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ASSESSMENT OF CELLULASE COMPLEX SECRETORY CAPACITY OF *TRICHODERMA* STRAINS AND MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF THE ISOLATE WITH THE HIGHEST ENZYMATIC SECRETION CAPACITY

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ABSTRACT

The bioconversion of lignocellulosic biomass into monomeric sugars is a key economic difficulty hindering the profitable use of plant biomass as energy. The production of cellulase is a main factor in the cellulose hydrolysis. Among the main cellulase producers are the filamentous fungi. Therefore, many efforts have been made in obtaining new microorganisms with high cellulase secretion capacity. The cellulase secretory capacity of 28 isolates of *Trichoderma* was qualitatively and quantitatively evaluated. The detection of cellulolytic fungi was correlated with both Congo red and the dinitrosalicylic acid reagent methods. Based on qualitatively assays, sixteen of the isolates revealed carboxymethyl cellulose degradation ability, where the *Trichoderma* POS7 isolate showed the highest increase in filter paper activity, endo-1,4-β-glucanases and β-glucosidases activities ($p<0.05$) in a short incubation time. This isolate was molecularly identified as *Trichoderma koningiopsis*, based on internal transcribed spacer sequences. Our results provide new information and reveal new microorganism in the hydrolysis of cellulose material. The phylogenetic analysis revealed close positioning of *T. koningiopsis* clade with *T. viride*, *T. viridescens* and *T. petersenii* clades in a closely related group, in concordance with the current taxonomic classification of *Trichoderma* genus.

Keywords: fungal isolate; cellulase activity; morphological characters; ribosomal DNA; *Trichoderma koningiopsis*

INTRODUCTION

Our planet is facing an energy crisis as a result of the use of nonrenewable and limited energy resources (**Doolotkeldieva and Bobusheva, 2011; Amin and Rahman, 2019**). The production of bioenergy from renewable and less costly lignocellulosic materials could bring benefits to the local economy, environment and national energy security (**Doolotkeldieva and Bobusheva, 2011; Nigam and Singh, 2011; Akinyele *et al*., 2014**).
Lignocellulosic biomass, a renewable, cheap and abundant resource, has also the advantage that it does not compete with food production (**Bandikari *et al*., 2015**). Cellulose is a type of homogeneous polysaccharide that exists in nature as units of cellobiose connected by β-1,4-glycosidic bonds, and is the most abundant renewable biomass (**Zhang *et al*., 2014**). Second-generation biofuels production from lignocellulosic materials includes three main steps: (a) pretreatment, (b) enzymatic hydrolysis of cellulose and hemicellulose to glucose, and (c) ethanol fermentation (**Kawai *et al*., 2013**). Hydrolysis of cellulose to glucose requires synergistic action of the cellulase complex (**Sadhu *et al*., 2012**), composed of three groups of enzymes: endo-1,4-β-glucanases (EGs - EC 3.2.1.4) randomly cut β-1,4-bonds of cellulose chains generating new ends; cellobiohydrolases (CBHs - EC 3.2.1.91) act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains liberating either cellobiose or soluble cellodextrins as major products; and β-glucosidases (BGLs - EC 3.2.1.21) hydrolyze soluble cellodextrins and cellobiose to glucose (**Zhou *et al*., 2008; Zhang *et al*., 2014**).
In recent years the interest in cellulase complex has been increased due to many potential applications for these types of enzymes (**Rathnan *et al*., 2013; Adrio and Demain 2014; Patel *et al*., 2019**). In the production of bioethanol, the cost of the enzymes used for hydrolysis needs to be reduced. Also the enzyme's efficiency needs to be modified in order to economically improve the process. The production of cellulase is a main factor in the cellulose hydrolysis (**Zhou *et al*., 2008; Dos Santos Castro *et al*., 2014**). Among the main cellulase producers many filamentous fungi are reported, which can produce an extracellular non-composite cellulase complex. They are one of the most important sources of commercial cellulase available (**Zhang *et al*., 2014**).
Trichoderma genus (**Persoon, 1794**) comprises an important number of many saprotrophic species with a wide ecological distribution. They are capable of growing on soil, bark, wood, other fungi and inother types of substrates demonstrating their opportunistic potential and their adaptability to different ecological conditions (**Druzhinina *et al*., 2011**). Many members in *Trichoderma* genus are key microorganism in biotechnologically due to their ability to secrete a wide spectrum of enzymes and bioactive compounds (**Mandels and Reese, 1957; Doolotkeldieva and Bobusheva 2011; Druzhinina *et al*., 2011**). Therefore, many efforts have been made in obtaining new microorganisms of this genus with high specific cellulase activity and outstanding efficiency (**Zhou *et al*., 2008**). The morphological identification can become difficult and even ambiguous because many fungi are microscopic or have cryptic life cycles (**Druzhinina and Kubicek, 2005; Samuels, 2006; Hibbett *et al*., 2017**). Molecular approaches and techniques have been studied and developed to improve fungal identification related to traditional phenotypic methods. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is one of the most reliable approach to classify a strain at the species level (**Leaw *et al*., 2006; Hassan *et al*., 2015; Hibbett *et al*., 2017**).
Nonetheless, in Argentina the studies related to the screening of qualitative and quantitative cellulolytic activities and molecular identification of outstanding biotechnological fungal isolates is scarce.
The objective of this work was to evaluate native *Trichoderma* fungi with significant cellulase secretion. This work includes: (a) the isolation of new strains of *Trichoderma* searching for optimal enzyme secretion; (b) the analysis of the cellulase secretion of these strains by means of qualitative and quantitative methods; (c) the evaluation of the congruence between the qualitative and quantitative results; (d) the selection of the *Trichoderma* isolate with the highest

cellulase secretion capacity and the morphological and molecular identification of this *Trichoderma* isolate.

MATERIALS AND METHODS

Microorganisms

Twenty-eight *Trichoderma* isolates were collected from natural ecosystems of Misiones, Argentina. All isolates were identified at genus level by standard macro-micro morphological techniques (**Rifai, 1969; Samuels, 2006; Druzhinina *et al*., 2011; Piontelli, 2015**). The isolates were coded as: PROF1, PROF2, PROF3, PROF4, PROF5, PROF6, PROF7, PROF8, NAN9, NAN11, NAN12, NAN13, TEYU14, POS1, POS2, POS3, POS5, POS6, POS7, POS8, POS9, POS10, POS11, TN1, TN2, TN3, TN4 and TN5. The isolates coded as "PROF", "NAN" and "TEYU" were provided by the culture collection of the Universidad Nacional de Misiones. The isolates coded as "POS" and "TN" were isolated from rotten wood samples from non-anthropic environments of Posadas city (Misiones) (27°24'31.8''S, 55°53'48.5''W; and 27°22'12.2''S, 55°57'46.6''W respectively). For the isolation of new native *Trichoderma* fungi small pieces of rotten wood were placed in potato-dextrose agar plates 3.9% (w/v) (PDA – Britania SA). After an incubation step for 3 to 5 days at 28 ± 1°C, the single fungal colonies were picked using an inoculating rod and inoculated onto a new PDA plate. All the isolates were deposited in the culture collection of the Universidad Nacional de Misiones (Argentina).

Culture conditions

All the isolates were reactivated in PDA plates for 5-7 days at 28 ± 1°C under constant photoperiod (24 h light). A white light tube (6500 K, 18 w) was used as supplementary light with alight/dark periodregulated with a Zurich XTIM03205 digital timer.

To prepare the fungal inocula for qualitative assays, 5 mm-agar plugs from each fungal isolate grown in PDA were cut and transferred to agar plates containing solid Mandels' medium (**Mandels and Reese, 1957**) with the following modifications: 1.7% (w/v) agar-agar and 0.5% (w/v) sodium carboxymethyl cellulose (CMC) as only carbon source. The agar plates were incubated for 5 days at 28 ± 1°C under constant photoperiod (24 h light).

To prepare the fungal inocula for quantitative assays, spore suspensions with 10^7 spores/mL concentration were used as initial inoculum for each experiment and transferred to 250 mL-Erlenmeyer flasks containing 50 mL of liquid Mandels' medium with 0.5% (w/v) CMC as only carbon source. The Erlenmeyer flasks were incubated under static conditions for 5 days at 28 ± 1°C under constant photoperiod (24 h light)[8]. Daily aliquots of 1.5 mL of supernatant were taken and used as crude enzyme extract to assay extracellular cellulase secretion in quantitative screening assays.

