Characterization of ligninolytic enzyme production in white-rot wild fungal strains suitable for kraft pulp bleaching

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Abstract Fungal strains identified by phylogenetic analysis of the ITS rDNA region as *Trametes versicolor* (CMU-TA01), *Irpex lacteus* (CMU-84/13), and *Phlebiopsis* sp. (CMU-47/13) are able to grow on and bleach kraft pulp (KP) in a simple solid-state fermentation (SSF) assay conducted in Petri dishes. Kappa number reductions obtained with *Phlebiopsis* sp. (48.3%), *T. versicolor* (43%), and *I. lacteus* (39.3%), evidence their capability for lignin breakdown. Scanning electron microscopy images of KP fibers from SSF assays demonstrated increased roughness and striation, evidencing significant cell wall modification. *T. versicolor* produces laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) in potato dextrose broth (PDB), PDB + CuSO₄, and PDB + KP, whereas *Phlebiopsis* sp. and *I. lacteus* showed no Lac and low LiP activities in all media. Compared to PDB, the highest increase in Lac (7.25-fold) and MnP (2.37-fold) activities in PDB + CuSO₄ occur in *T. versicolor*; for LiP, the greatest changes (6.95-fold) were observed in *I. lacteus*.

Incubation in PDB + KP shows significant increases in Lac and MnP for *T. versicolor*, MnP and LiP for *Phlebiopsis* sp., and none for *I. lacteus*. SSF assays in Petri plates are a valuable tool to select fungi that are able to delignify KP. Here, delignification by *Phlebiopsis* sp. of this substrate is reported for the first time, and MnP activity was strongly associated with the delignification ability of the studied strains. The obtained results suggest that the studied fungal strains have biotechnological potential for use in the paper industry.

Keywords White-rot fungi · Ligninolytic enzymes · Kraft pulp bleaching · *Trametes* · *Phlebiopsis* · *Irpex*

Introduction

Biotechnology applied to pulping and bleaching in the paper industry has been developed to reduce the environmental impact of these processes, while maintaining high pulp quality (Martín-Sampedro et al. 2015; Kumar et al. 2016). In particular, stages involving oxidative fungal enzymes laccase (Lac), Mn peroxidase (MnP), and lignin peroxidase (LiP) have shown promising results for pulp bleaching (Bajpai 2012; Upadhyay et al. 2016). This biotechnological approach is currently being used with excellent results in pulp and paper at mill-scale production (Bajpai 2012). The use of microbial lignocellulolytic enzymes may enhance the bleaching effect of chemical reagents, resulting in reduced consumption and lower amounts of required energy (Bajpai 2012; Martín-Sampedro et al. 2015). Extracellular oxidative enzymes that degrade lignin are produced by bacteria and fungi (Hatakka and Hammel 2011; Cragg et al. 2015), and have been studied for their use in pulping and bleaching (Bajpai

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2012); Basidiomycota taxa are a particularly important source of fungal strains for these processes (Hatakka and Hammel 2011).

White-rot basidiomycetes efficiently degrade the lignin within the plant cell wall using extracellular ligninolytic enzymes, such as Lac, MnP, and LiP (Hatakka and Hammel 2011). The main fungal species/strains examined produce extracellular ligninolytic enzymes that can be used in wood treatment and kraft pulp biobleaching; these belong to the *Phanerochaete* and *Trametes* genera. Although strains of the genera *Phlebia*, *Physisporinus*, *Pleurotus*, *Pycnoporus*, and *Bjerkandera* (Hatakka and Hammel 2011), among others, have also been analyzed, they represent a small percentage of the diverse white-rot fungi (Berrin et al. 2012). Moreover, some screening studies have identified physiological variability and different processing results on bleaching and biopulping among strains belonging to the same genus or species, even from apparently well-studied taxa (Levin et al. 2007; Berrin et al. 2012; Daâssi et al. 2016). In addition, lignin degradation by different fungal species or strains depends on the monomer composition of this complex structure (Skyba et al. 2013). Therefore, searching for new fungi from different geographical areas, new types of wood, or different life styles allows the discovery of strains with different enzymatic capabilities (Oses et al. 2006; Berrin et al. 2012; Daâssi et al. 2016), which vary both quantitatively and qualitatively in substrate affinity and processing (Levin et al. 2007; Liew et al. 2011; Martín-Sampedro et al. 2015).

The aim of the present study was to identify and characterize three wild fungal strains, and determine their potential for use in biobleaching of kraft pulp. To support the assessment of the biotechnological potential of the studied strains, this work combined approaches not commonly found together in the previous biobleaching reports. These approaches included: (1) making a robust phylogenetic analysis of studied strains based on Maximal Parsimony and Bayesian inference criteria, (2) applying a simple solid-state fermentation (SSF) screening process assays in Petri dishes, (3) visualizing kraft pulp fiber modifications caused by fungal treatment by means of scanning electron microscopy (SEM), and (4) determining basal and induced ligninolytic enzyme activities in the studied strains. One of the studied strains belongs to *Phlebiopsis* sp., a genera for which no species has previously been reported to bleach and delignify kraft pulp. The other strain was identified as *Irpex lacteus*, a rarely studied taxon in that regard. The third fungal taxon studied is a *T. versicolor* strain that shows a high capability to secrete ligninolytic enzymes in the assayed conditions. The results from this approach were compared and discussed to evaluate their contribution to the

screening and characterization of fungi for use in bleaching kraft pulp.

