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Cellulases and xylanases production by endophytic fungi by solid state fermentation using lignocellulosic substrates and enzymatic saccharification of pretreated sugarcane bagasse

Natália Paganini Marques[a], Josiani de Cassia Pereira[b], Eleni Gomes[b], Roberto da Silva[c], Angela Regina Araújo[d], Henrique Ferreira[e], André Rodrigues[e], Kelly Johana Dussán[a], Daniela Alonso Bocchini[a,*]

[a] Department of Biochemistry and Chemical Technology, IQ/UNESP–Univ Estadual Paulista, Rua Prof. Francisco Degni, 55, 14800-060 Araraquara, São Paulo State, Brazil
[b] Department of Biology, IBILCE/UNESP–Univ Estadual Paulista, Rua Cristóvão Colombo, 2265, 15054-000 São José do Rio Preto, São Paulo State, Brazil
[c] Department of Chemistry and Environmental Sciences, IBILCE/UNESP – Univ Estadual Paulista, Rua Cristóvão Colombo, 2265, 15054-000 São José do Rio Preto, São Paulo State, Brazil
[d] Department of Organic Chemistry, IQ/UNESP–Univ Estadual Paulista, Rua Prof. Francisco Degni, 55, 14800-060 Araraquara, São Paulo State, Brazil
[e] Department of Biochemistry and Microbiology, IB/UNESP–Univ Estadual Paulista, Avenida 24A, 1515, 13506-900, Rio Claro, São Paulo State, Brazil

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ABSTRACT

Endophytic fungi are widely studied as producers of secondary metabolites of biotechnological interest. In recent years, the interest in these fungi as new sources of enzymes, especially hydrolytic, has increased. In the present study, 14 strains of endophytic fungi not yet explored as enzymes sources were randomly chosen and prospected for cellulases and xylanases production by solid-state fermentation. Initially, fungi were cultivated in a mixture (1:1 w/w) of sugarcane bagasse and wheat bran for 7 days, at 28 °C. In this initial screening, 4 fungi excelled in endoglucanase activity (U/g): *Cladosporium cladosporioides* PAJ 03 (88.51 ± 1.0), *Phomopsis stipata* SC 04 (83.44 ± 7.7), *Trichoderma viridae* PAJ 01 (64.56 ± 4.0) and *Botryosphaeria* sp. AM 01 (42.79 ± 1.6). On the other hand, the following 4 fungi stood out in relation to β-glucosidase activity (U/g): *Saccharicola* sp. EJC 04 (51.56 ± 2.7), *Paecilomyces* sp. SF 021 (33.19 ± 9.2), *Ustilaginoidea* sp. CV 04 (29.75 ± 0.8) and *Ustilaginoidea* sp. XYA 04 (21.72 ± 3.05). Among these fungi, *P. stipata* SC 04 and *Botryosphaeria* sp. AM 01 were the best producers of xylanase and β-xilosidase (694,33 and 4,87 U/g, respectively). These 8 fungi were then cultured in new mixtures (1:1 w/w) of lignocellulosic substrates. *Botryosphaeria* sp. AM01and *Saccharicola* sp. EJC04 stood out regarding endoglucanase and β-glucosidase activities (184.74 ± 6.0 and 92.28 ± 9.57 U/g, respectively) when cultivated on cotton seed meal and wheat bran and were selected to continue the study. The influence of time cultivation, inoculum amount and substrate initial moisture content was evaluated and the best condition for cellulases production was 192 h, six mycelial plugs and 65%, respectively, for both fungi. Cellulases and xylanases produced under these conditions were characterized and optimum pH and temperature values were between 4.5–6 and 60–75 °C, respectively. The enzymes were stable over a wide pH range and under 30–70 °C. β-glucosidase from both isolates retained about 75–80% of their activity in the presence of glucose at 6 mM. The presence of ethanol stimulated β-glucosidase activity from *Botryosphaeria* sp. AM01 (about 60% higher in the presence of ethanol at 15%). On the other hand, the activity of β-glucosidase produced by *Saccharicola* sp. EJC 04 was reduced at ethanol concentrations above 15%. A blend of the enzymatic extracts was used to saccharify pretreated sugarcane bagasse and a face-centered central composite design was used to find the best conditions. Under the predicted optimum condition (50 °C, 5% of sugarcane bagasse, 150 U g^{-1} of endoglucanase and 20 h), glucose and xylose concentrations obtained were 3.56 and 1.66 mg mL^{-1}, respectively. These results show that the 14 endophytic fungi studied have potential to be explored as producers of plant material degrading enzymes. *Botryosphaeria* sp. AM01 and *Saccharicola* sp. EJC 04 are promising in relation

