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Bioprospecting and enzymatic potential of filamentous fungi from the Bela Vista Biological Refuge in Itaipu, Brazil

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ABSTRACT. Disordered anthropic action causes relevant impacts on different ecosystems. This may endanger key species and the compounds they produce, which have potential for commercial development. We collected environmental samples from the Bela Vista Biological Refuge, belonging to ITAIPU/Brazil, over a period of three years. A total of 181 fungal species were isolated and evaluated for cellulases and xylanases, 74% of which were classified as good enzymatic producers, with a production of up to 50 U/mL of xylanase and 7 U/mL for cellulase. A total of 34 isolates were selected and identified by amplification of internal transcribed spacer regions and then analyzed in BLASTn with 89-99% similarity/identity with others deposited in GenBank; the genera found were *Aspergillus*, *Penicillium*, *Chaetomium*, *Clonostachys*, *Fusarium*, *Hypocrea*, *Paecilomyces*, *Thermoascus*, *Thermomyces*, and *Trichoderma*. The enzymatic data reveals details of the roles of this biological community. The ability of these fungal species to utilize plant cell wall compounds discovered based on bioprospecting analysis of this biome is a pioneering study for this purpose in this region and points out important microorganisms that have potential for enzymatic production in biological biomass depolymerization, resulting in biotechnologically useful products.

Key words: Biomass depolymerization; Internal Transcribed Spacer; Xylanolytic enzymes

INTRODUCTION

Fungi are considered key organisms in environments of high diversity as they are osmotrophic, feeding by secreting macromolecules into the extracellular environment, resulting in metabolites that are moved into the cell, thus presenting a great ability to adapt to various environmental conditions (Richards and Talbot, 2013). The global increase in energy consumption worldwide and the actual estimated depletion of fossil fuel reserves in the near future, coupled with the depletion of ecosystems by anthropogenic action, has lead to a search for knowledge of new resources for alternative fuels that target different biotechnological applications, such as enzymatic hydrolysis by environmental fungi (Arora et al., 2015). Some fungi, such as *Pseudocercospora spp.*, have been isolated from various different biomes, such as the semi-arid cerrado (Furlanetto and Dianese, 1999) and the Atlantic forest (Parreira et al., 2014). Although this genus has a wide distribution, it is quite genetically diverse, particularly in tropical and subtropical environments (Silva et al., 2016).

Phylogenetic analysis through the production of phylogenetic trees allows us to know the similarity of a given population of individuals and their possible ancestral relationships (Ney and Kumar, 2000; Felsenstein, 2004; Hall, 2011), which is currently being constructed by molecular data analysis, DNA sequences, or proteins. Environmental fungal diversity patterns are being more commonly studied and described using nucleotide sequences generated by next generation DNA sequencing, which produces a large number of extensive sequences that can be identified by automated bioinformatics tools (Caporaso et al., 2010).

In bioinformatics analyses, the nucleotide sequences are filtered and processed, and the Operational Taxonomic Units (OTUs) are collected using reference-based approaches. The taxonomy is attributed to representative OTU sequences and an array is created when there is an abundance of OTUs. In the sequence, OTU sequences are grouped together, and a taxonomy is assigned to the clusters obtained (Lindahl et al., 2013; Hart et al., 2015). The taxonomy attribution is particularly sensitive to the qualitative properties of the reference sequence affecting the amplitude of taxa, with sufficient information for inferences regarding intra- and inter-specific genetic variations.

The purpose of this study was to isolate fungi from various sources of native forest biomass for their application in future biotechnological processes. The microorganisms were isolated by conventional microbiological methods and submitted to molecular identification, such as amplification of ITS1-4/rRNA genes by PCR, followed by DNA sequencing by the Sanger et al. (1977) method.