Materials and methods

Pulp sampling and processing

Kraft pulp was supplied by the Scribe™ Paper Corporation Mexico at Morelia City in Michoacán State, Central Mexico. At the date of sampling (January 13th, 2015), wood used in the processing plant was from pine (*Pinus* sp.) and eucalyptus (*Eucalyptus* sp.) trees in a 50%/50% ratio. After kraft digestion, the pulp displayed a kappa number of 13.23 (Tappi T-264 om-88); normally, this process continues with a refining step, which occurs in the bleaching stages known as the acidic and alkaline sequences. The pulp was collected directly at the plant in sterilized bags and stored at room temperature until processing. Within the same week of collection, the pulp was dried at 40 °C in an oven and stored at room temperature until it was used for the bioassays.

Fungal strains

The three fungal strains CMU-TA01, CMU-84/13, and CMU-47/13 used in this work were isolated from different ecosystems in the state of Michoacán, Mexico, and deposited in the Michoacán Strain Culture Collection (CMU by its Spanish name) of the Laboratory of Microbial Biotechnology and Conservation from the Universidad Michoacana de San Nicolas de Hidalgo.

Solid and liquid media

Potato dextrose agar (PDA, Difco, USA) was used to maintain the strains and produce inocula. The decolorization/oxidation assays of the phenolic substances were performed both in PDA and malt extract agar (MEA, Difco, USA). Potato dextrose broth (PDB, Difco, USA) was used in assays of enzyme activity induction in the presence of kraft pulp. All media were prepared as indicated by the supplier and sterilized by autoclaving at 120 °C and 15 lb/in².

Fungal inoculum generation

All strains tested were stored in PDA at 4 °C until used. Inocula were generated using 6-mm cylindrical plugs removed with a cork borer from the margin of a mycelial colony in the log growth phase and inoculated in the center of a 90-mm PDA Petri dish. The dishes were incubated in darkness at 28 °C until the mid-log phase, and 6 mm of

inoculum was taken from the margin of the colony as described. These mycelial plugs were used for solid and liquid media assays.

DNA extraction, PCR assays, and sequencing

Genomic DNA was extracted from mycelial colonies growing on cellophane sheets overlaid on PDA medium in 90-mm Petri dishes using the Fastprep 24 system (MP Biomedicals, USA) with Matrix A for cell lysis according to the supplier instructions. The primer pair ITS-1 and ITS-4 (White et al. 1990) was used for amplification of the Internal Transcribed Spacer regions 1 and 2 (ITS1-5.8S-ITS2) of the rRNA gene cluster. The reaction mix used for all amplifications contained 10 mM Tris–HCl buffer pH 8.0, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 μM of each oligonucleotide, 0.5 U of *Taq* recombinant polymerase (Invitrogen, USA), and a total of 25 ng of template DNA, and the final volume was adjusted to 25 μL with sterile deionized H₂O. PCR reactions were performed in a Veriti thermal cycler (Applied Biosystems, USA) with the following amplification schedule: one initial denaturing cycle at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 1 min, alignment at 55 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Both the integrity of the isolated DNA and the amplification products were analyzed in 1% (w/v) agarose gels stained with ethidium bromide. The amplification products were purified and sequenced by Elim Biopharm (Hayward, CA, USA). The obtained ITS sequences of CMU-84/13, CMU-47/13, and CMU-TA01 were submitted to GenBank with the accession numbers KY448286, KY448287, and KY448288, respectively.

Phylogenetic analysis

The sequences of ITS from the three strains were individually analyzed within the NCBI GenBank database using the BLASTn algorithm. Only sequences from well-identified species that displayed a similarity of 97% or more with the sequences of the characterized strains were selected for phylogenetic analysis. Sequences obtained with the BLASTn search from non-cultivated samples or without species assignment were discarded. Based on the results obtained from the first set of sequences for each strain, additional sequences of the same genus or taxonomically related sequences were retrieved from GenBank. These additional sequences were necessary both to select output groups and to increase the number of well-identified species that were taxonomically related to the strains studied but not provided in the initial BLASTn search. These additional sequences allow for a robust phylogenetic analysis.

Sequences were aligned with SATé (Liu et al. 2009a) using MAFFT (Katoh et al. 2005) as the external sequence alignment tool and RaxML (Stamatakis 2006) as the tree estimator. The alignments improved by hand were used for maximum parsimony (MP) and Bayesian inference (BI) analyses to infer phylogenetic relationships. Parsimony analyses were performed in PAUP 4.0a150 (Swofford 2003) using a heuristic search with a TBR exchange branch option excluding gaps and non-informative characters, leaving a total of 359, 88, and 49 positions in the final matrix for the strains CMU-47/13, CMU-84/13, and CMU-TA01, respectively. Node support was determined for bootstrap values with 1000 replicates. Bayesian inference (BI) was conducted in MrBayes 3.2 (Ronquist et al. 2012), which performed a sampling to determine the model most appropriate for nucleotide substitution during the run, so it was not necessary to determine that parameter a priori with other packages. Four MCMC chains were run simultaneously starting from random trees for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities were determined from a majority-rule consensus tree generated with the remaining 7500 trees. Because no incongruences were observed between MP tree and Bayesian inference, the different matrices were combined for the final phylogenetic tree. Trees were edited and visualized with software tools cited elsewhere.

