Enzymatic Potential of Native Fungal Strains of *Agave* Residues

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Twelve strains of fungi from residues of *Agave durangensis* were isolated and identified by molecular techniques for evaluation of their hydrolytic enzyme production capability. A proportion (50%) of the fungal strains belonged to the *Aspergillus* genus and the other strains used belonged to *Alternaria*, *Neurospora crassa*, *Mucor* sp, *Rhizopus* sp., *Botryosphaeria* sp., and *Scytalidium* sp. The isolated strains were evaluated for their potential to produce extracellular enzymes using different substrates (cellulose, xylan, inulin, *Agave* fructans, starch, and tannic acid). It was observed that most of the tested strains were capable of simultaneously secreting cellulases, xylanases, inulinases, fructanases, and laccases. *Botryosphaeria* sp. ITD-G6 was selected for its evaluation in the production of inulinase, using different substrates. Showing high inulinase activities (5.22 U / mL for *Agave* waste, 4.37 U/ mL for inulin and 5.00 U / mL for *Agave* fructans).

Keywords: Inulinase; Fructanase; Molecular identification; Agave durangensis leaves; Botryosphaeria

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INTRODUCTION

Agave is the main genus of the Agavaceae family with about 200 different species. *Agave durangensis* Gentry is a plant belonging to this family that is used for the production of Mexican alcoholic beverages (Tequila and Mezcal), a process that generates various agroindustrial waste products. Approximately 1 million tons of *Agave durangensis* are produced and processed annually, generating large quantities of agricultural waste, of which about 1200 tons are *Agave* leaves; these are considered to have potential as agroindustrial residue (Contreras-Hernández *et al.* 2017). The waste leaves present a high content of cellulose (38 to 50%), whereas the lignin is 15 to 25% and the hemicellulose is 23% to 32% (Vieira *et al*. 2002; Sun *et al.* 2016). *Agave* leaves also have a high fructan content (Orozco-Cortés *et al.* 2015). This agroindustrial waste has characteristics making it a substrate for the production of fungal enzymes with biotechnological interest.

Fungi are remarkable microorganisms due to their plasticity and physiologic versatility. They have the capability to grow and develop in inhospitable habitats with extreme environmental conditions because of their efficient enzymatic system. Among the

varied mechanisms for adaptability of fungi to environmental extremes and for the utilization of their trophic niche, their ability to produce extracellular enzymes are of great survival value (Gopinath *et al.* 2005). New species research is of great importance for biotechnological research development. Approximately 1.5 to 3.5 million species of fungi have been identified in the world.

Filamentous fungi have been used to produce important substances for pharmaceutical, food, and biotechnological industries; they have proved to be useful to produce primary and secondary metabolites such as peptides, organic acids, antibiotics, and extracellular enzymes (Valencia and Chambergo 2013). The production of extracellular enzymes has gained importance due to its wide commercial and industrial applications, which is successfully used to catalyze different chemical processes. These enzymes are often chosen as replacements for chemical catalysts because they are cheaper and environmental friendly (Sohail *et al.* 2009). Recently, enzymes have also been exploited in the production of biofuels (Haghighi Mood *et al.* 2013)

Most extracellular enzymes in use are hydrolytic and are can degrade different natural agroindustrial residues. Proteases are the most employed enzymes due to their application in detergents and the dairy industry (Maurer 2004). The next most popular extracellular enzymes include amylases, pectinases, xylanases, and cellulases, which are used for the extraction and clarification of beverages and in the starch, textile, and detergent industries (Bhat 2000; Beg *et al.* 2001). The most studied fungi that have the capacity to produce these complex extracellular enzymes are *Aspergillus*, *Penicillium*, and *Trichoderma* (Chandra *et al.* 2007; Quintanilla *et al.* 2015; Novelli *et al*. 2016).

Inulin enzymes are an important class of industrial enzymes, which act on the β-2, 1 bonds of inulin to produce syrups with a high fructose content and fructooligosaccharides (Kango and Jain 2011). Fructose is used as a low-carbohydrate sweetener, and fructooligosaccharides are commonly used as a prebiotic source (Singh *et al.* 2015). The most studied fungi for inulinase production are *Aspergillus niger* and *Rhizopus* sp. (Kango 2008; Rawat *et al*. 2015).

Fungi are essential in nature because of their potential to produce extracellular enzymes, which are attributed to their ability to express and secrete proteins (Ferreira *et al.* 2013, Quintanilla *et al.* 2015); therefore the search for new fungi species with demonstrated abilities to secrete enzymes is of interest in the area of biotechnological research.

