Research article

Pure lignin induces overexpression of cytochrome P450 (CYP) encoding genes and brings insights into the lignocellulose depolymerization by *Trametes villosa*

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ABSTRACT

Trametes villosa is a remarkable white-rot fungus (WRF) with the potential to be applied in lignocellulose conversion to obtain chemical compounds and biofuels. Lignocellulose breakdown by WRF is carried out through the secretion of oxidative and hydrolytic enzymes. Despite the existing knowledge about this process, the complete molecular mechanisms involved in the regulation of this metabolic system have not yet been elucidated. Therefore, in order to understand the genes and metabolic pathways regulated during lignocellulose degradation, the strain *T. villosa* CCMB561 was cultured in media with different carbon sources (lignin, sugarcane bagasse, and malt extract). Subsequently, biochemical assays and differential gene expression analysis by qPCR and high-throughput RNA sequencing were carried out. Our results revealed the ability of *T. villosa* CCMB561 to grow on lignin (AL medium) as the unique carbon source. An overexpression of Cytochrome P450 was detected in this medium, which may be associated with the lignin O-demethylation pathway. Clusters of up-regulated CAZymes-encoding genes were identified in lignin and sugarcane bagasse, revealing that *T. villosa* CCMB561 acts simultaneously in the depolymerization of lignin, cellulose, hemicellulose, and pectin. Furthermore, genes encoding nitroreductases and homogentisate-1,2-dioxygenase that act in the degradation of organic pollutants were up-regulated in the lignin medium. Altogether, these findings provide new insights into the mechanisms of lignocellulose degradation by *T. villosa* and confirm the

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ability of this fungal species to be applied in biorefineries and in the bioremediation of organic pollutants.

1. Introduction

The lignocellulosic biomass (LB), one of the main sources of carbon and energy on Earth, has increased as a potential raw material to obtain biofuels, biochemicals, and biomaterials [1–3]. LB is also a promising input for replacing non-renewable fossil fuels (coal, oil, and natural gas) and reducing greenhouse gas emissions [4]. The estimated annual world production of lignocellulosic wastes is 181.5 billion tons; however, only a small portion of that amount (8.2 billion tons) is annually used [5,6]. Lignocellulose is mainly composed of cellulose, hemicellulose, and lignin, and the proportion of each biopolymer varies according to the plant species (source of biomass) [7–9].

Cellulose is a natural and linear carbohydrate composed of D-glucose residues linked by β-1,4-glycosidic bonds [7,10,11]. Hemicellulose is chemically characterized as a branched heteropolymer composed of pentoses (D-xylose and D-arabinose) and/or hexoses (D-mannose, D-glucose, and D-galactose). This polymer is often acetylated and its branches are composed of L-arabinose, D-xylose, D-galactose, uronic acid, ferulic acid, and fucose monomers [7,10,11]. Lignin is a phenolic polymer composed of three phenylpropane units called monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, linked by C–C, ester, and ether bonds [12]. This is the most recalcitrant and external compound of the plant cell wall and is associated with cellulose and hemicellulose through hydrogen and covalent bonds, forming a heterogeneous structure [7,10,13,14].

The depolymerization and conversion (e.g., fermentation) of lignocellulosic biomass can result in a plethora of marketable chemicals, such as biobased ethanol, biomethane, lactic acid, succinic acid, xylitol, sorbitol, phenols, vanillin, vanillic acid, among others, which have high-value for different economic sectors, such as in food, biofuels and pharmaceutical industries [3,15,16]. Nevertheless, the main challenge for lignocellulose to be widely used is the depolymerization of the highly recalcitrant lignin, which limits the saccharification of cellulose and hemicellulose to monomeric compounds (e.g., glucose and xylose) [3,7,15,17,18].

