Genome sequencing and functional characterization of a *Dictyopanus pusillus* fungal extract offers a promising alternative for lignocellulose pretreatment of oil palm residues

Andrés M. Rueda\(^1\), \(^2\), \(^3\), Yossef López de los Santos\(^1\), Antony T. Vincent\(^4\), Myriam Létourneau\(^1\), Inés Hernández\(^3\), Clara I. Sánchez\(^3\), \(^5\), Daniel Molina V.\(^6\), Sonia A. Ospina\(^2\), Frédéric J. Veyrier\(^4\) & Nicolas Doucet\(^1\), \(^7\), *

\(^1\)Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique (INRS), Université du Québec, Laval, Canada. \(^2\)Instituto de Biotecnología, Universidad Nacional de Colombia, Bogotá, Colombia. \(^3\)Centro de Estudios e Investigaciones Ambientales, Universidad Industrial de Santander, Bucaramanga, Colombia. \(^4\)Bacterial Symbionts Evolution Laboratory, Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique (INRS), Université du Québec, Laval, Canada. \(^5\)Escuela de Microbiología, Universidad Industrial de Santander, Bucaramanga, Colombia. \(^6\)Escuela de Química, Universidad Industrial de Santander, Bucaramanga, Colombia. \(^7\)PROTEO, the Québec Network for Research on Protein Function, Engineering, and Applications, Université Laval, Québec, Canada.

*To whom correspondence should be addressed: Nicolas Doucet, Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique (INRS), Université du Québec, 531 Boulevard des Prairies, Laval, QC, Canada, H7V 1B7; nicolas.doucet@inrs.ca.*
Abstract

The pretreatment of biomass is a critical requirement of bio-renewable fuel production from lignocellulose. Although current processes primarily involve chemical and physical approaches, the biological breakdown of lignin using enzymes and microorganisms is quickly becoming an interesting eco-friendly alternative to classical processes. As a result, bioprospection of wild fungi from naturally occurring lignin-rich sources remains a suitable method to uncover and isolate new species exhibiting ligninolytic activity. In this study, wild species of white rot fungi were collected from Colombian forests based on their natural wood decay ability and high capacity to secrete oxidoreductases with high affinity for phenolic polymers such as lignin. Based on high activity obtained from solid-state fermentation using a lignocellulose source from oil palm as matrix, we describe the isolation and whole-genome sequencing of *Dictyopanus pusillus*, a wild basidiomycete fungus exhibiting ABTS oxidation as an indication of laccase activity. Functional characterization of a crude enzymatic extract identified laccase activity as the main enzymatic contributor to fungal extracts, an observation supported by the identification of 13 putative genes encoding for homologous laccases in the genome. To the best of our knowledge, this represents the first report of an enzymatic extract exhibiting laccase activity in the *Dictyopanus* genera, offering means to exploit this species and its enzymes for the delignification process of lignocellulosic by-products from oil palm.

**Keywords:** Lignocellulose, *Dictyopanus*, Laccase, Oil palm, Pretreatment, Biomass, Bioprocesses, Fungi.
1. Introduction

The accumulation of agro-industry lignocellulosic postharvest by-products is a direct consequence of the global demand for crops employed in the food supply chain and bio-renewable fuel production. Following this trend, global palm oil production has increased 43% over the past 10 years to reach 52 million tons in 2015, primarily due to high biodiesel demand (1). As a result, the product-to-waste ratio for palm oil production remains significantly high (1:3), generating important lignocellulosic biomass accumulation (2). This represents a particularly pressing environmental issue for the largest producing countries such as Malaysia and Indonesia. One alternative to overcome the significant build-up of cellulosic biomass is the production of bioethanol by fermentation of syrups extracted from cellulose and hemicellulose hydrolysis. Lignocellulosic ethanol production is an eco-friendly alternative to current agro-industry by-products, in addition to offering an important source of renewable energy (3).

Lignocellulose is a raw material composed of lignin, cellulose, and hemicellulose, forming a complex aromatic polymer that provides rigidity and strength to plant cell walls. While cellulose represents an inestimable carbon energy source on a global scale, releasing cellulose from lignocellulose by lignin removal represents a major challenge in many industrial processes, including the bioethanol and pulp and paper industries (4-6). To this day, delignification is either performed by chemical strategies using environmentally damaging acids or alkaline solutions, and/or through physical processes such as high temperature and pressure conditions (7). A biological delignification process using ligninolytic enzymes that breakdown lignin through an oxidation mechanism would therefore offer a valuable alternative for the pretreatment of lignocellulose (8). Laccases (EC 1.10.3.2), manganese peroxidases (EC 1.11.1.13), and lignin peroxidases (EC 1.11.1.14) are the most promising ligninolytic catalysts for such biological pretreatment. These enzymes are primarily expressed and secreted from basidiomycete fungi, especially the *Agaricomycetes* class (9). Fungi are the main organisms associated to wood decay colonization due to their ability to secrete oxidoreductases and their high affinity for phenolic polymers such as lignin. Studies on fungi lignocellulose decomposition have thus demonstrated that species involved in wood decay produce a pool of many enzymes acting against the three primary lignocellulose components (10,11).
It has been established that co-evolution between white-rot fungi and angiosperms favored the specialization of ligninolytic enzymes to degrade lignin and a broad range of compounds derived from wood decay, turning these organisms into valuable biotechnological tools (12,13). Fungi enzymatic extracts exhibiting ligninolytic activities are thus currently positioned as a promising biotechnological tool for the management of recalcitrant pollutants such as dyes, pesticides, phenolic compounds, and agro-industry residues (14,15). Nevertheless, fungus-based lignocellulosic pretreatment processes for industrial applications is still hampered by the difficulty to produce large amounts of highly active enzymes. Luckily, these problems can partly be overcome by the use of recombinant organisms and/or screening of species with enhanced enzymatic ability (16,17). Additionally, new sequencing techniques used in combination with fungi bioprospecting can improve our understanding of the enzymatic delignification process performed by fungi during lignocellulose recycling. Such knowledge can then serve as basis to develop biotechnological alternatives to handle lignocellulosic residues from agro-industry, potentially leading to new developments in the production of bioethanol and/or organic compounds (18-20).

Herein, we describe the isolation, whole-genome sequencing of *D. pusillus*, and initial characterization of wild basidiomycete enzymatic extracts exhibiting ABTS oxidation as an indication of laccase activity. To shed light on potential enzymes involved in this ligninolytic activity, the genome of *D. pusillus* was sequenced using single-molecule real-time sequencing technology, *de novo* assembled, and annotated. Our main goal was to identify new fungal enzymatic tools capable of sustaining harsh experimental conditions for extended periods of time, such as higher temperatures and lower pH, while favoring an increase in the release of reducing sugars during simultaneous pretreatment and saccharification (SPS) processes of empty fruit bunch from oil palm trees. We found that laccase activity was the main enzymatic contributor to our fungal extracts, which included a highly active isolate from *D. pusillus* LMB4. In addition to characterizing potentially valuable biotechnological tools for the enzymatic lignocellulose pretreatment of palm tree residues, our results also present the first complete genome sequencing of a *Dictyopanus* fungus.

