High cellulolytic activities in filamentous fungi isolated from an extreme oligotrophic subterranean environment (Catão cave) in Brazil

CAIO C.P. DE PAULA[1], QUIMI V. MONTOYA[2], LUCAS A. MEIRELLES[2], CRISTIANE S. FARINAS[3], ANDRÉ RODRIGUES[2] and MIRNA H.R. SELEGHIM[1]

[1]Programa de Pós-Graduação em Ecologia e Recursos Naturais, Universidade Federal de São Carlos, Departamento de Ecologia e Biologia Evolutiva, Rodovia Washington Luiz, Km 235, 13565-905 São Carlos, SP, Brazil
[2]Universidade do Estado de São Paulo "Júlio de Mesquita Filho", Instituto de Biociências de Rio Claro, Departamento de Bioquímica e Microbiologia, Av. 24A, 1515, 13506-900 Rio Claro, SP, Brazil
[3] Empresa Brasileira de Pesquisa Agropecuária/Embrapa – Instrumentação, Rua XV de Novembro, 1452, 13560-970 São Carlos, SP, Brazil

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Abstract: Isolation and screening of new fungal strains from extreme and understudied environments, such as caves, is a promising approach to find higher yields enzyme producers. Cellulolytic fungal strains isolated from a Brazilian cave were evaluated for their enzymatic production after submerged (SmF) and solid-state fermentation (SSF). After SmF, three strains were selected for their high enzymatic activities: *Aspergillus ustus* for endoglucanase (4.76 U/mg), *Talaromyces bruneus* for β-glucosidase (11.71 U/mg) and *Aspergillus* sp. (CBMAI 1926) for total cellulase (1.70 U/mg). After SSF, these strains, showed better yields compared to the reference strain *Aspergillus niger* 3T5B8. *Aspergillus* sp. (CBMAI 1926) stood out as a new species that expressed activity of total cellulases (0.10 U/mg) and low protein concentration (0.44 mg/mL). In conclusion, these isolated strains have a more efficient and promising cellulolytic enzyme complex that can be used in fermentation and saccharification processes with a lower protein concentration and a higher enzymatic activity than the reference strain. Therefore, beside the new genetic material characterized, our study highlights the benefits of cave extreme environments exploitation to find new potentially valuable strains.

Key words: cellulases, fungu's cave, solid state fermentation, submerged fermentation, subterranean environment.

INTRODUCTION

Subterranean environments such as caves can be considered extreme environments that provide highly specialized niches (Engel 2007) but, so far, they have been overlooked regarding its potential for new genetic resources. For this reason, there are few reports assessing the cellulolytic potential of microorganisms from caves (Ogórek et al. 2016, Paula et al. 2016, Rautela et al. 2017). According to Lynd et al. (2002) in extreme environments, such as caves, microorganisms tend to use a broad range of carbohydrates, probably as a consequence of the

Correspondence to: Caio César Pires de Paula
E-mail: piresdepaula@yahoo.com.br
ORCid: https://orcid.org/0000-0002-4306-9839

small amount of cellulose input combined with the presence of few competing species in these habitats. Pioneering study in Catão cave (Brazil) isolated fungal strains and carried out the screening (plaque test) to select cellulolytic enzyme producing strains. From this study, the authors highlighted the great percentage of cellulolytic strains isolated from the subterranean environment in comparison to the surface environment (Paula et al. 2016).

Cellulose is the main constituent of plant cell walls and the most abundant renewable material on Earth (Lynd et al. 2002, Zhang and Lynd 2004). Its enzymatic hydrolysis is a promising alternative for the production of items of economic interest, such as ethanol (Qu et al. 2006), organic acids (Shen and Xia 2006) and other chemicals (Cao et al. 1997). Currently, the cellulolytic enzymes are already used in the production of food, animal feed, cleaning products, textile industry, among others (Jahangeer et al. 2005). Cellulolytic enzymes are produced by a wide variety of microbes, but those performed by aerobic fungi are characterized by high enzymatic activity and protein synthesis (Lynd et al. 2002, Singhania et al. 2010).

The cellulolytic complex comprises enzymes from different classes (exoglucanases, endoglucanases and glucosidases) acting in a coordinated way (Chandra et al. 2007, Lynd et al. 2002). The production of these enzymes can be carried out under submerged fermentation (SmF) and in solid-state fermentation (SSF) (Singhania et al. 2010, Cunha et al. 2012). The SmF is advantageous and widely used due to the easy control of variables during the process (Howard et al. 2003). On the other hand, the SSF is more advantageous from the economical point of view, since the by-products formed can be readily used. However, the operating parameters such as temperature, pH and moisture are difficult to control in SSF (Singhania et al. 2010, Singhvi et al. 2011, Cunha et al. 2012). Aiming to lower the costs of these processes, researchers seek microorganisms with high cellulolytic potential in

extreme understudied environments, such as caves (Lynd et al. 2002).

Thus, seeking for new highly efficient cellulose degraders, we here reveal cellulolytic activities (endoglucanases, β-glucosidases and total cellulase) under submerged and solid-state fermentation conditions for new fungal strains isolated from a Brazilian cave environment. Beside the new genetic material characterized, our study highlights the benefits of cave extreme environments exploitation to find new potentially valuable strains.

