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LEONARDO CORREA BERTONHA

CONTRIBUIÇÕES PARA A OBTENÇÃO DE AÇÚCARES FERMENTESCÍVEIS A PARTIR DO BAGAÇO DE CANA DE AÇÚCAR

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Tese apresentada ao Programa de Pós Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para obtenção do grau de Doutor em Ciências Biológicas.

Orientadora: Dra. Rosane Marina Peralta

Co-orientador: Dr. Rafael Castoldi

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Leonardo Correa Bertonha possui graduação em Farmácia. É mestre em Agronomia pela Universidade Estadual de Maringá.

APRESENTAÇÃO

Este trabalho foi realizado no Laboratório de Bioquímica de Microrganismos e Alimentos da Universidade Estadual de Maringá. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas a presente tese está apresentada na forma de dois artigos científicos e os mesmos foram redigidos de acordo com as normas dos periódicos correspondentes.

ARTIGO 1

Bertonha, L.C., Castoldi, R., Bracht, A., Peralta, R.M. Screening of *Fusarium* sp. for xylan and cel lulose hydrolyzing enzymes and perspectives for the saccharification of xylan f rom sugarcane bagasse. Este artigo foi submetido ao periódico científico Biocatalysis and Agricultural Biotechnology.

ARTIGO 2

Bertonha, L.C., Leal Neto, M., Castoldi, R., Peralta, R.M. Pré-tratamento do bagaço de cana-de-açúcar com extrato enzimático bruto de *Oudemansiella canarii* rico em lacase aumenta a sacarificação enzimática. Este artigo, após defesa, incorporação das sugestões da banca e versão para o inglês, será submetido a periódico da área.

RESUMO GERAL

INTRODUÇÃO - A geração de energia a partir da biomassa vegetal tem sido objeto de vários estudos. Resíduos lignocelulósicos incluindo bagaço de cana, casca de arroz, sabugo de milho, entre outros são abundantes, facilmente obtidos e de baixo custo. Estes resíduos são formados por 50% de celulose, 25% de hemicelulose e 25% de lignina. No Brasil, a produção de cana-de-açúcar (Saccharum officinarum L.) vem aumentando nos últimos anos e com isso, a disponibilidade de bagaço de cana-de-açúcar. Parte do bagaço de cana é utilizado nas caldeiras das próprias usinas, mas ainda há um grande superávit que deve receber uma destinação. Nas últimas três décadas, uma extensa pesquisa vem sendo conduzida para a conversão de materiais lignocelulósicos em etanol. A conversão inclui três processos, a). o pré-tratamento, em que são utilizados métodos físicos, químicos ou biológicos para remover pelo menos parte da lignina a fim de tornar os polissacarídeos mais acessíveis à hidrólise enzimática; b). hidrólise enzimática, onde enzimas celulolíticas e hemicelulolíticas são utilizadas para a hidrólise de polissacarídeos e a produção de açúcares fermentáveis, principalmente glicose e xilose; e (c) a fermentação propriamente dita, onde as leveduras são aplicadas para a produção de etanol. A fim de contribuir para esta tão importante área biotecnológica, esta tese de doutorado abordou dois processos: o pré-tratamento do bagaço de cana de açúcar e a hidrólise do bagaço de cana de açúcar. Os objetivos do primeiro trabalho foram (1) avaliar a capacidade de espécies de *Fusarium* sp. (isolados endofíticos saprófitas e fitopatogênicos) cultivados em cultivos em estado sólido utilizando bagaço de cana-de-açúcar como substrato produzir enzimas celulolíticas e xilanolíticas; (2) utilizar extratos enzimáticos selecionados na sacarificação do xilano do bagaço de cana-de-açúcar. O objetivo do segundo trabalho foi utilizar um extrato enzimático bruto *Oudemansiella canarii* rico em lacase no desenvolvimento de um pré-tratamento biológico do bagaço de cana para facilitar a posterior sacarificação da celulose e da hemicelulose por um coquetel enzimático comercial (CTec2-HTec2®).

MÉTODOS - No primeiro trabalho, espécies do gênero *Fusarium* (isolados endofíticos, saprófitos e fitopatogênicos) foram avaliadas quanto ao potencial de produção de enzimas capazes de hidrolisar os polissacarídeos do bagaço de cana. Os fungos foram cultivados em estado sólido utilizando uma mistura de bagaço de cana: farelo de trigo como substrato com umidade inicial de 82%. Os extratos enzimáticos brutos obtidos foram avaliados quanto às atividades endoglucanase, xilanase, beta-glucosidase e beta-xilosidase. No segundo trabalho, o bagaço de cana-de-açúcar foi pré-tratado com uma lacase bruta de *O. canarii* e a sacarificação do material foi realizada utilizando-se um coquetel enzimático comercial CTec2® + HTec2® gentilmente cedido pela Novozymes.

RESULTADOS E DISCUSSÃO - No primeiro trabalho, xilanases, beta-glucosidases e beta-xilosidases foram produzidas por todos os isolados, mas as atividades de endoglucanases foram baixas. *F. lateritum* var. *majus* e *F. sacchari* var. *subglutinans*, produziram mais xilanase do que F. oxyporum, F. graminearum e F. solani, espécies amplamente conhecidas como capazes de degradar materiais lignocelulósicos. Por esta razão, os extratos enzimáticos de F. lateritum var. majus e F. sacchari var. subglutinans foram utilizados na

sacarificação do bagaço de cana pré-tratado com peróxido de hidrogênio alcalino. Após hidrólise, foram obtidos 200,60 ± 10,60 mg/g e 280,00 ± 20,10 mg/g de açúcares redutores por g de bagaço de cana pré-tratado, respectivamente. Xilose foi o monossacarídeo mais abundante dos hidrolisados. Essas duas espécies de *Fusarium* são até agora pouco exploradas como fontes de enzimas capazes de degradar a biomassa vegetal, mas nossos resultados mostraram seus potenciais biotecnológicos para a obtenção de açúcares fermentáveis a partir do bagaço da cana. No segundo trabalho, após 48 h de hidrólise com celulases comerciais, uma quantidade de 0,35 g de açúcares redutores (0,21 g de glicose) foram obtidos a partir de 0,5 g de um bagaço de cana-de-açúcar pré-tratado com lacase de O. canarii. Nas mesmas condições de hidrólise, apenas 0,033 g de açúcares redutores (sendo 0,017 g de glicose) foram obtidos a partir do bagaço de cana não pré-tratado.