MATERIAL AND METHODS

Study area, collection and sample processing

The fungi species used in this study are data from a network project called Sisbiota, approved by the National Council for Scientific and Technological Development (CNPQ), 563260/2010-6-Brazil. Samples of litterfall, soil and water were collected on an Atlantic

Forest biome trail, located at the Bela Vista Biological Refuge, Foz do Iguaçu, Paraná, Brazil, at the coordinates 25°26'41.90" S, 54°33'06.09" W, with an elevation of 750 m (Figure 1). Litterfall samples were collected and deposited in plastic bags and water in sterile glass bottles, later taken to the laboratory for separation (leaves, stems, seeds and root) and cleaning to reduce bacterial load by contact with a 5% hypochlorite solution followed by distilled water. These components were then placed on a Petri dish containing potato dextrose agar (BDA) plus chloramphenicol (50 mg/L) to prevent bacterial and yeast growth. Later the plates with samples in duplicates were divided at temperatures of 28 and 46°C (microbiological greenhouse) for selection of mesophilic and thermophilic fungi.

The isolation of fungi from soil and water samples was performed by surface spreading directly on BDA solid medium according to the methodology described by Warcup (1950). All incubated samples were monitored daily for mycelial growth until obtaining isolated colonies. All strains isolated from these samples were transferred to glass tubes containing slanted BDA solid medium and refrigerated at 10°C.

Determination of enzymatic index values

To examine the enzyme production capacity of the xylanolytic complex, the isolated fungi were inoculated at the center of a Petri dish containing modified M5 solid medium (0.05% peptone, 0.1% yeast extract, 0.5% NaCl, 0.1% CaCl, 0.5% Xylan, 1.5% Agar), supplemented with 1% (w/v) beechwood xylan for xylanase activity analysis and cellulase in a Luria-Bertani LB medium plus 1% carboxymethylcellulose (w/v).

The fungi were grown for 24-72 h at 28 or 46°C and then incubated at 50°C for 16 h. The development of a halo was performed with 0.1% Congo red solution in 0.1 M Tris buffer, pH 8, for 30 min, washed with 1 M saline solution for 15 min (Neiroti and Azevedo 1988; Ruegger and Tauk-Tornisielo, 2004), allowing the visualization of hydrolysis halos of the substrate, and the diameter of the halo was measured with the aid of a pachymeter.

Enzymatic determination for cellulase and xylanase

Filamentous fungi were incubated with shaking at their respective isolation temperatures in Czapeck liquid mineral medium (Wiseman, 1975), the inoculum was prepared for a suspension of 10^5 spores / mL in 25 mL of medium supplemented with 1% glucose. The cultures were filtered and stored in sterile flasks with the aid of a Büchner funnel vacuum pump to obtain the crude enzyme extract.

Crude extracts were quantified for xylanase and cellulase enzymatic activities using standard beechwood xylane and carboxymethylcellulose (CMCase) substrates, respectively. Both enzymes were quantified by the formation of reducing sugars in the reaction medium containing 1% substrate in pH 6 sodium citrate buffer according to the 3,5-dinitrosalicylic acid - DNS method (Miller 1959). For the determination of the enzymatic activity, the reaction was carried out in a solution of 500 µL of enzyme from the crude extract and 500 µL of the substrate (sodium carboxymethylcellulose or beechwood xylan), and then incubated at 40°C for 30 min. Successive aliquots were taken (125 µL) at 10 min intervals and added to tubes containing 125 µL of the DNS solution. The tubes were then heated for 5 min in a water bath, added 1 mL of distilled water added, then read in a spectrophotometer at 540 nm, where the values obtained were compared to a standard

glucose curve (for cellulase calculation) or xylose curve (for xylanase determination). The enzyme activity unit in both enzymes was defined as the concentration of micromoles of reducing sugars (glucose or xylose) released per 1 mL in 1 min reaction (U mL-1) under the assay conditions.

Culture conditions and extraction of genomic DNA

The fungi were grown under suitable conditions in test tubes with BDA medium under defined conditions in isolation and then inoculated into 250 mL flask flasks in cultures containing 25 mL Czapek liquid medium (Wiseman 1975) supplemented with 1% glucose for 24-48 h or until obtaining apparent mycelial mass; the cultures were then vacuum-filtered for extraction of the genomic DNA. The mycelial mass obtained from the cultures was transferred to 1.5 mL conical tubes, an estimated amount of 200 uL or 0.2 g with 600 uL of the extraction solution (EDTA 0.05 M; 1% SDS) heated at 65°C for 20 min. They were the centrifuged at 12,000 rpm for 10 min at room temperature. The upper phase was transferred to a new tube and treated with 50 µL of RNase 20 mg/mL for 60 min at 37°C. Then 300 µL of chloroform: isoamyl alcohol (24:1) was added and mixed by manual inversion, with centrifugation at 12,000 rpm for 10 min at room temperature. The upper layer was recovered and precipitated with 40 µL 5 M potassium acetate, pH 4.8, and after mixing, packed on ice for 30 min. The sample was centrifuged again at 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube and precipitated with 1 mL of absolute ethanol, followed by centrifugation and re-insertion of 70% ethanol with centrifugation at 1,2000 rpm at 4°C for 5 min.