Biochemical analyses

Qualitative cellulase activities screening assays of Trichoderma isolates

The qualitative cellulase activity of fungal isolates was determined based on their ability to grow and form clear zones around colonies on solid Mandels' medium (**Mandels and Reese, 1957**). The surface of the plates with medium and grown fungal colonies was flooded with 0.1% (w/v) Congo red solution (BioPack SA) and incubated for 15 min at room temperature. The dye was removed with sterile distilled water followed by incubation for 10 more minutes at room temperature. The plates were further treated by flooding with 1M NaCl solution for 5 min. The ratio of the diameter of the clear zone to the diameter of the colony growth was measured (**Ouyang *et al*., 2006; Doolotkeldieva and Bobusheva 2011**). The cellulase activity of each *Trichoderma* isolate was determined using a qualitative scale, where the number of + represents qualitative enzymatic activity, ranging from null (-) to maximum (++++) enzymatic activity.

Quantitative cellulase activities screening assays of Trichoderma isolates

The quantitative cellulase activity of the fungal isolates was determined by their EGs, CBHs and BGLs activities. The synergic effect of the cellulase complex was determined by filter paper activity (FPA). These activities were performed as reported previously (**Rodriguez *et al*., 2019**). The FPA and EGs activities were determined according to the International Union of Pure and Applied Chemistry tests (**Ghose, 1987**). FPA and EGs were assayed by measuring the release of reducing sugars. FPA activity was assessed in a reaction mixture containing 0.1 mL of crude enzyme, 10 mg of Whatman No. 1 filter paper as substrate and 0.2 mL of 50 mM sodium acetate buffer (pH 4.8) incubated at 50°C for 60 min. EGs activity was assessed in a reaction mixture containing 0.1 mL of crude enzyme and 0.1 mL of 2% (w/v) of CMC solution in 50 mM sodium acetate buffer (pH 4.8) incubated at 50°C for 30 min. The CBHs activity was assessed according to **Wood and Bhat** (**1988**). CBHs activity was assessed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of crude enzyme and 0.1 mL of 1% (w/v) of cellulose in 50 mM sodium acetate buffer (pH 4.8) incubated at 50°C for 60 min and 125 rpm. Reducing sugars were assayed by dinitrosalicyclic acid method (**Miller, 1959**). One unit of FPA, EGs or CBHs activity was defined as the amount of enzyme required to liberate 1 µmol of glucose per minute from each substrate under the assay conditions (**Rodriguez *et al*., 2019**).

The BGLs activity was assessed using p-nitrophenyl-β-D-glucopyranoside method (**Bailey, 1981**). The release of p-nitrophenol was measured at 400 nm from a reaction mixture containing 0.9 mL of 0.03 M p-nitrophenyl glucopyranoside in 50 mM acetate buffer (pH 4.8) and 0.1 mL of suitably diluted enzyme, incubated at 50°C for 15 min and 125 rpm. One unit of BGLs activity was defined as the amount of enzyme required to liberate 1 µmol of p-nitrophenol per minute under the assay conditions (**Rodriguez *et al*., 2019**).

Congruence between qualitative and quantitative screening assays

To compare the congruence between qualitative and quantitative screening assays, the isolates classified as positives (++, +++ and ++++) and two isolates with low (+, PROF2 isolate) and null (-, NAN9 isolate) activities were selected and statistically compared among them. In all cases, the enzymatic activities (EGs – CBHs – BGLs – FPA) were measured by the methods previously mentioned and the isolate with the highest cellulase activities was selected (**Sazci *et al*., 1986; Florencio *et al*., 2012**).

Data Analysis

All experiments were conducted in triplicate. The experimental runs were designed, and the results were analyzed using the Statgraphic Centurion program (StatPoint, Inc., version 15.2.05) and GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was used for all data analysis. The Least Significant Difference (LSD) test was selected and used to establish differences among levels of a factor. A confidence level of 95% was applied. The results of quantitative assays represent the mean values ± the standard deviation of the mean (SD).

Morphological identification

The *Trichoderma* POS7 isolate with the highest cellulase secretion capacity was reactivated in PDA plates and incubated for 3 days at 28 ± 1°C in a 12 h light/12 h dark photoperiod. After the incubation period, the macroscopic and microscopic features of the selected isolate were described. The macroscopic features observed of the fungal colony were: growth rate, morphology and surface and reverse color in PDA and malt extract agar (MEA– Britania SA) culture media. The records of the colony diameter, shape, arrangement, color and texture were documented at 24, 48 and 72 hours using the ImageJ 1.4 program (**Schneider *et al*., 2012**).

For the microscopic identification the microculture technique and lactophenol cotton blue staining with the adhesive tape method were used to facilitate the observation of structural features like conidiophores and sizes, branching pattern, shape and arrangement of conidia, pigmentation, among others (**Samson *et al*., 1988; Kubicek and Harman, 2002; Prats, 2007; Piontelli, 2015**). For light microscopic observations the preparations were made from the edge of the growing colony. A Carl Zeiss - West Germany model 467065-9902-18VA light microscope was used. Examinations were made with the oil immersion augmentation to establish details of conidial shape and ornamentation. The mean values and standard deviation of at least 10 measured structures were calculated and indicated to determine the microscopic measurements of the reproductive structures.

Molecular identification and phylogenetic analysis

For DNA extraction mycelia of the *Trichoderma* POS7 isolate were grown in malt extract broth 1.27% (w/v) (ME - Britania SA) at 28 ± 1°C for 5 days in the dark. The extraction of genomic DNA was performed as reported previously (**Castrillo *et al*., 2012; Bich *et al*., 2017**). DNA was resuspended in 30 µL of sterile distilled free of DNAse water (Biopak®). DNA extracted was further examined by electrophoresis in 1% (w/v) agarose gels (InBio) and stained with Gel Red Solution (Biotium, 10,000 X). The DNA purity was determined by the A 260 /A 280 ratio (**Sambrook *et al*., 1989; Castrillo *et al*., 2016**).

For the analysis of this section the ITS1-5.8S-ITS2 region was evaluated. PCR amplifications were carried out in a 20 µL reaction mixture which included 1X PCR Buffer, 2.5 mM $MgCl_2$, 200 µM of dNTP mix, 10 pmol of each of the amplification primers, 0.5 u of Taq polymerase (InBio), and 1 µg of genomic DNA (**Castrillo *et al*., 2012; Bich *et al*., 2017**). The primers used were the ITS1 F- (5'-TCCgTAggTgAACCTgCgg-3') and ITS4 R- (5'-TCCTCCgCTTATTgATATgC-3') (**White *et al*., 1990**). The amplification protocol consisted of an initial denaturation at 94ºC for 4 min, followed by 35 PCR amplification cycles of 94ºC for 40 s, 53ºC for 40 s and 72ºC for 40 s. A final extension step of 72ºC for 10 min was included (**Bich *et al*., 2017**). The amplified fragment was examined by electrophoresis in 2% (w/v) agarose gels (InBio) and stained with Gel Red Solution (Biotium, 10,000 X). Both strands of

PCR products were sequenced by Macrogen Korea for further phylogenetic studies.

The ITS1-5.8S-ITS2 nucleotide sequence of the *Trichoderma* POS7 isolate was compared with deposited sequences in GenBank, Fungal barcoding and TrichOKEY databases for species identification. Sixty-nine ITS1-5.8S-ITS2 sequences were selected and retrieved from GenBank and Fungal barcoding databases representing species within the *Trichoderma* genus (Table 1). Nucleotide sequences retrieved in this study consisted of about 600 bp which correspond to the ITS1-5.8S-ITS2 and partial 18S and 28S regions (**Castrillo *et al.*, 2016**). A sequence of *Hypomyces subiculosus* (EU280093) was used as an outgroup to root the *Trichoderma* phylogenetic tree. DNA sequences were aligned using the Clustal W program (**Thompson *et al.*, 1994**). The analyses were based on a distance-based method (Neighbor joining, NJ) and two phylogenetic methods (Maximum Likelihood, ML and Maximum Parsimony, MP). Support for specific clades represented in the tree was estimated by bootstrap analysis of 1,000 replicates. Nucleotide divergences were estimated using Kimura's two-parameter method. The MEGA 6.0 package (**Tamura *et al.*, 2013**) was used for the analyses. Phylogenetic data have been submitted to TreeBase with submission number S22003 (http://purl.org/phylo/treebase/phylows/study/TB2:S22003).

Table 1 Genbank accession numbers retrieved from databases.