CONCLUSÕES - No primeiro trabalho, extratos enzimáticos de *F. lateritum* var. *majus* e *F. sacchari* var. *subglutinans* mostraram-se potencialmente úteis para a obtenção de açúcares fermentescíveis a partir do bagaço de cana-de-açúcar. No segundo trabalho, o pré- tratamento com extrato enzimático bruto rico em lacase de *Oudemansiella canarii* mostrou- se eficiente para tornar mais acessível os componentes celulose e xilano para a hidrólise enzimático pelo coquetel enzimático comercial (CTec2-HTec2 ®).

PALAVRAS-CHAVES: açúcares redutores, biomassa de plantas, celulases, lacases, pré-tratamento, sacarificação,

GENERAL ABSTRACT

INTRODUCTION – The generation of energy from plant biomass has been the object of several studies. Waste and agricultural residues, including sugar cane bagasse, rice husk, corn cob, among others, mostly containing lignocellulosic materials, are prominent among the available plant biomasses, mainly because they are low cost, renewable and abundant. These residues are composed of approximately 50% cellulose, 25% hemicellulose and 25% lignin. In Brazil, the production of sugarcane (Saccharum officinarum L.) has been increasing in recent years and with it, the production of sugarcane bagasse. Part of the sugar cane bagasse is used in the boilers of the mills themselves, but there is still a large surplus that must receive an allocation. In the last three decades, extensive research has been conducted on the conversion of lignocellulosic materials to ethanol. The conversion includes three processes: (a) the pre-treatment, where physical, chemical or biological methods are used to remove at least part of the lignin to make polysaccharides more accessible to enzymatic hydrolysis; (b) enzymatic hydrolysis, where cellulolytic and hemicellulolytic enzymes are used for the hydrolysis of polysaccharides and the production of fermentable sugars, mainly glucose and xylose; and (c), the fermentation itself, where yeasts are applied for the production of ethanol. The objectives of the first work were (1) to evaluate the capacity of species of *Fusarium* sp. (endophytic and phytopathogenic isolates) to grow in solid state cultures using sugarcane bagasse as substrate; (2) to evaluate the production of enzymes involved in the hydrolysis of xylan and cellulose of sugarcane bagasse; and (3), to use selected enzymatic extracts in the saccharification of xylan from sugarcane bagasse. The objective of the second work was to use a rich laccase crude enzymatic extract of *Oudemansiella canarii* for biological pre-treatment of sugar cane bagasse to facilitate the saccharification of cellulose and hemicellulose by a commercial enzymatic cocktail (CTec2- Htec2®)

METHODS – In the first work, species of the genus *Fusarium* (endophytic, saprophytic and phytopathogenic isolates) were evaluated for their potential to produce enzymes able to hydrolyse sugar cane bagasse. The fungi were grown under solid-state conditions using a mixture of sugarcane bagasse: wheat bran as substrate with an initial moisture of 82%. The enzymatic extracts were evaluated for endoglucanase, xylanase, beta-glucosidase and beta-xylosidase activities. In the second work, sugar cane bagasse was pre-treated using a crude laccase from *O. canarii* and the saccharification of material was carried out using the commercial enzymatic cocktails Cellic CTec2® + HTec2® from Novozymes.

RESULTS AND DISCUSSION – Results of the In the first work revealed that all isolates are able to produce xylanases, beta-glucosidases and beta-xylosidases, but not endoglucanases. *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans*, produced more xylanase than F. oxyporum, F. graminearum and F. solani, species largely known as able to degrade lignocellulosic materials. For this reason, F. lateritum var. majus and F. sacchari var. subglutinans extracts were used in the saccharification of cane bagasse pretreated with alkaline H_2O_2. Amounts of 200.60±10.60 mg/g and 280.00±20.10 mg/g of reducing sugars, mainly xylose, were obtained after enzymatic hydrolysis by xylanases These species of

Fusarium are until now barely explored as sources of enzymes capable to degrade plant biomasses, but our results suggests that they have biotechnological potential in the obtainment of fermentable sugars from sugarcane bagasse. In the second work, after 48 h of hydrolysis with commercial cellulases, an amount of 0.35 g of reducing sugars being 0.21 g of glucose from 0.5 g of a laccase pre-treated sugar cane bagasse. After hydrolysis of non pre-treated sugar cane bagasse under the same conditions, only 0.033 g of reducing sugars (being 0.017 g of glucose) were obtained.

CONCLUSION – In the first work, crude enzymatic extracts from F. lateritum var. majus and F. sacchari var. subglutinans showed to be potentially useful for the obtainment of fermentable sugars from sugarcane bagasse. In the second work, the pre-treatment using a laccase rich crude enzymatic extract from *Oudemansiella canarii* improved at least 10 times the liberation of reducing sugars from sugar cane bagasse.
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KEYWORDS: cellulases, laccases, plant biomass, pre-treatment, saccharification, xylanases, reducing sugars.

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.

Key words: *Fusarium* sp, endophytic fungi, phytopathogenic fungi, sugarcane bagasse, xylanase.

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 belonged to genera Trichoderma, and Aspergillus species 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 & Ning, 2013, Imazaki & 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 & 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 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 fiber neutral detergent (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 x 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 ºC. The supernatants were considered as crude enzymatic extracts and maintained at -20 ºC 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 ^{o}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 ^{o}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 layers.

2.9. Data handling

All analyses were performed in triplicate. The data were expressed as means±standard deviations and one-way analysis of variance (ANOVA) and Tukey test were carried out to assess for any significant differences between the means. Differences between means at the 5% (p<0.05) level were considered significant. Data analysis was performed using GraphPad Prism® software version 5.0 (Graph Pad Software, San Diego, USA).

3. Results and discussion

3.1. Evaluation of the production of cellulolytic and xylanolytic enzymes by *Fusarium*

The twenty one different endophytic, plant pathogenic and saprophytic isolates of several species of *Fusarium* were grown on a mixture of sugarcane bagasse and wheat bran. These conditions should be adequate for evaluating their potential as producers of enzymes involved in the hydrolysis of cellulose and xylan, endoglucanase (CMCase), xylanase, β-glucosidase and β-xylosidase (Table 1). In a preliminary attempt of introducing some degree of systematization the isolates were divided into three groups. The first one comprises those *Fusarium* species that have already been extensively studied . The second group includes those isolates found in healthy leaves of sugarcane (i.e. endophytes) and whose identification does not go beyond genus. The third group, finally, encompasses species that, up to now, have been but poorly evaluated as producers of enzymes capable of hydrolyzing polysaccharides of the plant cell wall.

