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Characterization of Cellulases of Fungal Endophytes Isolated from *Espeletia* spp.[§]

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Endophytes are microorganisms that asymptomatically invade plant tissues. They can stimulate plant growth and/or provide defense against pathogen attacks through the production of secondary metabolites. Most endophyte species are still unknown, and because they may have several applications, the study of their metabolic capabilities is essential. We characterized 100 endophytes isolated from *Espeletia* spp., a genus unique to the paramo ecosystem, an extreme environment in the Andean mountain range. We evaluated the cellulolytic potential of these endophytes on the saccharification of the oil palm empty fruit bunch (OPEFB). The total cellulolytic activity was measured for each endophyte on filter paper (FPA). In addition, the specific carboxymethyl cellulase (CMCase), exoglucanase, and β-glucosidase activities were determined. We found four fungi positive for cellulases. Of these fungi, *Penicillium glabrum* had the highest cellulolytic activity after partial purification, with maximal CMCase, exoglucanase and β-glucosidase enzyme activities of 44.5, 48.3, and 0.45 U/ml, respectively. Our data showed that the bioprospection of fungi and the characterization of their enzymes may facilitate the process of biofuel production.

Keywords: endophytes, antimicrobial activity, cellulases, OPEFB, *Penicillium*

Introduction

Endophytes are microorganisms that live inside the tissues of living plants without causing apparent harm to their host (Schulz *et al.*, 1999; Tian *et al.*, 2004; Kogel *et al.*, 2006; Schulz and Boyle, 2006; Naik *et al.*, 2009; Rodriguez *et al.*, 2009; Lahlali and Hijri, 2010; Zhao *et al.*, 2011). Endophytic fungi may produce plant-growth regulatory, antimicrobial, antiviral or insecticidal substances to enhance the growth and competitiveness of the hosts in nature (Park *et al.*, 2003; Kim *et al.*, 2007; Verma *et al.*, 2009). They have been recognized as a repository for novel metabolites of possible agricultural, pharmaceutical, and industrial importance. Thus, endophytes are potential sources of new bioactive molecules and may be useful as agents for biotechnological processes (Schulz *et al.*, 2002; Tian *et al.*, 2004; Yu *et al.*, 2010; Zhao *et al.*, 2011). Due to these features, endophytes are important from a bioprospection perspective, and with the use of biotechnological tools, it is plausible to develop products with economic potential from them (Strobel and Daisy, 2003; van den Burg, 2003; Suryanarayanan *et al.*, 2009).

Enzyme complexes produced by endophytes, mainly complexes that can contribute to the generation of economically interesting energy sources, have been the focus of multiple investigations (Karboune *et al.*, 2008). This is the case with cellulases, which allow the bioconversion of biomass to biofuels and mitigate dependence on fossil oil. Lignocellulosic biomass is the most abundant renewable source of sugars that can be fermented to biofuels such as ethanol (Sun and Cheng, 2002; Jiang *et al.*, 2011). The bioconversion of lignocellulolytic compounds to fuel requires biomass transformation into fermentable sugars by enzymatic hydrolysis, which requires a specific mix of cellulolytic enzymes (endoglucanases, cellobiohydrolases, and β-glucosidases) (Cianchetta *et al.*, 2010; de Castro *et al.*, 2010). Cellulase production is a very expensive process that increases the cost of ethanol production from cellulosic biomass. Cellulase production accounts for approximately 40% of the total cost of ethanol production. A significant cost reduction is required to enhance the commercial viability of cellulase production. For this reason, it is important to search for microorganisms with a high rate of cellulase production and to use a cheap lignocellulosic material (e.g., agricultural waste) to reduce the cost of ethanol production (Zhang *et al.*, 2006; Gao *et al.*, 2008). Colombia has an important agricultural area dedicated to oil palm production. This industry produces large amounts of biomass, as more than half of the processed product is the oil palm empty fruit bunch (OPEFB). This large volume of agricultural waste has not been effectively used, considering the amount of lignocellulosic materials, and it appears to be a viable alternative as a cheap substrate source for ethanol production (Umikalsom *et al.*, 1997a, 1997b, 1998).