Decolorization/oxidation assays

The assays were performed in 90-mm Petri dishes with PDA or MEA supplemented in independent experiments with indigo carmine (indigoid dye), methyl orange (azo dye), acid fuchsin (triphenylmethane dye), and guaiacol. The first three compounds were used at a final concentration of 250 μg/mL and added to the media as described elsewhere (Nyanhongo et al. 2002). The guaiacol substrate was utilized at a final concentration of 0.01% (w/v) (Kiskinen et al. 2004). All chemicals were purchased from Sigma-Aldrich (USA). The media with phenolic compounds were inoculated at the center of the plate with a 6-mm mycelium plug obtained as previously described and incubated in darkness at 28 °C. A control plate with media but without fungal inoculum was incubated to confirm that the color change was not induced by physicochemical factors during fungal growth. Mycelium growth was determined every 12 h by measuring colony diameter over 15 days. The decolorization of phenolic dyes was determined visually by the clearance and loss of color of the media; guaiacol oxidation was registered as the formation of a reddish-brown halo in the media (Nyanhongo et al.

2002; Kiiskinen et al. 2004). All experiments were performed in triplicate.

Kraft pulp treatment and analysis

To analyze pulp bleaching by the fungal strains, simple solid-state fermentation assays were designed using both pulp fibers as obtained from the processing plant and as a fine grain obtained by fragmentation in a blender. The fiber size was determined microscopically in a Leica DM750 microscope equipped with an ICC50 HD camera, and using the software Leica Application Suite (LAS EZ), Version 3.0.0. At least 100 fibers were measured for each fiber type and the range of longitude was 1.17–1.78 and 0.08–0.86 mm for intact and fragmented fibers, respectively; however, larger fibers were present in both samples. In independent assays, both intact and fragmented pulp fibers (5 g) extended to cover the surface of a 90-mm Petri dish and moistened with 10 mL of distilled sterilized water. For the two particle sizes, fungal bleaching by the three fungal strains was evaluated both in the absence and presence of a low concentration (0.5% w/v) of dextrose (Sigma-Aldrich, USA). Dextrose was dissolved in the water to moisten the fibers. After inoculation in the center of the plate with the 6-mm cylindrical mycelial plugs (obtained as previously described), the mixtures were incubated at 28°C for 15 days. Thus, three strains were assayed for kraft pulp bleaching in four different conditions (size particle/dextrose), generating 12 different treatments (Table 1). All assays were performed in triplicate.

The kappa numbers of the treated pulp were characterized following the Tappi method (Tappi T-264 om-88). The kappa number is defined as the volume of KMnO_4 (0.1 N) solution consumed by 1 g of moisture-free pulp in an acidic medium during the following reactions, depending on the acidity of the medium. The approximate % of lignin in kraft pulp samples before and after fungal treatments was determined using the formula: lignin level (%) = kappa number $\times$ 0.13 (<https://ipstesting.com/find-a-test/tappi-test-methods/tappi-t-236-kappa-number/>). All determinations were run in triplicate, the resulting data were averaged, and the standard error of the results was

calculated using GraphPad Prism version 6. Data were compared by analysis of variance (ANOVA) with Tukey's multiple comparisons test. The results are reported as the mean $\pm$ standard deviation (SD). p values <0.05 were considered significant.

Kraft pulp fibers inoculated with fungal strains were sampled after 7 and 15 days of fungal growth on the pulp. The samples were overlaid with copper and examined by SEM using a JEOL JSM-7600F microscope operated at 20–30 keV.

Determination of fungal extracellular ligninolytic enzyme activities

Basal Lac, MnP, and LiP activities were determined in 50 mL of PDB in 250-mL Erlenmeyer flasks. Induced activities were measured in the same conditions, but PDB was supplemented with 150 μM of CuSO_4 and 2% (w/v) of intact kraft pulp fibers in independent assays. In all these assays, the flasks were inoculated with three 6-mm diameter mycelium plug inocula, obtained as previously described, and incubated at 28 °C with agitation at 120 rpm for 15 days. Enzyme activities were determined every 24 h from aliquots of extracellular medium obtained under sterile conditions. All experiments were conducted in triplicate.

The determination of extracellular laccase enzyme activity was performed by quantifying the formation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) radical (ABTS^{1+}), according to (Nagai et al. 2002) using 100 μL of the supernatant of the centrifuged ($1500\times g$ for 10 min) culture medium as the enzyme source. Enzyme activity was quantified spectrophotometrically at 420 nm using an ϵ of 36/M/cm for ABTS. LiP enzyme activity was determined by quantifying the oxidation of veratryl alcohol to veratraldehyde according to (Tien and Kirk 1988) with 50 μL of 5 mM H_2O_2 as initiator and 200 μL of the supernatant of the centrifuged enzyme reaction culture medium, as described previously. The enzyme activity was quantified spectrophotometrically at 310 nm using an ϵ of 9.3/M/cm for veratraldehyde. MnP enzyme activity was determined by quantifying the formation of the manganese-

Table 1 Experimental design for kraft pulp bleaching assays

Treatment	CMU/TA01	CMU/84-13	CMU/47-13	Total ^a
Intact pulp	T01	T05	T09	
Fragmented pulp	T02	T06	T10	
Intact pulp + dextrose 0.5% (w/v)	T03	T07	T11	
Fragmented pulp + dextrose 0.5% (w/v)	T04	T08	T12	
Total by strain ^a	4 (12)	4 (12)	4 (12)	12 (36)

^a Each treatment was tested in triplicate. The numbers in parenthesis represent the total assays by strain and the total for all strains

tartrate complex in the presence of 10 mM 2,6-dimethoxyphenol according to Wariishi et al. (1992), with 50 μ l of 5 mM H₂O₂ as the initiator of the enzyme reaction and 300 μ l of the supernatant of the centrifuged culture medium as described previously. The enzyme activity was quantified spectrophotometrically at 334 nm using an ϵ of 18.3/M/cm for manganese tartrate.