The values of CMCase activity and β-glucosidase activity found in the crude enzymatic extracts of *Fusarium* ranged from 0.20±0.03 to 5.31±0.30 U/g of substrate and from 21.28±3.70 to 531.97±11.99 U/g of substrate, respectively. Solid state cultures using lignocellulosic wastes as substrate have been considered as an efficient method for the production of cellulolytic enzymes (Dutta et al., 2018, Ferreira et al., 2016). In general terms, however, the sugarcane bagasse-wheat bran medium used in this work was not efficient in providing conditions to the *Fusarium* species for the production of elevated amounts of CMCase. On the other hand, high activities of β-glucosidase were produced by several isolates, especially *F. graminearum* 23 (356.43±8.78 U/g of substrate), *Fusarium* sp. 126 (527.17±22.14 U/g substrate), and *Fusarium* sp. AR 206 (320.23±15.11 U/g of substrate).

The *Fusarium* species of group I in Table 1 are considered, at least in principle, able to

degrade cellulose (Pessoa et al., 2017). For example, *F. graminearum* produced efficient enzymatic cocktails in submerged cultures using different lignocellulosic biomasses (Debeire et al., 2014). *F. oxysporum* has been described as capable of producing ethanol directly from cellulose (Christakopoulos et al., 1989). On the other hand, analysis of the secretome of *F. verticillioides* revealed the absence of cellobiohydrolases (Ravalason et al., 2012). Consistently, in the latter, the saccharification of wheat straw was improved when its crude secretome was enriched with a commercial cellulase from *Trichoderma reesei.* A synergistic action of all the cellulolytic activities is required for a full degradation of cellulose, and β-glucosidases are considered as the key enzymes for this process as they are indispensable for releasing free glucose. Beta-glucosidases are generally found in small proportions in commercial preparations. Hence, many studies are focusing on finding robust β-glucosidases, since enzyme cocktails must be supplemented with this activity to increase the efficiency of cellulose saccharification (Singhania et al., 2017, Nishida et al., 2018). The *Fusarium* sp, described in this paper, particularly the endophytes, may thus be explored in the future as valuable sources of β-glucosidases after identification to the species level.

The levels of xylanase and β-xylosidase activities found in the crude enzymatic extracts of *Fusarium* ranged from 4.69±0.0.76 to 125.57±8.32 U/g substrate and from 7.23±0.27 to 40.69±1.26 U/g substrate, respectively. Several *Fusarium* species have been described as good xylanase and β-xylosidase producers, such as *F. oxysporum* (Panagiotou et al., 2003), *F. verticillioides* (Arabi et al., 2011), *F. solani* (Gupta et al., 2009), and *F. graminearum* (Sella et al., 2013). Our results show that, under the conditions used in this work, *F. lateritum var. majus*and *F. sacchari var. subglutinans* presented a high capability of producing both xylanase (124.44±8.25 and 112.70±7.16 U/g of substrate, respectively), and β-xylosidase (27.69±2.24 and 36.13±1.62 U/g of substrate, respectively).

3.2. Saccharification of delignified sugarcane bagasse by crude enzymatic extracts from *F. lateritum var. majus* and *F. sacchari var. subglutinans*

Taking into account the high capability of *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* in producing xylanase and β-xylosidase, these two species were used for testing their capabilities of hydrolysing delignified sugarcane bagasse. The delignified sugarcane bagasse used in the saccharification experiments contained a lignin content significantly lower than the crude sugarcane bagasse (from 23± 2% to 10±2%). The results of the experiments are shown in Fig. 1 in which the concentrations of reducing sugars (mg/g substrate) were represented against the time of incubation that was conducted for up to 48 h. Tests of the enzyme activity

revealed no significant changes during the whole incubation time. At the end of 48 h of saccharification, a total of 200.60±10.60 mg/g and 280.00±19.00 mg/g of reducing sugars were obtained by the action of the crude enzymatic extracts from *F. lateritum var. majus* and *F. sacchari var. subglutinans*, respectively. The amounts of reducing sugars were also evaluated by HPLC and compared with those obtained in the chemical analyses (DNS method) in Table 2. Amounts of 166.20±12.10 mg/g and 247.40±22.80 mg/g of xylose were obtained after enzymatic hydrolysis by *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* extracts, respectively. These results show, thus, that the xylan from sugarcane bagasse was considerably hydrolysed, whereas the low amounts of glucose that were released suggest that the cellulose was barely hydrolyzed. It should be noted that the amounts of xylose revealed by the HPLC analyses are lower than the amounts of reducing sugars detected by DNS method. This apparent discrepancy, however, is most likely the consequence of the simultaneous release of reducing sugars such as glucuronic acid and arabinose from the xylan structure (Voragen et al., 1992, Sporck et al., 2017).

Scanning electronic microscopy of the sugarcane bagasse was done to investigate the structural changes caused by the chemical delignification and by the hydrolysis carried out using the *F. sacchari var. subglutinans* crude extracts (Fig. 2). The non-treated samples exhibited rigid and highly ordered fibrils (Fig. 2A). After the alkaline peroxide pre-treatment and the subsequent saccharification, the structures were strongly modified to less ordered ones with the detachment of the fibers, cell wall collapse and, in several cases, with the formation of pores in the cell wall surfaces (Fig. 2B and Fig 2C, respectively). Such microscopic alterations in the fibers have already been described for other kinds of treatments and have been generally considered to result from lignin removal (Castoldi et al., 2014, 2017). The appearance of pores is usually considered as an indicative of increases in the surface area of the cellulose and xylan available for enzyme attack (Taherzadeh & Karimi, 2008).

4. Conclusion

The production of the enzymes CMCase, β-glucosidase, xylanase and β-xylosidase was evaluated in solid state cultures of 21 different isolates of *Fusarium*. High activities of β-glucosidase were produced by several isolates, especially *F. graminearum*, *Fusarium* sp. 126, and *Fusarium* sp. AR 206. These findings can be explored by further studies in terms of their potential industrial relevance. Enough xylanase and β-xylosidase were produced by *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* so that an efficient hydrolysis of the xylan component of alkaline peroxide-pretreated sugarcane bagasse was possible. The latter,

consequently, lead to xylose as the main hydrolysis product.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1. CMCase, xylanase, β-glucosidase and β-xylosidase of *Fusarium* crude enzymatic extracts.