2. Materials and Methods
2.1. Fungi isolation and growth conditions. Fruit bodies from basidiomycete fungi growing on decaying wood were collected in a tropical humid forest in Colombia, following previously published parameters to favor the presence of delignification enzymes (21,22). The main inclusion criteria were macroscopic properties belonging to the orders of Agaricales, Russulales, and Polyporales due to the possible ligninolytic activity of these organisms (23,24). Collected samples were kept in wax paper bags to prevent deterioration. Isolation of the collected fungi was performed in wheat bran extract agar composed of 18 g.L⁻¹ agar, 10 g.L⁻¹ glucose, 5 g.L⁻¹ peptone, 2 g.L⁻¹ yeast extract, 0.1 g.L⁻¹ KH₂PO₄, 0.1 g.L⁻¹ MgSO₄.7H₂O, 0.085 g.L⁻¹ MnSO₄, 1000 mL wheat bran extract, 0.1 g.L⁻¹ chloramphenicol, 0.1 g.L⁻¹ and 600 U.L⁻¹ nystatin. Pilei were adhered to the top cover of Petri dishes, allowing spores to fall and, eventually, to germinate on the culture media. Top covers were rotated every 24 h for 3 days and those containing the pilei were replaced by new sterilized ones (25). Sub-cultures in the same media were incubated at 25°C to obtain axenic strains from these isolates. The axenic cultures were determined by fungal slide culture technique (26). The presence of microscopic sexual basidiomycete properties was checked, including septate hyaline hyphae and clamps. Lactophenol cotton blue stain was used for all the microscopic observations. Twelve ligninolytic fungi belonging to genera Aleurodiscus, Dictyopanus, Hyphodontia, Mycoacia, Phellinus, Pleurotus, Stereum, Trametes, and Tyromyces were axenically isolated from 43 collected wild-type strains. Fungi collection was planned under the regulations of Colombia’s Environmental Ministry. The research permit in biological biodiversity was obtained from the Corporación Autónoma de Santander (file number 153-12 REB) and with the agreement of the Ministerio del Interior, certifying the absence of ethnic groups in the area (application number 1648, August 14, 2012).

2.2. Phylogenetical identification of selected isolates. Total genomic DNA was extracted from selected isolates following a standard phenol-chloroform protocol. Briefly, fungi were grown in wheat bran extract agar for 15 days and 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M NaCl₂, Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads. The aqueous fraction was collected, and the fungus DNA was precipitated with isopropanol. The DNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) (27). A pair of primers within the Internal Transcribed Spacer regions (ITS1/ITS4) was
used to amplify ribosomal DNA by PCR (28). PCR products were sequenced by the Sanger method using
the same amplification primers. ITS1 sequences were used as query to retrieve the most similar DNA
sequences from the NCBI database. A set of 36 curated sequences were extracted from the results
obtained through BLAST, after which the ITS1 sequences and the query sequence were used to create a
multiple sequence alignment. To infer the evolutionary history and obtain the genetic identity of the
fungus isolated and pre-identified as *Dictyopanus* sp., we applied the UPGMA protocol, where the best
tree hits arose after a bootstrap of 500 repetitions using the Maximum Composite Likelihood method to
obtain the evolutionary distances. All phylogenetic analysis were performed with the MEGA suite, version
10.0.5 (29).

2.3. Fiber analysis of oil palm by-products. Neutral Detergent Fiber (NDF), Acid Detergent
Fiber (ADF), and Acid Detergent Lignin (ADL) were determined by the Van Soest method using the
FiberCap™ system (Foss Analytical AB, Denmark). Cellulose and hemicellululose percentages were
estimated as the difference between ADF and ADL, and NDF and ADF respectively, while lignin
concentrations corresponded to ADL percentages in dry weight of oil palm by-products. Additionally,
values were used to estimate the total carbon concentration in fermentation assays. All assays were
performed in duplicate.

2.4. Basidiomycete screening by solid-state fermentation (SSF). The main selection criterion
of isolated wild-type fungi was ligninolytic activity observed in the crude extracts from SSF using
lignocellulosic material from oil palm by-products (30). SSF was performed in 250 ml flasks in sterile
conditions. Each flask contained 12 ml of basal media in deionized water, comprising 0.2 g.L⁻¹ yeast
extract, 0.76 g.L⁻¹ peptone, 0.3 g.L⁻¹ urea, 0.25 g.L⁻¹ CuSO₄·5H₂O, 1.4 g.L⁻¹ (NH₄)₂SO₄, 2 g.L⁻¹ KH₂PO₄, 0.3
g.L⁻¹ MgSO₄·7H₂O, 0.4 g.L⁻¹ CaCl₂·2H₂O, 0.005 g.L⁻¹ FeSO₄·7H₂O, 0.0016 g.L⁻¹ MnSO₄, 0.0037 g.L⁻¹
ZnSO₄·7H₂O, 0.0037 g.L⁻¹ CoCl₂·6H₂O, and 2.5 g.L⁻¹ of empty fruit bunch (EFB) chopped into chunks of
approximately 2 cm³. Each flask was inoculated with eight agar plugs cut from actively growing fungal
mycelium grown on wheat bran extract agar. Each SSF batch isolation contained thirty flasks and
fermentation was held without agitation at 25˚C for 30 days. Every three days, three flasks were used to collect crude enzymatic extracts.

2.5. Recovery of crude enzymatic extracts. Crude enzymatic extracts were obtained by addition of 30 ml of 60 mM sterile phosphate buffer into the fermentation flask, which was shaken for 24 h at 150 rpm. Whole flask contents were then collected in 50 mL tubes, vortexed in a Benchmark Scientific multi-tube vortexer for 15 minutes at 1500 rpm, and finally centrifuged twice at 8900g for 15 minutes to remove suspended solids. Supernatants were taken as crude enzymatic extracts (31) and concentrated by lyophilization to evaluate the effects of pH and temperature on enzymatic activity and the SPS.

2.6. Quantification of reducing sugars. Reducing sugars were quantified by oxidation of 3,5-dinitrosalicylic acid to 3-amino,5-nitrosalicylic acid (DNS) by measuring the release of the reducing extremity of sugars. The reaction was followed at 420 nm and a standard curve was obtained with glucose (0.1 to 1 mg.mL⁻¹) to quantify the concentration of reducing sugars (32).

2.7. Ligninolytic and cellulase assays. Crude enzymatic extracts obtained from SSF were assayed for laccase, lignin peroxidase, and manganese peroxidase activities. Laccase activity was followed by the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, USA) (33). Reactions were initiated by mixing 40 µL of culture supernatant, 150 µL of 50 mM acetate buffer (pH 4.5) and 10 µL of 1.8 mM ABTS; activity of mixtures was estimated by reading absorbance at 420 nm. Manganese peroxidase activity was measured by the formation of Mn³⁺-malonate complexes at pH 4.5 in 50 mM sodium malonate buffer containing 0.5 mM MnSO₄ (34). Reactions were performed by mixing 20 µl of culture supernatant, 100 µl of 20 mM citrate buffer at pH 4.5, 40 µl of sodium malonate buffer, and initiated with 40 µl of fresh 0.8 mM H₂O₂. Readings at 270 nm were used to estimate the transformation of Mn³⁺ to Mn²⁺ as manganese peroxidase activity. Lignin peroxidase activity was measured by the transformation of 3,4-dimethoxybenzyl alcohol (VA) (Sigma-Aldrich, USA) to veratryl aldehyde (VAD), which exhibits a yellow color (35). Reactions were performed by mixing 20 µl of culture supernatant, 100 µl of 20 mM citrate buffer at pH 3, 40 µl of 10 mM VA, and initiated with 40 µl of fresh
0.8 mM H₂O₂. Enzymatic activity was measured at 310 nm and expressed in units per liter (U.L⁻¹). One unit of enzymatic activity was defined as the quantity of enzyme needed to transform 1 µmol of substrate per minute. Absorbance readings were performed with a ThermoFisher Multiskan™ GO Microplate Spectrophotometer.