The objective of this study was to isolate and identify native strains of *Agave durangensis* agroindustrial residues (from Mezcal production process, typical Mexican alcoholic beverage) and to explore their enzymatic potential for possible future applications.

EXPERIMENTAL

Materials

Sample origin

The *Agave* leaves were donated by the Dolores Hidalgo Durango S.P.R. de R.L. Mezcal Production Company (Durango, México), and originated from Ejido Paura in Mezquital, Durango, Mexico. They were approximately 12 years old.

Fungal strains

The fungal strains were first isolated from the *Agave* leaves. *A. durangensis* leaves were moistened and incubated in the dark for ten days at 25 ºC. In places where fungal growth was observed, 0.1 cm^2 of leaf was cut and used for the isolation in a petri dish containing 1% dehydrated ground *Agave* leaves passed through a 40-mesh screen (0.42-mm), 2% agar-agar, and 0.000875% rose bengal (ALDRICH, Chemistry, St. Louis, MO, USA) and was incubated for 7 days at 37 ºC. Samples were reinoculated under the same conditions until isolated colonies were obtained, and these colonies were preserved in 10% glycerol.

Methods

Identification of fungal strains

Morphological identification of the isolated strains was achieved at both macroscopic and microscopic levels using the microculture technique, in potato dextrose agar (PDA) (Difco™ Becton, Dickinson Company, Le Pont de Claix, France) (Baltz *et al*. 2010).

Molecular identification of fungal strains

Fungal strains were inoculated in yeast extract peptone dextrose (YPD) media (1% yeast extract, 2% casein peptone, and 2% glucose; (BD Bioxon, México City, México) at 37 ºC for 3 days. Subsequently, DNA was extracted using the method reported by Sambrook and Russell (2001). Polymerase chain reactions (PCR) were performed in a 12.5 µL volume, using 0.50 µL of DNA template, and the following recombinant DNA primers 464 fglITRS1: (5´AACTCATTGCAATGCYCTATCCCC´3) and 463 fgIITSR2: (5´TCAAGTTAGCATGGAATAATRRAATAG´3). These primers encode a fragment of the ribosomal region 18s. Amplification was performed with an initialization step with one cycle at 95 ºC for 5 min followed by denaturation at 95 ºC for 1 min and then 30 cycles at 55 ºC for 1 min, at 72 ºC for 1 min, with a final elongation at 72 ºC for 10 min (Valinsky and Vedova 2002). The PCR product was observed by electrophoresis in 1% agarose gel with a TE-1X buffer (Promega Corporation, Madison, WI, USA), at 66 volts for 90 min. Gels were stained with ethidium bromide (Sigma-Aldrich Corporation, St. Louis, MO, USA) and observed with a UV transilluminator (Benchtop UV, Upland, CA, USA) (Zhang *et al*. 2000). The PCR product was purified using a Kit Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). The rRNA sequences were acquired using an ABI PRISM Model 3730XL sequencer (Applied Biosystems Inc., Foster City, CA, USA) in the National Genomics for Biodiversity Laboratory (Langebio) of Cinvestav Irapuato, México). The obtained sequences were aligned in the National Center for Biotechnology Information (NCBI) GeneBank using the Basic Local Alignment Search Tool (BLAST), which finds regions of local similarity between sequences.

Detection of extracellular enzymes

Agar media were inoculated with a 2-mm diameter agar plug from an actively growing culture. The incubation was at 25 °C to 37 °C for 2 to 5 days unless otherwise stated.

Inulinase and fructanase

The screening medium was a modification of that described by Kim (1975) and had the following composition: Chicory Inulin (Orafti®, Oreye, Belgium), 2%; potassium

phosphate (K_2HPO_4), 0.1%; magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.05%; sodium nitrate ($NaNO_3$), 0.15%; potassium chloride (KCl), 0.05%; ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 0.01%; ammonium phosphate ($NH_4H_2PO_4$), 0.2%; and Agar, 1.8% (J. T. Baker, México City, México). The initial pH was 6.0. Isolated samples were inoculated in the medium and incubated at 28°C for 5 days. *Agave* of fructans (*Agave tequilana* Weber (American Foods, Jalisco, México) 2% was used for fructanases activity determination.

