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Screening of *Fusarium* sp. for xylan and cellulose hydrolyzing enzymes and perspectives for the saccharification of delignified sugarcane bagasse

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ABSTRACT

Endophytic and phytopathogenic isolates of the genus *Fusarium* were evaluated for their potential to produce cellulolytic and xylanolytic enzymes. The fungi were grown under solid-state conditions using a mixture of sugarcane bagasse and wheat bran as substrate with an initial moisture of 82%. Endoglucanases (ranging from 0.20 ± 0.03 to 5.31 ± 0.30 U/g of substrate), xylanases (ranging from 4.65 ± 0.76 to 125.57 ± 8.25 U/g of substrate), β-glucosidases (ranging from 21.48 ± 3.70 to 527.17 ± 22.14 U/g of substrate) and β-xylosidases (ranging from 5.61 ± 1.25 to 40.69 ± 1.26 U/g of substrate) were produced by all isolates. Enzymatic extracts from the best xylanase producers, *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans*, were tested for their capacities in promoting saccharification of delignified sugarcane bagasse. Amounts of 200.60 ± 10.60 mg/g and 280.00 ± 19.00 mg/g of reducing sugars, mainly xylose, were obtained after enzymatic hydrolysis by *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* extracts, respectively. Up to now these species of *Fusarium* have been barely explored as sources of xylanolytic enzymes capable to degrade plant biomass, but our results open perspectives for their biotechnological use in the obtainment of xylose from delignified sugarcane bagasse.

1. Introduction

The generation of energy and valuable products from plant biomass has been the object of several studies. Waste and agricultural residues, including sugarcane bagasse, rice husk, corn cob, among others, mostly containing lignocellulosic materials, are prominent among the available plant biomasses, mainly because they are of low cost, renewable and abundant.

In Brazil, the production of sugarcane (*Saccharum officinarum* L.) has increased in recent years and with it the production of sugarcane bagasse. Part of the sugarcane bagasse is used in the boilers of the mills themselves, but there is still a large surplus ready to be allocated to other purposes. In the last decades, extensive research has been conducted aiming at the conversion of sugarcane bagasse into valuable products, including mono-, oligo- and polysaccharides, organic acids, organic solvents, fermentative products and soluble lignin derivatives (Ferreira et al., 2016; Mandegari et al., 2017). The obtainment of several of these products depends on the use of efficient pre-treatments and appropriate enzymatic cocktails able to hydrolyse selectively the main polysaccharides. A variety of filamentous fungi produce and secrete enzymes such as endo- and exo-glucanases, β-glucosidases, endo- and exo-xylanases and β-xylosidases, necessary for the enzymatic hydrolysis of cellulose and xylan, respectively. However, only a few number of species, generally belonging to the *Trichoderma* and *Aspergillus* genera, are used for obtainment of commercial enzymatic preparations (Ferreira et al., 2016)

The genus *Fusarium* encompasses more than 700 species. A large number of them are associated to agricultural productions, such as plant pathogens (Pollet et al., 2009; Hafezi et al., 2013), toxin producers on edible parts of the plants (Dorn et al., 2011) and biological control agents for plant diseases (Ghini et al., 2000). In an ecological perspective, *Fusarium* also includes epiphytes (Inácio et al., 2002) and endophytes (Zakaria and Ning, 2013; Imazaki and Kadata, 2015). In addition to these agriculturally and ecologically distinct strains, many are putative saprophytes (Demers et al., 2015).

Several species of *Fusarium* have been considered as potential sources of enzymes useful in the degradation of plant biomass due to their great capacity of growth on several vegetal substrates (Pessoa et al., 2017; Panagiotou et al., 2003, 2011; Xiros et al., 2008, 2009; Xiros and Christakopoulos, 2009; Dutta et al., 2018). Within this context, *F. oxysporum* is the most studied species. Its genome encodes a complete xylanolytic system and the species has a complete arsenal of

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cell wall degrading enzymes that allows it to convert efficiently plant biomasses (cellulose and xylan) into ethanol (Ali et al., 2012; Anasontzis et al., 2011; Lin et al., 2012; Gomes et al., 2016; Gupta et al., 2009; Arabi et al., 2011). *F. graminearum* (Debeire et al., 2014), *F. verticilioides* (Almeida et al., 2013; Ravalason et al., 2012) and *F. solani* (Obruca et al., 2012) are also exploited as sources of hydrolytic enzymes able to degrade plant biomass. It is probable, however, that the resources of the genus in terms of hydrolytic enzymes are still far from being exhausted. Considering, thus, the high number of potentially useful species of *Fusarium,* the objectives of this work were: (1) to evaluate the capacity of species of *Fusarium* sp. (endophytic and phytopathogenic isolates) of growing on solid state cultures using a mixture of sugarcane bagasse and wheat bran as substrate; (2) to evaluate the production of enzymes involved in the hydrolysis of xylan and cellulose; and (3) to evaluate the perspective of using enzymatic extracts of the species richer in xylanolytic enzymes in the saccharification of xylan from delignified sugarcane bagasse.

2. Material and methods

2.1. Substrates used in the fungal cultivation

Sugarcane bagasse was obtained from Usina Santa Terezinha, Iguatemi, PR, Brazil. It was dried in the sunlight, milled to give a particle size of 2–3 mm thickness and used as raw material in this study. The material was evaluated using the technique of acid detergent fiber (ADF) to obtain the percentages of cellulose, hemicellulose and lignin, and neutral detergent fiber (NDF) for the percentage of lignocellulose (Castoldi et al., 2017). The average composition was 42 ± 4% cellulose, 29 ± 2% hemicelluloses, 23 ± 2% lignin, 2.2% ashes, and 3.8% moisture. According to the commercial supplier the wheat bran contained (wt%) ~ 15 starch, ~ 50 fiber (cellulose and hemicellulose), ~ 16 protein, and ~ 19 of extractives besides some vitamins and minerals.