MATERIALS AND METHODS

SAMPLING

The sampling was carried out at the Catão cave (12° 22' 6" S, 44° 52' 3" W), located at the Conservation Unit of the Lagoa Azul Municipal Park, São Desidério, State of Bahia, Brazil, under the permit ICMBio / SISBIO (10215 license). A quadrant of approximately 0.25 m^2 of soil was sampled in two areas of the cave: Entrance Zone (EZ) and Twilight Zone (TZ) in the year of 2012. Compound sediment samples, obtained from 05 sub-samples, were collected (0-10 cm depth) in sterile plastic containers. At the laboratory samples were homogenized, sieved (2 mm mesh) and kept in refrigerator at 4.0°C until the analysis. Sampling details can be accessed at Paula et al. (2016).

FUNGAL ISOLATION AND CHARACTERIZATION

Strains isolation was carried out by serial dilution (1:10000) of the sediment samples in physiological saline (0.85% NaCl), with triplicates per dilution, followed by spread plate inoculation in Malt agar 3% medium (M3%, 30.0 g L^{-1} malt extract, 3.0 g L^{-1} soy peptone, 0.05 g L^{-1} Rose Bengal, 20.0 g L^{-1} agar and pH 5.5- 6.0). Plates were incubated for 15 days at 28.0°C. After incubation, colonies were transferred by streaking in M3% to obtain monosporic cultures. Preliminary identification

was carried out based on morphological characters of the colonies as well as by their microscopic reproductive structures observed in wet mount under an optical microscope. Classical taxonomic keys were used to assess all isolates genera (Domsch et al. 1980, Barnett and Hunter 1998). As described by Paula et al. (2016), as one step of their characterization, the strains were also pre-tested to evaluate their cellulolytic activity by the formation of a degradation zone revealed by the addition of a Congo red solution in colonies grown in carboxymethylcellulose plates.

SUBMERGED FERMENTATION (SmF)

The cellulolytic fungal strains were grown in submerged fermentation using the synthetic medium adapted from Mandels (Mandels and Weber 1969) (Urea 0.3 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $(NH_4)_2SO_4$ 1.4 g L^{-1}, $CaCl_2.2H_2O$ 0.4 g L^{-1}, $MgSO_4.7H_2O$ 0.3 g L^{-1}, yeast extract 0.6 g L^{-1}, $FeSO_4.7H_2O$ 5.0 mg L^{-1}, $MnSO_4.4H_2O$ 1.6 mg L^{-1}, $ZnSO_4.7H_2O$ 1.4 mg L^{-1}, $CoCl_2.6H_2O$ 2.0 mg L^{-1}, pH 5.6). To obtain a pre-culture, 10 g L^{-1} of glucose was added to the synthetic medium (50 mL) and was inoculated with a 10^6 spore mL^{-1} suspension before incubation in a shaker (130 rpm) at 28.0°C for three days. Then, 5.0 mL of the pre-culture was transferred to 700 mL of Mandels medium containing 10.0 g L^{-1} of microcrystalline cellulose and incubated in a shaker at 28.0°C (130 rpm) for 7 days. At 24 hour intervals, an aliquot (7.0 mL) of the culture was withdrawn and centrifuged at 3000 rpm for 20 min. The supernatant was used as crude enzymatic extract for the analysis of cellulases expression.

SOLID-STATE FERMENTATION (SSF)

The strains that stood out in terms of enzyme activity under submerged fermentation were cultivated under solid-state fermentation. For solid-state fermentation it was used, as substrate and sole carbon source, wheat bran (WB) in ammonium

sulfate solution $((NH_4)_2SO_4)$ without glucose. The selected strains were first grown on tubes containing Malt agar 3% tilted for seven days. After this period, a 10^7 spore mL^{-1} suspension was prepared and inoculated into 5.0 g of WB substrate in 250 mL Erlenmeyer flasks with humidity adjusted to 72%. All cultivations were performed in triplicate at 30.0°C for 48 hours without stirring. For comparison, we used a reference fungal strain known for its high cellulolytic enzyme production, the mutant strain *Aspergillus niger* 3T5B8 (Embrapa Collection Food Technology) (Pirota et al. 2013). After cultivation, 50.0 mL of 0.2 M sodium acetate (pH 4.5) were added and the flasks shaken at 200 rpm (30.0°C) for 40 minutes. To obtain the enzyme extract, the culture was filtered in vacuum pump (Whatman® n.1) and the filtrate was subsequently centrifuged for 15 minutes at 10,000 rpm (4.0°C). The supernatant was considered the crude enzymatic extract. For total extracellular protein content and enzymatic activity quantification (endoglucanase – EC; β-glucosidase BG and total cellulose on filter paper - FP), it was used the same substrates and methodologies described for submerged fermentation. We set up a control culture (CC) which consisted only of WB with $(NH_4)_2SO_4$ without glucose, in order to measure the concentration of protein released by the substrate during the SSF. The enzymatic activities were expressed in international units per gram of dry substrate (U g^{-1}).