Fusarium	Enzymatic activity (U/g substrate)			
	CMCase	xylanase	β-glucosidase	β-xylosidase
GROUP I[1]				
F. oxysporum oxysporum	4.26±0.07*	75.29±6.33	27.40±1.46	11.19±1.30
F. graminearum 8	2.90±0.54	66.36±3.26	77.62±1.83	20.55±1.70
F. graminearum 23	3.43±0.70	64.47±4.06	359.64±8.86*	38.39±2.90*
F. verticilioides ATCC1442	3.86±0.30	88.11±5.43	101.43±7.79	32.13±2.70
F. verticilioides	3.80±0.13	30.04±2.63	69.53±1.96	24.47±1.57
F. solani var. solani	1.83±0.07	15.75±1.93	41.56±0.77	7.23±0.27
F. solani	5.03±0.20*	54.08±2.46	7.74±1.26	40.69±1.26*
GROUP II [2]				
Fusarium sp. 126	3.59±0.37	29.24±1.03	531.97±22.34*	26.21±2.63
Fusarium sp.AR167	3.26±1.10	38.63±1.10	108.89±1.30	27.67±3.03
Fusarium sp.AR176	3.50±0.80	40.19±2.26	159.04±5.26	25.07±2.16
Fusarium sp.132	3.96±0.50	111.25±5.59*	69.86±2.06	21.41±1.86
Fusarium sp.AR101	5.36±0.30*	94.04±6.29	106.89±4.66	32.13±2.10
Fusarium sp. AR 206	3.30±0.17	45.72±1.80	323.14±15.25*	35.30±1.53*
GROUP III [3]				
F. acuminatum	3.03±0.70	54.71±4.63	28.30±2.26	27.87±2.03
F. culmorum	1.20±0.07	10.56±0.80	47.32±4.63	11.32±1.20
F. javanicum var. javanicum	0.57±0.40	4.69±0.76	21.48±3.73	5.61±1.25
F. melanochlorum	1.93±0.83	36.23±1.93	90.61±10.22	8.62±0.30
F. equiseti var. equiseti	0.20±0.03	3.90±1.20	97.43±7.26	14.12±1.97
F. merismoides var. crissum	2.86±0.27	12.05±3.80	172.23±9.29	29.90±1.93
F. lateritum var. majus	2.43±0.40	125.57±8.32*	65.10±9.92	27.94±2.26
F. saccharivar. subglutnans	3.83±0.20	113.72±7.23*	58.74±9.24	36.46±1.63*

[1] well-studied enzyme producing species; [2]endophytic isolates; [3] incompletely studied enzyme producing species. The cultures were developed on solid state conditions using sugarcane bagasse-wheat bran as substrate for 8 days at 28 °C. The highest values of enzymes are indicated by (*) and differ significantly from the other values in the same column ($p \leq 0.05$)

Table 2. Amounts of total reducing sugars, glucose, and xylose liberated by hydrolysis of alkaline peroxide sugarcane bagasse by crude enzymatic extracts of *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans*

Enzymatic extract	Reducing sugars* (mg/g)	Xylose** (mg/g)	Glucose** (mg/g)
F. lateritum var. *majus*	200.60±10.60	166.20±12.10	24.12±2.20
F. sacchari var. *subglutinans*	280.00±19.00	247.40±22.80	trace

*Chemical quantification by DNS method;**Chromatographic quantification

Figure Legends

Figure 1. Enzymatic hydrolysis of alkaline peroxide pre-treated sugarcane bagasse by *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* enzymatic extracts.

Figure 2. Scanning electron microscopy of sugarcane bagasse (A): non-treated material; (B): after alkaline peroxide pretreatment; (C): after saccharification using *F. sacchari* var. *subglutinans* enzymatic extracts. The white arrows show pores in the cell wall surfaces

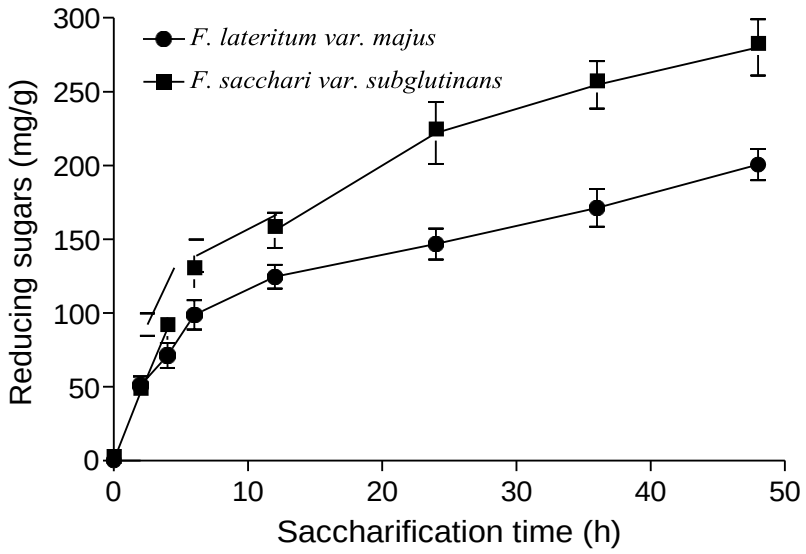

F. lateritum var. majus
F. sacchari var. subglutinans
Reducing sugars (mg/g)
Saccharification time (h)
0
50
100
150
200
250
300
0
10
20
30
40
50

Figure 1.

