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Identification of multienzymatic complexes in the *Clonostachys byssicola* secretomes produced in response to different lignocellulosic carbon sources

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ABSTRACT

Multienzymatic complexes with plant lignocellulose-degrading activities have recently been identified in filamentous fungi secretomes. Such complexes have potential biotechnological applications in the degradation of agro-industrial residues. Fungal species from the *Clonostachys* genus have been intensively investigated as biocontrol agents; however so far their use as producers of lignocellulose-degrading enzymes has not been extensively explored. Secretomes of *Clonostachys byssicola* following growth on different carbon sources (passion fruit peel, soybean hulls, cotton gin trash, banana stalk, sugarcane bagasse, orange peel, and a composition of soybean hulls: cotton gin trash:orange peel) were subjected to enzymatic assays. Remarkable differences were observed among the samples, especially regarding levels of mannanase and pectinase activities. Secretomes were then subjected to Blue Native PAGE in order to resolve putative protein complexes which subsequently had their composition revealed by trypsin digestion followed by LC–MS/MS analysis. The protein bands (named I, II, III and IV) were shown to be composed by holocellulolytic enzymes, mainly cellulases and xylanases as well as proteins involved in biocontrol processes, such as chitinases and proteases. The high diversity of proteins found in these multicatalytic assemblies confirms *C. byssicola* as a novel source of plant biomass-degrading enzymes.

1. Introduction

Lignocellulosic residues have potential for the generation of several value-added products, such as second generation biofuels. The degradation of plant cell wall polysaccharide constituents (holocellulose) is more attractive when carried out by enzymes, since conventional acid hydrolysis generates fermentation inhibitors, namely furfural and hydroxymethylfurfural. Holocellulose breakdown requires the coordinated and synergic action of cellulases, hemicellulases and pectinases, given the complex organization and interaction of polysaccharides in the plant cell wall that make it a very recalcitrant structure (Selig et al., 2009, 2008; Vardakou et al., 2004; Várnai et al., 2011).

Given the proximity between enzymes in multienzymatic associations they present an increased catalytic efficiency. This is clear when the performance of multienzymatic complexes is compared with free enzymes during lignocellulose digestion (Zhang, 2011). Some anaerobic bacteria, such as those belonging to the genus *Clostridium*, are able to organize their holocellulolytic enzymes into multienzymatic structures called cellulosomes. Organization of cellulosomes is scaffolding-protein dependent and anchorage to the bacterial cell wall is ensured by the structural proteins coesins and dockerins (Fontes and Gilbert, 2010).

Holocellulolytic systems produced by fungi and aerobic bacteria are often described as sets of free enzymes. However, previous studies have demonstrated the secretion of multienzymatic complexes was involved in lignocellulose breakdown in these organisms, such as the xylanosomes characterized from *Streptomyces olivaceoviridis* and *Chaetomium* sp., (Jiang et al., 2005; Ohtsuki et al., 2005). Similarly, a complex secreted by *Penicillium capsulatum* was characterized and found to be composed of β-glucosidase, laminarinase and lichenase activities (Connelly et al., 1991).

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Studies using Blue Native polyacrylamide gel electrophoresis (BN-PAGE) have revealed the presence of multienzymatic complexes secreted by filamentous fungi in response to lignocellulosic carbon sources. For instance, enzymatic complexes were described in secretomes produced by *Penicillium purpurogenum* when grown on sugar beet pulp, corn cob, or acetylated xylan, with the composition of these complexes varying according to the carbon source employed (Gonzalez-Vogel et al., 2011). Moreover, complexes composed of cellulases and hemicellulases were reported in *Trichoderma harzianum* grown in liquid media with sugarcane bagasse as the carbon source (Silva et al., 2012). Multienzymatic complexes have also been detected in media containing simple and defined carbon sources. For example, multienzymatic complexes, composed of glycosyl hydrolases, have been identified in secretomes of *T. reesei* following growth on lactose or galactose as carbon sources (Silva et al., 2015).

Within the order Hypocreales, a number of species in the genus *Trichoderma* have been widely employed in biological control (Schmoll and Schuster, 2010). Within the same order, the genus *Clonostachys* comprises both saprophytic and mycoparasitic organisms. Species such as *Clonostachys rosea* have been employed as biological control agents (Schroers, 2001; ten Hoopen et al., 2010) and others like *C. byssicola,* which is commonly found on cocoa (ten Hoopen et al., 2010), have been evaluated (Alvindia and Natsuaki, 2008; Krauss et al., 2013). Considering evidence in the genus *Trichoderma* that mycoparasitic species can also be efficient producers of lignocellulolytic enzymes, including multienzymatic complexes (Silva et al., 2015, 2012), characterization of such enzymes in *Clonostachys* species is warranted. Therefore, the objective of the current study was to detect and characterize multienzymatic complexes in the secretome of *C. byssicola* grown on several lignocellulosic carbon sources, based on BN-PAGE and mass spectrometry analyses.