ANALYTICAL MEASUREMENTS

All the fermentation processes were performed in triplicate. For each fermentation flask were measured: protein concentration and cellulolytic activities. The total extracellular protein content of the crude extracts was determined according to Bradford (1976). The endoglucanase activity (EC) was determined using 30 µL of enzyme extract (Ghose 1987) modified with 50 mL of 1%

solution of sodium carboxymethylcellulose, as substrate, in 20 µL of citrate buffer 0.05 mol L^{-1} (pH 5, 0). The reaction was incubated at 50.0°C for 15 minutes, interrupted by the addition of 300 µL of dinitrosalicilic acid (DNS). The amount of total reducing sugars (TRS) released was determined spectrophotometrically (Novaspec II) at 540 nm (λ). The β-glucosidase (BG) activity was determined by a commercial kit (Kit Bio Glucose Liquid, Laborclin, Campinas, Brazil), following the manufacturer's instructions, using 2% cellobiose as substrate prepared in citrate buffer 0.05 mol L^{-1} (pH 5, 0) with GOD (glucose oxidase-peroxidase) as a colorimetric reagent. The reducing sugar concentration was determined spectrophotometrically at 505 nm (λ). To determine the enzyme activity (total cellulase or FP) we used as substrate filter paper (Whatman® n.1, 7 mm diameter) with 50 mL of enzyme extract solution and 50 mL of 0.05 mol L^{-1} (pH 5.0) citrate buffer. The reaction was incubated for 60 minutes at 50.0°C and then 300 µL DNS were added. Reducing sugars were measured at 540 nm wavelength (λ) in the spectrophotometer.

For all enzymatic activities, separate controls were used to discount the absorbance values of the enzyme extract (enzyme control) and substrate (reaction control). The absorbance values were converted to equivalent amounts of glucose, using standard curve. One enzyme activity unit (U) was defined as the amount of enzyme required to produce 1 µmol of glucose mL^{-1} min^{-1} under the assay conditions (U = 1 µmol mL^{-1} min^{-1}). The parametric t-test was used to compare means for normal distribution data at 5% probability. For statistical analysis we used the PAST program v. 8.2.

MOLECULAR IDENTIFICATION OF FUNGAL STRAINS

DNA extraction was performed from mycelia of each isolate. For that, selected strains were grown on Potato Dextrose Agar (PDA) at 25.0°C for 7 days in the dark. Genomic DNA was extracted following Meirelles et al. (2015). The partial β-tubulin gene was amplified using the primers Bt2a (5'GGTAACCAAATCGGTGCTGCTTTC3') and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3') (Glass and Donaldson 1995, Samson et al. 2004). The PCR was performed in a final volume of 25 µL (4 µL of dNTPs [1.25 mM each]; 5 µL of 59 buffer; 1 µL of BSA [1 mg mL^{-1}]; 2 µL of MgCl$_2$ [25 mM]; 1 µL of primer [10 mM]; 0.2 µL of Taq polymerase [5 U lL^{-1}], 2 µL of diluted genomic DNA [1:100] and 8.8 µL of sterile ultrapure water). PCR conditions were: 94.0°C/3 min followed by 35 cycles at 94.0°C/1 min, 55.0°C/1 min and 72.0°C/2 min. Amplicons were purified using Wizard® SV Gel and PCR Clean-Up System Kit (Promega) following the manufacturer's protocol. Then, samples were quantified in NanoDrop® (Thermo Scientific) and subjected to cycle sequencing reaction using BigDye Terminator® v.3.1 Kit (Life Technologies), following the manufacturer's. The sequencing conditions were: 95.0°C/1 min followed by 28 cycles at 95.0°C/15 s, 50.0°C/ 45 s, and 60.0°C/4 min. Forward and reverse sequences were generated in ABI 3330xl sequencer (Life Technologies) and assembled into consensus using the program BioEdit v.7.0.5.3 (Hall 1999). For the phylogenetic analyses sequences from *Talaromyces* and *Aspergillus* were retrieved from the NCBI-GenBank database. In order to know the phylogenetic position of each isolate, three phylogenetic trees were built (one for each isolate), then they were merged into a unique tree that shows the lineages in a more comprehensible way (each sample maintained its initial phylogenetic position). The final phylogenetic tree was performed with a final dataset comprised by 21 B-tubulin sequences (447bp). For that purpose, our dataset was aligned using the program MAFFT v.7 (Katoh and Standley 2013) and the tree was built using the neighbor-joining algorithm with Kimura-2 parameter

substitution model. A phylogenetic tree was built using the MEGA v.7 software (Tamura et al. 2011) and the robustness of the tree was calculated using 1000 bootstrap pseudo-replicates. The final phylogenetic tree was edited manually using the Adobe Illustrator CC v.17.1 program.

The sequences of the β-tubulin gene for each strain were deposited in GenBank (SDC 1.2 (CBMAI 1894) - MF134411; SDC 2.7(CBMAI 1895) - MF134412 and SDC 2.8 (CBMAI 1926) - MF134413). GenBank accession numbers for each strain in the phylogenetic tree are included with each taxon.

RESULTS

Previous study isolated 20 cellulolytic fungal strains from the Entrance Zone (7) and the Twilight Zone (13). The fungal strains were morphologically identified (by microscopic analysis) as the following genera: *Aspergillus* (50.0%), *Penicillium* (25.0%), *Talaromyces* (10.0%), *Trichoderma* (5.0%), *Purpureocillium* (5.0%) and *Scopulariopsis* (5.0%) (Paula et al. 2016).

All cellulolytic fungal strains isolated by Paula et al. (2016) were submitted to submerged fermentation (data not shown). Only three fungal strains highlighted in this procedure: SDC 1.2 (*Aspergillus* sp.5), SDC 2.7 (*Talaromyces* sp.) and SDC 2.8 (*Aspergillus* sp.8) (codes defined in Paula et al. (2016)). These three strains showed protein content of the crude enzyme extract ranging from 0.027 to 0.041 mg mL^{-1} during submerged cultivation for 168 hours (Table I). In general, protein concentrations were higher after 96 hours of culture. The maximum enzymatic activity of endoglucanses (EC), β-glucosidases (BG) and total cellulases (FP) are shown in Table I. Due to the dissimilarities related to protein content of the crude enzyme extract for each strain, it was not possible to make a direct comparison among the volumetric values (U L^{-1}). Therefore, the enzymatic

activities were expressed as specific activity (U mg^{-1}), considering the protein concentration in the culture medium (Table II).