Species	Strain number	Isolation source	Country	ITS rDNA accession number
T. asperellum	GXNN1001	-	China	JQ040311
T. asperellum	DAOM 233975	-	Perú	EU280132
T. brevicompactum	PROF 1	Soil	Argentina	JX069193
T. brevicompactum	CIB T37	-	Colombia	EU280087
T. brevicompactum	SVPRT-TBC01	Soil	India	JX908732
T. brevicompactum	CEN522	Soil of guava crop	Brazil	KC561076
T. brevicompactum	T-28	Soil	-	KC884785
T. ceramicum	GJS 88-70	Decorticated wood	USA	AY737764
T. ceramicum	CBS 114576	-	USA	FJ860743
T. chlorosporum	PC4	-	-	AF275322
T. chlorosporum	GJS 98-1	Log	Costa Rica	AY737762
T. citrinoviride	EGE-K-128	Timber	Turkey	JX125615
T. citrinoviride	T196	-	-	HQ596981
T. citrinoviride	Tc-03	-	India	KF294838
T. citrinoviride	NBAII Tv-5	Cotton rhizosphere	India	JQ013145
T. citrinoviride	TN5 native	Decaying wood	Argentina	KT030886
T. citrinoviride	KUC1274	Bamboo	South Korea	HM008920
T. citrinoviride	KUC5160	-	-	HM534655
T. crassum	DAOM 164916	-	Canada	EU280067
T. crassum	DAOM 233775	-	Guatemala	EU280084
T. estonicum	GJS 96-129	*Hymenochaete tabacina*	Estonia	AY737767
T. estonicum	CBS 111147	-	-	FJ860752
T. hamatum	DAOM 237553	-	Peru	EU280136
T. hamatum	CiB T144	-	Colombia	EU280105
T. harzianum	PROF2 native	Soil	Argentina	JX069196
T. harzianum	PROF6 native	Soil	Argentina	JX069198
T. harzianum	TEYU14 native	Soil	Argentina	JX069201
T. harzianum	NAN13 native	Soil	Argentina	JX069200
T. harzianum	POS3 native	Decaying wood	Argentina	KT030877
T. harzianum	PROF3 native	Soil	Argentina	JX069197
T. harzianum	PROF7 native	Soil	Argentina	JX069199
T. harzianum	SZMC 20869	Soil	Hungary	JX173873
T. harzianum	OTU680	-	-	GU934533
T. koningiopsis	T-51	Soil	-	KC884808
T. koningiopsis	T-16	Soil	-	KC884773
T. koningiopsis	T-54	Soil	-	KC884811
T. koningiopsis	T-57	Soil	-	KC884814
T. koningiopsis	DMC 795b	-	-	EU718083
T. koningiopsis	CiB T01	-	Colombia	EU280108
T. koningiopsis	T-1	Soil	-	KC884758
T. koningiopsis	Dis3264	*Theobroma gileri*	Ecuador	DQ379015
T. koningiopsis	Tri021	-	-	HQ229944
T. koningiopsis	CQSQ1002	-	China	JQ040365
T. koningiopsis	DAOM 229982	-	Peru	EU280142
T. koningiopsis	DAOM 233971	-	Peru	EU280131
T. longibrachiatum	PRT-TL10	Soil	-	KC582841
T. longibrachiatum	T50	-	China	FJ459964
T. longibrachiatum	IHB F 539	Tea rhizosphere	-	HM461859
T. longibrachiatum	TNAU-TL1	-	-	DQ200259
T. longibrachiatum	POS5 native	Decaying wood	Argentina	KT030878
T. longibrachiatum	CIB T29	-	Colombia	EU280095
T. petersenii	GJS 04-351	Wood	USA	DQ323440
T. petersenii	GJS 04-355	-	USA	DQ323441
T. pulvinatum	NBRC 9385	-	Japan	JN943376
T. pulvinatum	NBRC 8543	-	Japan	JN943375
T. pulvinatum	NBRC 8544	-	Japan	JN943374
T. reesei	A1S3-D22	Beach soil	-	KJ767092
T. reesei	RSPG_24	Soil	Thailand:	KC478546
T. sinensis	JZ-129	Soil	China	HQ637329
T. sinensis	DAOM 230004	Bark	Taiwan	HQ260623
T. virens	DAOM 233974	-	Peru	EU280090
T. virens	TR039	*Trachymyrmex septentrionalis* nest	USA	HQ608079
T. viride	ATCC 28038	Soil	USA	AY380909
T. viride	GJS 90-95	Decorticaded wood	USA	DQ315455
T. viride	Tr21	Soil	USA	AJ230675
T. viridescens	DAOM 237554	-	Peru	EU280135
T. viridescens	DAOM 234234	-	Guatemala	EU280119
T. viridescens	DAOM 233967	-	Peru	EU280137
T. viridescens	CiB T10	-	Colombia	EU280104

RESULTS

Biochemical analyses

Qualitative cellulase activities screening assays of Trichoderma isolates

Six isolates, PROF5, PROF7, NAN13, TEYU14, POS3 and POS7, showed the highest qualitative enzymatic activity, and therefore were classified as ++++. Then the PROF1, PROF4, PROF6, PROF8, POS6, POS11 and TRICHO4 isolates were classified as +++, and POS8, POS10 and NAN11 isolates were classified as ++. All these isolates were classified as effective in the qualitative enzyme secretion. The isolates that showed low enzymatic activity were PROF2, PROF3, POS1, POS5, POS9, NAN12, TRICHO1 and TRICHO2, and as a consequence were classified as +, followed by NAN9, TRICHO3, TRICHO5 and POS2 isolates that showed null qualitative enzymatic activity, which were classified as - (Fig 1).

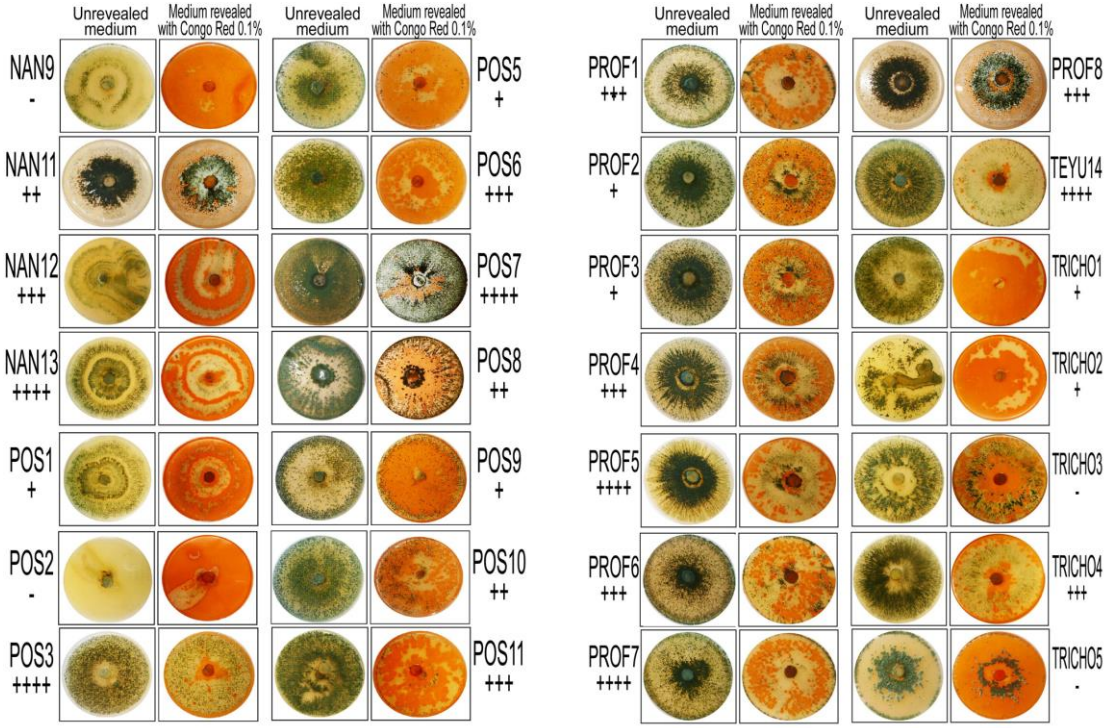

Figure 1 Qualitative enzymatic activity of the twenty-eight isolates of *Trichoderma* on solid Mandels' medium detected by Congo red method. Number of crosses (+ to ++++) indicates positive reaction intensity (weak to very strong); (-) indicates qualitative no detectable reaction.