Abbreviations: SCB, sugarcane bagasse; CSM, cottonseed meal; WB, wheat bran; OT, oat; SBM, soybean meal; SSF, solid-state fermentation; PDA, potato dextrose agar; FPase, cellulase activity on filter paper; FPU, filter paper unit; CCD, face centered central composite design; HPLC, high pressure liquid chromatography

* Corresponding author.
E-mail addresses: nataliapaganini@hotmail.com (N.P. Marques), josianipereira@gmail.com (J. de Cassia Pereira), eleni@ibilce.unesp.br (E. Gomes), dasilva@ibilce.unesp.br (R. da Silva), araujoar@iq.unesp.br (A.R. Araújo), henfer@rc.unesp.br (H. Ferreira), andrer@rc.unesp.br (A. Rodrigues), kelly.medina@iq.unesp.br (K.J. Dussán), danibocchini@iq.unesp.br (D.A. Bocchini).

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to the production of cellulases and xylanases and their enzymatic extracts have potential for application in pretreated sugarcane bagasse saccharification processes.

1. Introduction

Endophytic fungi are a very diverse group of microorganisms present in most (if not all) plants in the natural ecosystem without causing external symptoms (Kogel et al., 2006) and have been intensively studied due to their symbiotic relationship with plants and also due to their biotechnological potential as pest control agents and as sources of secondary metabolites, including bioactive substances (Chapla et al., 2014). More recently, they have been reported as producers of plant material degrading enzymes including hydrolases, such as cellulases, xylanases, amylases and pectinases, and oxidative ligninolytic enzymes (Amirita et al., 2012). By the production of these enzymes, they invade and colonize plant tissues and also obtain nutrients. However, most genera of endophytic fungi have been little explored as enzymes sources for biotechnological applications (Corrêa et al., 2014).

Cellulases especially have received attention in recent years, since they are used to saccharify cellulose of lignocellulosic materials releasing glucose that can be converted into cellulosic ethanol by fermenting microorganisms. Cellulosic ethanol is reported as the best alternative biofuel to be used as a substitute for fossil fuels, which besides not being renewable cause environmental impacts from its combustion (Raj and Krishnan, 2018; Nguyen et al., 2017; Harris et al., 2014; Pereira et al., 2016). Xylanases hydrolyze xylan, the main hemicellulosic polysaccharide, which associated to other hemicelluloses components binds to the surface of cellulose microfibrils by hydrogen bonding and hinders cellulase action during saccharification (Farinas et al., 2008). Therefore, the presence of xylanases in the enzymatic cocktail is very important to disrupt xylan and to facilitate the access of cellulases to their substrate, when the objective is to obtain glucose from this cellulose, in the context of sugarcane bagasse saccharification for second generation ethanol production (Kalim et al., 2015). Both cellulases and xylanases can be used in several other industrial processes, highlighting the textile, pulping, and animal nutrition sectors (Goswami and Rawat, 2015; Kuhad et al., 2011).

Enzymatic hydrolysis of cellulose, the main polysaccharide of lignocellulosic materials, to glucose involves the synergistic action of three enzymes: endoglucanases (EC 3.2.1.4) that hydrolyze glycosidic bonds randomly in the amorphous regions of cellulose, generating oligosaccharides with reducing and non-reducing ends for action of exoglucanases or cellobiohydrolases (EC 3.2.1.91), which cleave oligosaccharides to cellobiose, to be hydrolyzed by β-glucosidases (EC 3.2.1.21) to glucose (Juturu and Wu, 2014).

The hydrolysis of xylan, the second most abundant natural polysaccharide of lignocellulosic materials, is accomplished by the synergistic action of endo-xylanases (EC 3.2.1.8) which hydrolyze internal glycosidic bonds randomly on the main chain and β-xylosidases (EC 3.2.1.37) responsible for the hydrolysis of xylobiose and small xylooligosaccharides from the non-reducing end, releasing xylose (Moreira and Filho, 2016). In recent years, there has been increased the interest in ethanol production from xylose fermentation by some microorganisms (Duangwang et al., 2016). Efficiently mixed fermentation of both hexoses and pentoses may be a viable alternative for ethanol production (Novy et al., 2015).