The EC activity ranged from 0.11 to 4.76 U mg^{-1} (Table II). The highest enzyme activity was found for the strain *Aspergillus* sp. 5 (SDC 1.2). In general, the highest enzyme activity values of EC were expressed after 72 hours of culture (maximum peak in 144h). Regarding the BG activity, we verified that this activity was more pronounced than those for EC, with enzyme activity ranging from 0.52 to 12.77 U mg^{-1} (Table II). The enzymatic activities of BG were also obtained after 72 hours, especially for the strains *Aspergillus* sp. 5 (SDC 1.2) and *Talaromyces* sp. (SDC 2.7) that had the highest one (12.77 and 11.71 U mg^{-1}, respectively). The total cellulase (FP) activity ranged from 0.11 to 1.70 U mg^{-1} (Table II). Among all tested strains the *Aspergillus* sp. 8 (SDC 2.8) showed the highest activity of total cellulases (1.70 U mg^{-1}).

Analyzing the strains *Aspergillus* sp. 5 (SDC 1.2), *Talaromyces* sp. (SDC 2.7) and *Aspergillus* sp. 8 (SDC 2.8) (Fig. 1), we observed that only *Aspergillus* sp. 8 expressed a continuous enzymatic activity of the three enzyme classes throughout cultivation. We observed that the enzymatic activities of this strain reached their peak at 48 hours (for EC and FP) and 72 hours (for BG), reducing only slightly its enzymatic activity during the rest of the growth. *Aspergillus* sp. 5 expressed the FP activity only at the beginning of cultivation, and the BG activity showed increased values of enzyme activity after 72 hours of culture. Regarding the strain *Talaromyces* sp. the maximum activity occurred in 48 and 72 hours (EC and FP, respectively). *Talaromyces* sp. showed a peak of BG activity after 72 hours of cultivation, decreasing their activity after 120 hours of cultivation.

The strains *Aspegillus* sp. 5 (SDC 1.2), *Talaromyces* sp. (SDC 2.7), *Aspergillus* sp. 8 (SDC 2.8) and *Aspergillus niger* 3T5B8 (reference strain) were grown in a solid state fermentation (SSF),

TABLE I
Mean and standard deviation of the maximum protein concentration and maximum volumetric activities of endoglucanases, β-glucosidases and FP during submerged cultivation of fungal strains isolated from Catão cave (numbers in parenthesis corresponds to the cultivation time in hours).

Strains	Proteins (mg/L)		Endoglucanases (U/L)		β-Glucosidases (U/L)		FP (U/L)	
Aspergillus ustus	41.0 ± 2.2	(168)	109.0 ± 10.9	(144)	524.0 ± 63.7	(168)	4.0 ± 8.3	(24)
Talaromyces brunneus	27.0 ± 1.7	(144)	44.0 ± 27.4	(72)	260.0 ± 6.3	(168)	7.9 ± 7.9	(48)
Aspergillus sp. *(CBMAI 1926)*	36.0 ± 2.4	(168)	118.0 ± 29.4	(120)	16.0 ± 3.9	(144)	42.0 ± 6.5	(120)

TABLE II
Mean and standard deviation of the maximum specific activity of endoglucanases, β-glucosidases and FP during submerged cultivation of fungal strains isolated from Catão cave (numbers in parenthesis corresponds to the cultivation time in hours).

Strains	Endoglucanases (U/mg)		β-Glucosidases (U/mg)		FP (U/mg)	
Aspergillus ustus	4.76 ± 0.47	(144)	12.77 ± 1.55	(168)	0.11 ± 0.04	(24)
Talaromyces brunneus	1.87 ± 1.15	(72)	11.71 ± 0.28	(168)	0.44 ± 0.06	(48)
Aspergillus sp. *(CBMAI 1926)*	3.89 ± 0.20	(48)	0.52 ± 0.12	(72)	1.70 ± 0.12	(48)

using wheat bran as substrate and sole carbon source. Table III shows the results obtained after 48 hours of cultivation. Comparing the enzymatic activity of wild strains in with our reference strain, we found that the wild strains stood out in, at least, one of the evaluated enzyme classes. Strain *A. niger* 3T5B8 showed significantly high protein concentration in relation to wild strains; on the other hand, there was no statistical difference on the protein content among the wild strains. The control culture (CC) showed a protein content of 0.38 mg mL^{-1}, demonstrating the contribution of the wheat bran substrate on the estimated protein content at the end of cultivation. We considered the total protein concentration of the crude extract to estimate the specific enzymatic activity in order to compare the values obtained between SSF and SmF. All cellulolytic enzymes expressed lower values of specific enzymatic activity during SSF cultivation as compared to SmF cultivation.

Finally, we analyzed the amount of total reducing sugar (TRS) in crude enzyme extracts from the end of cultivation (Table III). We found that the average amount of TRS of the *Talaromyces* sp. was higher than the values for *A. niger* 3T5B8 (reference strain). However, there was no statistical difference on the amount of TRS estimated between *Aspergillus* sp. 5 and *Aspergillus* sp. 8 compared to the reference one.