Again, absolute ethanol was added, followed by 70% ethanol with the above-mentioned centrifugation between one and the other step. Subsequently, the ethanol was discarded, and the tube was dried at room temperature. Finally, the DNA precipitate was resuspended in 50 µL TE/Rnase. The theoretical extraction yield was obtained in a spectrophotometer at 260 nm.

Amplification of rDNA ITS 1 and 4 regions

For the identification of the fungal isolates, the ITS 1 and 4 regions of the ribosomal DNA (rDNA) were analyzed (White et al., 1990). The PCR reaction comprised a mixture of genomic DNA [100-200 ng]; Promega® buffer [1x]; MgCl 2 [1.5 mM]; ITS 1 and 4 primer [1 mM]; dNTP [2.5 mM]; Promega® Taq DNA polymerase [0.5 U], and distilled water to a final volume of 25 µL.

The amplification of the genomic DNA samples was performed in a thermocycler starting at 95°C for 2 min, followed by 35 cycles of 94°C for 45 s of denaturation; 50-60°C for 1 min of annealing; and 72°C for 45 s, with inactivation at 72°C for 5 min. Visualization of genomic DNA and PCR-amplified fragments were on agarose gel in 1X TAE buffer, stained with ethidium bromide. The amplified DNA fragments were purified using the Purelink PCF Purification Kit (Invitrogen®) and prepared for sequencing with the Big Dye Terminator 3.1 Kit (Applied Biosystems®). Sequencing was performed by the DNA Sequencing Service of the Institute of Chemistry of the University of São Paulo, SP, Brazil.

Analysis of gene sequences

Genetic sequences from the amplification of the ITS1-5.8S-ITS4 ribosomal DNA regions were analyzed in the NCBI using the BLASTn tool, in order to identify the strains at the genus and species levels.

Phylogenetic trees

The DNA sequences were appropriately aligned in the MAFFT (Multiple Alignment using Fast Fourier Transform) program. Following that, the phylogenetic trees were estimated by the Neighbor Joining method of the Mega 7.0 program.

RESULTS

Study area, collection and sample processing

The Atlantic Forest biome, located at the Bela Vista Biological Refuge, Foz do Iguaçu, Paraná, Brazil, located at coordinates 25°26'41.90" S, 54°33'06.09" W, with an elevation of 750 m (Figure 1). It is a forest remnant that has been extensively damaged by anthropic action. The loss of species is advancing year after year (Valencia and Chambergo 2013). This study included the isolation of filamentous fungal species with potential of applicability in biotechnological processes on plant biomass through the investigation of xylanolytic complex enzymes. In the period from 2011 to 2013, four collections were carried out in different weather stations in this Brazilian biome, which allowed the obtaining of 181 isolates. The viable fungal spores that gave rise to the isolates came from different environmental samples of this biome (Figure 2), the stem and soil standing out, with 26 and 27%, respectively.