The objective of this work was to identify fungal endophytes that produce active cellulases with high activity against palm empty fruit bunch (OPEFB). The importance of this work is based on the benefits that could be derived in the process of saccharification of the palm oil industry by-product,

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such as the facilitation of the coupling of the chemical and biological hydrolyses, augmenting the saccharification yields for biofuel synthesis and increasing the value of OPEFB.

Materials and Methods

Fungal collection and taxonomic identification

Fungi were selected from the fungal collection of the Mycology and Plant Pathology Laboratory at the Universidad de los Andes. In a previous study, Avila *et al.* (2012), isolated 609 endophytes from *Espeletia* spp. in the Paramo Cruz Verde, Choachi Cundinamarca. From this collection, we randomly selected 100 fungi and activated them in potato dextrose agar (PDA) for 4 weeks. Morphological characterizations were performed by microscopic observation of fungal preparations in water, lactophenol blue and lactophenol. Identifications were made following traditional taxonomy keys (Barnett, 1960; Domsch, 1980; Hanlin, 1998).

Enzymatic assays

In the first step, the cellulolytic capacity of the fungi was screened on solid agar with carboxymethyl cellulose (CMC), as the substrate using Congo red as an indicator dye. The selected fungi were grown on modified Fries medium consisting of 1% w/v NH_4NO_2, 1% w/v K_2HPO_4, 0.5% w/v $MgSO_4$, 0.1% w/v NaCl, 0.13% w/v $CaCl_2$, 0.01% w/v $MnSO_4$, 0.01% w/v boric acid, 0.001% w/v $CuSO_4$, 0.2% w/v $FeSO_4$, 0.001% w/v $ZnSO_4$, 10 g/L oil palm empty fruit bunch (OPEFB), 5 g/L glucose and 6 g/L peptone. Three plugs of each fungus were added to 30 ml of the modified Fries basal medium (100 ml Erlenmeyer flask) and incubated at room temperature with agitation (150 rpm) for 20 days. Each experiment was performed in duplicate. Sample aliquots (2 ml) were withdrawn at regular time intervals (3 times a week), centrifuged at 13,000 rpm at 4°C for 20 min, and the supernatant was filtered using Whatman N° 1 filter paper. The filtered raw enzyme extracts were used to determine the total activity on filter paper (FPA) and for other enzymatic assays.

For the endophytes showing cellulolytic activity, we measured the activity of the three main cellulase components, carboxymethyl cellulase (CMCase), β-glucosidase and cellobiohydrolase. CMCase activity was measured using 2% (w/v) CMC and quantifying the reducing sugars using the dinitrosalicylic (DNS) method (Miller, 1959; Ghose, 1987; Zhang *et al.*, 2006). β-Glucosidase activity was determined using the cellobiase assay with 1.5 M cellobiose as the substrate (Ghose, 1987; Zhang *et al.*, 2006). Cellobiohydrolase activity was determined using Avicel as the substrate and quantifying reducing sugars by the phenol-H_2SO_4 method (Zhang *et al.*, 2006). Total cellulolytic activity was determined by the filter paper activity (FPA) assay, and the amount of reducing sugars was determined by the DNS method (Miller, 1959; Ghose, 1987; Zhang *et al.*, 2006). The protein content was measured using the Lowry method, with bovine serum albumin as a standard (Waterborg and Matthews, 1996) and measuring the absorbance at 280 nm.

To carry out the cellulase purification process, we selected the fungal extract that showed the highest enzyme activity. The crude enzyme extract was precipitated with ammonium sulfate (90%). The preparation was incubated overnight at 4°C to allow the precipitated protein to sediment and was then recovered by centrifugation at 10,000 rpm for 30 min at 4°C and dissolved in buffer A (twice the volume of the precipitate). The enzyme solution was desalted using dialysis. The proteins obtained in the desalinated sample were quantified by Folin-Lowry methods. Total cellulolytic activity (FPA) was quantified.