Quantitative cellulase activities screening assays of Trichoderma isolates

The isolates classified previously as effective in the qualitative enzyme secretion (++++, +++ and ++), were selected to assess the quantitative cellulase activity. Most of the isolates revealed ability to CMC degradation, where the *Trichoderma* POS7 isolate showed the highest increase in FPA, EGs and BGLs activities ($p<0.05$). Regarding CBHs activity, the highest activity increase was observed in PROF7, PROF4 and POS7 isolates ($p<0.05$). Furthermore, in most cases the *Trichoderma* POS7 isolate showed the highest enzymatic activities in the shortest incubation time (Table 2). The *Trichoderma* POS7 isolate, based on the qualitative and quantitative cellulase activities assayed, was selected as a biotechnological isolate with outstanding enzymatic activity.

Congruence between qualitative and quantitative screening assays

It was clearly possible to verify a statistical congruence between the fast qualitative and the precise quantitative assays. All the isolates classified as ++++ had greater quantitative activities than the isolates classified as +++ and subsequently than the isolates classified as ++. Also, the PROF2 isolate with low qualitative activity showed greater quantitative activities than the NAN9 isolate with null qualitative activity ($p<0.05$), corresponding to the two isolates with the smallest quantitative activities (Table 2).

Table 2 Quantitative cellulase activities screening assays of 18 *Trichoderma* isolates from Misiones, Argentina.

Fungal isolate	Quantitative assays (U/L) (mean ± SD)[1]					
	FPA activity			EGs activity		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
PROF5	30.1 ± 2.8^{c}	$23.7 \pm 0.5^{d,e}$	$27.5 \pm 0.8^{c,d}$	$94.1 \pm 4.6^{c,d}$	104.4 ± 3.2^{c}	$\mathbf{120.8 \pm 9.2^{b}}$
PROF7	30.9 ± 2.6^{c}	$26.1 \pm 1.8^{c,d}$	35.3 ± 0.7^{b}	$79.6 \pm 1.6^{d,e}$	85.9 ± 6.9^{d}	70.2 ± 0.9^{f}
NAN13	$26.6 \pm 1.5^{c,d}$	$34.2 \pm 4.6^{b,c}$	$24.8 \pm 2.6^{c,d}$	$74.2 \pm 1.6^{e,f}$	$81.5 \pm 4.9^{d,e}$	100.4 ± 7.1^{c}
TEYU14	33.2 ± 1.7^{c}	35.9 ± 2.3^{b}	$29.7 \pm 4.1^{c,d}$	89.8 ± 1.9^{d}	99.4 ± 3.7^{c}	$\mathbf{130 \pm 7.2^{a,b}}$
POS3	$25.5 \pm 0.9^{c,d}$	$21.9 \pm 0.5^{e,f}$	$20.9 \pm 0.2^{e,f}$	$55.5 \pm 4.5^{g,h}$	$74.1 \pm 6.2^{e,f}$	$76.8 \pm 3.6^{e,f}$
POS7	$\mathbf{42.5 \pm 2.8^{a}}$	$\mathbf{41.2 \pm 1.5^{a}}$	$\mathbf{42.7 \pm 1.1^{a}}$	$\mathbf{143.6 \pm 8.7^{a}}$	$\mathbf{121.0 \pm 5.4^{b}}$	102.1 ± 3.9^{c}
PROF1	26.1 ± 0.6^{d}	15.0 ± 9.2^{g}	$29.0 \pm 1.9^{c,d}$	$68.9 \pm 9.5^{f,g}$	$75.2 \pm 9.7^{d,e,f}$	100.2 ± 13.4^{c}
PROF4	$24.2 \pm 0.6^{d,e}$	$25.0 \pm 1.3^{c,d}$	$20.2 \pm 0.9^{e,f}$	$48.7 \pm 1.3^{h,i}$	$74.9 \pm 2.3^{e,f}$	$83.2 \pm 7.3^{d,e}$
PROF6	$21.9 \pm 2.0^{e,f}$	$28.6 \pm 1.5^{c,d}$	$25.2 \pm 2.1^{c,d}$	$65.3 \pm 5.9^{f,g}$	95.1 ± 9.1^{c}	98.7 ± 8.9^{c}
PROF8	$25.3 \pm 1.7^{c,d}$	$26.8 \pm 1.7^{c,d}$	$22.7 \pm 4.6^{d,e}$	$77.2 \pm 8.5^{d,e,f}$	96.4 ± 15.6^{c}	$88.2 \pm 13.2^{c,d,e}$
POS6	$27.3 \pm 3.1^{c,d}$	$24.4 \pm 0.7^{d,e}$	32.6 ± 1.2^{c}	$61.3 \pm 4.3^{f,g}$	$64.2 \pm 6.5^{f,g}$	$82.9 \pm 2.0^{d,e}$
POS11	$28.7 \pm 0.7^{c,d}$	$28.4 \pm 0.4^{c,d}$	$27.8 \pm 7.8^{c,d}$	$51.8 \pm 10.7^{g,h,i}$	$49.3 \pm 7.4^{g,h,i}$	$66.1 \pm 9.7^{f,g,h}$
TRICHO4	$25.0 \pm 0.7^{c,d,e}$	$34.8 \pm 3.1^{b,c}$	30.9 ± 3.2^{c}	$51.7 \pm 4.9^{g,h,i}$	$93.0 \pm 4.9^{c,d}$	$79.9 \pm 7.8^{d,e}$
POS8	$23.1 \pm 0.2^{d,e}$	$28.7 \pm 0.4^{c,d}$	$24.8 \pm 1.2^{d,e}$	$69.2 \pm 0.6^{f,g}$	$66.9 \pm 11.2^{f,g}$	$64.3 \pm 6.1^{f,g}$
POS10	$25.4 \pm 2.3^{c,d}$	$22.3 \pm 4.6^{c,d,e}$	$25.5 \pm 2.6^{c,d}$	$47.1 \pm 5.5^{h,i}$	$65.7 \pm 9.2^{f,g}$	$58.9 \pm 9.2^{g,h}$
NAN11	$23.2 \pm 1.8^{d,e}$	$26.1 \pm 0.8^{c,d}$	$29.9 \pm 1.0^{c,d}$	$43.3 \pm 2.7^{h,i}$	$59.5 \pm 9.7^{g,h}$	$74.8 \pm 0.2^{e,f}$
PROF2	$21.3 \pm 0.3^{e,f}$	$21.5 \pm 0.7^{e,f}$	$22.7 \pm 1.7^{e,f}$	$47.9 \pm 2.0^{h,i}$	$59.8 \pm 8.7^{g,h}$	$49.4 \pm 11.7^{g,h,i}$
NAN9	18.1 ± 2.1^{g}	17.1 ± 2.4^{g}	19.3 ± 1.3^{g}	18.3 ± 0.9^{j}	17.9 ± 0.4^{j}	17.5 ± 0.1^{j}