An enzyme activity of 1 U is defined as the amount of enzyme that oxidizes 1 mmol of substrate per minute. All chemicals for enzyme activity assays were purchased from Sigma-Aldrich (USA), and the spectrophotometric scans occurred in a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). All enzyme activity assays were run in triplicate, and statistical analysis was conducted as previously described.

Results and discussion

Phylogenetic analysis

Phylogenetic analysis identifies strain CMU-84/13 as *Irpex lacteus* (Fig. 1a). The ITS sequence of strain CMU-84/13 displays high bootstrap/posterior probability (50.8/0.62) values with sequences of *I. lacteus* strains from different geographic origins. Phylogenetic analysis identifies the ITS sequence of strain CMU-47/13 as *Phlebiopsis* sp. (Fig. 1b). It is difficult to assign strain CMU-47/13 to a specific species, because the phylogenetic tree places it in an independent clade between *P. gigantea* and *P. flavidoalba* (Fig. 1b); thus, although this strain may be associated with the former species with high bootstrap/posterior probability (51.2/0.84), further analysis using additional genetic regions is necessary for more accurate identification of the strain. Finally, the CMU-TA01 strain groups with the sequences of various geographic isolates of *Trametes versicolor*, with values of 56.9 and 0.95 for bootstrap and posterior probability, respectively (Fig. 1c).

Phylogenetic analysis showed that all studied strains belong to the order Polyporales, a complex group including white-rot and brown-rot species for which six major clades have been identified (Floudas and Hibbett 2015). The analyzed strains are distributed within two of these clades: the CMU-TA01 (*T. versicolor* complex) strain lies within the Core Polyporoid clade, while the CMU-84/13 (*I. lacteus*) and CMU-47/13 (*Phlebiopsis* sp.) strains are within the phlebioid clade. The last group is further subdivided into the sub-clades *Phlebia*, *Byssomerulius*, and *Phanerochaete* (Floudas and Hibbett 2015), and strains CMU-84/13 and CMU-47/13 belong to these latter groups, respectively.

While phylogenetic analysis provides strong support for the species identification of the CMU-84/13 (*I. lacteus*)

strain, unambiguous species identification was not possible for the CMU-47/13 (*Phlebiopsis* sp.) strain. A recent study using four data set genes indicates that what was formerly the *T. versicolor* complex is now comprised of the species *T. versicolor*, *T. ochracea*, *T. pubescens*, and *T. ectypa* (Carlson et al. 2014). However, while the latter species has a restricted geographic distribution and is not found in Mexico, the previous three have a wide distribution in temperate forests of the northern hemisphere; *T. versicolor* is most common (Carlson et al. 2014). Furthermore, *T. versicolor* and *T. ochracea* have high ITS sequence similarity (98–99%), and the latter species has never been collected in the forest where the strains used in this study were obtained. Thus, taken together, both ecological and molecular data strongly favor the identification of CMU-TA01 as *T. versicolor*.

Kraft pulp modifications originated by fungal strains

Preliminary tests conducted in solid media were used to determine whether the studied strains were able to decolorize the three structurally different phenolic dyes indigo carmine (indigoid), methyl orange (azo), and acid fuchsin (triphenylmethane); strains that more efficiently decolorized the latter two compounds (Fig. 2) are also better for bleaching kraft pulp and reducing its kappa number, as shown below. The previous experimental evidence demonstrates that MnP is the main enzyme—not Lac or LiP—involved in the decolorization of azo dyes (Yao et al. 2013; Qin et al. 2017). In addition, crude extracts of MnP from *I. lacteus* can decolorize efficiently triphenylmethane dyes (Yang et al. 2016). These observations indicate that the use of azo/triphenylmethane dyes is adequate for selecting delignification fungi able to produce high levels of MnP. Recently, it has been shown the efficiency of decolorization assay in solid medium using Azure B to select white-rot fungi producers of LiP (Kinnunen et al. 2017); this clearly reflects the relevance of using a set of appropriate dyes for selecting fungi with the desired ligninolytic enzyme activities.

Fiber length is an important factor that influences paper strength properties (Carrillo et al. 2017). For this reason, the relationship between fiber length and other fiber parameters has been studied to optimize the pulping process to obtain paper with the desired characteristics, reduce energy consumption, or obtain novel fiber characteristics (Zhang et al. 2016; Mejía-Ballesteros et al. 2017). Thus, we analyzed fungal bleaching and kappa number reduction for two fiber sizes to determine whether there was a difference in both processes associated with that parameter. With simple solid-state fermentation assays, it was possible to observe that the tested fungal strains bleach kraft pulp with

Fig. 1 Phylogenetic analysis of studied fungal strains. The trees were generated according the maximum parsimony (MP) criteria and Bayesian inference (BI) using the ITS (ITS1-5.8S-ITS2) rDNA region. Only MP trees are shown, because BI trees are congruent with this grouping pattern. Shown is one of 20 and of 4 equally parsimonious trees for strains CMU-84/13 (a) and CMU-47/13 (b), respectively, and the single tree obtained for strain CMU-TA01 (c). The bootstrap values (1000 replicates)/Bayesian posterior probabilities above 50/0.5 are shown at each node. For each sequence retrieved from the GenBank, its accession number follows the name of the species

varying efficiency depending on the incubation conditions. While strains did not display significant bleaching in assays supplemented with dextrose (0.5% w/v), those grown in media without a carbon supply displayed the best bleaching results (Fig. 3). Particle size did not affect bleaching efficiency, because fragmented pulp fibers were decolorized with the same efficiency as that observed in intact fibers (Fig. 3). The strongest bleaching activity was obtained with *Phlebiopsis* sp. (CMU-47/13). The decreasing order of bleaching efficiency for the treatments was T9 (CMU-47/13) > T1 (CMU-TA01) > T3 (CMU-TA01).