Cellulases and xylanases

Cellulolytic and xylanolytic activity was detected using minimal medium containing 0.1% (w/v) ammonium chloride (NH_4Cl), 0.1% (w/v) potassium phosphate (KH_2PO_4), 0.04% (w/v) calcium chloride ($CaCl \cdot H_2O$), and 0.01% (w/v)magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (J.T. Baker, México City, México), supplemented with 1.5% (w/v) agar (J. T. Baker, México City, México) and 1% (w/v) Carboxymethylcellulase (CMC) (Sigma-Aldrich Corporation, St. Louis, MO, USA), for cellulases or 0.5% (w/v)xylan (Sigma-Aldrich Corporation, St. Louis, MO, USA), and 0.1% yeast extract (w/v)(MDC-LAB, México City, México) for xylanases detection (Leger *et al*. 1997). The initial pH of the medium was 6.0. After incubation for 48 h, the plates were flooded with Congo red (1mg/mL; 15 min) (Sigma-Aldrich Corporation, St. Louis, MO, USA) and destained with 1 M sodium chloride (NaCl) (J. T. Baker, México City, México). Activity was detected as a yellow halo around the mycelium (Teather and Wood 1982).

Amylases

Amylolytic activity was detected using starch agar (1% peptone (w/v) (Mayer, México City, México), 0.5% yeast extract (w/v) (MDC-LAB, México City, México), 0.5% sodium chloride (NaCl) (J. T. Baker, México City, México) (w/v), and 0.2% starch (National Starch Chemical Company, Bridgewater, NJ, USA) (w/v)). After incubation for 48 h, the plates were flooded with lugol solution (Sigma-Aldrich Corporation, St. Louis, MO, USA). Activity was detected as a yellow halo on a dark background (Hankin and Anagnostakis 1975).

Laccases

For laccase activity detection, strains were grown on a tannic acid malt extract medium (Rigling 1995). A solution of 1% tannic acid (Sigma-Aldrich Corporation, St. Louis, MO, USA) in water was sterilized separately and was added to 1.5% malt extract (Difco™Becton, Dickinson Company, Le Pont de Claix, France) and 2% agar (w/v) (J.T. Baker, México City, México), with a pH of 4.5, after cooling to 50 °C. After incubation for 48 h, activity was detected as a change of color (to brown) around the mycelium.

Submerged fermentation

Botryosphaeria sp. (ITD-G6) was selected to evaluate inulinase activity in the fermentation liquid using different carbon sources. Three cultivating media were prepared to contain each of the following carbon sources: 1) *A. durangensis* leaves, 2) *Agave* fructans, and 3) chicory inulin (3%) (Öngen-Baysal *et al.* 1994) with ammonium sulphate ($(NH_4)_2SO_4$) (0.5%) as the nitrogen source (Kim 1975; Cruz-Guerrero *et al.* 2006). The media were then sterilized and adjusted to a pH of 5; these were inoculated with 1×10^7 spores/mL of the selected strain. Agitation was done at 150 rpm and 30 °C for a period of 120 h (Zhang *et al.* 2004; Ge and Zhang 2005). The enzymes were extracted from the

fermented growth medium. The suspended materials and fungal biomass were separated by centrifugation (10,000 × g) (Eppendorf® AG 5452, Eppendorf Inc., Enfield, Connecticut, USA) for 15 min and the clarified supernatant was used for enzyme activity quantification. Experiments were conducted in duplicate.

Enzymatic activity quantification

Enzymes were assayed by measuring the concentration of reducing sugars released from inulin. The reaction mixture containing 1 mL of diluted crude enzyme and 15 mL of 5% Chicory inulin (dissolved in 0.05 M acetate buffer (Sigma-Aldrich Corporation, St. Louis, MO, USA) (, pH 5.0) was incubated at 50 °C for 30 min. Aliquots of 0.5 mL were then withdrawn and the increase in reducing sugar was estimated by a 3,5-dinitrosalicylic acid method (Miller 1959), a standard dextrose curve was used to quantify the enzymatic activity (Sigma-Aldrich Corporation, St. Louis, MO, USA) as standard. Absorbance was read at 540 nm. A higher absorbance indicated a high level of reducing sugar produced and, consequently, high enzyme activity. One inulinase unit is the amount of enzyme that forms 1 mmol of fructose per min (Jing *et al.* 2003).

RESULTS AND DISCUSSION

Twelve fungal strains were isolated from *A. durangensis* leaves and were labeled with the ITD-G1 code to ITD-G12 for identification (Fig. 1).