Quantitative assays (U/L) (mean ± SD)[1]						
Fungal isolate	CBHs activity			BGLs activity		
	Day 3	Day 4	Day5	Day 3	Day 4	Day 5
PROF5	35.1 ± 0.2^{h}	54,1 ± 4.9^{e,f}	65.5 ± 6.3^{d,e}	1.5 ± 0.2^{d}	2.3 ± 0.4^{c}	2.4 ± 0.7^{c}
PROF7	**100.9 ± 0.3^{a}**	**101.9 ± 0.4^{a}**	55.4 ± 1.3^{e,f}	0.4 ± 0.1^{f}	0.4 ± 0.1^{f}	0.6 ± 0.3^{e,f}
NAN13	**100.9 ± 8.8^{a,b}**	79.5 ± 1.2^{c,d}	75.6 ± 3.2^{c,d}	1.2 ± 0.2^{d,e}	1.4 ± 0.3^{d}	1.6 ± 0.2^{d}
TEYU14	65.5 ± 9.2^{d,e}	56.1 ± 5.3^{e,f}	76.3 ± 1.9^{c,d}	1.9 ± 0.6^{c,d}	3.1 ± 0.1^{c}	3.1 ± 0.1^{c}
POS3	71.9 ± 1.6^{c,d}	36.4 ± 1.2^{h}	40.2 ± 1.3^{g,h}	0.2 ± 0.1^{f}	0.3 ± 0.1^{f}	0.4 ± 0.1^{f}
POS7	35.1 ± 0.2^{h}	88.3 ± 12.3^{b,c}	**102.3 ± 2.5^{a}**	**7.7 ± 0.2^{b}**	**7.2 ± 0.2^{b}**	**9.2 ± 0.1^{a}**
PROF1	35.1 ± 0.2^{h}	42.7 ± 7.6^{g,h}	56.1 ± 5.6^{e,f}	0.5 ± 0.1^{e,f}	0.6 ± 0.1^{e,f}	1.1 ± 0.1^{e}
PROF4	52.9 ± 6.7^{e,f}	**104.3 ± 1.9^{a}**	65.1 ± 8.7^{d,e}	1.1 ± 0.1^{e}	1.5 ± 0.2^{d}	1.7 ± 0.2^{d}
PROF6	43.9 ± 8.8^{e,f,g}	63.0 ± 5.1^{d,e}	82.7 ± 0.9^{c}	0.3 ± 0.1^{f}	0.4 ± 0.1^{f}	1.2 ± 0.5^{d,e}
PROF8	65.5 ± 9.8^{d,e}	73.2 ± 7.9^{c,d,e}	68.2 ± 6.9^{d,e}	0.9 ± 0.2^{e}	1.3 ± 0.3^{d,e}	2.3 ± 0.2^{c}
POS6	69.4 ± 1.2^{d,e}	89.0 ± 13.5^{b,c}	73.1 ± 15.7^{c,d}	0.1 ± 0.1^{f}	0.1 ± 0.1^{f}	0.2 ± 0.1^{f}
POS11	50.3 ± 2.4^{e,f}	40.7 ± 2.6^{g,h}	47.5 ± 3.7^{g,h}	0.4 ± 0.2^{e,f}	0.9 ± 0.2^{e}	1.4 ± 0.1^{d}
TRICHO4	90.3 ± 13.4^{a,b,c}	64.3 ± 0.2^{d,e}	35.1 ± 0.1^{h}	0.6 ± 0.2^{e,f}	0.7 ± 0.1^{e}	1.5 ± 0.1^{d}
POS8	49.1 ± 11.2^{f,g,h}	45.9 ± 9.5^{f,g,h}	45.9 ± 9.3^{f,g,h}	0.8 ± 0.4^{e,f}	1.9 ± 0.3^{d}	2.3 ± 0.4^{c}
POS10	72.4 ± 9.5^{c,d}	41.4 ± 6.3^{g,h}	70.6 ± 5.7^{c,d}	0.3 ± 0.1^{f}	0.3 ± 0.2^{f}	0.2 ± 0.1^{f}
NAN11	52.2 ± 15.2^{e,f}	58.7 ± 7.8^{e,f}	41.5 ± 0.5^{h}	0.9 ± 0.1^{e}	1.2 ± 0.1^{e}	1.8 ± 0.3^{d}
PROF2	35.1 ± 0.2^{h}	35.1 ± 0.3^{h}	65.2 ± 7.6^{d,e}	0.7 ± 0.1^{e}	0.8 ± 0.1^{e}	1.1 ± 0.2^{e}
NAN9	15.1 ± 0.2^{j}	16.2 ± 0.3^{j}	25.7 ± 2.7^{i}	0.0^{g}	0.05 ± 0.02^{g}	0.05 ± 0.01^{g}

Legend: Data are the mean of three replicates; [1]Letters indicate significant differences between fungi grown on each culture medium (LSD test, $p<0.05$).

Morphological identification

After the incubation period, the colonies of the *Trichoderma* POS7 isolate showed rapid growth in both culture media. In MEA the mean growth rate for day was 2.19 cm (SD 0.12), and in PDA was 2.37 cm (SD 0.16). Regarding the color of the colonies it was observed a progressive change in their coloration, from a white and cottony color colony to a bright light green and light yellow color of velvety texture colony in both culture media. These features were more conspicuous in MEA culture medium (Fig 2).

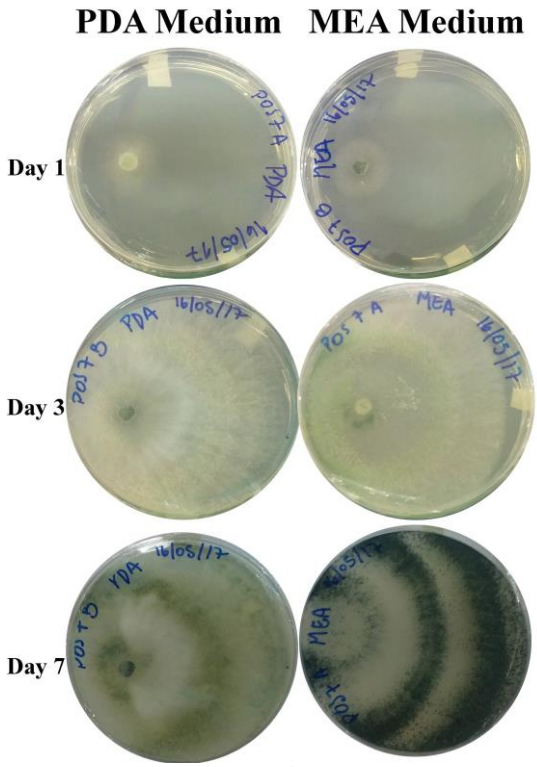

Figure 2 Macroscopic colonial features of the *Trichoderma* POS7 isolate grown in PDA and MEA culture media.

Microscopically, this isolate showed a more or less a regular pattern of indefinite branching, and a typical of *Trichoderma* terminal branches penicillated . Also, *Trichoderma* POS7 isolate showed regularly aculeate phialides on a relatively well-defined stipe. The conidiophore branching was regularly verticillate. Branches were relatively narrow and flexuous. It was observed that the phialides were disposed in regular verticils. The phialide shape lageniform to nearly cylindrical and elongated. Like in most *Trichoderma* species, the terminal phialides were elongated and narrowed. They showed a length of 7.40 µm (SD 0.73) after 72 h of incubation; the width of the observed base region was 1.46 µm (SD 0.33); and, the width of the apex region of the phialides presented a size of 0.93 µm (SD 0.24). Conidial shape varied from globose to ellipsoidal. The length of the conidia at 72 h of incubation was 2.77 µm (SD 0.44) with a width of 1.67 µm (SD 0.31) (Fig 3).

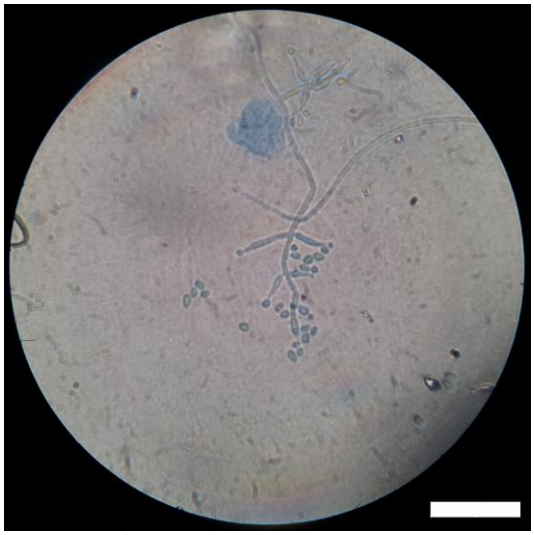

Figure 3 Microscopic features of the *Trichoderma* POS7 isolate. Microculture technique and lactophenol cotton blue staining with adhesive tape method were used to facilitate the observation. Bar = 20 µm.

Molecular identification and phylogenetic analysis

Genomic DNA of *Trichoderma* POS7 isolate had a 260 / 280 absorbance ratio of 1.75. The ITS1-5.8S-ITS2 nucleotide sequence obtained had a length of 534 bp after contig construction and was deposited in GenBank-NCBI under accession number KT030879.

The comparison and analysis of *Trichoderma* POS7 sequence with deposited sequences in genetic databases allowed us to define this sequence as belonging to *Trichoderma koningiopsis* species with 99% of identity with KC884811 and KC884814 accession numbers from Fungal barcoding and NCBI databases, respectively. TrichOKEY database, a specific and curated database for molecular identification of *Trichoderma* and *Hypocrea* species, also allowed us to define the *Trichoderma* POS7 sequence as belonging to *T. koningiopsis* species.

The trees obtained by the NJ, ML and MP methods showed a similar topography indicating that the datasets could be combined. The ITS1-5.8S-ITS2 phylogenetic trees revealed that the isolate under study belongs to the same monophyletic clade of *T. koningiopsis* species (85% bootstrap) (Fig 4).