2. Material and methods

2.1. Chemicals

Solvents employed in mass spectrometric analysis: acetonitrile (ACN), formic acid (FA), and LC-grade water were purchased from Sigma® Aldrich Chemical (St. Louis, USA). All substrates were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless indicated otherwise. Filter paper (Whatman No.1) was purchased from GE Healthcare, trypsin from Promega (Madison, WI, USA) and Poros R3 reverse-phase materials from Dr. Maisch (Ammerbuch, Germany).

2.2. Lignocellulosic substrates

Soybean hulls (SH), banana stalk (BS) and sugarcane bagasse (SB) were obtained from local farmers in the Brazilian Federal District region. These residues were autoclaved at 121 °C for two hours, extensively washed, dried at 60 °C during 72 h, and milled (Milanezi et al., 2012). Cotton gin waste (CG) was obtained from a cotton processing factory located at Blumenau, SC, Brazil, and subjected to the same pretreatment as SH, BS and SB. Oranges (OP) and passion fruits (PF) were purchased from local retailers in Brasília, Federal District, Brazil. Fruits were peeled, dried at 60 °C during 72 h, and milled.

2.3. Organism identification

The fungal isolate of *C. byssicola* used in this study, previously isolated from soil samples of Brazilian savanna, was deposited under strain code RCFS6 in the fungal culture collection at the Enzymology Laboratory, University of Brasilia, Brazil (genetic heritage number 010237/2015-1). The strain was also deposited in the bank of microorganism for control of plant pathogens and weeds of the Brazilian Agricultural Research Corporation (Embrapa). The collection is registered at the World Data Centre for Microorganisms (WDCM), under the code MCPPW 1128. Stocks were maintained at −80 °C in 50% glycerol.

Species confirmation was conducted through sequence analysis of ribosomal DNA Internal transcribed spacer (rDNA ITS) regions (White et al., 1990), together with a region of the β-tubulin gene (Glass and Donaldson, 1995). Genomic DNA was extracted using a standard phenol-chloroform method. Each PCR reaction contained 10 ng of template DNA, 0.4 μM of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.0 U Taq DNA polymerase and 1 × IB Taq polymerase buffer (Phoneutria, Belo Horizonte, MG, Brazil). Temperature cycling comprised an initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final elongation period at 72 °C for 5 min. Purification of amplification products was conducted with ExoSAP-IT® (USB, Cleveland, Ohio, USA) and forward and reverse-sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Purified sequences were analyzed on an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were compared with a nucleotide database (NCBI) using the BLAST algorithm and phylogenetic analysis conducted with the maximum likelihood method using the program MEGA, version 6.06 (Tamura et al., 2013).

The sequences used for identification of the strain were deposited on the GenBank with accession numbers KX259108, for the ITS sequence, and KX259107 for the β-tubulin sequence.

2.4. Cultivation conditions and enzyme production

Aliquots from the strain stored at −80 °C were plated on potato dextrose agar medium, and the plates were incubated at 28 °C during 15 days. After incubation, the spores were harvested by scraping the agar surface and transferred to a solution of 0.9% NaCl and 0.01% Tween 80.

C. byssicola was grown on liquid media containing agro-industrial wastes as carbon sources. Cultivation was carried out in 500 mL erlenmeyer flasks, with 250 mL of mineral medium (g/L: 7.0 KH_2PO_4; 2.0 K_2HPO_4; 0.5 $MgSO_4.7H_2O$; 1.0 $(NH_4)_2SO_4$; 0.6 yeast extract; pH 7.0), supplemented with 1% (w/v) of one of the carbon sources (passion fruit peel (PF), soybean hulls (SH), cotton gin trash (CG), banana stalk (BS), sugarcane bagasse (SB), orange peel (OP), and a composition of soybean hulls:cotton gin trash:orange peel (1:1:1) (SCO)) (Milanezi et al., 2012). An aliquot corresponding to 1% (v/v) of the spore suspension (10^7 spores/mL) was inoculated onto each medium and flasks were incubated at 28 °C under constant agitation at 120 rpm during 15 days. After this, samples were filtered under vacuum through Whatman n°1 filter paper, and the filtrates (hereafter called secretomes) were concentrated and stored at 4 °C for further analysis. Three independent biological replicates were cultivated for each carbon source used.

2.5. Sample concentration

Secretomes were concentrated 10-fold using an Amicon Ultrafiltration System (MILLIPORE, MA, USA) with a 10 kDa cut-off nitrocellulose membrane (MILLIPORE, MA, USA) at 4 °C and maximum pressure of 75 mPa. Concentrated fractions were dialyzed overnight (cellulose membrane, SIGMA, MO, USA) against distilled water, lyophilized and resuspended with Milli-Q water to a volume of 25 mL.

2.6. Enzymatic assays

Secretomes were screened for xylanase, mannanase, pectinase and CMCase activities using oat spelt xylan, galactoglucomannan, citrus pectin and carboxymethylcellulose (CMC) as substrates, respectively (Filho et al., 1993). Briefly, tubes containing 50 μL of secretome and 100 μL of substrate at a concentration of 1% (w/v) were incubated at 50 °C during 30 min. All activities were expressed in International Units