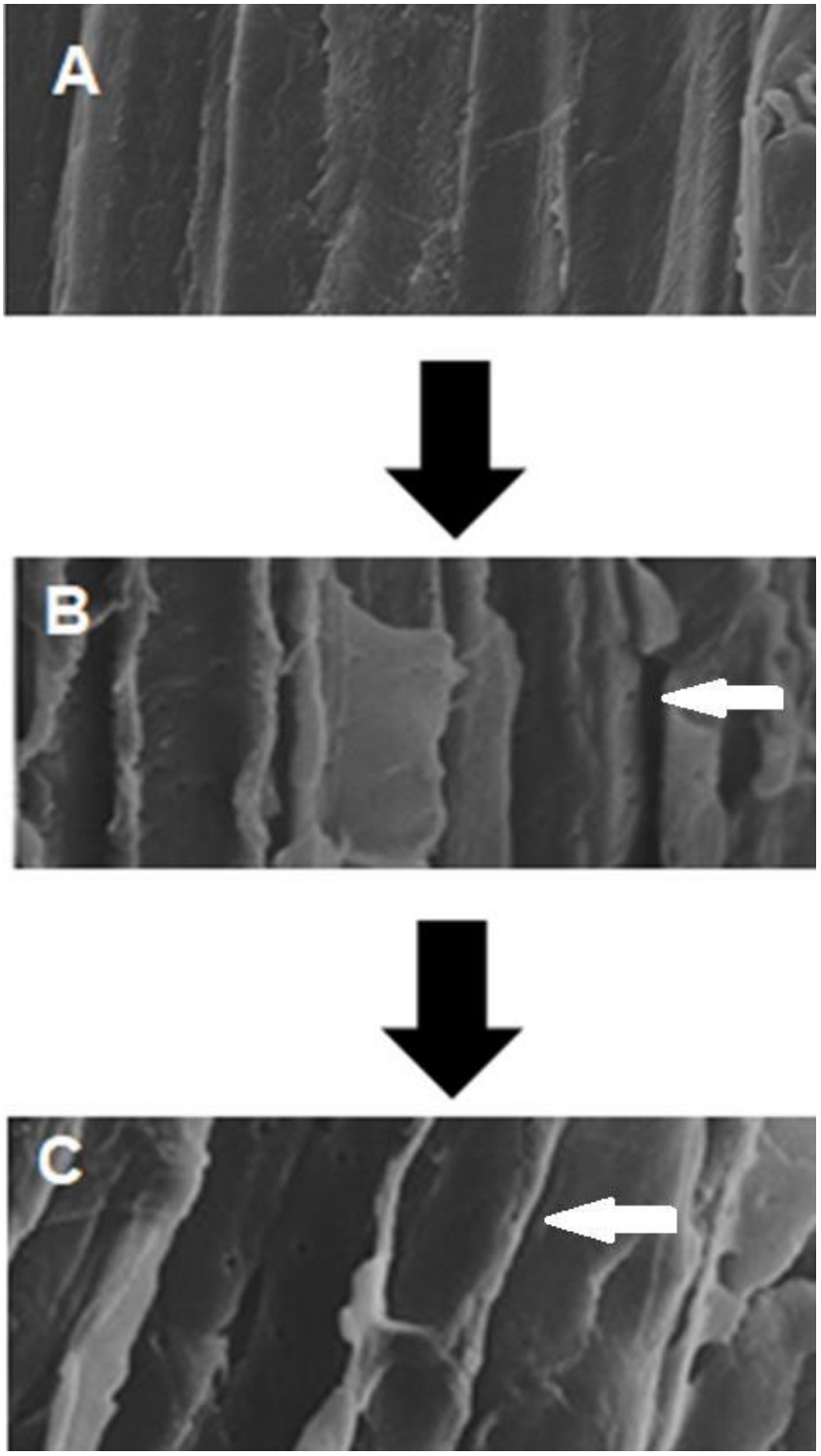
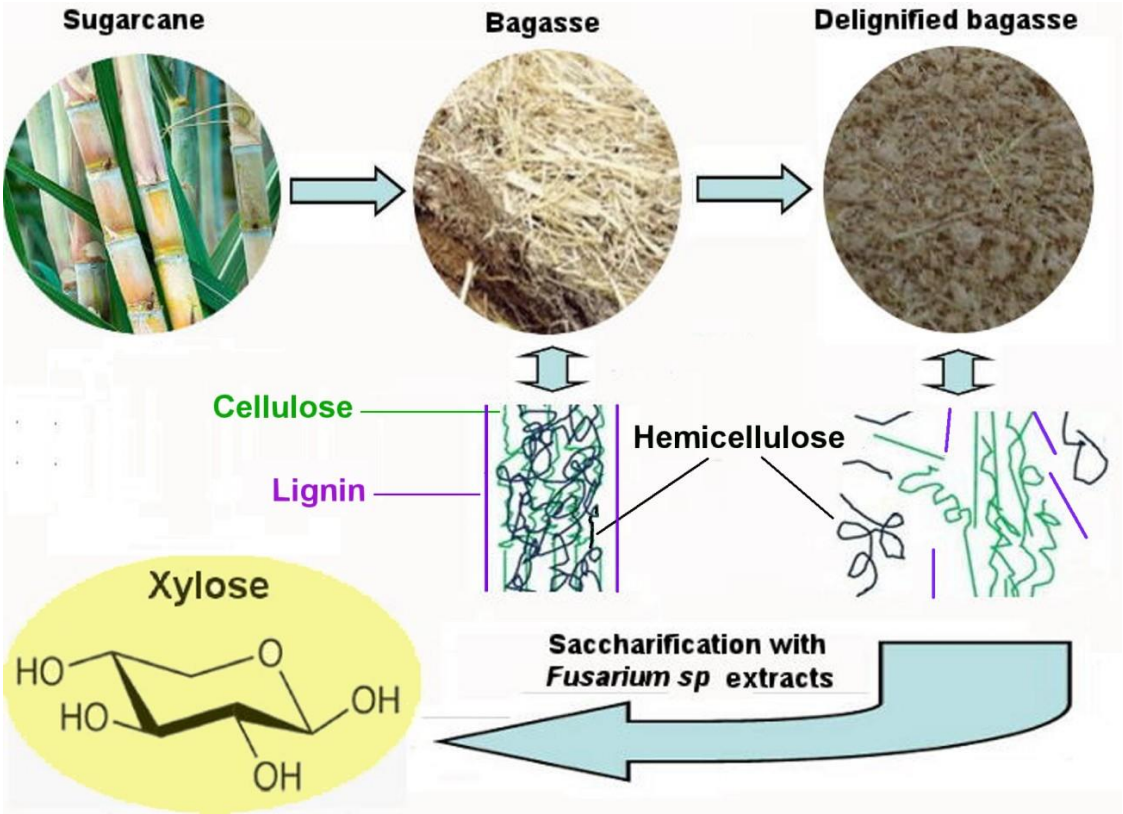


Figure 2.

