addition of a recently identified lytic polysaccharide monooxygenase, classified as GH61, has enabled a 2-fold reduction in the total protein required to hydrolyze pretreated corn stover (Harris et al., 2010). However, only few studies investigated the use of accessory enzymes to enhance enzymatic hydrolysis of sugarcane bagasse (Kovacs et al., 2009). Addition of accessory enzymes could result in a synergistic effect by increasing accessibility of cellulases to the substrate, improving the EH process and enabling the use of reduced enzyme dosages (Gao et al., 2011).

A recent study by de Souza et al. (2012) on the composition and structure of sugarcane cell wall revealed that cellulose accounts for ~30% of cell wall, hemicellulose ~50% and pectins ~10%. In this same study, it has been proposed that an enzyme set containing pectinases, endo-β-xylanase, arabinofuranosidase, acetyl xylan esterase, and ferulic acid esterase would be needed in order to make cellulose accessible to cellulases (de Souza et al., 2012). Steam explosion followed by alkaline delignification has been considered a potential pretreatment technology for sugarcane bagasse (Rocha et al., 2012). However, understanding the enzymes that are necessary for the complete hydrolysis of delignified steam-pretreated sugarcane bagasse (DSB) is still a challenge.

The present study was developed in order to find out which enzymes could enhance the hydrolytic efficiency of the enzymatic extract obtained from a newly-isolated strain of *T. harzianum*, cultivated using pretreated sugarcane bagasse as substrate. Initially, the performance of the enzymatic extract from *T. harzianum* was characterized in terms of its saccharification efficiency during hydrolysis of pretreated bagasse, and the results were compared to those obtained for enzymatic extracts from *T. reesei* and *Penicillium echinulatum*, cultivated under the same conditions, as well as two commercial preparations. The specific activities of these five enzymatic extracts were compared using a panel of different substrates. In addition, the secretome of *T. harzianum* grown on pretreated sugarcane bagasse was analyzed using tandem mass spectrometry (MS/MS). Finally, experimental design methodology was used to evaluate the influence on the effectiveness of pretreated sugarcane bagasse hydrolysis of supplementation of the *T. harzianum* enzymatic extract with two selected accessory enzymes (pectinase and α-L-arabinofuranosidase).

2. Methods

2.1. Microorganisms and cultivation conditions

Stock cultures of *T. harzianum* P49P11 were isolated from the Amazon rainforest as previously described by Delabona et al. (2012b), and identified by internal transcribed spacer (ITS) analysis (see supplementary data). This strain was deposited at the Embrapa Food Technology microorganism collection (Rio de Janeiro, Brazil) by the strain number BRMCTAA 115. *T. reesei* Rut 30, from the American Type Culture Collection, and *P. echinulatum* strain 9A02S1-DSM18942 (Dillon et al., 2006) were stored at 4 °C on slants of potato dextrose agar (PDA) (Difco, Detroit, USA). The strains were grown on PDA plates (90 × 15 mm) at 29 °C for 7 days. A conidia suspension, prepared by adding 20 mL of sterilized distilled water and Tween 80 to the grown PDA plates, was transferred to Erlenmeyer flasks containing 180 mL of pre-culture medium adapted from (Mandels and Weber, 1969). The composition of the medium was as follows: 1 mL Tween 80; 0.3 g L^{-1} urea; 2.0 g L^{-1} KH_2PO_4; 1.4 g L^{-1} $(NH_4)_2SO_4$; 0.4 g L^{-1} $CaCl_2.2H_2O$; 0.3 g L^{-1} $MgSO_4.7H_2O$; 1.0 g L^{-1} proteose peptone; 5.0 mg L^{-1} $FeSO_4.7H_2O$; 1.6 mg L^{-1} $MnSO_4.4H_2O$; 1.4 mg L^{-1} $ZnSO_4.7H_2O$; 2.0 mg L^{-1} $CoCl_2.6H_2O$; 10 g L^{-1} glucose (carbon source). The medium (pH 5.0) was incubated for 72 h at 29 °C on a rotary shaker at 200 rpm. A 100 mL volume of this pre-culture was transferred to a BioFlo 115 fermenter (New Brunswick Scientific Co., USA) equipped with automatic control of temperature, pH, agitation rate (Rushton impeller), and aeration rate. The working volume of the fermenter was 1.0 L, and the temperature was maintained at 29 °C. The medium used was the same as that employed for the pre-culture, except that the carbon source was delignified steam-exploded sugarcane bagasse (DSB), for all microorganisms. The aeration rate was adjusted so that the dissolved O_2 level in the culture medium did not drop below 30% of air saturation. The pH was controlled at pre-set values using either 0.4 M H_2SO_4 or aqueous NH_4OH solution (1:3). Foaming was controlled by the manual addition of previously sterilized polypropylene glycol antifoaming agent (P2000, Dow Chemical, Brazil). The antifoaming agent was added in a proportion of 1 mL per liter at the beginning of each cultivation.

2.2. Enzymatic hydrolysis (EH)

Enzymatic hydrolysis experiments were carried out using 2 mL Eppendorf tubes with 50 mM citrate buffer at pH 5, in a Thermomixer microplate incubator (Eppendorf, Germany) operated at an agitation speed of 1000 rpm and temperature of 50 °C. The pretreated sugarcane bagasse (DSB) was applied at a concentration of 5% (w/w) of substrate total solids (TS). The working volume was 1.0 mL, and all experiments were performed in triplicate. The same conditions were used in the synergy studies with DSB, employing α-L-arabinofuranosidase and pectinase combined with cellulases from *T. harzianum* (2.8 mg/mL) and the commercial NS 50010 β-glucosidase preparation (81.69 mg/mL) (Novozymes, Denmark). The resulting dosages were 0.75 FPU/g cellulose and 75 IU β-glucosidase/g cellulose. Supernatants were withdrawn after 24 h, the hydrolysis was stopped by boiling for 5 min, and the samples were then centrifuged (11,900g) at room temperature for 15 min. The EH was measured by quantification of the reducing sugars released (with glucose as standard) according to the DNS method (Miller, 1959). The sugarcane bagasse was pretreated by steam explosion followed by delignification, as described by Rocha et al. (2012), and its composition was as follows (% dry weight): 89.5 ± 1.6 (cellulose), 3.4 ± 0.3 (hemicellulose), and 5.5 ± 0.2 (lignin). The materials were thoroughly washed with water, dried at room temperature, milled, and classified using a 35 mesh sieve.

2.3. Characterization of enzymatic extracts

A "panel of enzymatic activity" was performed to characterize the activity profiles of the supernatants produced by the *T. harzianum*, *T. reesei* Rut C30, and *P. echinulatum* cultures. Cellulase from *Trichoderma viride* (C9422, Sigma–Aldrich, USA) and the cellulase