The total cellulosic activity was quantified by units of paper filter (UPF.ml⁻¹). In tubes, 500 µL of commercial cellulase solutions from *Trichoderma reesei* Sigma Aldrich C2730 Celluclast® (USA) were incubated with 500 µL of 50 mM citrate buffer at pH 4.8, 50 and 5 mg of filter paper for 1 h, at 50 °C. The concentration of reducing sugars released was measured by the oxidation of 3,5-dinitrosalicylic acid (DNS), as described above (36).

2.8. Effect of pH and temperature on ABTS oxidation as laccase activity. The effect of pH was examined for crude enzymatic extracts exhibiting the highest laccase activity. A pH range from 2 to 8 (50 mM hydrochloric acid buffer, pH 2; 50 mM citric buffer pH 3-4; 50 mM acetate buffer pH 4.5-5, and 50 mM phosphate buffer pH 6-8) was evaluated using ABTS as substrate. The effect of temperature on enzyme activity and stability was measured with crude enzymatic extracts in 50 mM acetate buffer pH 4.5 at 40°C, 50°C, and 60°C for 7 h. Finally, comparison of crude enzymatic extracts with a control laccase from *Trametes versicolor*, 53739 Sigma-Aldrich (Canada) was performed in triplicate using pH and temperature conditions exhibiting the highest activity. All components (except enzymes) were sterilized separately and mixed under environmentally sterile conditions.

2.9. Simultaneous pretreatment and saccharification of EFB. The simultaneous pretreatment was performed in 50 ml tubes containing 1.5 g EFB, 16 ml of 50 mM acetate buffer at pH 4.5 and combining either the laccase enzyme from *D. pusillus* or the commercial laccase from *T. versicolor* (53739 Sigma-Aldrich-Canada) with the cellulase from *T. reesei* (Sigma Aldrich C2730 Celluclast®). For the reaction mixture, both laccase and cellulase were added in a volume of 2 ml to reach a final concentration of 25 U*L⁻¹ and 50 UPF, respectively. Tubes were incubated at 40°C for 72 hours. The saccharification process was evaluated by the production of reducing sugars, measured by a DNS assay.
Assays were performed in triplicate and all components (except enzymes) were sterilized separately and mixed under environmentally sterile conditions.

Simultaneous pretreatment and saccharification of EFB was conducted with enzymatic extracts exhibiting laccase activity and cellulases according to a Plackett-Burman design to evaluate significant variables in the process. Five independent variables were evaluated: pH (3 to 5) using either 50 mM acetate buffer (pH 3 and 4) or 50 mM citrate buffer (pH 5), temperature (25, 35, and 45 °C), copper concentration (1, 3, and 5 mM), laccase (100, 200, and 300 U.L\(^{-1}\)), and cellulase (50, 100, 150 UPF.mL\(^{-1}\)) activities. SPS was performed in 50 ml tubes with 1.5 g EFB and 20 ml total volume, including 2 ml each of laccase enzymatic extract and cellulase concentrate. The mixture was incubated for 72 h and the concentration of reducing sugars was measured in each tube. Results represent four independent experiments and were analyzed using Statgraphics Centurion XVII.

2.10. Genome analysis of \(D.\) pusillus LMB4. Mycelium from \(D.\) pusillus grown on Potato Dextrose Agar (PDA) was used to extract the genomic DNA (gDNA) through a high salt phenol-chloroform cleanup protocol recommended by PacBio® systems. More precisely, 0.5 g of mycelium was placed in a tube with a lysis solution (0.1 M NaCl\(_2\), Tris-HCl pH 8, 5% SDS) and 0.5 mm diameter glass beads until mycelium was broken (visual evaluation) and centrifuged at 11,000 g for 10 minutes. The supernatant was mixed in the same proportion with a phenol-chloroform-isoamyl alcohol solution 25:24:1 and centrifuged at 11,000 g for 5 minutes. The new supernatant was mixed again in the same proportion with a chloroform-isoamyl alcohol solution (24:1) and centrifuged at 14,000 g for 10 minutes. Finally, the aqueous fraction was collected and fungal proteins were precipitated by adding absolute ethanol (10:3 aqueous fraction-ethanol). After centrifugation at 11,000 g for 15 minutes, the supernatant was mixed with ethanol (10:17 supernatant-ethanol) to precipitate DNA. The DNA pellet was obtained by centrifugation at 11,000 g for 15 minutes and dissolved in DEPC-treated DNase-free water. The genomic DNA of \(D.\) pusillus LMB4 was sequenced using five SMRT cells on a Pacific Biosciences RS II system at the Génome Québec Innovation Centre (McGill University, Montreal, Canada). Resulting sequencing reads were assembled de novo in contiguous sequences using Canu, version 1.7 (37). The heterozygous genome was then
reduced using Redundans, version 0.14a (38). This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession QVIE00000000.

2.11. Ligninolytic laccase annotations of the \textit{D. pusillus} genome draft. Protein encoding genes were predicted with WebAUGUSTUS (39) using \textit{Laccaria bicolor} as a training dataset. The resulting predicted gene sequences were annotated using the webserver of eggNOG-mapper (40). Each putative protein laccase sequence was submitted to the Basic Local Alignment Search Tool for proteins BLASTp tool from the database at National Center for Biotechnology Information (NCBI) server to find a correlation with other laccase enzymes reported on the Protein Data Bank server (PDB). Moreover, the four conserved copper-binding motifs, \textit{i.e.} Cu1 (HWHGFFQ), Cu2 (HSHLSTQ), Cu3 (HPFHLHG), and Cu4 (HCHIDFHL) (41), were searched into these putative protein sequences. Also, sequences corresponding to putative laccases were further analyzed using InterProScan (42) to verify the presence of multicopper oxidase signatures (PS00079 and PS00080 Prosite entries, ExPASy Bioinformatics Resource Portal) and Cu-oxidase Pfam domains (PF00394, PF07731, and PF07732 entries) (43). Comparisons with the Laccase and Multicopper Oxidase Engineering Database (44) was also used to validate that the identified sequences were laccases.

3. Results and Discussion

3.1. Fungi isolation. From all fruit bodies collected, twelve axenic cultures were obtained and thirty one isolations exhibited fungal contamination from biota mycoparasitism associated to basidiomycetes, mainly from \textit{Trichoderma} species (data not shown). These fungi possess fungicide and antagonistic activity against basidiomycete cell walls, in addition to releasing enzymes such as chitinases and glucanases (45,46). Moreover, basidiomycete recovery from collected samples can also suffer from competition with ascomycete fungi. Competition between these two fungi heavily relies on nutrient accessibility, growth factors favoring ascomycetes due to their faster growing pace in complete culture media, or even the presence of simple nutrient sources observed in advanced stages of wood decay (47). Based on macroscopic properties of fruiting body (front and back surface, color, texture, border margins, heights and widths), twelve fungi isolates were identified. Isolated strains belong to the orders: i)
Hymenochaetale: Hyphodontia (2 isolates), Phellinus (1 isolate); ii) Polyporales: Aleurodiscus (1 isolate), Mycoacia (2 isolates), Stereum (1 isolate), Trametes (1 isolate), Tyromyces (1 isolate); and iii) Agaricales: Dictyopanus (1 isolate), Pleurotus (2 isolates). Such orders are associated with oxidoreductase and hydrolase producers that cluster in the same evolutionary taxa (class Agaricomycetes). It is also worth mentioning that those fungi represent the most cited ligninolytic enzyme producers (48-50).