A select group of organisms known as wood-decay fungi has the potential to efficiently convert recalcitrant lignocellulosic biomass into monomers through the production of oxidative and hydrolytic enzymes [10,18–20]. Wood-decay fungi play a central role in carbon cycling in forest ecosystems and are mainly classified as white-rot or brown-rot fungi, depending on the type of degradation carried out [10,19,21].

Brown-rot fungi (BRF) depolymerize preferentially cellulose and hemicellulose, while the lignin fraction is modified only to some extent [19,22]. White-rot fungi (WRF) have the ability to breakdown all wood constituents simultaneously or selectively, with some species showing both degradation patterns [19,23]. WRF are considered the most effective lignocellulosic degraders due to their ability to depolymerize a large amount of lignin to CO_2 and H_2O, facilitating the access of cellulases and hemicellulases to the carbohydrates of the plant cell wall [22].

White-rot fungi degrade lignin by secreting oxidative enzymes that are classified as oxidases and peroxidases, which belong to auxiliary activity oxidoreductases families (AA) [24]. Oxidases include laccases (AA1 family), and peroxidases include H_2O_2-dependent enzymes (AA2): Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Versatile Peroxidase (VP). Cellulose is degraded by enzymes belonging to the glycoside hydrolases (GH) and AA families; hemicelluloses by enzymes belonging to the AA, carbohydrate esterases (CE), and GH families; and pectin by CE, GH, and pectate lyases (PL) families [11,24–26]. These enzyme families are classified according to the CAZy database (Carbohydrate Active Enzymes database) and besides acting in the degradation of biomass, they can also be applied to the bioremediation of organic pollutants and xenobiotics without producing toxic byproducts [25–27].

Trametes villosa is a WRF, and prior studies have demonstrated its amazing potential to effectively act in the degradation of lignin. Silva et al. (2014) [28] demonstrated that crude enzymatic extract produced from the isolate *T. villosa* CCMB561 can efficiently act in the delignification of agro-industrial residues. This activity was related to the high capacity of the fungi to produce Manganese Peroxidase (MnP), which was reached by optimizing the cultivation conditions. Similarly, Carneiro et al. [29] showed that *T. villosa* CCMB561 is able to produce Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) in culture media containing sugarcane bagasse as carbon source. Studies with other *T. villosa* isolates also demonstrated the potential of this fungal species to produce peroxidases and cellulases [30–32].

Recently, the complete genome of *T. villosa* CCMB561 was sequenced using the high-throughput sequencing platforms Illumina HiSeq and Oxford Nanopore MinION (hybrid assembly strategy) [33]. The obtained high-quality genome revealed an arsenal of genes encoding peroxidases, cellulases, hemicellulases, and pectinases that act synergistically in the degradation of all lignocellulose polymers. Furthermore, the comparative genomic analyses showed that *T. villosa* CCMB561 was the species with the highest number of genes encoding lignin-modifying enzymes when compared to other species of the same genus [33].

Therefore, to deeply understand the gene expression profile and metabolic pathways related to lignocellulose degradation in *T. villosa* CCMB561 we cultivated it in pure lignin, sugarcane bagasse, and malt extract. Subsequently, we performed biochemical assays, relative expression analysis by qPCR, transcriptome sequencing, differential gene expression analysis (DGE), and co-expression network analysis. Taking together, the results described here will contribute to the improvement of enzyme production and the use of agro-industrial residues for the production of chemical compounds and biofuels.