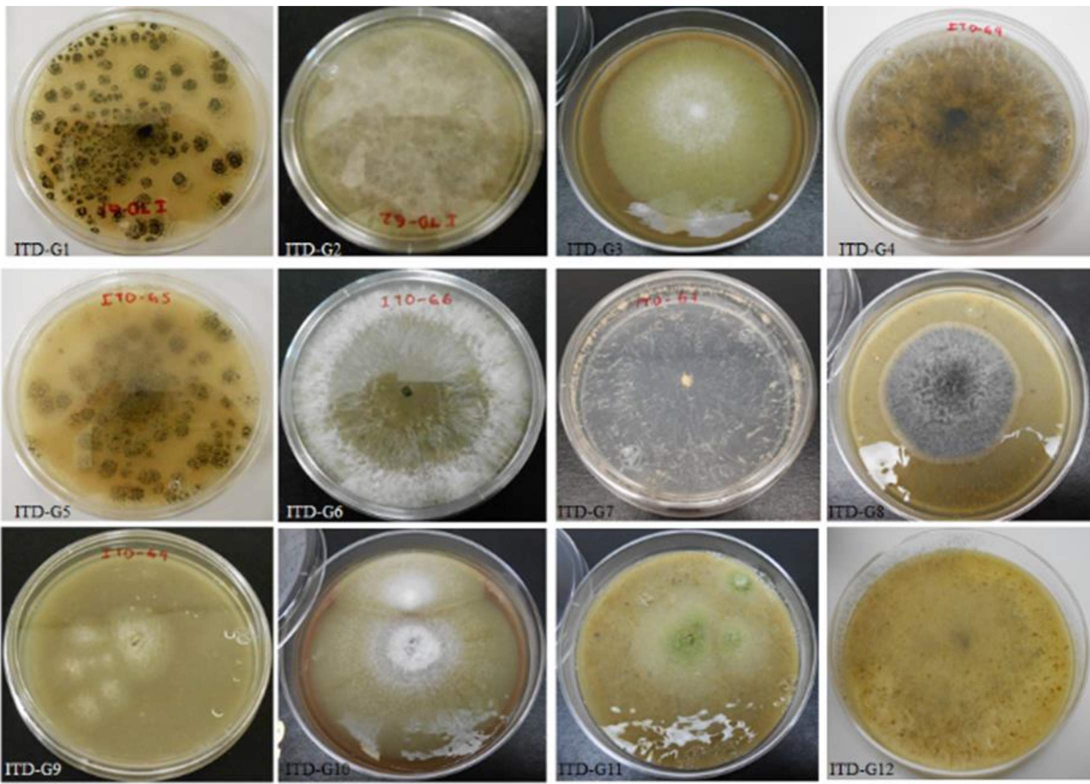


Fig. 1. Isolated fungi from *A. durangensis* (inoculated in *Agave* leaf medium as the carbon source)

Each of the strains showed different characteristics according to the morphological analysis. General characteristics and structures of sporulation of fungal strains are presented in Tables 1a, and 1b, which correspond to the macroscopic and microscopic (40×) images of fungal strains.