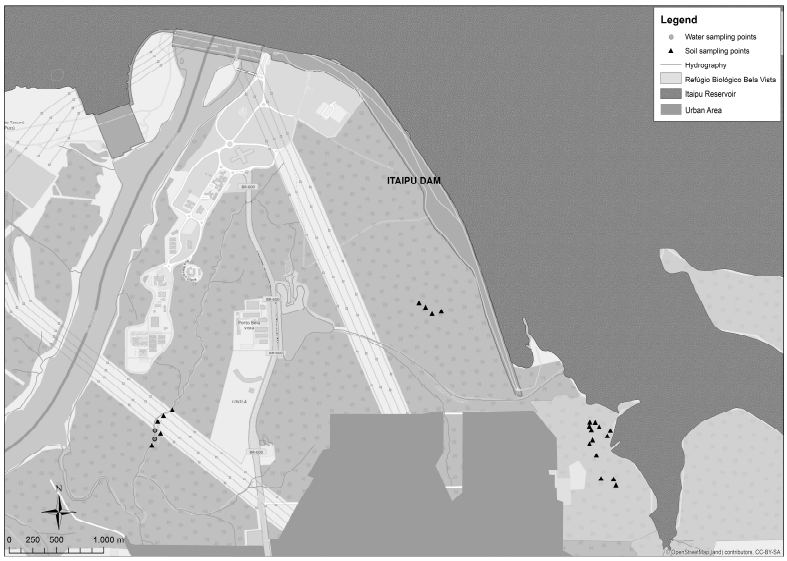

Figure 1: Origin of the isolation of filamentous fungi by type of environmental sample.

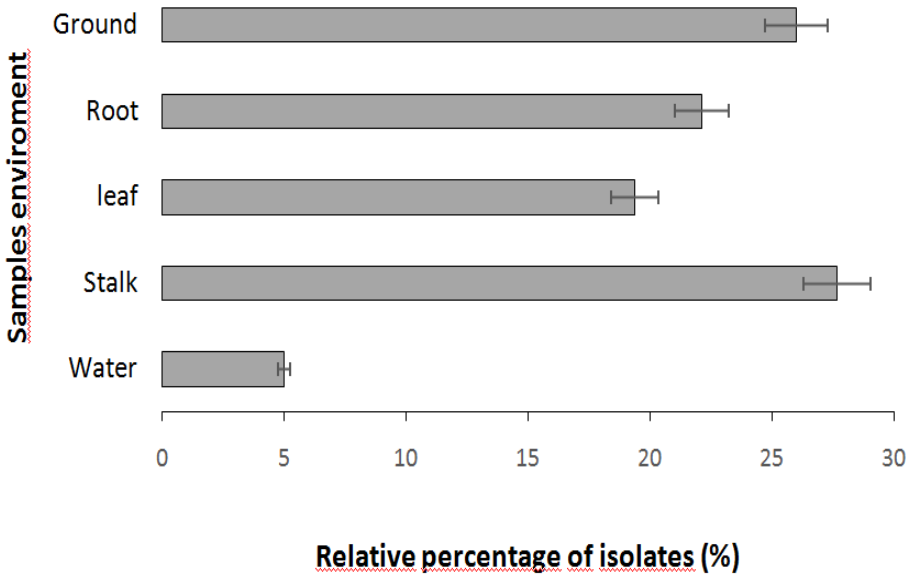

Figure 2. Origin of the isolation of filamentous fungi by type of environmental sample.

Species isolation was variable according to the collection season, with 64 species in winter, 37 species in spring, 48 species in autumn, and 32 species in summer (unpublished data). The enzymatic production capacity (xylanase and cellulase) was verified by means of individual tests of the isolates in a solid medium with halo hydrolysis development, characterized by a clear zone around the colony (Figure 3B), which allowed the determination of the enzymatic qualitative index of the strains. The occurrence of the two enzymes were observed in 134 isolates and in 47 the complete absence of activity due to the absence of a halo in the two tested situations (figure 3A). The enzymatic quantification in substrates for determination of the cellulase and xylanase activities of the fungi that presented the largest halos in the qualitative test are shown in Figures 4A, 4B and 4C. In general, xylanase activity was higher in all 34 species when cellulase activities that were below 7 U / mL were compared.

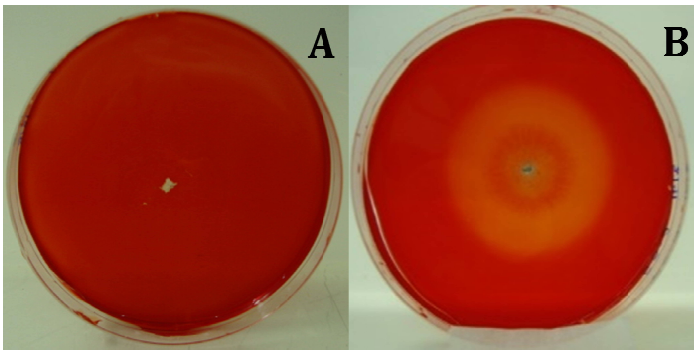

Figure 3. Enzymatic index test. Legend: A = Negative test; B = Positive test.