The kappa number of the untreated kraft pulp fiber was 13.23, and the fungal treatments resulted in kappa numbers ranging from 10.41 in T05 with strain CMU-84/13 of *I. lacteus* to 6.83 in T09 with strain CMU-47/13 of *Phlebiopsis* sp.; T09 was the best treatment overall, followed by T01 and T03 with the CMU-TA01 strain of *T. versicolor* (Table 2); these corresponded to reductions in the kappa number of 48, 43, and 41%, respectively. Other treatments with different strains did not reduce the kappa

number by more than 40%, and *I. lacteus* had its best result with a kappa number of 8.02 in T07, which represents a 39% reduction in the original value (Table 2). The approximate percentage of lignin content in treated pulp declines according with the reduction in kappa number, from 1.71% in the non-treated fiber to 0.88% in the best treatment result (T09) and to 1.35% in the worst case (T05), as shown in Table 2.

Solid-state fermentation assays for evaluating kraft pulp bleaching are usually conducted in Erlenmeyer flasks involving liquid cultures for inoculum preparation (Katagiri et al. 1995; Afrida et al. 2009), but this approach becomes complicated when handling large numbers of strains. Simpler solid-state fermentation bleaching assays, based on incubating strains directly on kraft pulp in a Petri dish, allow for daily visual corroboration of the progress of the bleaching process. Furthermore, the use of an inoculum plug taken from a fungal colony in the log phase of growth from solid medium avoids growing fungi in liquid medium, a procedure that involves more work and resources than the use of Petri dishes. The fungal evaluation protocol we present here uses solid medium and Petri dishes from inoculum generation to bleaching via intermediate dye decolorization/guaiacol oxidation assays. It is thus less expensive and simpler than previously reported protocols (Katagiri et al. 1995; Afrida et al. 2009), and is appropriate for evaluating a large number of strains. Recent strategies to select fungal strains able to bleach lignocellulose include variations in the composition of media, alternative plant biomass, and miniaturized activity assays, reflecting the

Fig. 2 Decolorization/oxidation of phenolic compounds by analyzed strains. Assays were conducted in potato dextrose agar (PDA) and malt extract agar (MEA) at 28 °C for 10 days. The key of the fungal strains is shown at the top of each column. Control dishes (C) without fungal inoculation are shown in the first column. The initial color for

each phenolic compound was the same for PDA and MEA, and thus, only one control plate was shown for both media. Phenolic compounds tested (250 µg/mL) were indigo carmine (IC), methyl orange (MO), acid fuchsin (AF), and guaiacol (G)

Fig. 3 Kraft pulp bleaching by studied fungal strains. Bleaching is shown in intact kraft pulp from a processing plant (IP) and in blended fragmented pulp (FP) alone or with 0.5% (w/v) of dextrose added (IP + G and FP + G). The CMU code strain is shown at the top of each column. Cultures were incubated at 28°C for 15 days

Table 2 Kappa number of kraft pulp after treatment with fungal strains

Treatments	Kappa no. ^a	% Lignin ^b	% Reduction ^c
Control	13.23 (0.13)	1.71	–
T01	7.53 (0.25)	0.97	43.08
T02	9.05 (0.04)	1.17	31.59
T03	7.78 (0.12)	1.01	41.19
T04	9.55 (0.11)	1.24	27.81
T05	10.41 (0.29)	1.35	21.31
T06	8.59 (0.14)	1.11	35.07
T07	8.02 (0.09)	1.04	39.38
T08	9.13 (0.07)	1.18	30.99
T09	6.83 (0.10)	0.88	48.37
T10	8.60 (0.09)	1.11	34.99
T11	8.25 (0.11)	1.07	37.64
T12	8.19 (0.09)	1.06	38.09

^a The values in parentheses are $\pm$ the standard deviation obtained from triplicate analyses

^b The value is the approximate content estimated by the formula: lignin level (%) = kappa number $\times$ 0.13, as described in “Materials and methods”

^c Values are the % decrease in kappa number with respect to the control. All treatments showed statistically significant reductions compared to control fiber ($p < 0.05$)

need for further simplification and improvement of protocols (Kinnunen et al. 2017).