2. Material and methods

2.1. Evaluation of Trametes villosa CCMB561 growth and ligninolytic enzymes production

The isolate *T. villosa* CCMB561 was cultivated in six culture media containing different concentrations of alkali lignin (Lot #: MKCD3898, Sigma-Aldrich, St. Louis, Missouri, USA) with or without supplementation (Table 1). The capacity to produce ligninolytic enzymes was evaluated using the Remazol Brilliant Blue R dye (RBBR, Sigma-Aldrich, St. Louis, Missouri, USA), which was added to all media at a final concentration of 0.02%. The lignin concentration was determined based on the amount of lignin present in sugarcane bagasse, according to Carneiro et al. [29]. The media were transferred to 90 × 15 mm Petri dishes and the fungal isolate was inoculated and incubated at 28 ± 2 °C for seven days. The inoculum was obtained from the CCMB561 isolated cultivated in MEA culture medium (2% malt extract, 2% dextrose, and 2% agar) for seven days at 28 ± 2 °C. After the growth, one culture piece with fungal mycelium (~5 mm diameter) was removed and transferred to the center of the new plate. All the experiments were carried out with six replicates per culture medium (36 experiments in total), and the discoloration halo was measured on the third, fifth, and seventh days of incubation. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA) applying analysis of variance (ANOVA) followed by the post-test of Bonferroni.

2.2. Trametes villosa CCMB561 growth conditions for enzymatic quantification, relative expression analysis by qPCR and transcriptome sequencing

The isolate *T. villosa* CCMB561 was inoculated into 50 mL Erlenmeyers flasks containing the following liquid culture media: AL (0.12 g of alkali lignin and 30 mL of water), BG (0.6 g of sugarcane bagasse and 30 mL of water) and MEA (0.015 of yeast extract, 0.06 g of malt extract and 30 mL of water). The inoculum was obtained from the CCMB561 isolated cultivated in MEA culture medium (2% malt extract, 2% dextrose, and 2% agar) for seven days at 28 ± 2 °C. After the growth, five culture pieces with fungal mycelium (~5 mm diameter) were removed from the Petri dish and transferred to the Erlenmeyer. The AL media was selected based on the results obtained from the previously described experiment (section 2.1). The BG media was used as a model condition, since agro-industrial residues are a cheap and available source of lignocellulose to be used on an industrial scale, and MEA, a widely used media for fungal growth, worked as a control condition since it is a non-inducing substrate. After fungal inoculation, the Erlenmeyers were incubated without rotation at 28 ± 2 °C. All experiments were performed with five replicates per culture media.

Biochemical assays, quantitative PCR, and transcriptome sequencing were carried out on the seventh day of growth. This choice was based on the findings of Miyauchi et al. [34] who described that common molecular functions of the fungus *Pycnoporus coccineus* (Current name: *Trametes coccinea*) were triggered in the initial phase of growth (day 3) while specific responses to the lignocellulosic substrates were identified at a later stage (day 7).

2.3. Manganese Peroxidase and laccase quantifications

On the seventh day of cultivation, an aliquot of 10 mL from the culture media (AL, BG, and MEA) was removed from each Erlenmeyer flask and transferred to microtubes of 2 mL. The tubes were centrifuged at 15,300 g for 2 min at 4 °C and the supernatant was used to quantify the enzymes. The Laccase (Lac) activity was determined according to the method described by D'Agostini et al. using the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS-1 mmol. 1–1, $C_{18}H_{24}N_6O_6S_4$, Sigma-Aldrich®) as substrate [35]. The Manganese Peroxidase (MnP) activity was determined through the oxidation of phenol red in the presence of hydrogen peroxide, following the method described by Kuwahara et al. [36]. Absorbances of 420 nm and 610 nm were used to evaluate the activity of Lac and MnP, respectively. The production of both enzymes was expressed in U/L by applying equation U/L = ΔAbs x 10^6/Ɛ x R x t, according to Almeida De Menezes et al. [37]. The statistical analyses were carried out using the software GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA) applying analysis of variance (ANOVA) followed by Tukey's post-test.

2.4. RNA extraction

On the seventh day of cultivation, the fungal mycelium was separated from the culture medium, transferred to 2 mL microtubes, and macerated using zirconium beads in FastPrep (MP Biomedicals, Santa Ana, California, USA). Total RNA was extracted using the Fastzol reagent (Quatro G, Porto Alegre, Rio Grande do Sul, Brazil) according to the manufacturer's recommendations. RNA samples

Table 1

Different culture media compositions used to evaluate *T. villosa* CCMB561 growth capacity and ligninolytic enzymes production.