2.2. Microorganisms

Twenty one isolates of the genus *Fusarium* were used in this work: endophytes *Fusarium* isolated from sugarcane identified as 126, 132, AR 167, AR 176, AR 101 and AR 206, were kindly provided by EMBRAPA-Brasília. The phytopathogenic species *F. oxysporum* var. *oxysporum*, *F. solani* var. *solani, F. culmorum*, *F. javanicum* var. *javanicum*, *F. melanochlorum*, *F. equiseti* var. *equiseti*, *F. merismoides* var. *crassum*, *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* were kindly provided by Fiocruz, Manguinhos, Rio de Janeiro. *F. acuminatum*, *F. graminearum* 8, *F. graminearum* 23, *F. verticillioides* ATCC 1442, *F. verticillioides* and *F. solani* were kindly provided by the Department of Agronomy of the State University of Maringá, PR, Brazil. All isolates were maintained in test tubes containing potato agar dextrose (PDA) culture medium. After 7 days at 28 °C, the media were completely colonized. These cultures were used to obtain the spore suspension for solid-state cultivation.

2.3. Culture conditions

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 mL) containing 4.5 g of sugarcane bagasse plus 1.5 g of wheat bran and 25 mL of mineral solution (Vogel, 1956). Prior to use, the mixtures were sterilized by autoclaving at 121 °C for 15 min. Inoculation was done directly in the Erlenmeyer flasks. Erlenmeyer flasks received 1.6×10^5 spores per gram of substrate of each fungus and were incubated statically for 8 days at 28 °C and in complete darkness.

2.4. Obtainment of crude enzymatic extracts

A volume of 20 mL of distilled water was added to the cultures and the mixtures were agitated at 100 rpm at room temperature for 30 min. The materials were firstly filtered in gauze and then centrifuged at 8000 rpm for 10 min at 4 °C. The supernatants were considered as crude enzymatic extracts and maintained at − 20 °C for further analyses.

2.5. Enzyme assays

Endo-β-D-1,4 glucanase, also designated as carboxymethyl cellulase (CMCase) activity (EC 3.2.14), and endo-β-D-1,4-xylanase (EC.3.2.18) activities were determined by measuring the reducing sugars released from carboxymethylcellulose and oat xylan, respectively, as substrates, in 50 mM sodium acetate buffer, pH 5.0. The released reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) using glucose or xylose as standards. The activities of β-glucosidase (EC 3.2.1.21), and β-xylosidase (EC 3.2.1.37) were determined in 50 mM sodium acetate buffer, pH 5.0, by measuring *p*-nitrophenol release from *p*-nitrophenyl-β-D-glucopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside, respectively (Lenartovicz et al., 2003). All enzyme activities were determined at 40 °C. Enzyme activities were expressed as international enzymatic units (U).

2.6. Alkaline peroxide pre-treatment of sugarcane bagasse

Sugarcane bagasse was submitted to alkaline peroxide pre-treatment to remove lignin as described previously (Yamashita et al., 2010) with a few modifications. An amount of 5 g dry weight of sugarcane bagasse (50 mesh) solid material was added to 500 mL of a 1% (w/v) sodium hydroxide solution containing 2% (v/v) hydrogen peroxide. The mixture was agitated at 120 rpm at 50 °C for 1 h. The solid material was separated by filtration and washed with distilled water several times until neutral pH, dried at room temperature and stored at 4 °C until use.

2.7. Saccharification of delignified sugarcane bagasse

An amount of 0.5 g of pre-treated sugarcane bagasse was added to a 250 mL Erlenmeyer flask. A volume of 9 mL of *F. lateritum* var. *majus* or *F. sacchari* var. *subglutinans* enzymatic extracts was added to each flask. Subsequently, each flask received a volume of 1 mL of 500 mmol/L citrate buffer, pH 5.0. The mixtures were maintained on a rotary shaker at 130 rpm at 37 °C for up to 48 h. Samples were withdrawn periodically and filtered under vacuum. Total reducing sugars present in the filtrates were estimated by the 3,5 dinitrosalicylic method (Miller, 1959) using D-xylose as standard and expressed as mg of reducing sugar per g of pre-treated sugarcane bagasse.

2.8. Quantification of glucose and xylose by high performance liquid chromatography (HPLC)

The amounts of glucose and xylose obtained by hydrolysis of the alkaline peroxide pre-treated sugarcane bagasse by *F. lateritum* var. *majus* or *F. sacchari* var. *subglutinans* enzymatic extracts were quantified using a HPLC system (Shimadzu, Japan) equipped with a refractive index detector and a NH_2 Supelco column (4.6 mm × 250 mm, 5 μm), thermostated at 40 °C. The mobile phase was (25:75, v/v) water: acetonitrile at a flow rate of 1 mL/minute. Standard stock solutions of glucose and xylose were prepared in water. Working solutions were prepared by diluting the stock solutions with the mobile phase. Linearity was established by triplicate injections of different concentrations of the standards.

2.9. Scanning electron microscopy of sugarcane bagasse

Scanning electron microscopy (SEM) (Shimadzu SS-550 Superscan) was used to characterize the sugarcane bagasse before and after chemical pretreatment and after enzymatic saccharification. treatment. For the imaging procedures the samples were sputter coated with gold