3.2. Screening of isolates. Enzymatic extracts were screened for enzymes known to participate in the delignification process, i.e. laccase, manganese peroxidase, and lignin peroxidases. From the crude enzymatic extracts obtained by SSF, only five isolates exhibited laccase activity in our screening assay. Surprisingly, we were unable to measure peroxidase activity other than through the ABTS assay. Since peroxidases are common enzymes present during fungi-catalyzed wood decay, peroxidase activity was either negligible in our isolates or the enzymatic assay was not sensitive enough to quantify such activity. It has been reported that variations in the concentrations of lignin, carbon, nitrogen, and the presence of chemical compounds such as inducers in the culture media could affect the profile of ligninolytic enzymes expressed and secreted during fermentation (51-53). While current experiments cannot explain whether the lack of peroxidase activity is related to the composition of the culture media, the abovementioned results confirm previous reports suggesting that laccase activity is the most prevalent ligninolytic activity observed during fermentation with lignocellulose as substrate (54,55).

Isolates exhibiting ligninolytic activity were identified as Dictyopanus sp. LMB4 (22.3 U.L\(^{-1}\)), Pleurotus sp. LMB2 (69.5 U.L\(^{-1}\)), and Pleurotus sp. LMB3 (57.2 U.L\(^{-1}\)) (Fig. 1). Laccase activity of the Hyphodontia and Trametes isolates was considered too low to warrant further characterization. For the three most active isolates, the highest laccase activity was detected after 20 days of fermentation. Using these 3 isolates, laccase activity conditions were optimized by increasing copper concentration and carbon-to-nitrogen ratios (C/N) (56,57). As a result, the isolate exhibiting the highest laccase activity under these newly optimized conditions was Dictyopanus sp. LMB4 (267.6 U.L\(^{-1}\) after 28 days of fermentation). To the best of our knowledge, this represents the first observation of significant laccase activity in a crude enzymatic extract from a Dictyopanus fungus. Furthermore, this activity is similar to a previously reported Trametes sp. laccase activity evaluated under comparable fermentation conditions.
using lignocellulosic by-products from oil palm (218.6 U.L⁻¹) (58). The maximal laccase activities of the
*Pleurotus* isolates were at least 5 times lower than the one observed in *Dictyopanus* sp. LMB4, with 98
U.L⁻¹ for *Pleurotus* sp. LMB2, and 66.9 U.L⁻¹ for *Pleurotus* sp. LMB3 (Fig. 1).

**Figure 1.** Laccase activity of SSF isolates. ABTS oxidation activity was tested for three culture
supernatants from *Dictyopanus* LMB4 (circles), *Pleurotus* LMB2 (squares), and *Pleurotus* LMB3 (triangles) isolates. With a C/N ratio of 1.9 and in the absence copper, the *Pleurotus* spp. isolate exhibited the highest laccase activity (see inset). However, a 12-fold increase in laccase activity was observed in the *Dyctiopanus* sp. isolate with a 10-fold increase in the carbon-to-nitrogen ratio (19 C/N) and 5 mM copper (main histogram). Axes and units are the same for both histograms. The *Dictyopanus* LMB4 isolate is highlighted by an asterisk in both histograms.

Upon growth condition optimization, the crude enzymatic activity of *Dictyopanus* sp. LMB4
increased 6- and 12-fold after 20- and 28-day incubation, respectively, highlighting the importance of
copper and carbon source accessibility for proper enzyme expression. The increase in laccase activity for
enzymatic extracts upon copper and glucose addition has been reported for *Colorios versicolor* and
Ganoderma lucidum. These reports suggested that copper and glucose could respectively stimulate laccase expression and mycelial growth, further correlating with a proportional increase in the amount of laccase secreted by the fungi (59,60). For the enzymatic extract of D. pusillus, the calculated laccase activity obtained per gram of oil palm by-products was 31.5 U.g⁻¹ after 12 days of SSF. It is worth mentioning that this activity is four times higher than the previously reported laccase activity of a Pycnoporus sanguineus enzymatic extract obtained under similar SSF conditions using EFB as substrate (7.5 U.g⁻¹) (61).

3.3. Molecular identification of Dictyopanus sp. In contrast to most organisms genetically identified using 16S ribosomal RNA sequencing, using Internal Transcribed Spacer regions (ITS) is considered a more appropriate method to identify species in the fungi kingdom (62). In the past, mycologists have used an arbitrary sequence similarity cutoff ranging between 3-5% ITS identity as a threshold for species differentiation. However, the natural variability of ITS sequences at the phylum level within the fungi kingdom complicates the use of such cutoff (62). For instance, in Basidiomycota (to which the Dictyopanus genus belongs), the infraspecific ITS variability was reported to average at 3.3%, with a standard deviation of 5.62% (62). This significantly limits the use of GenBank BLAST searches as the only source to properly identify fungi species, especially considering the fact that more than 27% of ITS sequences were submitted with insufficient taxonomic identification (63). In addition, until 2003, nearly 20% of all fungal species listed in GenBank were incorrectly annotated (64). As a result, using BLAST searches to categorize fungal species can lead to serious misidentification and characterization. Consequently, fungal specimen identification requires a careful, systematic, and multi-source process.

To overcome some of these limitations, we first performed preliminary in situ morphological identification of the samples collected in the Colombian forest. Genus level inspection was performed in the laboratory using macroscopic and microscopic examination, followed by final phylogenetic identification through DNA extraction and sequencing of ITS regions 1 and 4 (28). This allowed identification of the pusillus species, to which the Dictyopanus LMB4 fungus sample belongs (Fig. 2). The same analysis also allowed us to differentiate the evolutionary history for some members of the Panellus genus, with which members of the Dictyopanus genus are often confused. Results presented in Figure 2
support the usefulness of taxonomic classification performed during fungi sample collection, selection, and isolation. The *Dictyopanus* genus belongs to the *Agaricomycetes* class, and its genus is known to include species capable of bioluminescence, which have been suggested to be linked to delignification processes through the use of secondary compounds produced during lignin degradation (65). *Dictyopanus* isolates were also reported as an alternative for the pretreatment of remazol brilliant blue R (66) and bamboo in ethanol production (67), further supporting the potential use of this fungus in large-scale biomass degradation.

**Figure 2.** Phylogenetic analysis of the pre-identified isolates labeled as *Dictyopanus* sp. We used the ITS region 1 as the genetic marker to infer the evolutionary history of this fungus using the UPGMA protocol (see Materials and Methods for details). The optimal tree analysis shows a branch length of 0.60, with clustering of species after a bootstrap of 500 replicates using the Maximum Composite Likelihood method to obtain evolutionary distances between members. The species was identified as *Dictyopanus pusillus*. The phylogenetic tree was drawn to use the same branch length units as those of the evolutionary distances. This analysis was performed using the standalone MEGA software, version 10.0.5.
3.4. Effect of pH and temperature on the enzymatic extracts obtained from *D. pusillus*.

Characterization of crude enzymatic extract isolates showed that pH values between 3 and 5 provided the highest laccase activity for *D. pusillus* LMB4, with a maximum activity at pH 3 (Fig. 3a). This pH range corresponds to other laccase preferences in fungi (68). Moreover, thermal stability of the crude *D. pusillus* LMB4 enzymatic extract was found to be quite robust, with reduced activity only observed at 60°C (46% activity loss after 6 hours of incubation). This behavior is quite different from that observed with the *T. versicolor* commercial laccase under the same experimental conditions, showing 28% and 78% activity loss after a 6h incubation at 50°C and 60°C, respectively (Fig. 4). Thus, *D. pusillus* LMB4 appears to express laccases with enhanced thermostability and higher tolerance to lower pH values. However, long incubation of this crude enzymatic extract at low pH resulted in an important activity loss (Fig. 3b). Previous studies have shown that a laccase from *Physisporinus rivulosus* remained stable at 50°C with optimal activity at pH 3.5 (69). Similarly, a laccase from *Trametes trogii* was shown to sustain temperatures up to 75°C, although only for short 5-min incubations (70). Nevertheless, our results suggest that the laccase activity from the *D. pusillus* LMB4 extract has higher tolerance to acidic and thermally induced perturbations than previously identified fungal laccases.