The phylogenetic analyses revealed close positioning of *T. koningiopsis* with *T. viride*, *T. viridescens* and *T. petersenii* in a closely related group, in concordance with the actual taxonomic classification of Viride clade in Trichoderma section. *T. asperellum* and *T. hamatum* revealed close positioning in the Hamatum clade in the same Trichoderma section. *T. longibrachiatum*, *T. reesei*, and *T. citrinoviride* showed close positioning in the Longibrachiatum clade in Longibrachiatum section, close to the Trichoderma section. Moreover, the Hypocreamun section included the Ceramica, Megalocitrina and Chlorospora clades and the Pachybasidium section included the Virens and Harzianum clades, these two sections revealed a closely related group. Finally, *T. brevicompactum*

species, belonging to the Lutea clade (Lone Lineages Section) showed high genetic distance with the other species of *Trichoderma* (Fig 4).

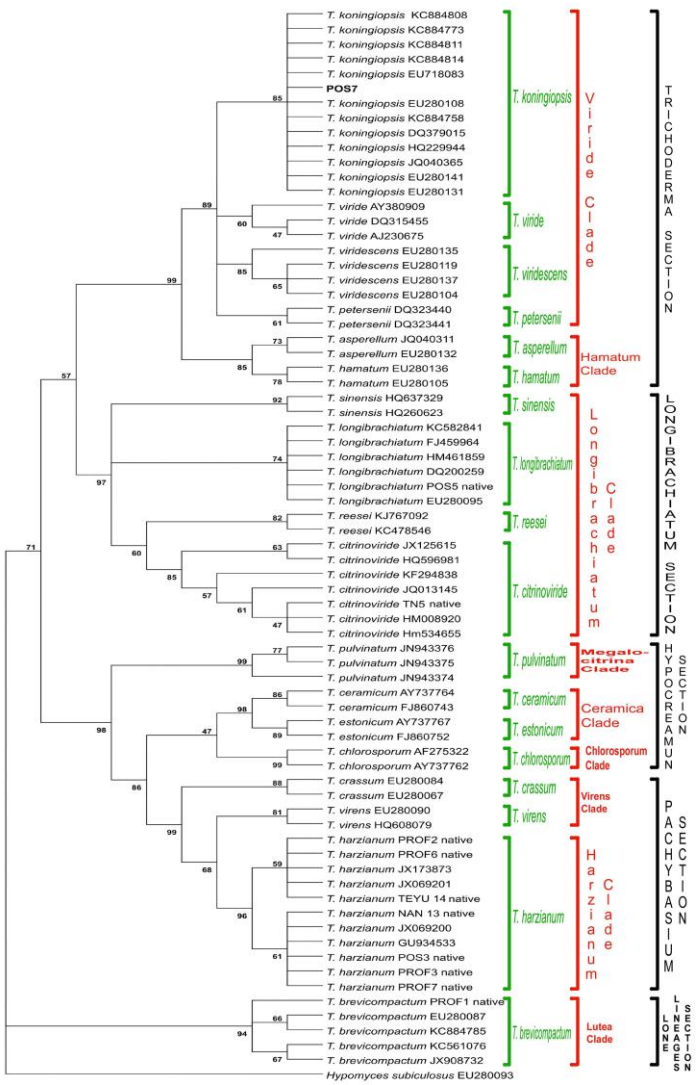

Figure 4 Phylogenetic tree based on MP method of the ITS1-5.8S-ITS2 sequence of *Trichoderma* POS7 isolate and the obtained sequences of GenBank and Fungal barcoding databases.

DISCUSSION

The fungal enzymatic degradation of cellulose has been suggested as a feasible alternative to produce bioethanol (**Akinyele *et al*., 2014; Ma and Ruan, 2015**). Therefore, different studies on cellulase produced by fungi, particularly by *Trichoderma* strains were developed and many signs of progress have been made (**Mandels and Reese, 1957; Doolotkeldieva and Bobusheva, 2011; Druzhinina *et al*., 2011**). In spite of some present successes, the task of finding new highly active cellulase or efficient producers of cellulase remains topical (**Zhou *et al*., 2008; Zhang *et al*., 2014**).

The province of Misiones (Argentina), an ecoregion of the Atlantic Forest, is one of the most valuable sites of wet subtropical ecosystems on biodiversity. The biota of the Atlantic Forest is extremely diverse and the assessment of the current state of this region´s biodiversity is still poorly known (**Bich *et al*., 2015; Fernandez *et al*., 2017**). For this reason, in this study twenty-eight *Trichoderma* isolates were evaluated to reveal new players in cellulose degradation.

Biochemical analyses

Various authors reported that the Mandels' medium is a complex nitrogenous source that induces cellulase secretion in microorganisms (**Mandels and Reese, 1957; Doolotkeldieva and Bobusheva, 2011; de Eugenio *et al*., 2017**). CMC substrate is a water-soluble cellulose product and is a suggested substrate for detection of cellulase secretion because it is degraded easily by microorganisms (**Mandels and Reese, 1957; Mandels and Andreotti, 1976; Sazci *et al*., 1986; Doolotkeldieva and Bobusheva, 2011; Ray *et al*., 2013; Akinyele *et al*., 2014; de Eugenio *et al*., 2017**).

The Congo red dye was used as an indicator for β-1,4-glycosidic bonds degradation in an agar medium. This simple diffusion technique provides a rapid and sensitive screening test for cellulolytic microorganisms (**Sazci *et al*., 1986; Doolotkeldieva and Bobusheva, 2011; Florencio *et al*., 2012**).

In this study, all isolates of *Trichoderma* were classified into five categories based on the qualitative enzymatic activity. This method, conducted in plates, resulted rapid, simple, and well adapted for the screening high amount of samples of the same genus. Likewise, **Hankin and Anagnostakis** (**1975**), **Doolotkeldieva and Bobusheva** (**2011**) and **Florencio *et al*.** (**2012**) reported that the extracellular enzymes can be produced in solid and liquid media, nonetheless the use of solid media enables rapid assays and can be useful for the isolation and detection of cellulase-producing organisms from natural materials.

The most widely accepted mechanism of enzymatic hydrolysis proposes that synergistic cooperation of EGs, CBHs and BGLs is a prerequisite for the efficient degradation of cellulose (**Sun and Cheng, 2002; Ouyang *et al*., 2006; Zhang *et al*., 2014**). Thus, when there is a lack of synergism, an incomplete hydrolysis can occur due to an incomplete cellulase system or an insufficient enzyme loading (**Martinez-Anaya *et al*., 2008**). In this study, the enzymatic synergism was evidenced when the maximum EGs activity was increased at early days of incubation while the maximum CBHs activity was increased at the end of the incubation period. Hence, the action of CBHs occurred after the action of EGs. The conventional mechanistic interpretation of the synergism between randomly acting EGs and chain end-specific processive CBHs is that the new chain end on cellulose surface generated by the EGs action serves as starting points for the CBHs action. Considering the crystalline nature of a substrate like cellulose, the number of reducing chain ends available for CBHs is expected to be low. The conventional explanation of EGs-CBHs synergism assumes that the chain end availability is rate-limiting for CBHs so that generation of new chain end by EGs will increase the population of productive CBHs cellulose complexes (**Jalak *et al*., 2012**).

In many isolates (NAN13 – PROF4 – PROF7) the maximum CBHs activity increase was observed before the maximum EGs activity ($p<0.05$). This could be due to the number of reducing chain ends available in the substrate (**Martinez-Anaya *et al*., 2008; Jalak *et al*., 2012**).

The BGLs hydrolyze cellobiose and cello-oligosaccharides to form glucose, so their effects must take place after the EGs and CBHs action (**Zhang *et al*., 2014**). In this study, it was observed that the maximum BGLs activity occurred after the maximum EGs and CBHs activities in all isolates. These results evidence the synergism among cellulase complex enzymes.

The correlation between qualitative screening with Congo red technique and quantitative screening with dinitrosalicylic acid reagent method was first reported by **Sazci *et al*.** (**1986**) and **Florencio *et al*.** (**2012**). In this study, the congruence between both methods was verified; the clear zones around fungal colonies in solid Mandels' medium with CMC substrate had congruence with the FPA, EGs and CBHs activities. Therefore, the qualitative assays can be used in a sensitive, cheap, reproducible and rapid way for screening a large number of cellulase-producing fungi (**Sazci *et al*., 1986; Doolotkeldieva and Bobusheva, 2011; Druzhinina *et al*., 2011**).

Morphological and molecular identification and phylogenetic analyses

Trichoderma strains usually can be identified to genus level by a distinctive morphology that includes rapid growth, bright green or white conidial pigments, and a repetitively branched conidiophore (**Kubicek and Harman, 2002**). The morphological characters used to identify the *T. koningiopsis* POS7 isolate were in agreement with the morphological characters typical of this species (**Samuels, 2006**).