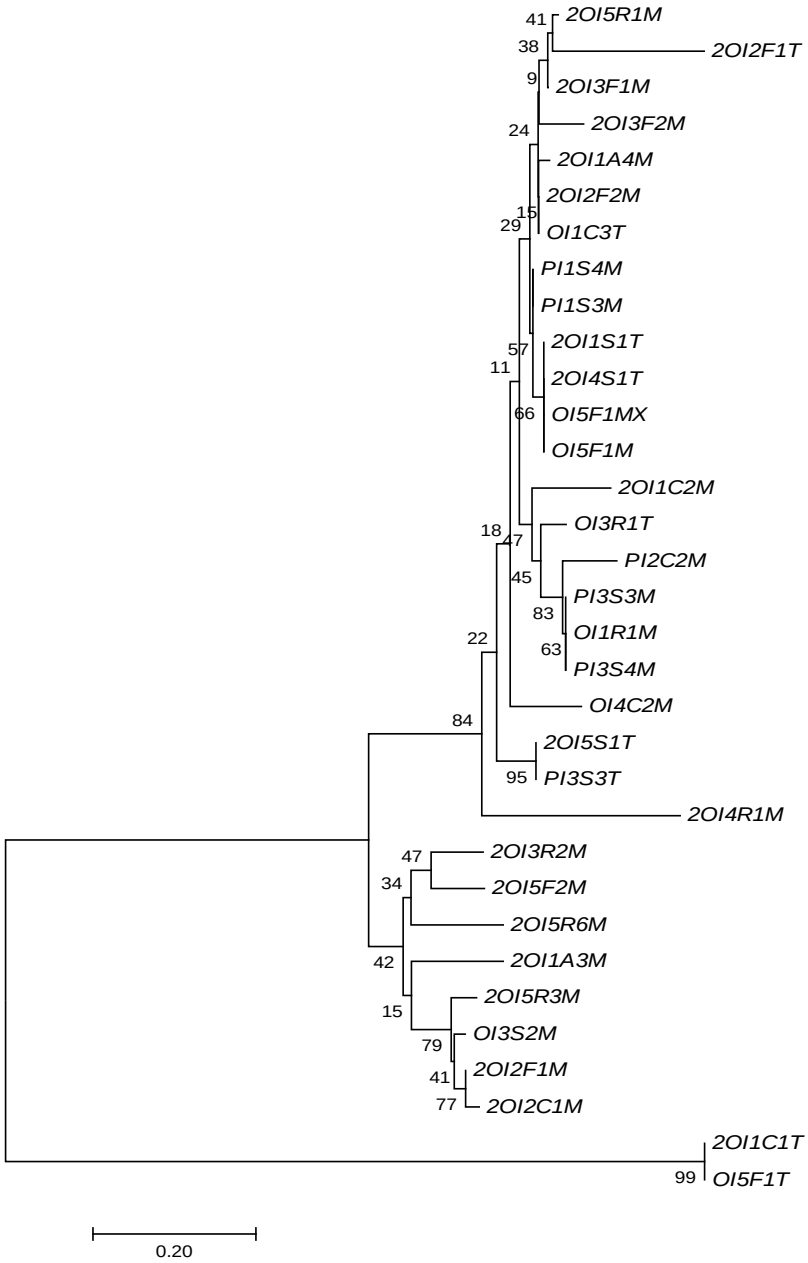

Figure 4. List of evolutionary rates of the fungal community belonging to the Atlantic Forest Biome of the Bela Vista Biological Refuge, Paraná, Brazil.

A. fumigatus KM382061 exhibited 50 U.mL^{-1} of xylanase (Fig. 5A). P. crysogenum KY549809 62 U.mL^{-1} showed a higher xylanase activity than Trichoderma virens KM516021 with 27 U.mL^{-1} (Fig 5B).