Culture media	Composition
AL20	2.4 g of agar, 0.48 g of alkali lignin and 120 mL of distilled water.
AL30	2.4 g of agar, 0.72 g of alkali lignin and 120 mL of distilled water.
ALG20	2.4 g of agar, 0.48 g of alkali lignin, 0.6 g of glucose and 120 mL of distilled water.
ALG30	2.4 g of agar, 0.72 g of alkali lignin, 0.6 g of glucose and 120 mL of distilled water.
ALSM	2.4 g of agar, 0.48 g of alkali lignin, 3.048 g of manganese sulfate and 120 mL of distilled water.
ALSA	2.4 g of agar, 0.48 g of alkali lignin, 0.6 g of ammonium sulfate and 120 mL of distilled water

were qualitatively analyzed by agarose gel electrophoresis 1%, and quantitatively by spectrophotometry Nanodrop 1000 ND (Thermo Scientific, Waltham, Massachusetts, USA) and automated capillary electrophoresis TapeStation System (Agilent Technologies, Santa Clara, California, USA).

2.5. Relative expression of genes encoding ligninolytic enzymes by real-time quantitative PCR (qPCR)

2.5.1. Primers design and synthesis

Specific primers (Table 2) for the amplification of Laccase (Lac), Lignin peroxidase (LiP), Manganese peroxidase (MnP), and Actin (actin1) were designed based on the genome and functional annotations of *T. villosa* CCMB561 [33]. The Geneious® software version 9.0.5 (Auckland, New Zealand) and the OligoAnalyzer™ tool (https://www.idtdna.com/) were used to design and analyze the primers, respectively. Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) and tested by conventional PCR and qPCR.

2.5.2. Quantitative polymerase chain reaction (qPCR)

RNA samples were treated with DNase I enzyme (New England BioLabs, Ipswich, Massachusetts, USA), and the complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). Quantitative PCR (qPCR) was performed using the SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, California, USA) and the primers described in Table 2. The PCR reaction was carried out in the Applied Biosystems 7900HT Fast Real-Time PCR System equipment (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the following amplification cycles: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation stage for recording the melting curve. For each growth condition, quantitative PCR was performed for five biological and two technical replicates.

2.5.3. Relative gene expression quantification and statistical analyses

The relative expression of the target genes was analyzed by the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene encoding actin (actin1) as an endogenous reference. Statistical analyses of gene expression were performed using the GraphPad Prism software version 7.00 (GraphPad Software, San Diego, California, USA) applying the analysis of variance (ANOVA) and the Tukey post-test.

2.6. RNA library preparation and transcriptome sequencing

The library preparation was carried out using 1 μg of total RNA from each sample (section 2.4). Initially, the NEBNext Poly(A) mRNA Magnetic Isolation Module kit (New England BioLabs) was used for messenger RNA (mRNA) selection (enrichment) and ribosomal RNA (rRNA) depletion. Non-strand-specific library construction was performed using the NEBNext Ultra™ II RNA Library Prep Kit for Illumina and the NEBNext Multiplex Oligos for Illumina. Single-end sequencing was performed on the NextSeq 500 Sequencing System platform. A total of nine libraries were sequenced, corresponding to the growth of the isolate *T. villosa* CCMB561 in the three different culture media (AL, BG, and MEA) in triplicate.

