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Chrysoporthe cubensis: A new source of cellulases and hemicellulases to application in biomass saccharification processes

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HIGHLIGHTS

- *Chrysoporthe cubensis* is new fungal source of cellulases and hemicellulases.
- The enzymes showed pH and temperature optimum appropriate to biomass conversion.
- *C. cubensis* extract was more efficient than commercial cellulases for biomass hydrolysis.
- *C. cubensis* extract can be used as β-glucosidase supplement.

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ABSTRACT

The plant pathogenic fungus *Chrysoporthe cubensis* was cultivated under solid state employing different substrates and the highest endoglucanase (33.84 U g^{-1}), FPase (2.52 U g^{-1}), β-glucosidase (21.55 U g^{-1}) and xylanase (362.38 U g^{-1}) activities were obtained using wheat bran as carbon source. Cellulases and xylanase produced by *C. cubensis* showed maximal hydrolysis rate at pH 4.0 and in a temperature range of 50–60 °C. All enzymatic activities were highly stable at 40 and 50 °C through 48 h of pre-incubation. Saccharification of alkaline pretreated sugarcane bagasse by crude enzyme extract from *C. cubensis* resulted in release of 320.8 mg/g and 288.7 mg/g of glucose and xylose, respectively. On another hand, a similar assay employing commercial cellulase preparation resulted in release of 250.6 mg/g and 62.1 mg/g of glucose and xylose, respectively. Cellulolytic extract from *C. cubensis* showed a great potential to be used in biomass saccharification processes.

1. Introduction

There is currently great interest in the degradation of lignocellulosic materials to monomeric sugars through the concerted action of cellulolytic enzymes, since sugars can serve as raw materials for production of valuable products such as ethanol, acid lactic, methane, hydrogen and others (Juhasz et al., 2005). Growing concerns over the potential consequences of a worldwide shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion has resulted in an increased focus on the production of bioethanol from lignocellulosics and especially the possibility of using cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic materials (Camassola and Dillon, 2007; Kumar et al., 2009)

The biotechnological conversion of cellulose into fermentable sugars requires a cooperative and synergistic action among three main enzyme types. Endoglucanases (E.C. 3.2.1.4) randomly attack cellulose chains generating reducing and non-reducing ends. Cellobiohydrolases (E.C. 3.2.1.91) act over the reducing and non-reducing ends, releasing cellobiose units which are converted to glucose by the action of β-glucosidase enzymes (E.C. 3.2.1.21) (Lynd et al., 2002). Moreover, hemicellulolytic enzymes such as xylanases (E.C. 3.2.1.8), mannanases (E.C. 3.2.1.78), β-xylosidases (E.C. 3.2.1.37), β-mannosidases (E.C. 3.2.1.25), α-Arabinofuranosidase (E.C. 3.2.1.55) and α-galactosidases (E.C. 3.2.1.22), also play an important roles in the cellulose depolymerization process. These enzymes hydrolyze and remove the hemicellulose fragments that coat the cellulose fibers, increasing cellulose accessibility and boosting the action of cellulases (Berlin et al., 2007; Juhasz et al., 2005).

The major bottleneck to lignocellulosic bioethanol production is the high cost of the cellulolytic enzymes. Large-scale application of cellulases for lignocellulosic material degradation processes

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demand microorganisms with improved activity and productivity as well as cellulases with better resistance to product inhibition (Jiang et al., 2010). Enzymes for degradation of the polysaccharide portion of biomass have been predominantly produced from fungi belonging to the genus *Trichoderma*. The fungi *Trichoderma reesei* and *Trichoderma viride* are able to secrete large amounts of endoglucanase and cellobiohydrolase enzymes; however, the amount of β-glucosidase secreted by *Trichoderma* species is very low and this may lead to accumulation of cellobiose and incomplete cellulose hydrolysis when cellulolytic extracts produced by these fungi are employed in saccharification processes (Jiang et al., 2010). Other important cellulase producing fungi, including those belonging to the genera *Penicillium* and *Aspergillus*, are able to secrete large amounts of β-glucosidase; however the total cellulase activity (FPase) found in their enzymatic extracts is relatively low. As a result, production of glucose from waste cellulose is not yet commercially feasible (Jiang et al., 2010). Considering this scenario, there is a constant search for new cellulolytic microorganisms that are able to produce cellulases in large quantities and in balanced proportions, so that it can be used for efficient saccharification of lignocellulosic biomass.