![Figure 3](image)

**Figure 3.** pH tolerance of a *D. pusillus* LMB4 extract exhibiting laccase activity. A) Laccase activity from a crude *D. pusillus* LMB4 enzymatic extract at different pH values. B) pH stability assay performed with the
crude enzymatic extract from *D. pusillus* LMB4. Laccase activity was evaluated at 40°C under different pH conditions: pH 3 (circles), pH 4 (squares), and pH 5 (triangles).

![Figure 4](image.png)

**Figure 4.** Thermal stability of a *D. pusillus* LMB4 extract exhibiting laccase activity. Laccase activity was measured after different temperature incubations: 40°C (triangles), 50°C (circles), and 60°C (squares). Solid lines represent the crude enzymatic extract from *D. pusillus* LMB4, while dashed lines represent the commercial laccase from *T. versicolor*, 53739.

### 3.5. Using *D. pusillus* for the simultaneous pretreatment and saccharification of EFB.

Fiber analysis of palm empty fruit bunch revealed a composition of 77.53% NDF, 58.32% ADF, and 17.15% ADL (see Materials and Methods for details). These values indicate that EFB composition of the lignocellulosic polymer used for SSF was 40.79% cellulose, 19.21% hemicellulose, 17.15% lignin, and 22.47% impregnated oil and ashes. These results are in accordance with typically reported EFB composition, with cellulose being the main component, followed by hemicellulose and lignin (71). Reducing sugar release was observed when the cellulolytic enzymatic extract from *T. reesei* was used alone (20.84 ± 0.7 g. g⁻¹). Higher reducing sugar release from EFB was also observed when the cellulolytic enzymatic extract from *T. reesei* was used with the commercial laccase enzyme from *T. versicolor* (46.47 ± 5.9 g. g⁻¹) or the enzymatic extract from *D. pusillus* (44.80 ± 5.21 g. g⁻¹), confirming that ligninolytic enzymes such as laccases favor cellulose hydrolysis, as previously reported (72,73). These results suggest that a combination of cellulolytic and ligninolytic enzymes enhance the release of
reducing sugars. However, production of reducing sugars was not significantly different when the commercial laccase from *T. versicolor* or enzymatic extracts from *D. pusillus* were mixed with the cellulolytic enzymatic extract from *T. reesei* (Fig. 5).

To identify the dominant variables affecting the reducing sugar release, we compared the effects of pH, temperature, copper concentration, and laccase (U.L\(^{-1}\)) or cellulase (UPF) concentration using a Plackett-Burman analysis (P value <0.05 with a confidence level of 95%) (Fig. 6). In both cases (SPS with laccase from *T. versicolor* and *D. pusillus*), pH was the dominant variable affecting activity, followed by temperature and copper concentration. Our results suggest that high pH values and temperatures (up to 45°C) promote sugar release by SPS. These results confirm what was observed in our stability experiments, where the activity of the enzymatic extract was compromised at pH values lower than 4 (Fig. 3B). These results are also in agreement with prior observations suggesting that basic pH is a desirable property for laccases used in biotechnological processes, since low pH values were linked to increased enzyme degradation (74). It is worth mentioning that cellulase is the third most important contributing factor to activity when SPS is performed with the commercial laccase (Fig. 6A), a result we do not observe with the enzymatic extract from *D. pusillus* (Fig. 6B). The requirement of a cellulase activity in the case of the commercial laccase are perhaps due to the combined production of ligninolytic and cellulolytic enzymes in the basidiomycete fungi during wood decay processes (75,76). Some authors have also demonstrated the efficiency of enzymatic extracts from basidiomycetes for the SSF production of ligninolytic and cellulolytic enzymes using wheat straw as substrate (77). These results further highlight the importance of *D. pusillus* as an efficient, accessible, and economical source of relevant biotechnological assets in the field of delignification processes.

The highest reducing sugar concentration obtained with the enzymatic extract of *D. pusillus* was 65.87 g.g\(^{-1}\) (pH 4.5, 45°C, 2:1 laccase-to-cellulase ratio). In the same conditions, reducing sugar production reached 64.13 g.g\(^{-1}\) for the commercial laccase from *T. versicolor*. These results confirm that the enzymatic extract from *D. pusillus* exhibits similar ligninolytic efficiency than the purified commercial laccase from *T. versicolor*. Additionally, EFB represent a good lignocellulose source for reducing sugar production since palm oil bunches are subjected to a first round of “sterilization” to extract oil palm fruits from the bunch, which effectively acts as a pretreatment during palm oil extraction. As a result, this initial
pretreatment might improve the delignification process performed by the enzymes. Lignocellulose breakdown of EFB and EFB pulp was previously reported using the white rot fungi *T. versicolor* TISTR 3224, *Phanerochaete chrysosporium* CECT 2798, and *Pleurotus ostreatus* CEC20311. These fungi were also used as efficient pretreatments for lignin removal in EFB (78,79). To the best of our knowledge, only one study reported the use of enzymatic extracts with laccase activity from *Pycnoporus sanguineus* UPM4 as a pretreatment of EFB to increase production of reducing sugars in similar conditions (80). This report and the results presented here on the use of a crude enzymatic extract exhibiting laccase activity from a white-rot fungi reinforce the relevance of using ligninolytic enzymatic extracts as a valuable tool for the pretreatment of lignocellulose in EFB.

**Figure 5.** Comparative production of reducing sugars from EFB using enzymatic extracts alone or in combination. Reducing sugar release was observed: A) without any enzymatic extract, B) with a cellulolytic extract from *T. reesei*, C) with a commercial laccase from *T. versicolor*, D) with the enzymatic extract from *D. pusillus*. Combinations of B+C and B+D were also tested.
Figure 6. Pareto charts from Plackett-Burman multilevel analysis. A) Cellulolytic extract from *T. reesei* mixed with commercial laccase from *T. versicolor*. B) Cellulolytic extract from *T. reesei* mixed with enzymatic extract from *D. pusillus*. Parameters: A, pH; B, temperature; C, copper concentration; D, U.L\(^{-1}\) of laccase, and E, UPF of cellulase. The red line represents the statistically significant threshold of 95% confidence, while grey and blue bars highlight positive and negative effects, respectively.