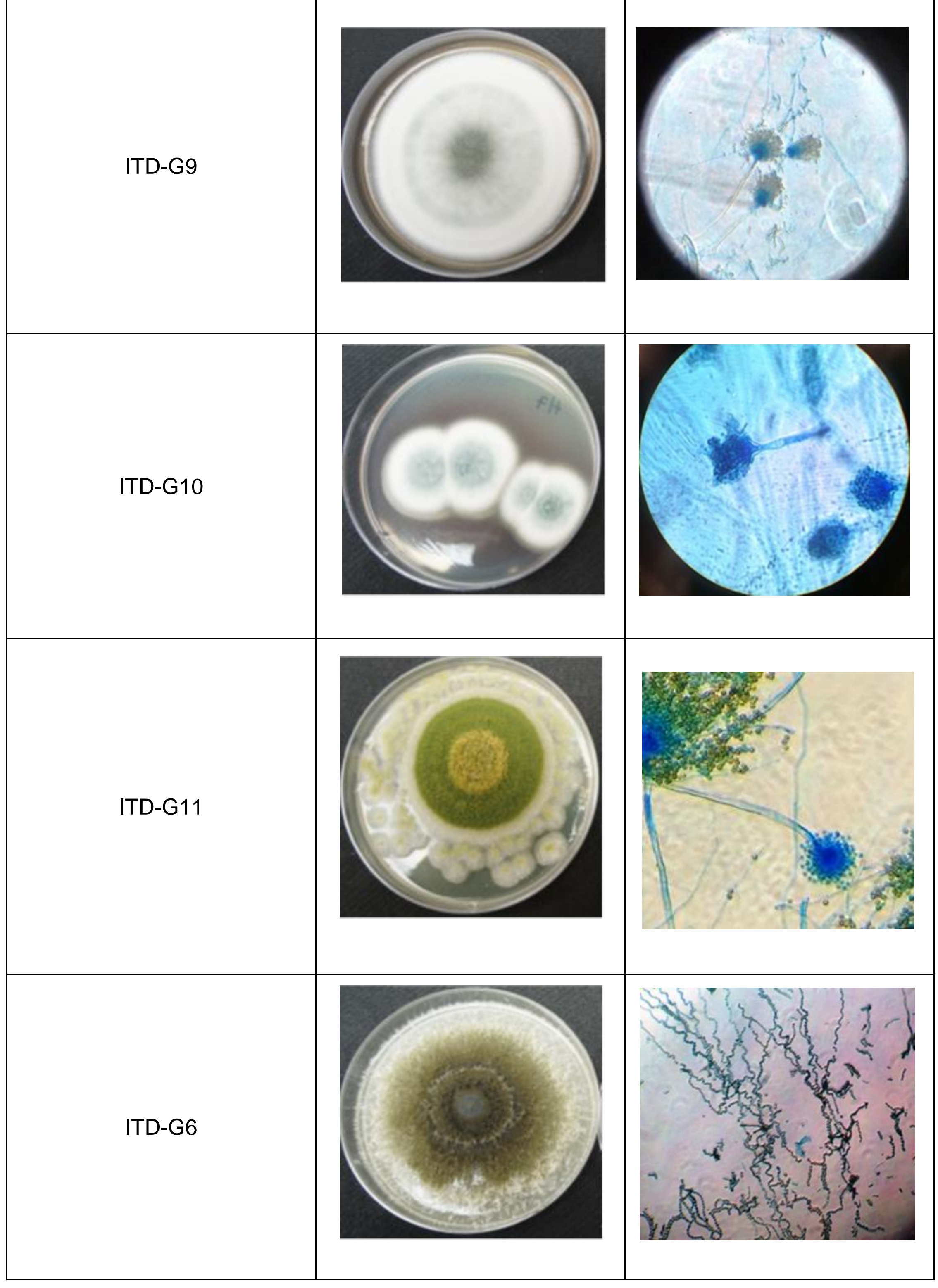
The predominantly isolated microorganism was *Aspergillus* sp. with a presence percentage of 50%, of which 25% of the strains presented physical and microscopical features typically assigned to the genus *Aspergillus fumigatus*. *Scytalidium* sp. (16.6%) and *Mucor* sp. (16.6%), *Neurospora* (8.3%) and *Alternaria* sp. (8.3%), taking into account that the 12 isolated strains were 100%.

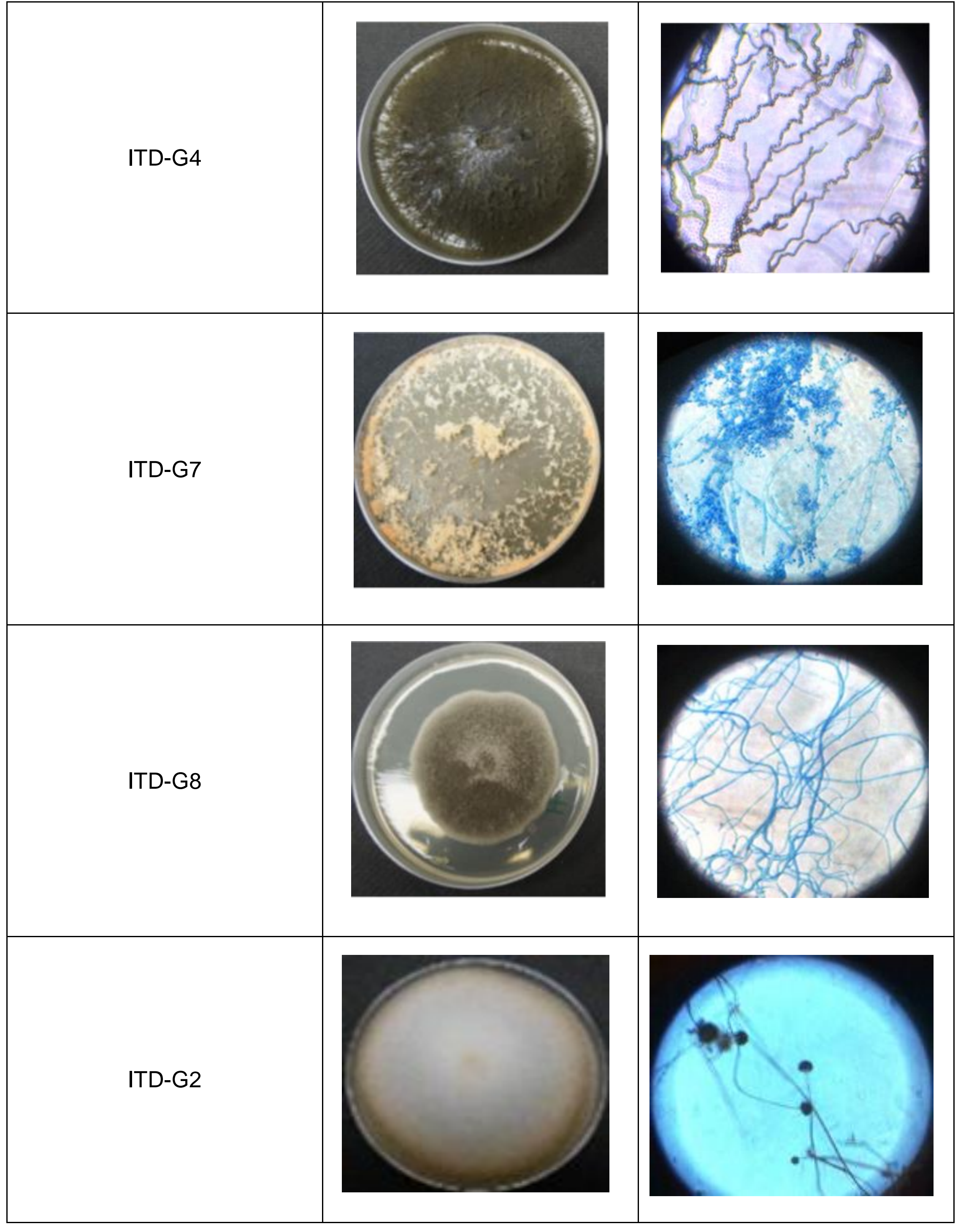
Table 1a. Morphological Identification of Fungal Strains Isolated from *Agave durangensis* Leaves