Plant pathogenic fungi produce extracellular enzymes which can degrade the cell wall components of plants. These fungi do not only digest plant cell wall polymers to obtain important nutrients but also degrade the cell wall enabling cell penetration and spread through the plant tissue (Kikot et al., 2009). Plant pathogenic species such as *Fusarium graminearum* (Kikot et al., 2009), *Bipolaris sorokiniana* (Geimba et al., 1999) and *Sclerotinia sclerotiorum* (Yajima et al., 2009) have been evaluated with relation to secretion of hemicellulases, pectinases and cellulases, and it was observed that there is a close correlation between the capacity to secrete some hydrolases and the virulence of these microorganisms. However, despite the proven ability of plant pathogenic fungi to produce cell wall-degrading enzymes, few works report the utilization of these fungi in production of cellulases and hemicellulases destined for converting biomass in monomeric sugars.

In the present study, the production of cellulases and hemicellulases by *Chrysoporthe cubensis*, a well-documented pathogen of various tree species, was examined in solid state fermentation using different carbon sources. The produced cellulolytic complex was characterized in terms of pH, temperature and thermal stability. For the first time, an attempt was made to apply the cellulolytic complex from *C. cubensis* in a biomass saccharification process using sugarcane bagasse as substrate. Assays of saccharification using a commercial cellulases complex were carried out in parallel in order to establish a comparison between both extracts.

2. Methods

2.1. Materials

Substrates including ρ-nitrophenyl-β-D-glucopyranoside (pNPGlc), ρ-nitrophenyl-β-D-xylopyranoside (pNPXyl), ρ-nitrophenyl-β-D-mannopyranoside (pNPMan), ρ-nitrophenyl-β-D-galactopyranoside (pNPGal), ρ-nitrophenyl-α-D-arabinofuranoside (pNPAra), cellobiose, carboxymethylcellulose (CMC), xylan from birchwood, locust bean gum, polygalacturonic acid and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). Chemical reagents including NaOH, H_2SO_4 and potassium sodium tartrate were obtained from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). A commercial cellulase complex, Multifect® CL, was purchased from Genencor International Inc. (Rochester, NY, USA). Kraft pulp was kindly supplied by the Pulp and Paper Laboratory of the Federal University of Viçosa, Viçosa, MG, Brazil. Sugarcane bagasse, corn cobs and wheat bran were obtained at the local market. All others reagents used in this study were of analytical grade.

2.2. Microorganism and inoculum preparation

The fungus *C. cubensis* LPF-1 used in this study was obtained from the mycological collection of the Forest Pathology Laboratory, Federal University of Viçosa, MG, Brazil. The fungus was maintained on PDA plates at 28 °C and subcultured periodically. The inoculum was prepared by growing the fungus under submerged fermentation in 250 mL Erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2.0. Each flask was inoculated with 10 agar plugs cut out of a 5 day-old colony of *C. cubensis* grown on PDA plates and incubated in a rotary shaker for 5 days, at 150 rpm and 28 °C. The culture obtained was aseptically homogenized with Polytron® and immediately used to inoculate the solid culture media. This inoculum preparation routine was employed for all experiment developed in this work.

2.3. Culture condition

In order to evaluate the effect of carbon sources on fungal growth and enzyme production, *C. cubensis* was cultured under solid state fermentation (SSF) using Kraft pulp, wheat bran or milled corn cob as support and the main carbon source. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 5 g (dry weight) of substrate moistened with 12 mL of culture media (final moisture of 70%) containing the following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$ (0.1 mg L^{-1}), H_3BO_3 (0.075 mg L^{-1}), Na_2MoO_4 (0.02 mg L^{-1}), $FeCl_3$ (1.0 mg L^{-1}) and $ZnSO_4$ (3.5 mg L^{-1}) also were added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 3 mL (containing 1.5×10^7 spores mL^{-1}) of inoculum obtained as aforementioned. The flasks were maintained at 30 °C in a controlled temperature chamber and the enzymatic extraction was performed after 4, 8 and 12 days of fermentation. Enzymes secreted during SSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate), under agitation of 150 rpm for 60 min at room temperature. Solids were separated by filtration through nylon cloth followed by centrifugation at 15000*g* for 10 min; and the clarified supernatants were frozen and stored for subsequent enzymatic analysis. Experiments were carried out with three replicates for each medium composition and for each incubation time.

2.4. Enzymatic assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50 °C. All were performed in triplicate and the mean values were calculated. Relative standard deviations of measurements were below 5%. FPase and endoglucanase activities were determined using Whatman No. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions (Ghose, 1987). The total reducing sugar liberated during the enzymatic assays were quantified by the dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose as a standard.

Xylanase, mannanase and pectinase activities were determined using xylan from birchwood (1% w/v at final concentration), locust bean gum (0.4% w/v) and poligalacturonic acid (0.2% w/v) as