3.6. Genome sequencing of *D. pusillus* LMB4 and laccase sequence annotation. Given the striking ligninolytic activity of *D. pusillus* LMB4 and lack of genomic data available to identify and compare potential enzyme homologs promoting such activity in this organism, we used long-reads single-molecule real-time technology (PacBio) to perform genomic DNA sequencing of *D. pusillus* LMB4. This allowed analysis and annotation of a number of putatively encoded laccases in this genome, offering means to predict potential enzymes involved in this ligninolytic potential. After de novo assembly, we estimated genome heterozygosity at 13.53%. Reduction in homozygous genome allowed the assembly of 49.37 Mbp distributed in 3463 contigs (N50 = 23,741 bp) (Table 1). After splicing of the 95,174 annotated introns, a total of 16,866 coding sequences (CDSs) were predicted to be encoded in the genome of *D. pusillus* LMB4. Of this number, we confidently annotated a total of 13 CDSs as complete putative laccase sequences, which were further aligned with a previously reported laccase homolog from *Trametes* to identify consensus regions and conserved motifs (Fig. 7, Table S1). Our results show that all putative laccases encoded in the *D. pusillus* genome preserve the four conserved copper-binding motifs normally
observed in this enzyme family, i.e. Cu1 (HWHGFFQ), Cu2 (HSHLSTQ), Cu3 (HPFHLHG), and Cu4 (HCHIDFHL) (41). These make them potentially promising candidates for future functional investigation of new laccases exhibiting interesting properties with respect to activity, stability, and industrial tolerance.

| Feature                        | Value  |
|-------------------------------|--------|
| Genome assembly size (Mbp)    | 49.37  |
| Number of contigs             | 3463   |
| N50 (bp)                      | 23,741 |
| GC (%)                        | 53.08  |
| Number of CDSs                | 16,866 |
| Number of introns             | 95,174 |
| Heterozygosity (%)            | 13.53  |
Figure 7. Multiple sequence alignment (MSA) of the 13 putative laccases identified in the genome of *Dictyopanus pusillus* LMB4. The four conserved copper-binding motifs are highlighted by red dashed rectangles. The laccase sequence of the *Trametes* genus was used as reference to perform the MSA. Consensus sequence is presented on the bottom of the alignment. Putative laccase genes are identified as in Table S1.
4. Conclusion

The present work demonstrates that a crude enzymatic extract from a wild Colombian source of *D. pusillus* LMB4 exhibits significant laccase activity (267 ± 18 U.L\(^{-1}\)). This crude enzymatic extract was probed for the successful pretreatment of low-cost lignocellulosic raw materials (oil palm by-products), suggesting that an upscaling of this process could potentially help with the delignification of starting materials in cellulosic bioethanol production. An increase in copper and glucose concentration during solid-state fermentation proved beneficial, resulting in a 12-fold increase in laccase activity and suggesting that ligninolytic enzyme expression can further be induced to improve enzyme production in *D. pusillus* LMB4. The SPS of EFB also illustrated that the enzymatic extract from *D. pusillus* exhibits good ligninolytic capacity at basic pH, in addition to demonstrating higher pH and thermal stability than the purified commercial laccase from *T. versicolor*. These properties demonstrate the efficiency of such crude enzymatic extract from *D. pusillus* as a versatile biotechnological tool for lignocellulose pretreatment such as for cellulosic bioethanol production. Genome sequencing of *D. pusillus* LMB4 also revealed 13 laccases and a significant number of other putative enzymes that could be exploited and/or engineered to develop more efficient delignification pre-treatments. These results thus present the first few stages in the implementation of a strategy that combines genome data mining and computational modelling as efficient approaches to identify promising new protein engineering candidates as a new set of catalysts with applications in delignification processes.

Acknowledgements. This work was partially supported by a grant from Universidad Industrial de Santander, Vicerrectoría de Investigación y Extension (Grant number 5199) (to C.I.S. and D.M.V.), Industrias Acuña LTDA, and a Natural Sciences and Engineering Research Council of Canada (NSERC), via Discovery Grant RGPIN-2016-05557 (to N.D.). A.T.V. received a Postdoctoral Fellowship from NSERC. A.M.R. was supported by a doctoral scholarship from the Colombian Departamento Administrativo de Ciencia, Tecnología e Innovación (Colciencias) (PhD scholarship 567, 2012), and was the recipient of a scholarship from the Emerging Leaders in the Americas Program (ELAP) from the
Government of Canada. F.V. and N.D. hold Fonds de Recherche Québec-Santé (FRQS) Research Scholar Junior 1 and Junior 2 Career Awards, respectively (numbers 35038 and 32743).
References