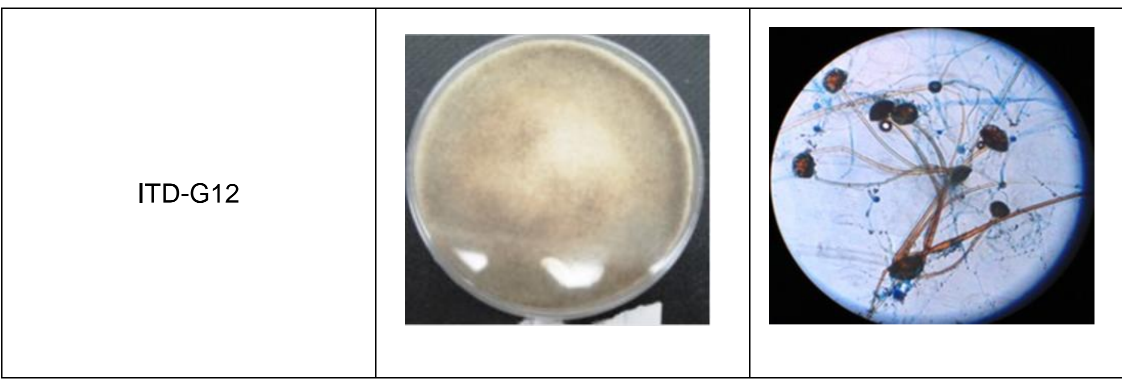
Fungal Isolated	General Characteristics of Fungi Isolated			Genus
	Color of Mycellium	Spore color	Pigmenta-tion	
ITD-G1	Black	Black	Yellow	*Aspergillus*
ITD-G5	Black	Black	Yellow	
ITD-G11	Yellow-Green	Yellow-Green	-	
ITD-G3	White	White	Purple	*Aspergillus fumigatus*
ITD-G9	Green	Green	Purple	
ITD-G10	Green	Green	Purple	
ITD-G6	Green-White	Green	-	*Scytalidium*
ITD-G4	Dark Green	Dark Green	-	
ITD-G7	Orange	Orange	-	*Neurospora crassa*
ITD-G8	Brown	Brown	Blank	*Alternaria*
ITD-G2	Yellow	Grey	-	*Mucor*
ITD-G12	Grey	Grey	-	

Table 1b. Macro and Microscopic Images of Isolated Fungal Strains of *A. durangensis* Leaves

Fungal Isolate	Observation of Fungi Isolated (PDA)	
	Macroscopic	Microscopic (40×)
ITD-G1		
ITD-G3		
ITD-G5		


ITD-G9
ITD-G10
ITD-G11
ITD-G6


ITD-G4
ITD-G7
ITD-G8
ITD-G2

ITD-G12		

The macro and microbiological identification data obtained supports strain identification by molecular techniques, corroborated by phylogenetic analysis (data not shown). When the isolated fungal strains 18s DNA ribosomal (rDNA) sequences were obtained, they were compared to those previously reported in GeneBank, and most were very similar to strains associated with their morphologic characterization (Table 2).