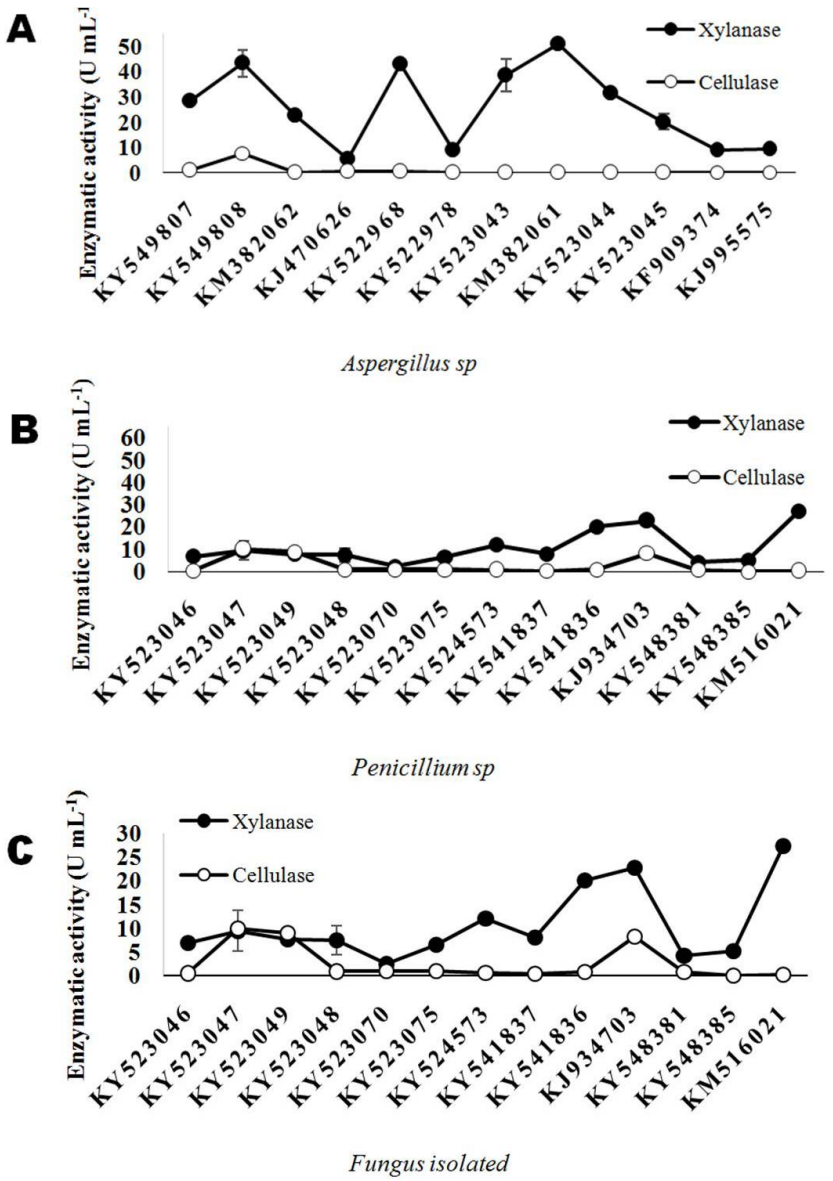

Figure 5: Quantitative enzymatic determination for xylanase and cellulase of Atlantic forest biome isolates. A- Isolated fungal group Aspergillus sp. B- Isolates fungal group Penicillium sp. C- Other isolated group.

Culture conditions and extraction of genomic DNA

Among the isolated species, 34 were selected for molecular identification, as they presented desirable colony and hydrolytic capacity characteristics. In these isolates, genomic DNA extraction and subsequent amplification at temperatures of 50-60°C with ribosomal ITS 1 and 4 primers were performed, and amplification resulted in inserts of 520-

1,007 bp (Table 1). The sequences were purified and prepared for sequencing, which generated identified 13 fungi of the genus Aspergillus, 9 Penicillium, 3 Trichoderma and Thermoascus, 2 Fusarium, and 4 of miscellaneous genera (Table 1). When analyzing the sequences of the isolates in NCBI by means of BLASTn, the similarity was 88-99%. All study sequences were deposited in the same database under individualized codes (Table 1).

Table 1: Data on the identified fungi of the Atlantic Forest Biome.