1. FAO. (2016) Food and agriculture data. (Division, S. ed.
2. Sulaiman, F., Abdullah, N., Gerhauser, H., and Shariff, A. (2011) An outlook of Malaysian energy, oil palm industry and its utilization of wastes as useful resources. *Biomass and Bioenergy* 35, 3775-3786
3. Gupta, V. K., Kubicek, C. P., Berrin, J.-G., Wilson, D. W., Couturier, M., Berlin, A., Filho, E. X. F., and Ezeji, T. (2016) Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. *Trends in Biochemical Sciences* 41, 633-645
4. Bhuuto, A. W., Qureshi, K., Harijan, K., Abro, R., Abbas, T., Bazmi, A. A., Karim, S., and Yu, G. (2017) Insight into progress in pre-treatment of lignocellulosic biomass. *Energy* 122, 724-745
5. Bilal, M., Asgher, M., Iqbal, H. M. N., Hu, H., and Zhang, X. (2017) Biotransformation of lignocellulosic materials into value-added products—A review. *International Journal of Biological Macromolecules* 98, 447-458
6. Kumar, A. K., and Sharma, S. (2017) Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresources and Bioprocessing* 4, 7
7. Chen, H., and Fu, X. (2016) Industrial technologies for bioethanol production from lignocellulosic biomass. *Renewable and Sustainable Energy Reviews* 57, 468-478
8. Munk, L., Sitarz, A. K., Kalyani, D. C., Mikkelsen, J. D., and Meyer, A. S. (2015) Can laccases catalyze bond cleavage in lignin? *Biotechnology Advances* 33, 13-24
9. Wong, D. W. S. (2009) Structure and Action Mechanism of Ligninolytic Enzymes. *Applied Biochemistry and Biotechnology* 157, 174-209
10. Abbas, A., Koc, H., Liu, F., and Tien, M. (2005) Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate. *Current Genetics* 47, 49-56
11. Martínez, Á. T., Ruiz-Dueñas, F. J., Martínez, M. J., del Río, J. C., and Gutiérrez, A. (2009) Enzymatic delignification of plant cell wall: from nature to mill. *Current Opinion in Biotechnology* 20, 348-357
12. Krah, F.-S., Bässler, C., Heibl, C., Sohgijian, J., Schaefer, H., and Hibbett, D. S. (2018) Evolutionary dynamics of host specialization in wood-decay fungi. *BMC Evolutionary Biology* 18, 119
13. Savinova, O. S., Moiseenko, K. V., Vavilova, E. A., Chulkin, A. M., Fedorova, T. V., Tyazhelova, T. V., and Vasina, D. V. (2019) Evolutionary Relationships Between the Laccase Genes of Polyporales: Orthology-Based Classification of Laccase Isozymes and Functional Insight From Trametes hirsuta. *Frontiers in Microbiology* 10
14. Maqbool, Z., Hussain, S., Imran, M., Mahmood, F., Shahzad, T., Ahmed, Z., Azeem, F., and Muzammil, S. (2016) Perspectives of using fungi as bioresource for bioremediation of pesticides in the environment: a critical review. *Environmental Science and Pollution Research* 23, 16904-16925
15. Hyde, K. D., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A. G. T., Abeywickrama, P. D., Aluthmuhandiram, J. V. S., Brahamanage, R. S., Brooks, S., Chaiyasen, A., Chethana, K. W. T., Chomnunti, P., Chepkirui, C., Chuankid, B., de Silva, N. I., Doilom, M., Faulds, C., Gentekaki, E., Gopalan, V., Kakumyan, P., Harishchandra, D., Hemachandran, H., Hongsanan, S., Karunarathna, A., Karunarathna, S. C., Khan, S., Kumla, J., Jayawardena, R. S., Liu, J.-K., Liu, N., Luangharn, T., Macabeo, A. P. G., Marasinghe, D. S., Meeks, D., Mortimer, P. E., Mueller, P., Nadir, S., Nataraja, K. N., Nontachaiyapoom, S., O’Brien, M., Penkhrue, W., Phukhamsakda, C., Ramamani, U. S., Rathnayaka, A. R., Sadaba, R. B., Sandargo, B., Samarakoon, B. C., Tennakoon, D. S., Siva, R., Sriprom, W., Suryanarayanan, T. S., Sujarit, K., Suwannarach, N.,
16. Alcalde, M. (2015) Engineering the ligninolytic enzyme consortium. *Trends in Biotechnology* **33**, 155-162
17. Upadhyay, P., Shrivastava, R., and Agrawal, P. K. (2016) Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech* **6**, 15
18. Gasser, C. A., Hommes, G., Schäffer, A., and Corvini, P. F.-X. (2012) Multi-catalysis reactions: new prospects and challenges of biotechnology to valorize lignin. *Applied Microbiology and Biotechnology* **95**, 1115-1134
19. Madhavan, A., Sindhu, R., Parameswaran, B., Sukumaran, R. K., and Pandey, A. (2017) Metagenome Analysis: a Powerful Tool for Enzyme Bioprospecting. *Applied Biochemistry and Biotechnology* **183**, 636-651
20. Rodgers, C. J., Blanford, C. F., Giddens, S. R., Skamnioti, P., Armstrong, F. A., and Gurr, S. J. (2010) Designer laccases: a vogue for high-potential fungal enzymes? *Trends in Biotechnology* **28**, 63-72
21. Blanchette, R. A. (1984) Screening Wood Decayed by White Rot Fungi for Preferential Lignin Degradation. *Applied and Environmental Microbiology* **48**, 647-653
22. Worrall, J. J., Anagnost, S. E., and Zabel, R. A. (1997) Comparison of wood decay among diverse lignicolous fungi. *Mycologia*, 199-219
23. Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henriassat, B., Martínez, A. T., Otiliar, R., Spatafora, J. W., Yadav, J. S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P. M., de Vries, R. P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Görecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J. A., Kallen, N., Kersten, P., Kohler, A., Kües, U., Kumar, T. K. A., Kuo, A., LaButti, K., Larrondo, L. F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D. J., Morgenstern, I., Morin, E., Murat, C., Nagy, L. G., Nolan, M., Ohm, R. A., Patyshakulyeva, A., Rokas, A., Ruiz-Dueñas, F. J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J. C., St. John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D. C., Martin, F., Cullen, D., Grigoriev, I. V., and Hibbett, D. S. (2012) The Paleozoic Origin of Enzymatic Lignin Decomposition Reconstructed from 31 Fungal Genomes. *Science* **336**, 1715-1719
24. Peláez, F., Martínez, M. J., and Martínez, A. T. (1995) Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. *Mycological Research* **99**, 37-42
25. Choi, Y. W., Hyde, K. D., and Ho, W. H. (1999) Single spore isolation of fungi. *Fungal Diversity* **3**, 29
26. Harris, J. L. (1986) Modified method for fungal slide culture. *Journal of Clinical Microbiology* **24**, 460-461
27. Plaza G. A., U. R., Brigmon R. L., Whitman W. B. and Ulfig K. (2004) Rapid DNA Extraction for Screening Soil Filamentous Fungi Using PCR Amplification. *Polish Journal of Environmental Studies* **13**, 315 - 318
28. Gardes, M., and Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113-118
29. Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* **35**, 1547-1549
30. Rodríguez Couto, S., and Sanromán, M. A. (2005) Application of solid-state fermentation to ligninolytic enzyme production. *Biochemical Engineering Journal* **22**, 211-219
31. Lim, S.-H., Lee, Y.-H., and Kang, H.-W. (2013) Efficient Recovery of Lignocellulolytic Enzymes of Spent Mushroom Compost from Oyster Mushrooms, Pleurotus spp., and Potential Use in Dye Decolorization. *Mycobiology* **41**, 214-220
32. Miller, G. L. (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* **31**, 426-428
33. Johannes, C., and Majcherzyk, A. (2000) Laccase activity tests and laccase inhibitors. *Journal of Biotechnology* **78**, 193-199
34. Järvinen, J., Taskila, S., Isomäki, R., and Ojamo, H. (2012) Screening of white-rot fungi manganese peroxidases: a comparison between the specific activities of the enzyme from different native producers. *AMB Express* **2**, 62
35. Khindaria, A., Yamazaki, I., and Aust, S. D. (1995) Veratryl alcohol oxidation by lignin peroxidase. *Biochemistry* **34**, 16860-16869
36. Ghose, T. (1987) Measurement of cellulase activities. *Pure and applied Chemistry* **59**, 257-268
37. Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., and Phillippy, A. M. (2017) Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research* **27**, 722-736
38. Pryszczy, L. P., and Gabaldón, T. (2016) Redundans: an assembly pipeline for highly heterozygous genomes. *Nucleic Acids Research* **44**, e113-e113
39. Hoff, K. J., and Stanke, M. (2013) WebAUGUSTUS—a web service for training AUGUSTUS and predicting genes in eukaryotes. *Nucleic Acids Research* **41**, W123-W128
40. Huerta-Cepas, J., Forslund, K., Coelho, L. P., Szklarczyk, D., Jensen, L. J., von Mering, C., and Bork, P. (2017) Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Mol Biol Evol* **34**, 2115-2122
41. Kumar, S. V. S., Phale, P. S., Durani, S., and Wangikar, P. P. (2003) Combined sequence and structure analysis of the fungal laccase family. *Biotechnology and Bioengineering* **83**, 386-394
42. Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.-Y., Lopez, R., and Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30**, 1236-1240
43. Moreno, L. F., Feng, P., Weiss, V. A., Vicente, V. A., Stielow, J. B., and de Hoog, S. (2017) Phylogenomic analyses reveal the diversity of laccase-coding genes in Fonsecaea genomes. *PLoS ONE* **12**, e0171291
44. Sirim, D., Wagner, F., Wang, L., Schmid, R. D., and Pleiss, J. (2011) The Laccase Engineering Database: a classification and analysis system for laccases and related multicopper oxidases. *Database: The Journal of Biological Databases and Curation 2011*, bar006
45. Colavolpe, M. B., Mejía, S. J., and Albertó, E. (2014) Efficiency of treatments for controlling Trichoderma spp during spawning in cultivation of lignicolous mushrooms. *Brazilian Journal of Microbiology* **45**, 1263-1270
46. Schubert, M., Fink, S., and Schwarze, F. W. M. R. (2008) Evaluation of Trichoderma spp. as a biocontrol agent against wood decay fungi in urban trees. *Biological Control* **45**, 111-123
47. Boddy, L. (2000) Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiology Ecology* **31**, 185-194
48. Eastwood, D. C. (2014) Evolution of Fungal Wood Decay. in *Deterioration and Protection of Sustainable Biomaterials*, American Chemical Society. pp 93-112
49. Kim, N. K., Park, J. Y., Park, M. S., Lee, H., Cho, H. J., Eimes, J. A., Kim, C., and Lim, Y. W. (2016) Five New Wood Decay Fungi (Polyporales and Hymenochaetales) in Korea. *Mycobiology* **44**, 146-154

50. Floudas, D., Held, B. W., Riley, R., Nagy, L. G., Koehler, G., Ransdell, A. S., Younus, H., Chow, J., Chiniquy, J., Lipzen, A., Tritt, A., Sun, H., Haridas, S., LaButti, K., Ohm, R. A., Kües, U., Blanchette, R. A., Grigoriev, I. V., Minto, R. E., and Hibbett, D. S. (2015) Evolution of novel wood decay mechanisms in Agaricales revealed by the genome sequences of Fistulina hepatica and Cylindrobasidium torrendii. *Fungal Genetics and Biology* **76**, 78-92