The advent of molecular tools for investigations in fungal taxonomy prompted the reassessment of the morphology-based taxonomy in *Trichoderma* species (**Druzhinina and Kubicek, 2005**). Molecular methods do not replace morphological characterization; conversely, they complement morphological methods. New DNA sequence analysis has clarified the taxonomy of many *Trichoderma* species, and even several new species have been described with multigenic phylogenies (**Samuels, 2006; Druzhinina *et al*., 2012**). In this study, we decided to use and contrast the information provided by a specific database for the genus *Trichoderma* (TrichOKEY), a primary database (NCBI) and a curated database (Fungal barcoding). These identification analyses generated a greater approach to taxonomic identification of the isolate under study.

For identification of *Trichoderma* strains, TrichOKEY and TrichoBLAST (www.isth.info) are suggested as appropriate tools freely available online (**Hassan *et al*., 2015**). The GenBank database contains many sequences of *Trichoderma* isolates which had been incorrectly identified and several of them may be redundant (i.e. synonyms of other described species) or not correctly placed in the genus. Hence, to make a more accurate identification of the *Trichoderma* POS7 sequence, it was decided to use only duly published sequences from Genbank and Fungal barcoding databases. The sequences indexed in different databases which alignments showed high score percent identity and low E-value were selected to use in the construction of the nucleotide sequence trees.

In this study, the *Trichoderma* POS7 sequence showed a close position with other *T. koningiopsis* deposited sequences. The generated trees showed high bootstrap values of 85% for *T. koningiopsis* clade. Several authors reported that values of

70% or higher are expected to indicate reliable groupings (**Baldauf, 2003**). Moreover, the phylogenetic analyses revealed close positioning of the *T. koningiopsis* clade with *T. viride*, *T. viridescens* and *T. petersenii* clades in a closely related group, in concordance with the current taxonomic classification of *Trichoderma* genus (**Druzhinina *et al*., 2012; Gupta *et al*., 2014**).

CONCLUSION

The cellulase secretory capacity of twenty-eight native fungi of *Trichoderma* genus was evaluated with the aim to explore new fungal isolates with high cellulase secretory capacity in Argentina. The morphological and molecular identification allowed us to classify the isolate that showed the highest cellulase activity as *T. koningiopsis* POS7. This isolate showed the highest enzymatic activity and was selected for further studies. Our results provide new information and reveal new microorganism in the hydrolysis of cellulose material. *T. koningiopsis* POS7 isolate can be used in further studies to optimize its cellulase secretion, leading to a better use and application enzimatic secretion like in lignocellulose saccharification for bioethanol production.

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REFERENCES

ADRIO, J.L., DEMAIN, A.L. 2014. Microbial Enzymes: tools for biotechnology processes. *Biomolecules Journal*. 4(1), 117-139. https://doi.org/10.3390/biom4010117

AKINYELE, J.B., FALADE, O.E., OLANIYI, O.O. 2014. Screening and optimization of culture conditions for cellulase production by *Aspergillus niger* nspr012 in submerged fermentation. *Journal of Microbiology, Biotechnology and Food Sciences*. 4(3), 189-193. http://dx.doi.org/10.15414/jmbfs.2014-15.4.3.189-193

AMIN, S.B., RAHMAN, S. 2019. Bioenergy: The Hope that Is After Gas. In Energy Resources in Bangladesh, Springer, 63-66. https://doi.org/10.1007/978-3-030-02919-7_12

BAILEY, M.J. 1981. The effect of β-glucosidase on some assays for cellulolytic enzymes. *Biotechnological Letters.* 3, 695–700. https://doi.org/10.1007/BF00134846

BALDAUF, S.L. 2003. Phylogeny for the faint of heart: a tutorial. *Review Trends in Genetics.* 19(6), 345-351. https://doi.org/10.1016/S0168-9525(03)00112-4

BANDIKARI, R., MANNEPULA, S., POLUDASU, R., REDDY OBULAM, V. 2015. Supplementation of fruit processing waste for endoxylanase production by *Trichoderma koeningi* isolate and its optimization using central composite design: application of produced endoxylanase in mango juice clarification. *Journal of Microbiology, Biotechnology and Food Sciences*. 5(2), 162-166. http://dx.doi.org/10.15414/jmbfs.2015.5.2.162-166

BICH, G.A., CASTRILLO, M.L., SILVA, M.R., DÍAZ, G.V., VILLALBA, L.L., ZAPATA, P.D., MEDVEDEFF, M.G. 2015. Hongos entomopatógenos en la provincia de Misiones: Revisión. *Recyt*. 23(5), 54-58.

BICH, G.A., CASTRILLO, M.L., VILLALBA, L.L., ZAPATA, P.D. 2017. Isolation of the symbiotic fungus of *Acromyrmex pubescens* and phylogeny of *Leucoagaricus gongylophorus* from leaf-cutting ants. *Saudi Journal of Biological Science*. 24(4), 851–856. https://doi.org/10.1016/j.sjbs.2016.05.010

CASTRILLO, M.L., FONSECA, M.I., BICH, G.A., JERKE, G., HORIANSKI, M.A., ZAPATA, P.D. 2012.Taxonomy and phylogenetic analysis of *Aspergillus* section nigri isolated from yerba mate in Misiones (Argentina). *Journal of Basic and Applied Genetics*. 23(2), 19-27.

CASTRILLO ML, BICH GA, ZAPATA PD, VILLALBA LL. 2016. Biocontrol of *Leucoagaricus gongylophorus* of leaf-cutting ants with the mycoparasitic agent *Trichoderma koningiopsis*. *Mycosphere.* 7, 810-819.

DE EUGENIO, L.I., MÉNDEZ-LÍTER, J.A., NIETO-DOMÍNGUEZ, M., ALONSO, L., GIL-MUÑOZ, J., BARRIUSO, J., PRIETO, A., MARTÍNEZ, M.J. 2017. Differential β-glucosidase expression as a function of carbon source availability in *Talaromyces amestolkiae*: a genomic and proteomic approach. *Biotechnology for Biofuels*. 10, 161. https://doi.org/10.1186/s13068-017-0844-7

DOOLOTKELDIEVA, T., BOBUSHEVA, S. 2011. Screening of Wild-Type Fungal Isolates for Cellulolytic Activity. *Microbial Insights*. 4, 1-10.

DOS SANTOS CASTRO, L., RAMOS PEDERSOLI, W., CAMPOS ANTONIÊTO, A., STECCA STEINDORFF, A., SILVA-ROCHA, R., MARTINEZ-ROSSI, N., ROSSI, A., ANDREW BROWN, N., GOLDMAN, G., FAÇA, V., PERSINOTI, R., SILVA, R. 2014. Comparative metabolism of cellulose, sophorose and glucose in *Trichoderma reesei* using high-throughput genomic and proteomic analyses. *Biotechnology for Biofuels*. 7, 1-41. https://doi.org/10.1186/1754-6834-7-41

DRUZHININA, I.S., KUBICEK, C.P. 2005.Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters?. *Journal of Zheijang University SCIENCE*. 6(2), 100-112. https://doi.org/10.1631/jzus.2005.B0100

DRUZHININA, I.S., SEIDL-SEIBOTH, V., HERRERA-ESTRELLA, A., HORWITZ, B., KENERLEY, C., MONTE, E., MUKHERJEE, P., ZEILINGER, S., GRIGORIEV, I., KUBICEK, C.P. 2011. *Trichoderma*: the genomics of opportunistic success. *Nature Review*. 9(12), 749-759. https://doi.org/10.1038/nrmicro2637

DRUZHININA, I.S., KOMON-ZELAZOWSKA, M., ISMAIEL, A., JAKLITSCH, W., MULLAW, T., SAMUELS, G., KUBICEK, C.P. 2012. Molecular phylogeny and species delimitation in the section Longibrachiatum of *Trichoderma*. *Fungal Genetics and Biology*. 49(5), 358-368. https://doi.org/10.1016/j.fgb.2012.02.004

FERNANDEZ, R.D., BULACIO, N., ALVAREZ, A., PAJOT, H., ARAGON, R. 2017. Fungal decomposers of leaf litter from an invaded and native mountain forest of NW Argentina. *Antonie Van Leeuwenhoek*. 110(9), 1207–1218. https://doi.org/ 10.1007/s10482-017-0893-8

FLORENCIO, C., COURI, S., SANCHEZ FARINAS, C. 2012. Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase production by *Trichoderma* strains. *Enzyme Research*. 2012, 1-7. http://dx.doi.org/10.1155/2012/793708

GHOSE, T. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry*. 59(2), 257–268.