To the best of our knowledge, information about the bleaching and kappa number reduction of kraft pulp treated with *Phlebiopsis* spp. had not been previously reported, and

we found that this taxa could be as efficient for kappa number reduction (48.3%, Table 3) as the well-studied white-rot species of the genera *Trametes*, *Phanerochaete*, and others (Takano et al. 2001; Afrida et al. 2009). Even the kappa number reductions obtained with the CMU-84/13 strain of *I. lacteus* were comparable to previously reported values (Table 3). This result strengthens the argument in favor of searching for new species/strains for biopulping and biobleaching, an approach that has contributed to the discovery of promising strains for this biotechnological application, even from taxa not ecologically associated with wood decomposition (Osés et al. 2006; Martín-Sampedro et al. 2015). The strain CMU-84/13 bleaches kraft pulp and reduces its kappa number by approximately 39%. The single previous study of *I. lacteus* showed that a strain was able to reduce by 46% the kappa number of kraft pulp from *A. mangium* wood (Afrida et al. 2009). However, the fungal-treated pulp was oxygen-delignified, and the treatment time was 1 week longer than in the present work (Table 3). In the case of *T. versicolor*, the reported percentages of kappa number reduction for solid-state fermentation assays range from 9.7 to 49.6%, depending on the type of pulp and whether the pulp was chemically treated (Table 3). It should be noted that the study with *T. versicolor* that achieved a better kappa number reduction than that obtained in the present work (Table 3) used a micronutrient- and vitamin-supplemented medium (Kata-giri et al. 1995) and longer incubation times (Afrida et al. 2009). Interestingly, the studied strain of *I. lacteus* showed a higher reduction in kappa number under low dextrose and intact pulp, whereas *T. versicolor* and *Phlebiopsis* sp. showed a higher reduction for intact pulp without added dextrose. These results indicate that the white-rot fungi which we evaluated might delignify kraft pulp with minimal processing, without special nutrient conditions or added mediators. Recent work performed with *T. versicolor* (sin. *Coriolus versicolor*) shows their ability to grow in commercial kraft lignin-based agar medium supplemented with dimethyl sulfoxide (DMSO) to solubilize lignin (Brzonova et al. 2017). This antecedent and the results here obtained demonstrate that white-rot basidiomycetes could grow and delignify kraft pulp in solid supports with very harsh nutrimental conditions. Overall, the previously discussed data clearly indicate that further study is needed to evaluate the minimal nutrimental conditions in which white-rot fungi can efficiently bleach kraft pulp under SSF.

Microscopic observations indicate extensive colonization of the cellulose kraft fiber surface by the studied fungi. SEM images show that the fungi initially colonize the fiber to cause erosion; eroded areas are subsequently filled with mycelia. Fiber surface modifications are observed after 1 week of fungal growth, with structural changes including

Table 3 Kappa number reductions reported for strains of the same species used in this work

Fungal taxa	Tree pulp	Incubation time	Kappa number			References
			Control	Treated ^a	−Δ (%)	
<i>T. versicolor</i>	Hardwood UKP	6 days	15.5	7.8	49.6	Katagiri et al. (1995)
	Hardwood UKP	10 days	15.7	12.9	17.8	Takano et al. (2001)
	<i>Acacia mangium</i> ODKP	3 weeks	7.6	4.1	46	Afrida et al. (2009)
	<i>Pinus/Eucalyptus</i> UKP	2 weeks	13.2	7.5	43	This study
<i>I. lacteus</i>	<i>Acacia mangium</i> ODKP	3 weeks	7.6	4.1	46	Afrida et al. (2009)
	<i>Pinus/Eucalyptus</i> UKP	2 weeks	13.2	8	39.3	This study
<i>Phlebiopsis</i> sp.	<i>Pinus/Eucalyptus</i> UKP	2 weeks	13.2	6.8	48.3	This study

ODKP oxygen-delignified kraft pulp, UKP unbleached kraft pulp

^a Only included are the mean best (lowest) value of kappa numbers after fungal treatment in solid-state fermentation assays reported in each reference

roughness and striation of the otherwise smooth fiber surface (Fig. 4); other obvious modifications include the presence of clefts and grooves distributed through the fiber surface (Fig. 4). For *Phlebiopsis* sp. (CMU-47/13), a loosening of the fiber is observed (Fig. 4c).

Kraft pulp structural modifications similar to those described here were previously observed by SEM using laccase with different mediator systems. An increase in roughness and striation was observed in kraft pulp when treated with the laccase of *Aspergillus fumigatus* and the mediator *N*-hydroxybenzotriazole (Vivekanand et al. 2008). Kraft pulp treated with the commercial laccase of *Aspergillus oryzae* and the mediator methyl syringate became rough and increased its surface adhesion (Liu et al. 2009b). Treatment of eucalyptus kraft pulp with the laccase of the white-rot basidiomycete *Panus conchatus* with the mediator 2,2,6,6-tetramethylpiperidine-1-oxy radical

(TEMPO) changes the fiber surface from smooth to textured and increases its fibrillation (Zhang et al. 2016). Thus, the observed structural modifications to kraft pulp fibers are consistent with those previously documented to improve fiber characteristics for paper production.

Ligninolytic enzyme activities

The upstream regulatory regions of genes coding for ligninolytic enzymes have been analyzed in different white-rot fungal species, but there are more detailed descriptions in this regard for Lac and MnP than for LiP (Janusz et al. 2013). In particular, both the metal response (MREs) and xenobiotic-responsive (XREs) elements have been described for the regulatory regions of Lac and MnP genes; the former respond to divalent cations, and the latter will induce transcriptional levels of Lac and MnP in

Fig. 4 SEM microphotographs of kraft pulp sections treated with the studied fungal strains. Fibers were incubated at 28°C for 7 (upper pictures) and 15 (bottom pictures) days with the strains CMU-84/13 (b), CMU-47/13 (c), and CMU-TA01 (d). **a** Control fiber incubated at

the same temperature without fungal inoculation. Roughness, clefts, holes, and striation not present in control fiber are evident in fungal-treated fibers. Bar = 1 μm

Fig. 5 Ligninolytic enzyme activities of studied fungal strains. Strains CMU-TA01 (**a**), CMU-47/13 (**b**), CMU-84/13 (**c**) were incubated in PDB (**A**), PDB plus 100 μ M of CuSO_4 (**B**), and PDB

supplemented with 0.5% (w/v) of kraft pulp (**C**) at 28°C and 120 rpm. Each experiment was conducted in triplicate

response to phenolic, lignin-related compounds. In addition, the catabolite repressor (CreA) element has been found in the regulatory regions of both genes and is responsible for low enzyme activities in the presence of glucose. Thus, in the present work, we evaluate the induction of ligninolytic enzymes in the studied strains to determine their respective responses to both Cu^{2+} and kraft lignin.