2.7. Transcriptome analyses

The raw reads quality was assessed using the FastQC software (https://github.com/s-andrews/FastQC). The BBDuk tool was used to remove the adapters and trimming bases with a Phred score <30 (https://sourceforge.net/projects/bbmap/). The trimmed reads were mapped to the genome of *T. villosa* CCMB561 to verify the percentage of reads mapped using the Bowtie2 software [38]. Differential gene expression (DGE) analysis was performed according to the protocol described by Yalamanchili et al. [39]. Briefly, reads were mapped to the reference genome using the Tophat2 software [40] with the support of the Bowtie2 program. Subsequently, the HTSeq script was used to quantify the number of reads mapped to each gene [41]. The genes with counts less than ten were excluded, and the differential expression analysis was performed in the R software using the DESeq2 package for normalization, differential analysis of the high-dimensional count data, and visualization. The abundance of transcripts was also obtained using transcript expression levels as TPM (Transcript per Million) using the Kallisto software [42] since this metric normalizes the sequencing data by both gene length and sequencing depth, which improves the comparison between samples [43]. Co-expression networks were built

Table 2
Primers used to evaluate gene expression of Laccase (Lac), Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) enzymes.

Primer ID	Sequence 5'- 3'
Lac_F	5'- ACCCCTTCCAYYTGCACGGB – 3'
Lac_R	5'- ATGTGGCAGTGGAGGAACCA – 3'
LiP_F	5'- TTCCACACBCCCGACCAGATC – 3'
LiP_R	5'- SGAAGAACTGCGTGTCCCAV – 3'
MnP_F	5'- TTCGACACSCAGTTCTTCAT – 3'
MnP_R	5'- TTGACSAAVGACTGCCACTCGC – 3'
actin1_F (constitutive gene)	5'- ACAACTCGATCTACAAGTGC – 3'
actin1_R (constitutive gene)	5'- CGACGATCTTGACCTTCATA- 3'
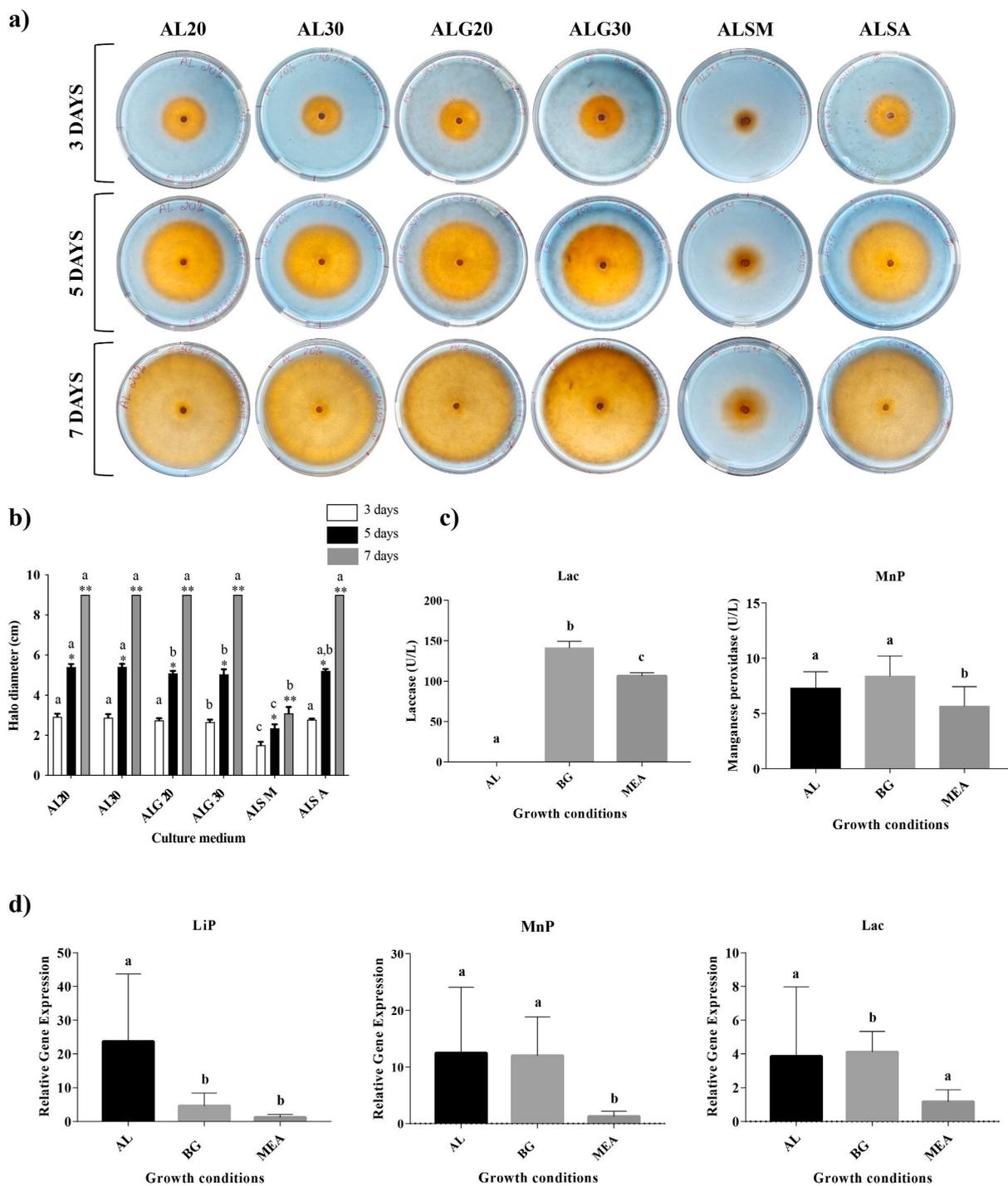
Fig. 1. Biochemical assays and relative gene expression analysis of ligninolytic enzymes of *Trametes villosa* CCMB561. a) RBBR dye discoloration halo on days 3, 5, and 7. Photos were taken from the bottom of the plate to better visualize the discoloration halo. b) Diameter of the discoloration halo in RBBR dye. Different letters (a, b, and c) indicate statistically significant differences (p < 0.05) between groups, and symbols (* and **) indicate a statistically significant difference between the days. c) Production of Laccase (Lac) and Manganese Peroxidase (MnP) enzymes by *T. villosa* CCMB561 in AL, BG, and MEA media. d) Relative expression quantification of Lignin Peroxidase (LiP), MnP, and Lac encoding genes by *T. villosa* CCMB561 in AL, BG, and MEA. Letters in the graphs from **c** and **d** indicate a statistically significant difference in enzyme production and gene relative expression among the culture media on the seventh day of growth.