HIGHLIGHTS

- Screening of *Fusarium sp*. for xylan and cellulose hydrolyzing enzymes
- *F. lateritum* var. *majus* and *F. sacchari* var. *subglutinans* were the best xylanase producers
- The xylans from delignified sugarcane bagasse were efficiently hydrolysed
- Xylose was the main reducing sugar released from delignified sugarcane bagasse



ARTIGO 2

Pré-tratamento do bagaço de cana-de-açúcar com extrato enzimático bruto de *Oudemansiella canarii* rico em lacase aumenta a sacarificação enzimática

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Resumo

Resíduos agroindustriais são fontes de polissacarídeos que podem ser hidrolisados a monossacarídeos constituintes e fermentados para a obtenção de moléculas combustíveis, incluindo o etanol de segunda geração. Pré-tratamentos que possibilitem a redução da lignina são fundamentais para uma sacarificação eficiente. O presente trabalho teve por objetivo avaliar os extratos brutos de *Oudemansiella canarii* ricos em lacase (EEB) no pré-tratamento de bagaço de cana. A sacarificação do bagaço de cana pré-tratado com EEB de *O. canarii* foi realizada utilizando-se o coquetel enzimático comercial Cellic CTec2® + HTec2® da Novozymes. Após 48 h de reação foram obtidos por grama de bagaço de cana, 0,35 g de açúcares redutores, dos quais 0,21 g era glicose. Quando se utilizou no pré-tratamento o extrato enzimático desnaturado, foram obtidos apenas 0,033 g de açúcares redutores dos quais 0,017 g era glicose. Estes dados sustentam que o pré-tratamento do bagaço de cana com EEB de *O. canarii* rico em lacase aumentou mais de 10 vezes a sacarificação do bagaço de cana.

Palavras-chave: bagaço de cana, basidiomicetos da podridão branca, *Oudemansiella canarii*, sacarificação.

Introdução

A crise energética vem atingindo setores importantes da economia global, forçando a busca por tecnologias sustentáveis de produção de combustíveis renováveis. Uma alternativa encontrada é a utilização de biomassa lignocelulósica como fonte de açúcares fermentescíveis. Um dos grandes gargalos ainda é o pré tratamento que visa reduzir o teor de lignina das fibras, favorecendo a posterior sacarificação. Diversos métodos físicos, químicos e biológicos visando reduzir a quantidade de lignina para tornar mais acessível os componentes celulose e hemicelulose à hidrólise vem sendo investigados. O uso de enzimas oxidativas de fungos ligninolíticos (lacases e peroxidases) capazes de degradar a lignina encaixa-se dentro do contexto de pré-tratamentos biológicos e são menos poluentes, requerendo um gasto energético menor que os métodos físicos e químicos (Couto & Sanromán, 2005, 2006, Panagiotou et al, 2003, Farinas, 2015, Winquist et al., 2008). As lacases (EC. 1.10.3.2) são fenol-oxidases que catalisam a redução de oxigênio molecular a água pela retirada de um elétron do substrato aromático. Lacases fúngicas tem sido consideradas como hábeis em modificar e/ou degradar a lignina dos materiais lignocelulósicos. Pré-tratamento de diferentes fibras lignocelulósicas já foram realizados com sucesso utilizando-se lacases de Picnoporus cinnabarinus (Rencoret et al., 2016, Rico et al., 2015), Trametes versicolor (Chem et al., 2012), Coriolopsis rigida e Trametes villosa (Jurado et al., 2009). Considerando o exposto, o presente trabalho teve como objetivo utilizar extratos enzimáticos brutos ricos em lacase obtidos de cultivos em estado sólido do fungo *Oudemansiella canarii* no pré-tratamento do bagaço de cana para facilitar a posterior sacarificação utilizando um coquetel enzimático comercial.

Material e métodos

Microrganismo.

O fungo ligninolítico *Oudemansiella canarii* utilizado pertence à Coleção de Basidiomicetos do Laboratório de Bioquímica de Microrganismos do Departamento de Bioquímica da Universidade Estadual de Maringá. O isolado de *Oudemansiella canarii* foi gentilmente cedido pela Embrapa-Florestas, Colombo, PR. Para manutenção do fungo em laboratório, repiques sucessivos são realizados em ágar extrato de malte (glicose 2%, extrato de malte 2%, peptona 0,1% e ágar 2%). Discos miceliais de 10 mm de diâmetro foram obtidos das placas de Petri cobertas por micélio e utilizados como inóculo.

Materiais.

Bagaço de cana utilizado neste trabalho foi gentilmente cedido pela Usina Santa Terezinha, Iguatemi, PR. Farelo de trigo foi adquirido no comércio local. Substratos utilizados para avaliação das atividades enzimáticas foram adquiridos da Sigma-Aldrich Co.

Cultivo e obtenção do extrato enzimático bruto.

O meio de cultivo consistiu de 3,0 g de bagaço de cana e 1,5 g de farelo de trigo, suplementados com solução mineral (Vogel, 1956) para se obter uma umidade inicial de 82%. Após esterilização por autoclavação, 3 discos de micélio de *O. canarii* com diâmetro de 1 cm foram adicionados aos frascos que foram mantidos a 28 ^{o}C por 13 dias. As culturas foram interrompidas pela adição de 20 mL de água. Após 30 min sob agitação de 100 rpm em temperatura ambiente, as misturas foram filtradas em gaze e posteriormente submetidas à

centrifugação (8000 rpm por 10 minutos). Os sobrenadantes foram considerados como sendo os extratos enzimáticos brutos (EEB).

Avaliação das atividades enzimáticas do EEB.