Sequences from this study have been deposited in GenBank under the accession numbers KM057746 through KM057754.

Table 2. Molecular Identification of Fungal Isolate

Fungal Isolate	Molecular Identification	GeneBank Accession No.	Closest Identified Relative Species	% Identity
ITD-G1	*Aspergillus niger*	KM057746	*Aspergillus niger* ANOS12 KP036601	99
ITD-G2	*Rhizopus sp.*	KM057747	*Rhizopus oryzae* A2 KM527239	99
ITD-G4	*Scytalidium sp.*	KM057748	*Scytalidium hyalinum* IP151783 AF258606	99
ITD-G5	*Aspergillus niger*	KM057746	*Aspergillus niger* ANOS12 KP036601	99
ITD-G6	*Botryosphaeria sp.*	KM057750	*Botryosphaeria Agaves* MFLUCC 11-0125 JX646825	98
ITD-G7	*Neurospora crassa*	KM057753	*Neurospora crassa* OR74A XR898033	99
ITD-G8	*Alternaria sp.*	KM057752	*Cochliobolus eragrostidis* NBRC 100188 JN941622	99
ITD-G11	*Aspergillus flavus*	KM057751	*Aspergillus flavus* HKF30 HM773230	99
ITD-G12	*Mucor sp.*	KM057754	*Mucoromycotina* clone S2-070 JQ321018	98

There were two exceptions to the foregoing description, namely Isolate ITD-G6, which presented a similarity with several *Botryosphaeria* sequences, and ITD-G2, which presented a sequence similarly with *Rhizopus* sequences.

The ITD-G6 present strain similarity of 98% with the strain reported in the database as *Botryosphaeria Agaves* MFLUCC 11-0125. However, this strain morphology is different from that previously reported; therefore, complementary studies should be carried out to achieve its identification. This type of microorganism belongs to the

Botrysphaeriaceae family, most of which are endophytic and phytopathogenic (Phillips *et al.* 2013). Taxonomic studies have shown that a morphological characterization is insufficient for the clear identification of this species; thus, sequencing methods should be used as an important tool for their identification. Other researchers (Phillips *et al.* 2013; Abdollahzadeh *et al.* 2013; Liu *et al.* 2012) made a reclassification of these fungi by undertaking other regions of highly conserved ribosomal DNA genes, such as: 5.8s, 28s, and also the β-tubulin gene, so it is recommended to perform a deeper identification study in this strain.

The strains ITD-G12 and ITD-G2 were identified as of the genus *Mucor* at the macroscopic and microscopic morphology levels (Table 1a and 1b), while strain ITD-G2 molecular analysis showed a similarity of 99% with respect to *Rhizopus oryzae* A2 (Table 2). This may be due to the morphological similarity that exists between these two species; however, by molecular identification, these fungi are separated into two distinct species.

Fungal species are characterized by their abilities to produce and secrete enzymes to the external environment. Their potential to produce different enzymes depends significantly on the natural environment where they are obtained. The enzymatic activities evaluated in the different filamentous fungi isolated from residues of *A. durangensis* are presented in Table 3.

In the evaluation of extracellular enzyme expression of the isolated strains, it was observed that most of the tested strains were capable of simultaneously secreting cellulases, xylanases, inulinases, fructanases, and laccases. This can be attributed to the cellulose, hemicellulose, lignin, and fructans contents in the *Agave* leaves where they were isolated, using it as source of carbon for its growth (Mancilla-Margalli and López 2006; Contreras-Hernandez *et al.* 2017). The least frequently detected enzymes were amylases (including proteases, data not shown), this is probably due to the absence of these compounds in the leaves of *Agave*, such that there would be no evolutionary advantage to production of amylases.