Acess NCBI	Identification of isolate	Isolated source	Growth condition	Size (pb)	Homology (%)	Enzymatic determination					
						Xylanase			Cellulase		
						Ø C	Ø H	Ie	Ø C	Ø H	Ie
KY549807	*Aspegillusflavus*	caule	mesophile	570	99	3,9	4,95	1,27	2,75	3,4	1,24
KY549808	*Aspegillusflavus*	ground	mesophile	567	99	1,95	4,5	2,31	2,8	4	1,43
KM382062	*Aspergillusaculeatus*	stalk	mesophile	466	99	1,15	1,9	1,65	1,35	2,75	2,04
KJ470626	*Aspergillusflavus*	root	mesophile	529	98	2,4	4,55	1,9	1,2	2,75	2,29
KY522968	*Aspergillusfumigatus*	stalk	termophilic	570	99	0,7	-	-	1	3	3
KY522978	*Aspergillusfumigatus*	ground	termophilic	670	99	2,6	4	1,53	4	4,5	1,05
KY523043	*Aspergillusfumigatus*	ground	termophilic	573	98	2	5	2,5	2	3	1,5
KM382061	*Aspergillusfumigatus*	leaf	mesophile	521	98	2,95	3,2	1,1	1,1	1,3	1,18
KY523044	*Aspergillusfumigatus*	leaf	termophilic	840	91	0,35	1,3	3,75	1,75	2,15	1,23
KY523045	*Aspergillusfumigatus*	ground	mesophile	978	89	1,6	3,45	2,16	1,85	-	-
KF909374	*Aspergillusniger*	root	termophilic	533	99	1,25	2,45	1,96	1,3	2,3	1,77
KJ995575	*Aspergillustamarii*	ground	mesophile	543	98	2,75	3,8	1,38	2,1	3,5	1,67
KY523046	*Chaetomiumglobosum*	water	mesophile	547	99	2,7	3,5	1,29	2	-	-
KY523047	*Clonostachysrosea*	water	mesophile	748	98	2,6	3,9	1,5	0,8	1,9	2,37
KY523049	*Fusariumdecemcellulare*	root	mesophile	546	98	1,9	3,6	1,89	1,9	-	-
KY523048	*Fusariumequiseti*	leaf	mesophile	520	98	2,2	4,4	2	4,3	4,5	1,05
KY523070	*Hypocrealixii*	leaf	mesophile	574	96	3,1	-	-	1,85	3,75	2,03
KY523075	*Paecilomycessp*	stalk	mesophile	534	96	5,6	-	-	2,8	3,1	1,1
KU560628	*Penicilliumbrefeldianum*	ground	mesophile	530	99	2,95	3,55	1,2	2,45	-	-
KY524459	*Penicilliumchrysogenum*	leaf	termophilic	565	88	3,4	6,4	1,88	0,9	1,5	1,66
KY549809	*Penicilliumchrysogenum*	leaf	mesophile	683	97	2	4,6	2,3	2	6,1	3,05
KY524484	*Penicilliumchrysogenum*	root	mesophile	635	90	3,8	4,6	1,21	2,2	-	-
KY524460	*Penicilliumchrysogenum*	leaf	mesophile	760	98	3,4	3,7	1,08	2,5	-	-
KY524485	*Penicilliumcommune*	water	mesophile	559	99	2,2	-	-	0,9	2,2	2,44
KY549810	*Penicilliumcommune*	leaf	mesophile	1007	97	3,1	5,5	1,77	2,5	2,6	1,04
KY524487	*Penicilliumcommune*	root	mesophile	577	88	5,8	6,4	1,1	3,3	-	-
KM065878	*Penicilliumcrustosum*	stalk	termophilic	543	98	2,25	4,35	1,93	0,45	0,65	1,44
KY524573	*Purpureocilliumlilacinum*	root	mesophile	563	98	1,9	2,6	1,36	1,4	1,9	1,35
KY541837	*Thermoascusaurantiacus*	ground	termophilic	726	99	1,5	4	2,66	-	-	-
KY541836	*Thermoascusaurantiacus*	ground	termophilic	768	99	6,9	7	1,01	6,1	6,3	1,01
KJ934703	*Thermomyceslanuginosus*	leaf	mesophile	563	99	2,95	3,2	1,1	1,1	1,3	1,18
KY548381	*Trichodermaharzianum*	stalk	mesophile	599	99	3	4	1,33	3,1	5,8	1,87
KY548385	*Trichodermalongibrachiatum*	root	mesophile	613	98	1,8	2,9	1,61	3,9	4,7	1,2
KM516021	*Trichodermavirens*	ground	mesophile	559	98	3,6	3,8	1,05	2,6	-	-

Legend: ØD = Colony diameter (centimeters); ØH = Diameter of the halo (centimeters); Ie = Enzymatic index result of the ratio between ØD / ØH; (-) = Activity not detected based on absence of a halo.