As atividades de celulase e xilanase foram avaliadas como descrito previamente (Corrêa et al., 2016) utilizando carboximetilcelulose e xilano como substratos. Os açúcares redutores liberados foram quantificados utilizando-se o método do ácido 3,5 dinitrosalicílico (Miller, 1959), utilizando curvas de calibração com glicose e xilose, respectivamente. Lignina peroxidase (LiP), peroxidase dependente de manganês (MnP), peroxidase versátil (VP) e lacase (lac) foram avaliadas conforme descrito previamente (Mota et al., 2015). A atividade da lacase (EC 1.10.3.2) foi mensurada com 2,2´-azino-bis (3-etilbenzotiazolina-6-sulfônico) (ABTS) em 50 mM de tampão acetato de sódio (pH 5.0). A oxidação do ABTS foi determinada pelo aumento da absorbância a 420 nm (ε = 36 $mM^{-1}cm^{-1}$). A atividade da peroxidase dependente de manganês (MnP; EC 1.11.1.13) foi mensurada espectrofotometricamente, seguindo a oxidação de $MnSO_4$ 1 mM em tampão malonato de sódio 50 mM (pH 4.5) na presença de $H_2O_2$0.1 mM. Os íons manganês formam um complexo com malonato que absorve a 270 nm (ε= 11,590 M^{-1} cm^{-1}). A atividade da Lignina peroxidase (EC 1.11.1.14) foi determinada espectrofotometricamente a 310 nm, através da avaliação da formação de veratraldeído dependente de H_2O_2 (ε = 9300 M^{-1} cm^{-1}) a partir de álcool veratílico. Todas as atividades enzimáticas foram determinadas a 40 °C e os resultados foram expressos em unidades enzimáticas internacionais.

Aplicação dos EEB de *O. canarii* no pré-tratamento de bagaço de cana.

Para o pré tratamento, utilizou-se bagaço de cana seco a 40 ^oC até atingir peso constante e triturado para obtenção de pós (50 mesh). Experimentos para avaliar o efeito do extrato

enzimático de *O. canarii* sobre o bagaço-de-cana foram conduzidos em frascos Erlenmeyer de 250 mL, contendo 5 g do bagaço-de-cana, 90 mL do extrato bruto rico em lacase de *O. canarii* e 10 mL de uma solução 500 mmol/L de tampão acetato pH 5,0 para obtenção de uma concentração final de 50 mmol/L. As misturas foram mantidas por 24 h, a 120 rpm e temperatura de 28º C. As biomassas foram filtradas com auxílio de bomba a vácuo e a parte insolúvel foi lavada com água destilada. Foram realizados 4 ciclos de pré-tratamento com EEB. Ao final, as biomassas residuais foram secas em estufa a 40 ºC até atingir peso constante. Como controles negativos foram usados EEB previamente fervidos a 100 ºC durante 3 minutos. Cada pré tratamento foi realizado em quadruplicata.

Sacarificação do bagaço de cana pré-tratado.

Para a sacarificação, bagaço de cana pré-tratado enzimaticamente (500 mg) foi colocado em frasco Erlenmeyer de 125 mL e adicionados 9 mL de tampão citrato de sódio 50 mM pH 5,0 e 1 mL de extrato das enzimas comerciais Novozymes Cellic Ctec2® e Htec2® na proporção de 9:1 (v/v). As misturas foram mantidas sob agitação de 120 rpm a 42 ºC por períodos variáveis até 48 horas. Amostras de 500 µL de extrato foram periodicamente retiradas para análises de açúcares redutores pelo método do ácido 3,5 dinitrosalicílico (Miller, 1959) e glicose pelo método da glicose oxidase-peroxidase utilizando kit comercial.

Análises estatísticas.

As análises foram realizadas em quadruplicata para cada amostra. Os resultados foram expressos com as médias ± desvio padrão (DP). A análise estatística foi realizada utilizando-se o software Graphpad Prism.

Resultados e discussão

Nas condições de cultivo utilizadas neste trabalho, foi possível obter um extrato enzimático bruto (EEB) de *O. canarii* com atividades de CMCase 0,12 U/mL, xilanase 0,37 U/mL e lacase 6,2 U/mL. Não foram detectadas as enzimas lignina peroxidase, peroxidase dependente de manganês e peroxidase versátil.

Os cultivos em estado sólido permitem o uso de subprodutos e resíduos de indústrias alimentícias e agrícolas como, por exemplo, farelo de trigo, bagaço de cana de açúcar, farelo de arroz, espiga de milho, entre outros. A escolha do resíduo deve ser baseada na abundância deste na região, o que reflete no custo do mesmo e também suas características físico-químicas e nutricionais devem ser de interesse. No Brasil, a cana de açúcar é uma das maiores monoculturas agrícolas, fornecendo assim uma enorme quantidade de resíduos, porém possui baixo valor nutricional. Devido a essa questão, o farelo de trigo que é uma boa fonte de nitrogênio, foi utilizado como substrato junto com o bagaço de cana na produção de enzimas por O. canarii.

A Figura 1 apresenta os resultados obtidos para a sacarificação do bagaço de cana pré tratado com EEB de *O. canarii* rico em lacase com o coquetel enzimático comercial Cellic CTec2® + HTec2® da Novozymes. Para fins de comparação, a sacarificação nas mesmas condições de um bagaço de cana que foi pré-tratado em paralelo utilizando o EEB de *O. canarii* desnaturado é apresentado. Após 48 h de sacarificação, foram obtidos 96,44 µmoles/mL de açúcares redutores e 57,86 µmoles/mL de glicose a partir do bagaço pré-tratado com o EEB de *O. canarii* rico em lacase. Por outro lado, quando se utilizou o extrato desnaturado no pré- tratamento, foram obtidos 9,19 µmoles/mL de açúcares redutores e 4,68 µmoles/mL de glucose.

A Figura 2 apresenta o rendimento final da sacarificação com o kit celulolítico comercial do bagaço de cana pré-tratado, 0,35 g/g de açúcares redutores e 0,21 g/g de glicose na sacarificação do bagaço de cana pré-tratado com EEB nativo. O pré-tratamento

aumentou em mais de 10 vezes a liberação de açúcares fermentescíveis a partir do bagaço de cana.