GUPTA, V.K., SCHMOLL, M., HERRERA-ESTRELLA, A., UPADHYAY, R.S., DRUZHININA, I.S., TUOHY, M.G. 2014. Biotechnology and Biology of *Trichoderma*. Elseiver, Amsterdam, The Netherlands. 650 p. ISBN: 9780444595942

HANKIN, L., ANAGNOSTAKIS, S. 1975. The use of solid media for detection of enzyme production by fungi. *Mycology*. 67(3), 597-607. https://doi.org/10.2307/3758395

HASSAN, G., EL-FEKY, Z., HEMADA, N., SAYED, M. 2015. Isolation and molecular characterization of egyptian *Trichoderma* and assessment of their antagonistic potential against *Rhizoctonia solani*. *Journal of Microbiology, Biotechnology and Food Sciences*. 4(6), 496-502. http://dx.doi.org/10.15414/jmbfs.2015.4.6.495-502

HIBBETT, D., ABARENKOV, K., KÕLJALG, U., ÖPIK, M., CHAI, B., COLE, J., WANG Q., CROUS P., ROBERT V., HELGASON T., HERR, J. R., KIRK P., LUESCHOW S., O'DONELL K., NILSSON R.H., OONO R., SCHOCH C., SMYTH C., WALKER D., PORRAS-ALFARO A., TAYLOR J., GEISER D. M. (2017). Sequence-based classification and identification of Fungi. *Mycologia*, 108(6), 1049-1068.

JALAK, J., KURAŠHIN, M., TEUGJAS, H., VÄLJAMÄE, P. 2012. Endo-exo synergism in cellulose hydrolysis revisited. *Journal of Biological Chemistry*. 287, 28802-28815. https://doi.org/10.1074/jbc.M112.381624

KAWAI, T., NAKAZAWA, H., IDA, N., OKADA, H., OGASAWARA, W., MORIKAWA, Y., KOBAYASHI, Y. 2013. A comprehensive analysis of the effects of the main component enzymes of cellulase derived from *Trichoderma reesei* on biomass saccharification. *Journal of Industrial Microbiology and Biotechnology*. 40(8), 805–810. https://doi.org/10.1007/s10295-013-1290-6

KUBICEK, C.P., HARMAN, G.E. 2002. *Trichoderma* & *Gliocladium.* Volumen 1. Basic biology, taxonomy and genetics. Taylor & Francis. London, 300 p. ISBN 9780748405725

LEAW, S.N., CHANG, H.C., SUN, H.F., BARTON, R., BOUCHARA, J.P., CHANG, T.C. 2006. Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. *Journal of Clinical Microbiology*. 44(3), 693-699. https://doi.org/10.1128/JCM.44.3.693-699.2006

MA, K., RUAN, Z. 2015. Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi. *Bioresource Technology*. 175, 586–593. https://doi.org/10.1016/j.biortech.2014.10.161

MANDELS, M., REESE, E. 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon source and metals. *Journal of Bacteriology*. 73(2), 269-278.

MANDELS, M., ANDREOTTI, R.C. 1976. Measurement of saccharifying cellulase. *Biotechnology and Bioenginering Symposium Journal*. 6, 21-33.

MARTÍNEZ-ANAYA, C., BALCÁZAR-LÓPEZ, E., DANTÁN-GONZÁLEZ, E., FOLCH-MALLOL, J. 2008. Celulasas fúngicas: aspectos biológicos y aplicaciones en la industria energética. *Revista Latinoamericana de Microbiología*. 50, 119-131.

MILLER, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*. 31(3), 426–428. https://doi.org/10.1021/ac60147a030

NIGAM, P., SINGH, A. 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*. 37(1), 52-68. https://doi.org/10.1016/j.pecs.2010.01.003

OUYANG, J., YAN, M., KONG, D., XU, L. 2006. A complete protein pattern of cellulase and hemicellulase genes in the filamentous fungus *Trichoderma reesei*. *Biotechnology Journal*. 1(11), 1266-1274.

PATEL, A.K., RANI SINGHANIA, R., JUN SIM, S., PANDEY, A. 2019. Thermostable cellulases: review and perspectives. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2019.01.049

PERSOON, C. 1794. Disposita methodica fungorum. Römer's Neues Mag. *Journal of Botany*. 1, 81-128.

PIONTELLI, E. 2015. Manual de microhongos filamentosos comunes. Chile. 462 p.

PRATS, G. 2007. Microbiología Clínica. Editorial Médica Panamericana, Buenos Aires, Argentina. 366 p. ISBN 847903971x

RATHNAN, R., JOHN, D., BALASARAVANAN, T. 2013. Isolation, screening, identification and optimized production of extracellular cellulase from *Bacillus subtilis* using cellulosic waste as carbon source. *Journal of Microbiology, Biotechnology and Food Sciences*. 2(6), 2383-2386.

RAY, R., DUTTA, W., SUR, D., KUNDU, A. 2013. Optimization of fermentation parameters for the production of extracellular endoglucanase, β–glucosidase and endoxylanase by a chromium resistant strain of *Trichoderma pseudokoningii*. *Journal of Microbiology, Biotechnology and Food Sciences*. 3(1), 54-58.

RIFAI, M. 1969. A revision of the genus *Trichoderma*. *Commonw Mycol Inst Mycol Pap*. 116, 1-56.

RODRIGUEZ M.D., ALONSO PAIVA I.M., CASTRILLO M.L., ZAPATA P.D., VILLALBA L.L. 2019 KH_2PO_4 improves cellulase production of *Irpex lacteus* and *Pycnoporus sanguineus*. *Journal of King Saud University – Science*. https://doi.org/10.1016/j.jksus.2018.07.009

SADHU, S., SAHA, P., MAYILRAJ, S., MAITI, T. 2012. Characterization of a *Bosea* sp. strain sf5 (mtcc 10045) isolated from compost soil capable of producing cellulase. *Journal of Microbiology, Biotechnology and Food Sciences*. 2(2), 576-591.

SAMBROOK, J., FRITSCHI, E.F., MANIATIS, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York. 2028 p. ISBN:978-1-936113-42-2

SAMSON, R.A., EVANS, H.C., LATGÉ, J.P. 1988. Atlas of entomopathogenic fungi. Springer-Verlag Berlin Heidelberg. 198 p. ISBN 978-3-662-05890-9

SAMUELS, G. 2006. *Trichoderma*: Systematics, the sexual state and ecology. The nature and application of biocontrol microbes II: *Trichoderma* spp. *Phytopathology Symptoms*. 96(2), 195-206. https://doi.org/10.1094/PHYTO-96-0195

SAZCI, A., RADFORD, A., ERENLERR, K. 1986. Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with dinitrosalicyclic acid reagent method. *Journal of Applied Bacteriology*. 61(6), 559-562. https://doi.org/ 10.1111/j.1365-2672.1986.tb01729.x

SCHNEIDER, C.A., RASBAND, W.S., ELICEIRI, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature. Methods*. 9(7), 671-675.

SUN, Y., CHENG, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*. 83(1), 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A., KUMAR, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*. 30(12), 2725-2729. https://doi.org/10.1093/molbev/mst197

THOMPSON, J.D., HIGGINGS, D.G., GIBSON, T.J. 1994. Clustal W improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. 22(22), 4673-4680.

WHITE, T., BURNS, T., LEE, S., TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. PCR Protocols: A guide to methods and applications. Academy Press, San Diego, 315-322.

WOOD, T.M., BHAT, K.M. 1988. Methods for Measuring Cellulase Activities. *Methods in Enzymology*. 160, 87–112. https://doi.org/10.1016/0076-6879(88)60109-1

ZHANG, Z., LIU, J.L., LAN, J.Y., DUAN, C.J., MA, Q.S., FENG, J.X. 2014. Predominance of *Trichoderma* and *Penicillium* in cellulolytic aerobic filamentous fungi from subtropical and tropical forests in China, and their use in finding highly efficient b-glucosidase. *Biotechnology for Biofuels*. 7, 107-121. https://doi.org/10.1186/1754-6834-7-107

ZHOU, J., WANG, Y.H., CHU, J., ZHUANG, Y.P., ZHANG, S.L., YIN, P. 2008. Identification and purification of the main components of cellulase from a mutant strain of *Trichoderma viride* T 100-14. *Bioresource Technology*. 99(15), 6826-6833. https://doi.org/10.1016/j.biortech.2008.01.077