Considering the potential for biotechnological applications of cellulases and xylanases, the prospect of new microbial sources is important, especially regarding filamentous fungi that are excellent protein secretors. In this sense, endophytic fungi are a promising group considering that most genera have been little explored as hydrolases producers and they most likely produce enzymes with interesting characteristics in terms of access and attack to the polysaccharides of plant cell walls, since they have to invade and colonize plant tissues (Corrêa et al., 2014).

Most of the studies regarding the production of plant degrading enzymes by endophytic fungi involve cultivation on solid media and qualitative evaluation of substrates hydrolysis (Sunitha et al., 2013) (Katoch et al., 2014). Quantitative analysis by submerged fermentation is also cited (Katoch et al., 2014). However, studies using solid-state fermentation (SSF) for this purpose are scarce and involve only some of the most commonly studied endophytic genera such as *Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium* and *Penicillium* (Almeida et al., 2011; Onofre et al., 2013). SSF is advantageous when compared to submerged fermentation in some aspects, including higher yields in a shorter time and the use of widely available and inexpensive lignocellulosic residues as substrates (Ghoshal et al., 2012).

In this context, the aim of the present study was to explore the production of cellulases and xylanases by 14 strains of endophytic fungi, belonging to 13 genera, by SSF using different mixtures of lignocellulosic materials as substrates. *Botryosphaeria* sp. AM01 and *Saccharicola* sp. EJC04 were selected and the influence of cultivation time, inoculum concentration and substrate initial moisture content was evaluated, since they are important parameters for enzymes production (Bansal et al., 2012; Yoon et al., 2014). Cellulases and xylanases from the selected fungi were characterized and the crude enzymatic extracts were used to saccharify sugarcane bagasse submitted to alkaline hydrothermal pretreatment.

2. Material and methods

2.1. Microorganisms, maintenance and inoculum

The endophytic fungi used in this study belong to the working collection of the Center for Bioassays, Biosynthesis and Ecophysiology of Natural Products (NUBBE), IQ/UNESP, Araraquara, São Paulo State, Brazil. Stock cultures were maintained on PDA, in cryovials, at −80 °C, under a 20% aqueous glycerol solution. Fourteen strains of the different genera were randomly chosen (Table 1), cultured on potato dextrose agar (PDA) at 28 °C, until complete mycelial growth (about 7 days) and

Table 1
Enzymes activities obtained by endophytic fungi cultivation, by SSF, at 7 days, under 28 °C, using a mixture (5 g; 1:1 w/w) of sugarcane bagasse and wheat bran as substrates, with the initial moisture of 70% and 5 mycelial discs as inoculum. EG: endoglucanase; βG: β-glucosidase; XYL: xylanase; βX: β-xylosidase.

Fungi	Enzymes activities (U g^{-1})				
	EG	βG	Fpase	XYL	βX
Acremonium sp. CSF 17	11.66	4.22	0.07	144.87	0.20
Myrothecium gramineum CSF 23	17.90	1.03	0.08	145.48	0.02
Colletotrichum crassipes CSY 02	5.32	0.43	0.08	8.33	ND
Coniothyrium minitans CV 03	13.02	8.27	0.06	407.24	0.08
Ustilaginoidea sp. CV 04	18.70	29.74	0.08	367.43	1.20
Trichoderma viridae PAJ 01	64.56	2.97	0.26	351.74	0.53
Cladosporium cladosporioides PAJ 03	88.50	11.88	0.20	569.48	1.52
Phomopsis stipata SC 04	83.43	24.76	0.16	694.33	0.24
Paecilomyces sp. SF 021	1.43	33.19	0.02	7.06	2.15
Chaetomium sp. TCF 01	12.13	3.81	0.09	39.75	0.21
Coniella petrakii PM 02	14.66	16.58	0.10	21.54	13.07
Botryosphaeria sp. AM01	42.78	13.76	0.25	424.73	4.87
Saccharicola sp. EJC04	39.22	51.56	0.155	103.80	4.24
Ustilaginoidea sp. XYA 04	14.61	21.72	0.06	299.28	0.76

*ND: not detected.