Analysis of gene sequences

The sequences were submitted to a phylogenetic analysis to examine evolutionary history by inference using the Neighbor-Joining method (Saitou and Nei, 1987). The ideal tree with the sum of the branch length = 3.00864461 is shown in Figure 4. The percentage of replicate trees in which the associated taxa grouped in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branching lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were calculated using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in units of the number of amino acid substitutions per site. The analysis involved 34 amino acid sequences. The coding data were translated assuming a standard genetic code table. All positions with gaps and missing data

were deleted. There were 74 positions in the final data set. Evolutionary analyses were performed on MEGA® 7.0 (Kumar et al., 2016).

DISCUSSION

We found various fungi with relevant enzymatic production indices for xylanase and cellulase enzymes. The review of Valencia and Chambergo (2013) includes a systematic and organized survey of studies reporting the biodiversity of Brazil; the native fungi obtained through collections from different regions of the country showed important characteristics for efficient hydrolysis of biomass with potential biotechnological applications.

The isolation of strains with the purpose of exploration of the production of composites was developed by Celestino et al. (2014). They were able to isolate 50 species of fungi in the Amazon capable of producing pigments. Additionally, with different procedures for growth, with alterations in the culture medium, it was possible to produce a large number of natural pigments. As used in our project, Pandey et al. (2003) used the ITS technique to identify the endophytic fungi of trees in India, which allowed the identification of various tropical species that cause infection in the leaves and the establishment of a correlation with the genetic variation between the isolates.

The 34 fungi we identified showed considerable diversity, grouping in several clades. The identification by analysis of the greater dominance of *Aspergillus* and *Penicillium* species was made possible by the analysis of a repeated unit of ribosomal DNA, which is a useful way of examining polymorphism due to the juxtaposition of conserved and variable regions (Bruns et al., 1991). In phylogeny, these species present several clades, suggesting considerable intra-species variability. The amplification of the rRNA/ITS region as a tool for molecular identification of fungal species from various sources has been applied in regions with high biological diversity and is a useful tool for analysis of phylogenetic evolution (Xu et al., 2007).

From this perspective, some of these isolates reported have already been studied in the study of Andrades et al. (2016), who investigated the influence of alternative carbon sources as inducers of β-galactosidase and fructofuranosidase by filamentous fungi *Aspergillus aculeatus* KM382062, *Aspergillus fumigatus* KY522978 and *Trichoderma longibrachiatum* KY548385. The highest levels of intracellular β-galactosidase activity were obtained using orange peel residues (56.31 U / mL) with *A. aculeatus*, *A. fumigatus* (17.26 U / mL) and *T. longibrachiatum* (17.53 U / mL). The most effective activity of intracellular β-fructofuranosidase was obtained by *A. aculeatus* using trub (409.46 U / mL), showing that these agroindustrial substrates and fungi are excellent producers.

Another study using related isolates was conducted by Corrêa et al. (2016); *Thermomyces lanuginosus* KJ934703 exhibited high production of Beta-xylosidase, reaching production levels in corn straw of 1003 U/mL. Farnsworth et al. (2015) demonstrated the need and difficulty to determine the value of biodiversity. They argue that this must be performed through an objective method, which can be achieved by a quantification of the relationship between the ecosystem and the services they can provide, achieving a biodiversity-function relationship. The diversity and the enzyme activities of the fungi that we found has potential for future exploration in biotechnology.

CONCLUSIONS

The analysis of the biological diversity of our study area showed a richness of species that have potential for hydrolysis of plant biomass. The fungi that we identified will serve as a basis for biotechnological applications and increase knowledge regarding the filamentous fungi of a conservation area with remnants of the Atlantic Forest biome.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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