using the R package WGCNA [44,45] and the count matrices (from each sample) were generated with the HTSeq python script. The CAZymes encoding genes differentially expressed had their predicted proteins analyzed using WoLF PSORT and SignalP 6.0 software for subcellular location prediction and signal peptide identification, respectively [46,47].

3. Results and discussion

3.1. Growth conditions analysis of Trametes villosa CCMB561

The RBBR discoloration test confirmed that *T. villosa* CCMB561 can grow in culture media containing only lignin as a carbon source and to produce ligninolytic enzymes (AL20 and AL30) (Fig. 1a and b). Studies with *Trametes versicolor*, a species phylogenetically close to *T. villosa* [33], demonstrated its ability to grow in a medium with lignin as the unique source of energy [48]. Nevertheless, a basal medium containing different nutrients, such as magnesium sulfate, ammonium nitrate, and dipotassium hydrogen phosphate was used for supplementation. Herein, the AL20 and AL30 media had only distilled water and lignin (Table 1).

The discoloration halo measurement results revealed that lignin media supplemented with glucose (ALG20 and ALG30) and ammonium sulfate (ALSA) did not increase the fungal growth and size of the discoloration halo when compared with media containing only lignin (AL20 and AL30). Still, medium supplemented with manganese sulfate (ALSM) inhibited the fungal growth and production of ligninolytic enzymes, which can be observed by the reduced halo of discoloration throughout the days (Fig. 1a and b).