The desalted enzyme solution was placed on a DEAE-Sephadex A-50 column previously equilibrated with buffer A. The fractions were eluted with a linear NaCl gradient and analyzed for protein concentration by measuring the absorbance at 280 nm using a Nanodrop® ND-1000 spectrophotometer. Total cellulolytic activity (FPA) and the protein concentrations of the fractions were also measured.

The fractions with cellulolytic activity were pooled and concentrated by ultrafiltration with a Centriprep device (Amicon) with a 10 kDa molecular mass cut-off. The concentrated enzyme was dried in a freeze-dryer and then the powder was dissolved in buffer A. Concentrated samples were analyzed to quantify proteins by the Folin-Lowry method and to determine the cellulolytic activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of the enzymes under denaturing conditions, as described by Laemmli (1970), using a standard molecular weight marker (BioRad 161-0318).

Molecular identification

Fungal strains that showed significant antagonistic or cellulolytic activity were identified by sequence analysis. For these strains, total DNA was obtained by extraction using a previously described protocol (Gardes and Bruns, 1993), and these samples were then amplified by polymerase chain reaction (PCR) using ITS5 and ITS4 primers (White *et al.*, 1990). Amplification reactions were performed in a final volume of 25 µl in a reaction mix with 1× Green GoTaq® Flexi buffer, 2.5 mM $MgCl_2$, 10 mM of each dNTP, 0.2 µM of forward and reverse primers, 1.25 U GoTaq® DNA Polymerase (Promega, USA) and 1 µl of total DNA. The PCR products were sequenced on an ABI Prism 3730XL (Applied Biosystems, USA) automated sequencer. Sequence analyses were performed using the GenBank BLAST online program (Blastn software) (Altschul *et al.*, 1990).

Results

Morphological and molecular characterization of the isolates

Most fungal isolates were identified using traditional taxonomy keys and macroscopic observations. We found 24 sterile mycelia and a large diversity of fungal endophytes. Fungal endophytes with cellulolytic activities were identified using the ITS sequence. All endophytes were shown to share 98–100% sequence identity with the species determined by morphology (Supplementary data Table S1).