Este resultado sugere que o EBB de *O. canarii* atuou na fibra lignocelulósica do bagaço de cana, tornando a hemicelulose e a celulose mais expostas ao ataque das enzimas hidrolíticas CTec2® + HTec2®. Este pool enzimático tem sido utilizado na hidrólise de várias fibras lignocelulósicas, mas os resultados obtidos são melhores quando métodos de pré-tratamento reduzem o teor de lignina e/ou afrouxam a estrutura dos polissacarídeos (Castoldi et al., 2017)

Os cogumelos do gênero Oudemansiella são comestíveis e consumidos ao redor do mundo. Tal gênero inclui as espécies O. radicata, O. canarii, O. orientalis, O. hongoi entre outras (Magingo et al., 2004). Eles são comuns no território brasileiro e são responsáveis por colonizar muitas plantas como, por exemplo, a Araucária (Ruegger et al., 2001). Muitas espécies desse gênero possuem compostos bioativos e alguns deles atuam como estimuladores imunológicos, anti-hipertensivos, anti-câncer e antibiótico (Xu et al., 2016). O gênero Oudemansiella é considerado um decompositor da madeira mas quase nada é conhecido a respeito de suas enzimas ligninolíticas, incluindo as lacases. As exceções são a descrição da produção de celulases e lacases por Oudemansiella radicata (Balaraju et al., 2010) e de uma piranose oxidase geradora de H_2O_2 por Oudemansiella mucida (Daniel et al., 1994). A espécie *Oudemansiella canarii* é comum em vários biomas brasileiros incluindo a Mata Atlântica (Rosa & Capelari, 2009), Floresta Amazônica e Pantanal (Bononi et al., 2008).

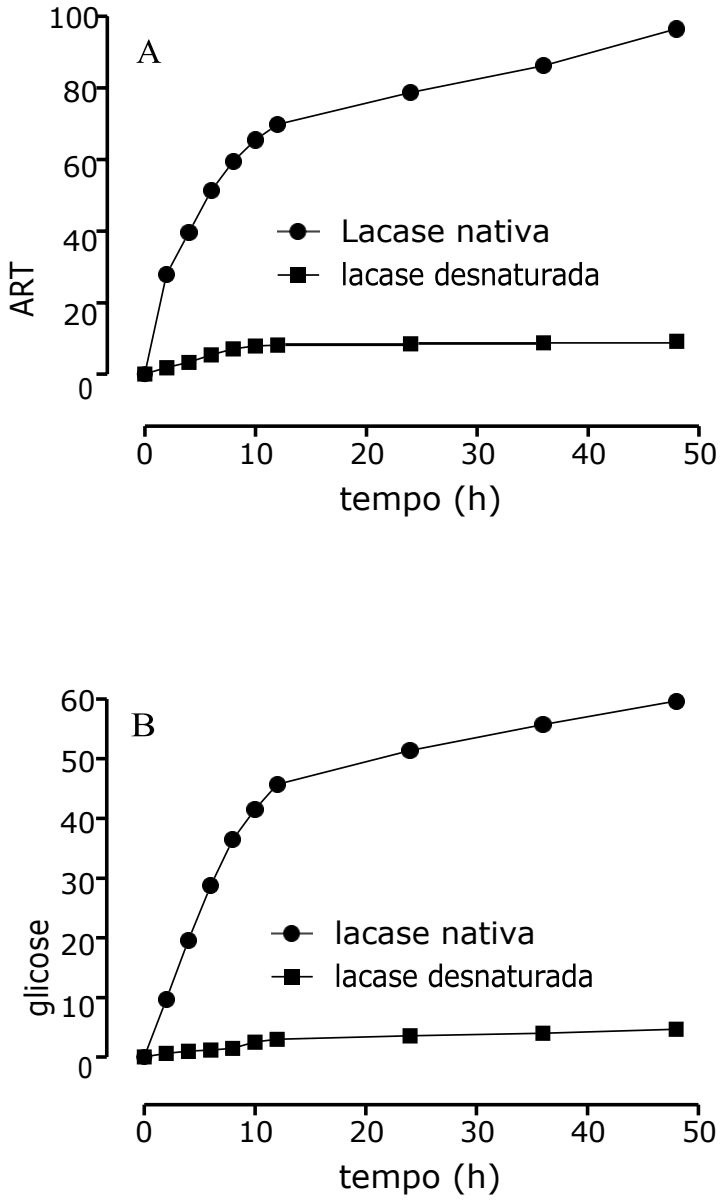

Figura 1: Sacarificação de bagaço de cana de açúcar pré-tratado. O bagaço de cana de açúcar foi pré-tratado com lacase bruta de *Oudemansiella canarii* nativa e desnaturada e sacarificados utilizando o coquetel enzimático comercial Cellic CTec2® + HTec2® da Novozymes. Em A: ART= açúcares redutores totais; Em B: glicose

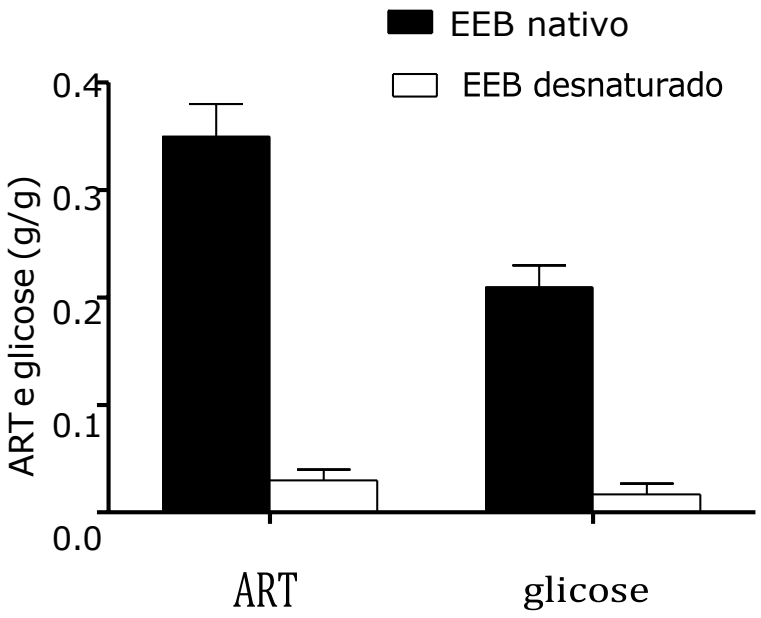

Figura 2. Avaliação da produção de açúcares redutores totais (ART) e glicose utilizando o bagaço de cana pré-tratado com EEB de *O. canarii* rico em lacase nativa e desnaturada.

Conclusões

Os dados obtidos neste trabalho suportam o uso do pré-tratamento enzimático do bagaço de cana com lacase bruta de *O. canarii* para aumentar a sacarificação e produção de açúcares fermentescíveis, incluindo glicose. Com isto, ampliamos o número de prováveis aplicações tecnológicas de O. canarii, um fungo ligninolítico comum no território brasileiro e ainda pouco explorado.

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