Molecular identification and phylogenetic analysis of the fungal strains that stood out in relation to their enzymatic activity, resulted in *Aspergillus* sp. 5 (SDC 1.2) identified as *Aspergillus ustus* (CBMAI 1894), *Talaromyces* sp. (SDC 2.7) identified as *Talaromyces brunneus* (CBMAI 1895) and *Aspergillus* sp. 8 (SDC 2.8) identified as *Aspergillus* sp. (CBMAI 1926) (Figure S1 - Supplementary Material). Strains were deposited in the Brazilian Collection of Microorganisms of Environment and Industry (CBMAI).
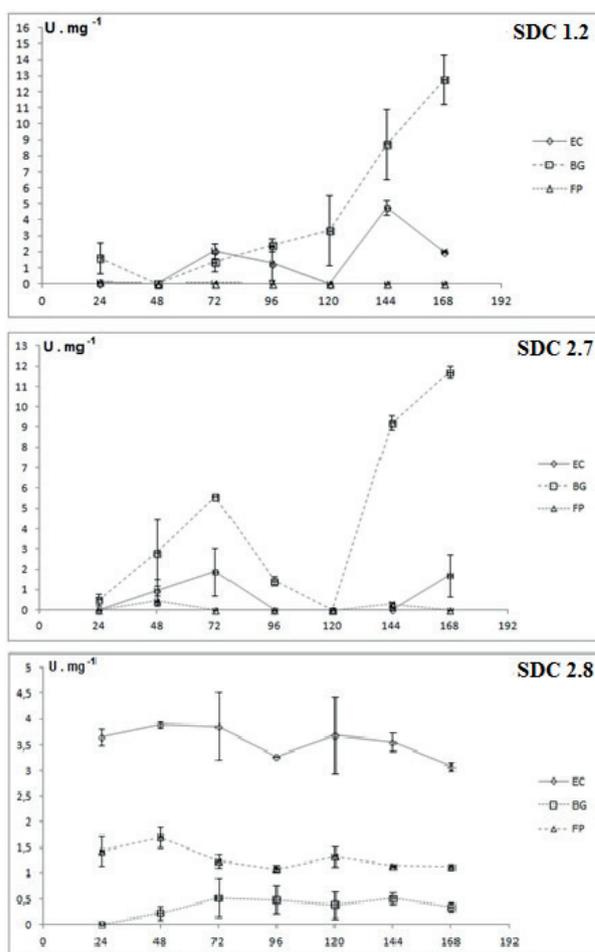
Figure 1 - Enzymatic kinetics of the cellulolytic complex (EC = endoglucanase, BG = β-glucosidase and FP = cellulose total) from the three wild type fungal strains *Aspergillus ustus* (SDC 1.2), *Talaromyces brunneus* (SDC 2.7) and *Aspergillus* sp. (CBMAI 1926- SDC 2.8) grown in submerged fermentation using microcrystalline cellulose as sole carbon source.

DISCUSSION

Caves are extreme oligotrophic environments overlooked regarding their diversity and that have its food chain based in the external source material degradation. Wherefore we expected to found fungal strains with cellulolytic activity isolated from the studied cave. Fungal genera isolated in this work are the most commonly isolated in several environments and are already known to have lytic activity (Ruegger and Tauk-Tornisielo 2004, Jahanger et al. 2005, Fernandes et al. 2008).

Paula et al. (2016) previously noted that 90% of the isolated fungi strains in Catão cave expressed cellulolytic activity. The percentage of cellulases producing strains isolated from the cave was higher as compared with other studied environments. (Ruegger and Tauk-Tornisielo 2004, Jahanger et al. 2005, Delabona et al. 2012).

After cultivation in SmF, three strains stood out for their high enzymatic activity: *Aspergillus ustus*, *Talaromyces brunneus,* and *Aspergillus* sp. (CBMAI 1926). *Aspergillus ustus* stood out in EC activity, which it was 1.34 to 2.80 times higher than the values expressed by *Aspergillus* sp. (CBMAI 1926) and *Talaromyces brunneus*, respectively. The SSF confirmed for this strain high EC activity. Jahangeer (2005), studying wild fungal strains, obtained EC activity values for *Aspergillus* strains similar to those reported in our study. The species *Aspergillus ustus* is known to have the cellulolytic enzyme complex, especially endoglucanases production (Macris and Galiotou-Panayotou 1986, Shamala and Sreekantiah 1986, Saleem et al. 2013).

The strains *Aspergillus ustus* and *Talaromyces brunneus* showed, respectively, BG activity 38.69 and 35.48 times higher compared to *Aspergillus* sp. (CBMAI 1926). When analyzing the results obtained in the SSF, *Talaromyces brunneus* showed greater BG activity in relation to *Aspergillus* sp. (CBMAI 1926). However, the enzymatic activity of BG in both strains (*Apergillus ustus* and *Talaromyces brunneus*) increased after 120h, reaching its maximum activity in 168 hours of culture. On the other hand, *Aspergillus* sp. (CBMAI 1926) decreases the enzymatic activity of BG after 120h of culture. This decrease of BG activity in *Aspergillus* sp. (CBMAI 1926) is common because this enzyme (BG) is inhibited by the product of the catalyzed reaction, the glucose (Sorensen et al. 2013). Studies with *Talaromyces*, known as good producer of BG (Moloney et al. 1983, El-Naggar et al. 2015), showed lower or similar BG activities than those expressed by *Talaromyces*

TABLE III

Mean and standard deviation of the total reducing sugar (TRS) concentrations (g/L), protein concentration (mg/mL) and specific activity (U/ mg) and activity per mass of dry substrate (U/g) of endoglucanases, β-glucosidases and FP during cultivation in solid state fermentation of fungal strains after growth in solid state using wheat bran as substrate (30°C; 48 hours) (bold values highlight strains which showed peak values).