The supplementation of culture media with chemical compounds to stimulate fungal growth and enzyme activity is still a controversial issue, with some species requiring supplementation and others not [30,49,50]. Yamanaka et al. reported that the initial Mn^{2+} concentration did not induce the production and activity of the MnP enzyme, nor stimulate the activity of laccase in *T. villosa* CCB176 [30].

Therefore, these results suggested that the AL20 medium, which contains the lowest amount of lignin and no supplementation, induced the production of ligninolytic enzymes equal to or better than the other conditions. This is a promising result since lignin is the most recalcitrant biopolymer of the plant cell wall, and consequently, the main limiting factor for the wide use of plant biomass and lignin valorization [18,51]. Furthermore, the ability to grow on lignin without supplementation decreases the costs for ligninolytic enzymes production and reveals the potential of this isolate to grow in plant biomass residues.

3.2. Quantification of lac and MnP

From the screening performed by the RBBR dye discoloration assay (section 3.1), the AL20 culture medium was selected as the most suitable condition to investigate the production of ligninolytic enzymes. In addition, with the aim to evaluate the fungus response to other carbon sources, culture media composed of sugarcane bagasse (BG) and malt extract (MEA) were included in the analyses. Sugarcane bagasse is a largely available and cheap agro-industrial residue in Brazil. Furthermore, previous studies have demonstrated the ability of the isolate *T. villosa* CCMB561 to grow on this substrate [28,29]. The malt extract is a non-inducing medium since it is mainly constituted by easily metabolized sugars.

The medium containing only lignin (AL20) did not induce the production of laccase (Lac) at detectable levels until the seventh day of growth (Fig. 1c). The highest production of Lac was observed in the BG medium (average of 141 U/L), followed by MEA (average of 106 U/L) (Fig. 1c). A statistical difference in Lac production between BG and MEA conditions was observed (p < 0.05). The highest production of MnP was observed in the BG culture medium (average of 8 U/L), followed by AL20 (average of 7 U/L), with no statistical difference between these conditions (Fig. 1c). In MEA, a smaller amount of enzyme was produced (5.7 U/L), which differed statistically (p < 0.05) from the amounts produced in AL and BG (Fig. 1c). The MnP production in BG is supported by previous studies that detected this enzyme in culture media containing lignocellulosic substrates as a carbon source [28,29].

Our results revealed the medium containing only lignin (AL) induced the MnP production; however, inhibited the secretion of Lac (Fig. 1c). Previous studies with *Trametes togrii* showed that supplementation of culture medium with lignocellulose (1%) increases the production of β-glucosidase, endoglucanase (EG), cellulase, xylanase, LiP, and MnP [52]. In contrast, supplementation with glucose (1%) decreases the activity of these enzymes and increases the activity of laccase [52]. Thus, the absence of Lac in AL media may be related to the lack of glucose, which is present in BG and MEA media.

3.3. Relative gene expression analysis by qPCR

The dissociation curves of the target genes (Actin1, Lac, LiP, and MnP) had homogeneous melting temperatures for all the samples amplified (Supplementary Figure 1), demonstrating that qPCR reactions were specific and without primer-dimer.

The relative expression of the LiP-encoding gene in *T. villosa* CCMB561 was significantly increased (p < 0.05) when the fungus was cultivated in the AL medium (Fig. 1d). Regarding the MnP encoding gene, there was an approximately 9-fold increase in the relative expression in AL and BG media, compared to MEA. Laccase gene expression was statistically increased in sugarcane bagasse and no significant difference was observed between AL and MEA (Fig. 1d).

The results of relative expression by qPCR applying the $2^{-\Delta\Delta Ct}$ method showed higher expression of LiP and MnP in the AL condition (Fig. 1d). In BG, higher expression of MnP and Lac encoding genes was observed. This result corroborates those obtained in the biochemical assays for MnP and Lac production, reinforcing the capacity of *T. villosa* CCMB561 to grow and produce ligninolytic enzymes in pure lignin and sugarcane bagasse. Besides, these results suggest that specific metabolic responses can be triggered according to the carbon source.