Table 3. Strains Used in this Study and Detection of Extracellular Activity

Strains	Cellula-ses	Xyla-nases	Inulina-ses	Fructana-ses	Amyla-ses	Lacca-ses
Aspergillus niger ITD-G1	+	+	+++	+++	+	-
Rhizopus sp. ITD- G2	++	+	ND	ND	-	-
Aspergillus fumigatus ITD-G3	+	+	++	++	++	+
Scytalidium sp. ITD- G4	+	+	+++	+++	+	+++
Aspergillus niger ITD-G5	+	+	+++	+++	++	-
Botryosphaeria sp. ITD-G6	++	+	+++	+++	-	+++
Neurospora crassa ITD-G7	+++	-	+++	+++	-	+
Alternaria sp ITD-G8	+	+	++	+	-	+
Aspergillus fumigatus ITD-G9	+	+	++	+	-	++
Aspergillus fumigatus ITD-G10	+	+	++	+	+	++
Aspergillus flavus ITD-G11	+	+	++	++	+	-
Mucor sp. ITD-G12	+	+	+++	+++	-	-

The *Aspergillus* genus is the most studied and reported fungi in industrial enzyme production, mainly *Aspergillus niger* for amylases and proteases expression (Lubertozzi and Keasling 2009). In recent years, *Aspergillus niger* and *Aspergillus oryzae* have been used to produce cellulases and inulinases (de Vries and Visser 2001; Percival Zhang *et al*. 2006; Chi *et al*. 2009).

Mucor sp. and *Rhizopus* sp. are fungi characterized by their ability to express a diverse variety of enzymes (amylases, cellulases, xylanases, proteases, and lipases) that are necessary to degrade plant carbohydrates (Ferreira *et al.* 2013). Studies have reported the use of *Neurospora crassa* for the production of cellulases (Yazdi *et al*. 1990; Dogaris *et al.* 2009), and it is one of the most commonly used species in industry.

Alternaria sp. ITD-G8 secreted most of the enzymes evaluated, demonstrating its ability to degrade different substrates and setting the stage for in depth studies in the biotechnology area; this species has only been studied for the production of secondary metabolites (Andersen *et al*. 2015).

The strains *Scytalidium* sp.ITD-G4 and *Botryosphaeria* sp.ITD-G6 isolated from *A. durangensis* demonstrated high percentages of enzymatic activity (cellulases, inulinases, amylases, xylanases, and laccases). This type of microorganism is related to the production of lacasses and cellulases mainly associated with the degradation of lignin and cellulose (Bahrin *et al.* 2011; Barbosa *et al.* 1996). It is well known that there are few studies on this type of microorganism concerning their capability to express extracellular enzymes. Such information is reported in this work for the first time, noting its potential to express the enzymes inulinase and fructanase.

From the isolates, strain *Botryosphaeria* sp. ITD-G6 was selected to evaluate its capability to produce inulinase enzymes using waste of *Agave*, commercial inuline, and *Agave* fructans as the sole carbon source. The strain was evaluated in three different mediums previously mentioned and incubated at 30 °C for 120 h at 150 rpm. The activity of inulinase in different substrates is shown in Table 4.

Table 4. Quantification of Inulinase Activity of the *Botryophaeria* sp. ITD-G6 Strain Using Different Substrates

Strains	Substrate	Inulinases Activity (U/mL)
Botryosphaeria sp. ITD-G6	*Agave* leaves	5.22 ± 0.37
	Agave fructans	4.37 ± 0.19
	Chicory inulin	5.00 ± 0.06

This type of enzyme activity has already been reported in other microorganisms such as *Aspergillus niger, Rhizopus*, and *Penicillium* (Huitron *et al*. 2008; Flores-Gallegos *et al.* 2015; Rawat *et al* 2015). And it is currently reported in native strains of *Agave durangensis* residues (Contreras-Hernández *et al.* 2017). Filamentous fungi are known for being excellent producers of industrial enzymes (Li *et al.* 2014; Quintanilla *et al*. 2015). The ability of this strain to secrete inulinase enzymes is advantageous over other microorganisms currently in literature, making it a strong candidate for application in the food and pharmaceutical industries. Inulinases have many biotechnological applications, from medical ones to the food industry (Pandey *et al.* 1999; Kirk *et al.* 2002; Bonciu and Bahrim 2011), and they represent almost 60% of the industrial enzyme world market (Singh and Gill 2006; Chi *et al.* 2009).

This research has added interesting strains. This is the first report on inulinase activity for a *Botryosphaeria* strain. Among them, *Botryosphaeriales* strains are able to isolate enzymes with industrial and biotechnological application potential. To make strides towards food industry applications, future studies should focus on increasing the production rate and characterization of these enzymes. However, most of these enzymes are obtained from *Aspergillus*, *Penicillium*, *Rhizopus*, and *Thricoderma*. Researching and characterizing new and more efficient microorganisms for enzyme production is a growing demand.

CONCLUSIONS

1. Twelve fungi were isolated and identified from *Agave durangensis* leaves.
2. The native fungal strains of *A. durangensis* residues have promising enzymatic potential by fructanase and inulinase.
3. The *Botryosphaeria* sp.ITD-G6 is reported for the first time was potential production of inulinase enzymes.

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