	Aspergillus ustus		Talaromyces brunneus		Aspergillus sp. (CBMAI 1926)		Aspergillus niger 3T5B8	
	(U/mg)	(U/g)	(U/mg)	(U/g)	(U/mg)	(U/g)	(U/mg)	(U/g)
Endoglucanase	**0.77**[a] ± 0.047	**3.66**[a] ± 1.708	0.52[b] ± 0.021	2.38[b] ± 1.259	0.68[b] ± 0.341	2.98[a,b] ± 2.279	0.49[b] ± 0.084	2.51[b] ± 0.738
β-glucosidase	1.23[a] ± 0.604	5.79[a] ± 2.777	**3.39**[b] ± 0.489	**15.31**[b] ± 2.598	1.29[a] ± 0.336	5.73[a] ± 1.389	0.79[a] ± 0.684	3.80[a] ± 2.943
FP	0.03[a] ± 0.012	0.19[a] ± 0.121	0.06[a,b] ± 0014	0.11[a] ± 0.083	**0.10**[c] ± 0.017	**0.45**[b] ± 0.236	0.09[b,c] ± 0.072	0.24[a] ± 0.132
Proteins (mg/mL)	0.46[a] ± 0.045		0.45[a] ± 0.048		0.44[a] ± 0.042		**0.50**[b] ± 0.044	
TRS (g/L)	3.02[a,c] ± 0.072		**3.09**[a] ± 0.093		2.67[b,d] ± 0.295		2.94[c,d] ± 0.102	

* Values followed by the same overwritten letters in the same line for each enzyme and protein, do not differ statistically (p < 0.05).

brunneus from this study. The cellulase complex of several fungi is limited by the low BG production, hydrolysis inhibition by glucose and, in most cases, by the BGs inhibition by their own substrate, the cellobiose (Schimid and Wandrey 1987). Analyzing enzyme kinetics of the selected fungi (Fig. 1), the profile described above, such as substrate inhibition, does not fit for *Talaromyces brunneus*, reinforcing its potential as a good BG producer. Few studies have evaluated the cellulolytic activity of the *Talaromyces* genus. Overall, *Talaromyces emersoni* is the most studied species within this genus and it is known to have multiple BG enzymes with high activity related to its cellulolytic complex (McHale and Coughlan 1981, Murray et al. 2004). This is the first report on cellulolytic activities in *Talaromyces brunneus*.

The strain *Aspergillus* sp. (CBMAI 1926) expressed high FP and EC activity and low BG in SmF and SSF. During SmF, this strain showed FP specific enzyme activity 15.45 and 3.86 times higher than *Aspergillus ustus* and *Talaromyces brunneus*, respectively. The FP activity of *Aspergillus* sp. (CBMAI 1926) is higher than that found by other authors in the published literature (Ruegger and Tauk-Tornisielo 2004, Krogh et al. 2004). Berlin (2005) obtained a maximum FP specific enzymatic activity of only 1.04 U mg^{-1} protein of an enzyme extract produced by seven mutant strains of *Trichoderma* sp. and *Penicillium* sp., known as good producers of cellulolytic enzymes, results much lower than those obtained for *Aspergillus* sp. (CBMAI 1926), indicating its high potential. Even using two genetic markers for identification (β-tubulin), it was not possible to identify this strain to species level. Analyzing the phylogenetic tree (Figure S1), we suggested that this fungus probably belong to a new species, due to the low similarity of the gene sequence with other copies from GenBanck database (< 97.0%) and the low bootstrap values when building the phylogenetic tree (being difficult to place it in the

phylogenetic tree). Although it is an unidentified species, there are several studies on the xylanolytic and cellulolytic potential of the genus *Aspergillus* (Carmona et al. 1997, Somera et al. 2009, Qaisar et al. 2014).

In contrast with the literature data, all the fungal strains studied in this work expressed a higher specific enzymatic activity during SmF than SSF (Saqib et al. 2010, Cunha et al. 2012). We must emphasize that the SSF was performed in only one temperature and pH conditions, which may not be the best for all these fungi to synthesize the enzymes in solid state growth. The enzymatic activities of wild-type strains found after SSF were higher, in comparison to the reference strain, at least in one of the evaluated enzyme classes. Only the protein content of the wild strains enzyme extract was less than *A. niger* 3T5B8. The wild strains studied showed a pattern with a lower protein synthesis and higher enzyme activity in the raw extract in SSF. So, we can consider that despite the fact they produce smaller amounts of enzymes, the efficiency of their cellulolytic enzymes for hydrolysis is higher in relation to that found for *A. niger* strain 3T5B8. Chandra (2007), evaluating different plant substrates in SSF, reported similar EC and FP values for *A. niger* strain, although this strain showed lower ones for BG, compared with our results. Using the same growth conditions than used in our work, Pirota et al. (2013), *A. niger* 3T5B8 and *Trichoderma reesei* Rut-C30 strains reached enzyme activity values for FP lower than those expressed by *Aspergillus* sp. (CBMAI 1926), proving, once again, the high cellulolytic potential of this strain.