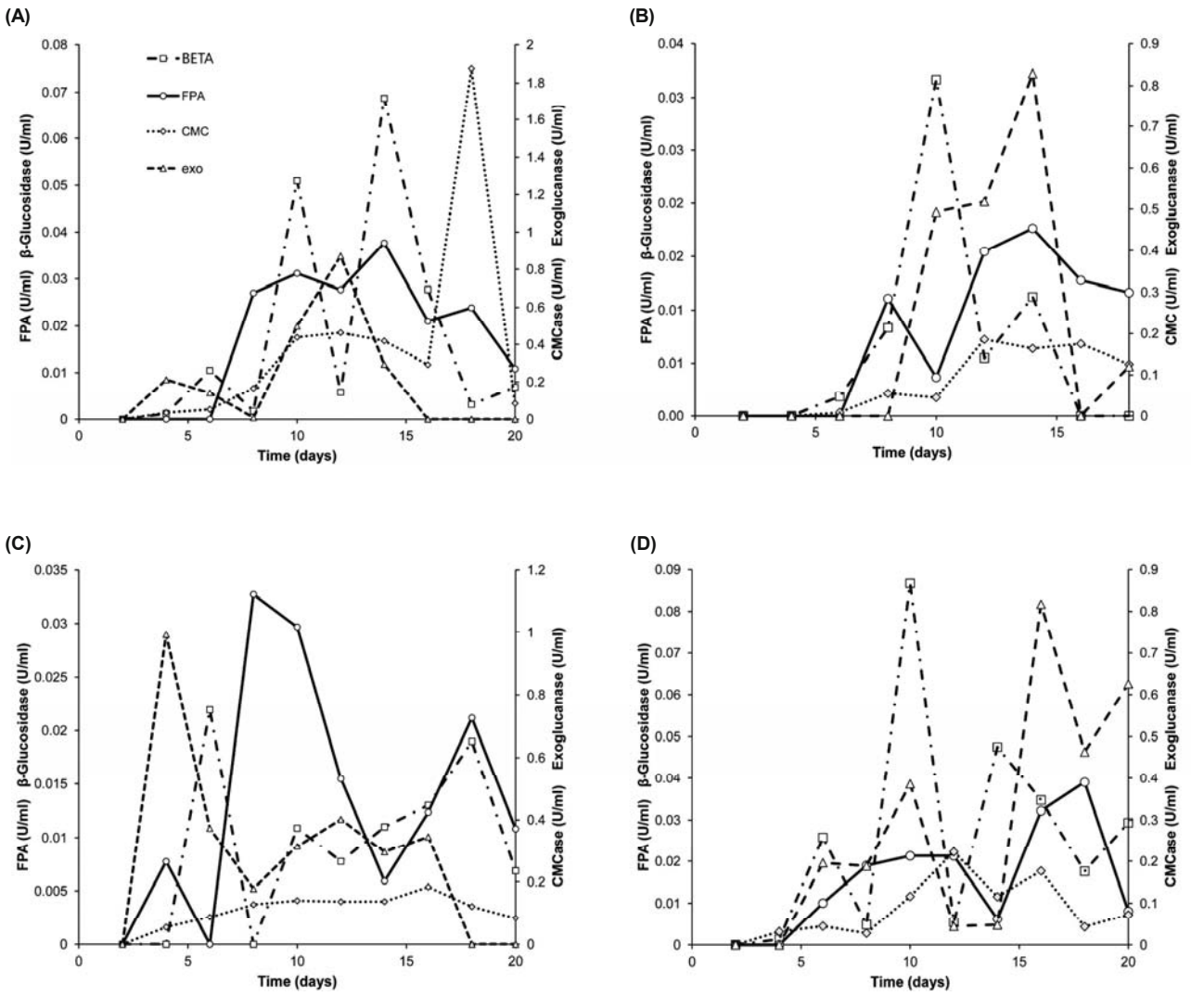

Fig. 1. Cellulase production by fungal endophytes. Total cellulolytic activity on filter paper (FPA) and β-glucosidase, carboxymethyl cellulase (CMCase) and exoglucanase activities are shown over time. (A) *Penicillium glabrum*, (B) Strain 2830, (C) *Aspergillus* sp., (D) *Chaetomium* sp.

Cellulolytic activity of the isolates

Cellulolytic assays with Congo red showed 28 positive fungal endophytes that were then evaluated for total cellulolytic activity on filter paper (FPA). Of these isolates, 17 endophytes showed FPA greater than 0.05 U/ml (Supplementary data Table S2), and four were selected for further quantification of the different cellulolytic activities (β-glucosidase, endoglucanase, and cellobiohydrolase). The profiles for *Aspergillus* sp. 2796, *Penicillium glabrum* 2399, Strain 2830 (Sterile mycelium) and *Chaetomium* sp. 2779, showed an increased cellulolytic activity over time, but there were no clear trends for the four endophytes (Fig. 1). *P. glabrum* had the highest cellulolytic activity, reaching maximal activities of 1.87 U/ml for CMCase, 0.035 U/ml for FPA, 0.06 U/ml for β-glucosidase and 0.87 U/ml for exoglucanases (Fig. 1A). *Aspergillus* sp. exhibited maximal activity for the first few days and then

Table 1. Total cellulolytic activity on filter paper (FPA) and carboxymethyl cellulase, β-glucosidase and exoglucanase activities for the extract and the purified enzyme

	Activity (U/ml)		Total activity (U)	
	Extract	Concentrated fraction	Extract	Concentrated fraction
FPA	0.04	1.21	39.25	1.81
CMCase	0.36	44.52	349.29	66.78
β-Glucosidase	0.09	0.45	89.04	0.68
Exoglucanase	1.13	48.36	1104.98	72.54

decreased activity in the last days (Fig. 1C). Surprisingly, although this isolate grew in a mycelial form as early as the third day, the total cellulolytic activity was very low and only measurable after the eighth day. However, after ten days, this endophyte had the highest total activity on filter paper (FPA).