The *T. versicolor* (CMU-TA01) strain was the only one showing activity of three ligninolytic enzymes in all assayed culture conditions; *I. lacteus* (CMU-84/13) and *Phlebiopsis* sp. (CMU-47/13) showed no Lac activity and low LiP activity (Fig. 5). *T. versicolor* also shows the highest levels of all ligninolytic activities in most assayed

conditions, including basal (68.37 ± 4.35 U/mL) and CuSO_4 -induced (495.76 ± 9.71 U/mL) Lac activity, as well as basal (54.73 ± 2.34 U/mL) and CuSO_4 -induced (130.08 ± 14.41 U/mL) MnP activity (Table 4). The *I. lacteus* strain was the poorest producer of the three ligninolytic enzymes in most liquid culture assays, but showed the highest CuSO_4 -induced (71.00 ± 5.11 U/mL) LiP activity. Addition of CuSO_4 to PDB significantly increases the activity of the ligninolytic enzymes detected in each of the studied strains (Fig. 5). In this culture condition, a maximal increase in Lac activity occurs in the *T. versicolor* (CMU-TA01) strain (7.25-fold); for MnP, the greatest increase was in the *T. versicolor* (CMU-TA01) strain (2.37-fold), and for LiP, the greatest increase was for the *I.*

Fig. 5 continued

lacteus (CMU-84/13) strain (6.95-fold) (Table 4). The addition of kraft pulp to the medium differentially increases the ligninolytic enzyme activities, but at a lower magnitude than CuSO_4 ; Lac and MnP show significant increases for *T. versicolor*, MnP and LiP for *Phlebiopsis* sp., and none for *I. lacteus* (Fig. 5; Table 4).

Irpex lacteus has been reported to show three distinct ligninolytic activities measured here when grown in liquid media (Rothschild et al. 2002; Kasinath et al. 2003). The absence of extracellular Lac activity that we observed in the presence of lignocellulosic substrates was previously reported (Xu et al. 2009), and some studies documented low levels or no activity of extracellular LiP and MnP in the presence of lignocellulosic substrates (Afrida et al. 2014). In the only previous work reporting ligninolytic enzyme activities of *I. lacteus* grown with kraft pulp, the

used strain produced high MnP, low Lac, and no LiP (Afrida et al. 2014), in agreement with our results. Although in *I. lacteus*, MnP produced preferentially over Lac and LiP, some cultivation conditions increase LiP activity significantly (Rothschild et al. 2002; Kasinath et al. 2003; Sklenar et al. 2010). It was recently reported that two heterologously expressed MnP isoenzymes from *I. lacteus* are able to efficiently oxidize non-phenolic lignin model compounds; the obtained results suggest that this fungal species may use its MnPs with a particular organic acid(s) that it excretes, possibly malonate or oxalate, to degrade more recalcitrant lignin (Qin et al. 2017). A bioinformatics analysis of the upstream regulatory region of genes for both isoenzymes shows the presence of CreA- and XRE-binding sites (Qin et al. 2017), which explains the low levels of MnP in PDB basal medium and the

Fig. 5 continued

increase in activity in PDB supplemented with KP that we observed. Although no MREs were reported by Qin et al. (2017) in the regulatory region of both MnP genes, we conducted an evaluation of the 1-kb upstream regions that they reported and found the motif 5'-TGCGCAAC-3' (position -193 to -186) for the *IIMnP1* and 5'-TGCA-CAA-3' (position -128 to -122) for *IIMnP2* isozyme genes, which resembles the 5'-TGCRNC-3' (R = A/G) consensus described as MRE in the regulatory region of the MnP gene of several white-rot fungi (Janusz et al. 2013). These putative MREs could explain the significant increase with respect to the basal PDB medium of MnP activity in the CMU-84/13 strain in the presence of CuSO₄. No genomics analysis in the available genome has been conducted to determine the number of LiP isoenzymes and

their regulatory regions; this information might improve their experimental production. Thus, although *I. lacteus* is the poorest producer of extracellular ligninolytic enzymes of the three studied strains, genomics and other omics tools might help to improve its performance in biopulping and biobleaching.