At the end of the SSF growth, *Aspergillus* sp. (CBMAI 1926) showed the lowest TRS concentration in the enzyme extract, compared to the other wild and the reference strain. So, we can conclude that even if the *Aspergillus* sp. (CBMAI 1926) has a lower potential in the saccharification process of the substrate, it can be used in enzyme production processes, due to its high enzyme activity expressed more strongly in submerged culture. On the other hand, *Talaromyces brunneus* showed higher TRS concentrations than the reference strain, highlighting a good potential for the saccharification process. The enzyme production at large scale is usually performed in SmF because greater stability and control of the culture conditions can be obtained compared to SSF (Howard et al. 2003, Singhania et al. 2010). Thus, such a situation favors the use of *Aspergillus* sp. (CBMAI 1926) in industrial production of cellulolytic enzymes compared to the other strains tested in this work.

This is the first report on the growth in SmF and SSF of filamentous fungi strains isolated from an extreme oligotrophic subterranean environment with genetic identification and it show a new strain of *Aspergillus* sp. (CBMAI 1926) to be described. In SmF three wild strains stood out, *Aspergillus ustus* (SDC 1.2) with high EC and BG activity values, *Talaromyces brunneus* (SDC 2.7) with high BG activity and *Aspergillus* sp. (CBMAI 1926) (SDC 2.8) with high EC and FP activities with a clear synergism among the three evaluated enzymes. Considering the SSF, the wild strains exceeded the reference strain in relation to the cellulolytic activity and the best was *Aspergillus* sp. (CBMAI 1926) for its high EC and FP values. We observed that, with a lower protein concentration and a higher enzymatic activity than the reference strain, these strains have a more efficient cellulolytic enzyme complex that can be used in biotechnological processes.

AUTHOR CONTRIBUTIONS

Caio César Pires de Paula and Mirna Helena Regali Seleghim originally formulated the idea and designed the experiments. Quimi Vidaurre Montoya and Lucas Andrade Meirelles gave support about the morphologically and genetic identified of the strains. André Rodrigues assisted in the phylogeny of strains and Cristiane Sanchez Farinas collaborated with the experiment of solid state fermentation. Mirna Helena Regali Seleghim assisted Caio César Pires de Paula in the discussion of the results. Caio César Pires de Paula performed the experiments and wrote the manuscript.

REFERENCES

BARNETT HL AND HUNTER BB. 1998. Illustrated genera of imperfect fungi. American Phytopathological Society Press, Saint Paul- Minnesota.

BERLIN A ET AL. 2005. Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates–evidence for the role of accessory enzymes. Enzyme Microb Technol 37(2): 175-184.

BRADFORD MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 255-260.

CAO NJ, XIA YK, GONG CS AND TSAO GT. 1997. Production of 2,3-butanediol from pretreated corn cob by *Klebsiela oxycota* in the presence of fungal cellulase. Appl Biochem Biotechnol 63-65: 129-139.

CARMONA EC, PIZZIRANI-KLEINER AA, MONTEIRO RTR AND JORGE JA. 1997. Xylanase production by *Aspergillus versicolor*. J Basic Microb 37(6): 387-393.

CHANDRA MS, VISWANATH B AND REDDY BR. 2007. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. Indian J Microbiol 47: 323-328.

CUNHA FM, ESPERANÇA MN, ZANGIROLAMI TC, BADINO AC AND FARINAS CS. 2012. Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase. Bioresour Technol 112: 270-274.

DELABONA PDS, PIROTA RD, CODIMA CA, TREMACOLDI CR, RODRIGUES A AND FARINAS CS. 2012. Using Amazon forest fungi and agricultural residues as a strategy to produce cellulolytic enzymes. Biomass Bioenerg 37: 243-250.

DOMSCH KH, WALTER G AND TRAUTE-HEIDI A. 1980. Compendium of soil fungi. Volume 2. Academic Press (London) Ltd.

EL-NAGGAR NEA, HAROUN SA, OWEIS EA AND SHERIEF AA. 2015. Identification of newly isolated *Talaromyces pinophilus* and statistical optimization of β-glucosidase production under solid-state fermentation. Prep Biochem Biotechnol 45(7): 712-729.

ENGEL AS. 2007. Observations on the biodiversity of sulfidic karst habitats. J Caves Karst Stud 69: 187-206.

FERNANDES S, MURRAY PG AND TUOHY MG. 2008. Enzyme systems from the thermophilic fungus *Talaromyces emersonii* for sugar beet bioconversion. Bioresources 3(3): 898-909.

GHOSE TK. 1987. Measurement of cellulase activities. Pure Appl Chem 59: 257-268.

GLASS NL AND DONALDSON GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61: 1323-1330.

HALL TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95-98.

HOWARD RL, ABOTSI E, JANCEN EL AND HOWARD R. 2003. Lignocellulosic biotechnology: issue of bioconversion and enzyme production. Afr J Biotechnol 12: 602-619.

JAHANGEER S, KHAN N, JAHANGEER S, SOHAIL M, SHAHZAD S, AHMAD A AND KHAN SA. 2005. Screening and characterization of fungal cellulases isolated from the native envionmental source. Pak J Bot 37(3): 739-748.

KATOH K AND STANDLEY DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30: 772-780.

KROGH KBR, MORKEBERG A, JORGENSEN H, FRISVAD JC AND OLSON L. 2004. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. Appl Biochem Biotechnol 113: 389-401.

LYND LR, WEIMER PJ AND VAN ZYL WH. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66: 506-577.