51. Kachlishvili, E., Penninckx, M. J., Tsiklauri, N., and Elisashvili, V. (2006) Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World Journal of Microbiology and Biotechnology* **22**, 391-397

52. Henske, J. K., Springer, S. D., O'Malley, M. A., and Butler, A. (2018) Substrate-based differential expression analysis reveals control of biomass degrading enzymes in Pycnoporus cinnabarinus. *Biochemical Engineering Journal* **130**, 83-89

53. Peng, M., Aguilar-Pontes, M. V., Hainaut, M., Henrissat, B., Hildén, K., Mäkelä, M. R., and de Vries, R. P. (2018) Comparative analysis of basidiomycete transcriptomes reveals a core set of expressed genes encoding plant biomass degrading enzymes. *Fungal Genetics and Biology* **112**, 40-46

54. Fernández-Fueyo, E., Ruiz-Dueñas, F. J., López-Lucendo, M. F., Pérez-Boada, M., Rencoret, J., Gutiérrez, A., Pisabarro, A. G., Ramírez, L., and Martínez, A. T. (2016) A secretomic view of woody and nonwoody lignocellulose degradation by Pleurotus ostreatus. *Biotechnology for Biofuels* **9**, 49

55. Mali, T., Kuuskeri, J., Shah, F., and Lundell, T. K. (2017) Interactions affect hyphal growth and enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes. *PLOS ONE* **12**, e0185171

56. Baldrian, P., and Gabriel, J. (2002) Copper and cadmium increase laccase activity in Pleurotus ostreatus. *FEMS Microbiology Letters* **206**, 69-74

57. Li, P., Wang, H., Liu, G., Li, X., and Yao, J. (2011) The effect of carbon source succession on laccase activity in the co-culture process of Ganoderma lucidum and a yeast. *Enzyme and Microbial Technology* **48**, 1-6

58. Singh, P., Sulaiman, O., Hashim, R., Peng, L. C., and Singh, R. P. (2013) Evaluating biopulping as an alternative application on oil palm trunk using the white-rot fungus Trametes versicolor. *International Biodeterioration & Biodegradation* **82**, 96-103

59. Kajita, S., Sugawara, S., Miyazaki, Y., Nakamura, M., Katayama, Y., Shishido, K., and limura, Y. (2004) Overproduction of recombinant laccase using a homologous expression system in Coriolus versicolor. *Applied Microbiology and Biotechnology* **66**, 194-199

60. Hailei, W., Ping, L., Yuhua, Y., and Yufeng, L. (2015) Overproduction of laccase from a newly isolated Ganoderma lucidum using the municipal food waste as main carbon and nitrogen supplement. *Bioprocess and Biosystems Engineering* **38**, 957-966

61. Vikineswary, S., Abdullah, N., Renuvathani, M., Sekaran, M., Pandey, A., and Jones, E. B. G. (2006) Productivity of laccase in solid substrate fermentation of selected agro-residues by Pycnoporus sanguineus. *Bioresource Technology* **97**, 171-177

62. Raja, H. A., Miller, A. N., Pearce, C. J., and Oberlies, N. H. (2017) Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *J Nat Prod* **80**, 756-770

63. Bridge, P. D., Roberts, P. J., Spooner, B. M., and Panchal, G. (2003) On the unreliability of published DNA sequences. *New Phytologist* **160**, 43-48
64. Nilsson, R. H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.-H., and Kõljalg, U. (2006) Taxonomic Reliability of DNA Sequences in Public Sequence Databases: A Fungal Perspective. *PLOS ONE* **1**, e59

65. Bechara, Etelvino J. H. (2015) Bioluminescence: A Fungal Nightlight with an Internal Timer. *Current Biology* **25**, R283-R285

66. Machado, K. M. G., Matheus, D. R., and Bononi, V. L. R. (2005) Ligninolytic enzymes production and Remazol brilliant blue R decolorization by tropical brazilian basidiomycetes fungi. *Brazilian Journal of Microbiology* **36**, 246-252

67. Suhara, H., Kodama, S., Kamei, I., Maekawa, N., and Meguro, S. (2012) Screening of selective lignin-degrading basidiomycetes and biological pretreatment for enzymatic hydrolysis of bamboo culms. *International Biodeterioration & Biodegradation* **75**, 176-180

68. Baldrian, P. (2006) Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews* **30**, 215-242

69. Hildén, K., Mäkelä, M. R., Lundell, T., Kuuskeri, J., Cherrykh, A., Golovleva, L., Archer, D. B., and Hatakka, A. (2013) Heterologous expression and structural characterization of two low pH laccases from a biopulping white-rot fungus Physisporinus rivulosus. *Applied Microbiology and Biotechnology* **97**, 1589-1599

70. Yan, J., Chen, Y., Niu, J., Chen, D., and Chagan, I. (2015) Laccase produced by a thermotolerant strain of Trametes trogii LK13. *Brazilian Journal of Microbiology* **46**, 59-65

71. Chang, S. H. (2014) An overview of empty fruit bunch from oil palm as feedstock for bio-oil production. *Biomass and Bioenergy* **62**, 174-181

72. Davidi, L., Morais, S., Artzi, L., Knop, D., Hadar, Y., Arfi, Y., and Bayer, E. A. (2016) Toward combined delignification and saccharification of wheat straw by a laccase-containing designer cellulosome. *Proceedings of the National Academy of Sciences* **113**, 10854-10859

73. Mukhopadhyay, M., Kuila, A., Tuli, D. K., and Banerjee, R. (2011) Enzymatic depolymerization of Ricinus communis, a potential lignocellulosic for improved saccharification. *Biomass and Bioenergy* **35**, 3584-3591

74. Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D. A., and Holliger, C. (2013) Bacterial versus fungal laccase: potential for micropollutant degradation. *AMB Express* **3**, 63

75. Zhang, J., Presley, G. N., Hammel, K. E., Ryu, J.-S., Menke, J. R., Figueroa, M., Hu, D., Orr, G., and Schilling, J. S. (2016) Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus *Postia placenta*. *Proceedings of the National Academy of Sciences* **113**, 10968-10973

76. Presley, G. N., and Schilling, J. S. (2017) Distinct Growth and Secretome Strategies for Two Taxonomically Divergent Brown Rot Fungi. *Applied and Environmental Microbiology* **83**

77. Xu, X., Lin, M., Zang, Q., and Shi, S. (2018) Solid state bioconversion of lignocellulosic residues by Inonotus obliquus for production of cellulolytic enzymes and saccharification. *Bioresource Technology* **247**, 88-95

78. Kamcharoen, A., Champreda, V., Eurwilaichitr, L., and Boonsawang, P. (2014) Screening and optimization of parameters affecting fungal pretreatment of oil palm empty fruit bunch (EFB) by experimental design. *International Journal of Energy and Environmental Engineering* **5**, 303-312

79. Piñeros-Castro, Y., and Velásquez-Lozano, M. (2014) Biodegradation kinetics of oil palm empty fruit bunches by white rot fungi. *International Biodeterioration & Biodegradation* **91**, 24-28

80. Zanirun, Z., Bahrin, E. K., Lai-Yee, P., Hassan, M. A., and Abd-Aziz, S. (2015) Enhancement of fermentable sugars production from oil palm empty fruit bunch by
ligninolytic enzymes mediator system. *International Biodeterioration & Biodegradation* **105**, 13-20