The absence of Lac, low LiP, and high MnP activities in the *Phlebiopsis* sp. strain documented in this study is supported by recent genomic evidence that shows the absence of Lac sensu stricto genes in the related species *P. gigantea* but the presence of 3 and 7 genes for LiP and MnP, respectively (Hori et al. 2014). When this species is incubated with grass biomass, it presents similar enzymatic activity patterns (Baker et al. 2015) as those shown here for *Phlebiopsis* sp. in the presence of kraft pulp. However, no

Table 4 Maximal basal and induced enzyme activities (U/mL) in the fungal studied strains

Media	Enzyme	Strain ^a		
		<i>T. versicolor</i> (CMU-TA01)	<i>Phlebiopsis</i> sp. (CMU-47/13)	<i>I. lacteus</i> (CMU-84/13)
PDB	Lac	68.37 (±4.35)	ND ^b	ND
	MnP	54.73 (±2.34)	46.75 (±0.87)	12.65 (±0.73)
	LiP	33.90 (±1.99)	12.97 (±0.40)	10.21 (±0.65)
PDB + CuSO ₄	Lac	495.76 (±9.71) 7.25 ^b	ND	ND
	MnP	130.08 (±14.41) 2.37	103.44 (±3.24) 2.21	18.65 (±1.82) 1.47
	LiP	43.58 (±3.75) 1.28	38.85 (±3.36) 2.99	71.00 (±5.11) 6.95
	Lac	99.94 (±4.58) 1.46	ND	ND
	MnP	82.96 (±2.99) 1.5	75.69 (±7.94) 1.61	12.75 (±0.99) ^c 1.00
	LiP	39.06 (±4.66) ^c 1.15	22.83 (±1.05) 1.76	8.99 (±1.27) ^c 0.88

ND non-detected

^a Mean ± standard deviation are shown

^b Numbers below enzymatic activity data in PDB + CuSO₄ and PDB + KP media correspond to the times each activity increased in comparison with the PDB basal medium

^c Cases in which no significant differences were found relative to their respective controls in the PDB basal medium ($p < 0.05$)

previous work has been conducted on the ability of species of *Phlebiopsis* to produce ligninolytic enzymes in the presence of kraft pulp; thus, more research is necessary to explore the biotechnological potential of these taxa in biopulping and biobleaching.

The presence of Cu²⁺ significantly increases Lac and MnP activities with respect to PDB not supplemented with this cation in *T. versicolor* (Lebrun et al. 2011). In contrast, transcriptome analysis in the presence of poplar wood has shown up-regulation of eight LiP genes, and down-regulation of one Lac and MnP gene each (Zhang et al. 2017). However, genomics analysis has documented the presence of 26 class II peroxidase (MnP- and LiP-related) and 10 multicopper oxidase (Lac-related) genes in *T. versicolor* (Floudas et al. 2012), clearly indicating that different culture conditions in the presence of wood and kraft lignin must be used to increase the ligninolytic potential in such species. *T. versicolor* secretes LiP, MnP, and Lac enzymes in the presence of wood substrates (Liew et al. 2011; Martínez-Morales et al. 2015), but there is a little research into the induction of these ligninolytic enzymes when grown in the presence of kraft pulp. Solid-state fermentation studies of *T. versicolor* conducted in kraft pulp supplemented with nitrogen-limited, but glucose-rich medium resulted in an increase in MnP activity (Katagiri et al. 1995). It has been documented that *T. versicolor* grown in kraft pulp attains very high Lac and MnP activities (Bermek et al. 2002).

Brzonova et al. (2017) showed that *T. versicolor* was able to grow in liquid media containing only commercial kraft pulp, using and modifying lignin, and that such modification significantly increased by adding dimethyl sulfoxide (DMSO) to the culture medium to increase lignin solubility. DMSO did not inhibit mycelial growth and enhanced Lac, MnP, and LiP activities. This remarkable kraft pulp transformation with excellent extracellular ligninolytic enzyme production was achieved using a strategy named by Brzonova et al. (2017) as quasi-immobilized conditions, consisting in using as inoculant agar blocks containing mycelium pre-growth in solid lignin medium at low agitation velocity. Such previous work, and the SSF assays we present here, highlights that it is important to conduct kraft pulp bleaching and delignification assays using renewed experimental approaches. It thus seems that kraft pulp bleaching optimization for economically and efficiently applying fungi for lignin treatment is far from complete.

Conclusion

Both the decolorization/oxidation and solid-state fermentation bleaching assays we conducted in Petri plates are good indicators to select fungi that can delignify kraft pulp.

The *Phlebiopsis* sp. strain exhibited a high delignification capability for kraft pulp, which is described here for the first time. Our results are consistent with previous work indicating that, in some culture conditions, MnP is more relevant for the bleaching and kappa number reduction of kraft pulp than are LiP and Lac (Katagiri et al. 1995; Afrida et al. 2014). However, better results were observed with *Phlebiopsis* sp. than *T. versicolor*, which produces similar levels of MnP; this could indicate that other enzyme activities not evaluated in this study have a role in the delignification process. The white-rot fungi which we evaluated might delignify kraft pulp with minimal processing, without special nutrient conditions, and without added mediators. However, further study is needed to evaluate the optimal conditions for pulp bleaching by the studied strains; for example, it will be important to increase production of MnP using a well-designed protocol for manipulating the composition of culture medium and growth conditions, as has been done for other white-rot fungi (Martani et al. 2017) and for ascomycetes to produce hydrolytic enzymes (Kumar et al. 2017). Treatment with oxidative enzymes in combination with thermostable hydrolytic enzymes from other fungal species (Kumar and Shukla 2016) might also be evaluated for improvement of kraft pulp bleaching. Taken together, the data that we present here suggest that fungal strains CMU-TA01, CMU-47/13, and CMU-84/13 have biotechnological potential in the wood and paper industries. This strengthens the argument for a continued search for new species, and strains of previously studied species, for biopulping and biobleaching, an approach that has contributed to the discovery of promising tools for this biotechnological application.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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