MACRIS BJ AND GALIOTOU-PANAYOTOU M. 1986. Enhanced cellobiohydrolases production from *Aspergillus ustus* and *Trichoderma harzianum*. Enzyme Microb Technol 8(3): 141-144.

MANDELS M AND WEBER J. 1969. Production of cellulases. Adv Chem Ser 95: 391-414.

MCHALE A AND COUGHLAN MP. 1981. The components of the cellulase system of *Talaromyces emersonii* with emphasis on β-glucosidase. Biochim Biophys Acta 662: 145-159.

MEIRELLES LA, SOLOMON SE, BACCI MJR, WRIGHT AM, MUELLER UG AND RODRIGUES A. 2015. Shared Escovopsis parasites between leaf-cutting and non-leaf-cutting ants in the higher-attine fungus-growing ant symbiosis. R Soc Open Sci 2(9): 150257.

MOLONEY AP, CONSIDINE PJ AND COUGHLAN MP. 1983. Cellulose hydrolysis by the cellulases produced by *Talaromyces emersonii* when grown on different inducing substrates. Biotechnol Bioeng 25(4): 1169-1173.

MURRAY P, ARO N, COLLINS C, GRASSICK A, PENTTILA M, SALOHEIMO M AND TUOHY M. 2004. Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. Protein Expr Purif 38: 248-257.

OGÓREK R, DYLĄG M AND KOZAK B. 2016. Dark stains on rock surfaces in Driny Cave (Little Carpathian Mountains, Slovakia). Extremophiles 20(5): 641-652.

PAULA CCP, MONTOYA QV, RODRIGUES A, BICHUETTE ME AND SELEGHIM MHR. 2016. Terrestrial filamentous fungi from Gruta do Catão (São Desidério, Bahia, Northeastern Brazil) show high levels of cellulose degradation. J Caves Karst Stud 78(3): 208-217.

PIROTA RDPB, FARINAS CS AND BALEEIRO FCF. 2013. Saccharification of biomass using whole soild-state fermentation medium to avoid additional separation steps. Biotechnol Prog 29(6): 1430-1440.

QAISAR S, ZOHRA RR, AMAN A AND UL QADER SA. 2014. Enhanced production of cellulose degrading CMCase by newly isolated strain of *Aspergillus versicolor*. Carbohydr Polym 104: 199-203.

QU Y, ZHU M, LIU K, BAO X AND LIN J. 2006. Studies on cellulosic ethanol production for sustainable supply of liquid fuel in China. Biotechnol J 1: 1235-1240.

RAUTELA R, RAWAT S, RAWAT R, VERMA P AND BHATT AB. 2017. Microbial diversity of Gumki cave and their potential role in enzyme production. Environment Conservation Journal 18(3): 115-122.

RUEGGER MJ AND TAUK-TORNISIELO SM. 2004. Atividade da celulase de fungos isolados do solo da Estação Ecológica de Juréia-Itatins, São Paulo, Brasil. Rev Bras Bot 27(2): 205-211.

SALEEM A, EL-SAID AHM, MOHARRAM AM AND ABDELNASER EG. 2013. Cellulolytic activity of fungi isolated from anise and cumin spices and potential of their oils as antifungal agents. J Med Plants Res 7(17): 1169-1181.

SAMSON RA, SEIFERT KA, KUIJPERS AFA, HOUBRAKEN JAMP AND FRISVAD JC. 2004. Phylogenetic analysis of *Penicillium subgenus Penicillium* using partial β-tubulin sequences. Stud Mycol 48: 175-200.

SAQIB AA, HASSAN M, KHAN NF AND BAIG S. 2010. Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). Process Biochem 45(5): 641-646.

SCHMID G AND WANDREY C. 1987. Purification and partial characterization of a cellodextrin glucohydrolase (β-glucosidase) from *Trichoderma reesei* strain QM 9414. Biotechnol Bioeng 30(4): 571-585.

SHAMALA TR AND SREEKANTIAH KR. 1986. Production of cellulases and D-xylanase by some selected fungal isolates. Enzyme Microb Technol 8: 178-182.

SHEN X AND XIA L. 2006. Lactic acid production from cellulosic material by synergistic hydrolisis and fermentation. Appl Biochem Biotechnol 133: 252-262.

SINGHANIA RR, SUKUMARAN RK AND PATEL AK. 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb Technol 46: 541-549.

SINGHVI MS, ADSUL MG AND GOKHALE DV. 2011. Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 nad its application. Bioresour Technol 102: 6569-6572.

SOMERA AF, PEREIRA MG, GUIMARÃES LHS, POLIZELI MDTD, TERENZI HF, FURRIEL RPM AND JORGE JA. 2009. Effect of glycosylation on the biochemical properties of β-xylosidases from *Aspergillus versicolor*. J Microbiol 47(3): 270-276.

SORENSEN A, LÜBECK M, LÜBECK PS AND AHRING BK. 2013. Fungal β-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomolecules 3: 612-631.

TAMURA K, PETERSON D, PETERSON N, STECHER G, NEI M AND KUMAR S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

ZHANG YHP AND LYND LR. 2004. Toward an aggregated understanding of enzymatic hydrolisys of cellulose: Noncomplexed cellulase systems. Biotechnol Bioeng 88: 797-824.

SUPPLEMENTARY MATERIAL

Figure S1 - The neighbor-joining tree obtained based on phylogenetic analysis of β-tubulin sequence data. *Talaromyces convolutus* was chosen as out-group. Numbers above branches are bootstrap values and in parentheses are the accession numbers in GenBank. Only values above 50% are indicated.[T] = ex type.