A partial purification of the cellulolytic enzyme complex from *P. glabrum* 2399 was performed using ammonium sulfate precipitation and desalting. Fourteen-day-old cultures demonstrated the highest total and specific cellulolytic activity. Fractions determined in each process were then grouped according to experimental plots obtained (Supplementary data Fig. S1). Each purification step showed a decrease in total protein and total activity. The greatest loss occurred after protein precipitation with ammonium sulfate, as compared to the loss in the subsequent steps on DEAE-Sephadex columns and fraction concentration (data not shown). However, the total activity and cellulolytic complex increased at each purification step. CMCase activity had a significant increase compared to the other two enzymes with 0.36 U/ml to 44.52 U/ml in the concentrated fraction (Table 1).

The dialysis product, sample column and purified extract revealed a high concentration of proteins with a molecular weight range from 30 to 100 kDa. This high concentration and weight of the purified protein sample may indicate the presence of the three enzymes as constituents of a cellulolytic complex.

Discussion

Endophytic fungi are ubiquitous in higher plants and produce a vast repertoire of natural bioactive compounds that constitute a reservoir for bioprospection (Verma *et al.*, 2009). Our results support these findings by revealing that some endophytic fungi isolated from *Espeletia* spp. have cellulolytic potential for saccharification of oil palm empty fruit bunch (OPEFB) as a source of second generation biofuels.

The bioprospection potential of *Espeletia* spp. endophytes was evidenced by the cellulolytic activities observed in seventeen of the isolates tested, some of which were statistically significant. *Penicillium glabrum* exhibited the highest cellulolytic activity, consistent with previous reports in the literature (Sukumaran *et al.*, 2005; Karboune *et al.*, 2008; de Castro *et al.*, 2010). However, compared to these reports, the FPA, exoglucanase and β-glucosidase activities determined for the isolate in this study were low. It has been proposed that the reason for the low secretion of β-glucosidase is that the major part of this enzyme is tightly bound to the cell wall of the fungus during culturing, and some parts of the enzyme may be found inside the cell (Jiang *et al.*, 2011). Another reason for the low activity of cellulases is that the cellobiohydrolase does not act synergistically with endoglucanase to hydrolyze crystalline cellulose and produce cellobiose, which β-glucosidase transforms into fermentable sugars (Umikalsom *et al.*, 1997a; Karboune *et al.*, 2008). Therefore, one way to alleviate the effect of cellobiose limitation on the overall hydrolysis is to supplement with exogenous β-glucosidase. In addition, it is recommended that the medium be supplemented with cellulase inducers and to use other modes of fermentation (Barros *et al.*, 2010; Cianchetta *et al.*, 2010; Jiang *et al.*, 2011). We suggest that strain complementation in combination with medium optimization may greatly enhance the cellulolytic enzyme yield and its biomass hydrolysis capacity. Finally, we found that the molecular weights obtained cover the range from 30–100 kDa, which, according to previous reports, is in agreement with the molecular weight of endoglucanase, cellobiohydrolase and β-glucosidase; the molecular weights of these proteins have been reported to be 83–94 kDa, 66 kDa, and 85 kDa, respectively (Zeikus, 1981; Wei *et al.*, 1996; Zhang *et al.*, 1998).

In this study, we showed that some tested fungal endophytes produce fermentable sugars. *Penicillium glabrum* 2399 was the best isolate at producing fermentable sugars, with significant cellulolytic activity. These enzymes should be explored for their potential for hydrolysis under various physicochemical conditions, including pH and temperature. The aim is to find the extreme conditions in which they could facilitate integration with a typical chemical-biological process in second generation biofuel production. Additionally, a kinetics characterization could enhance the understanding of the interaction of the enzyme with the substrate. This study could also be complemented by sequencing the genes with subsequent molecular docking studies. The major goal of current research is to screen these fungal resources for novel metabolites and enzymes and determine their application in environmentally friendly, technological development.

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