3.4. Transcriptome sequencing data and overall gene expression analysis

The RNA samples from *T. villosa* CCMB561 cultivated in AL, BG, and MEA were initially evaluated based on the total RNA capillary electrophoresis gel (TapeStation System). All samples, except for one AL replicate, showed the three characteristic bands corresponding to the 5.8S, 18S, and 28S ribosomal regions (Supplementary Figure 2). The presence of these bands confirms the RNA integrity. Samples for RNA-seq were selected based on quantification by Nanodrop (minimum of 1 μg per sample) and stability of the Cycle Threshold of the actin-encoding gene (CT ≤ 16).

For high-quality differential gene expression (DGE) analysis using the Illumina platform, 10–30 million reads per sample are required [53]. Herein, the trimmed libraries had a total number of reads ranging from 36,609,047 to 43,947,185 (Table 3). An important parameter for assessing the overall accuracy of RNA sequencing is the rate of reads mapped to the reference genome or transcriptome. For the human genome, a mapping ratio of 70–90% is expected [54]. In this study, the sequences mapping rate in the reference genome of *T. villosa* CCMB561 ranged from 84.8 to 88.1% (Table 3).

In order to visualize the transcriptomes clustering from the different culture conditions tested, Principal Component Analysis (PCA) and correlation heatmap plots were generated (Fig. 2a and b). The heatmap exhibited that the triplicates of each condition tested (AL, BG, and MEA) grouped together (white color) and differed from the other conditions. In the color scale, the bluer, the greater the difference in the expression profile among the samples (Fig. 2a). The PCA graph corroborates the results depicted in the heatmap, showing that the triplicates of each condition differed less among them when compared with others coming from a different cultivation media (Fig. 2b). It was also observed that the transcriptomes of the fungus cultivated in AL showed greater variation (PC1 with 60% variation) than the transcriptomes from BG and MEA (PC2 with 22% of variation) (Fig. 2b).

The MA plot is a scatterplot of log2 fold change values (y-axis) versus the average of normalized counts (x-axis) (Fig. 2c and d). From the analysis of this plot, it was possible to globally evaluate the differentially expressed genes in all analyzed samples. Fig. 2c shows the MA-plot of the transcriptome of *T. villosa* CCMB561 cultivated in AL versus MEA media. In red, all genes differentially expressed considering an adjusted P-value <0.01 are highlighted. Comparing AL and MEA, 2283 genes were up-regulated (17%) and 2260 genes were down-regulated (17%). Comparing BG versus MEA (Figs. 2d), 1426 genes were up-regulated (11%), and 1401 down-regulated (11%). The most differentially expressed genes among the media tested had no assigned function and were named uncharacterized proteins (Fig. 2c and d).

3.5. Differential gene expression (DGE) and co-expression network analyses

Comparing the transcriptomes of *T. villosa* CCMB561 sequenced in AL and MEA media (Fig. 3a), 19 up-regulated genes were detected in AL. Among these, genes encoding Homogentisate 1,2-dioxygenase (n = 1), Nitroreductase (n = 2), Cytochrome P450 (n = 3), Salicylate hydroxylase (n = 1), Pirin (n = 1), Inorganic phosphate transporter (n = 1) and Heat shock protein (n = 1). The Cytochrome P450 (CYP450) encoding gene (GENE 5632) had the highest value of log2 fold change (9.03), TPM (2886.41), and number of counts (192,208 reads). Two other genes encoding CYP450 (GENE 202 and 13169) are among the most differentially expressed (up-regulated) in the AL medium (Fig. 3b and c).

The weighted correlation network analysis (WGCNA) applying a minimum correlation of 0.8 generated a co-expression network only when the AL and MEA conditions were compared. The obtained network displays 38 genes and 68 connections (Fig. 4). The Cytochrome P450-encoding gene (GENE 5632) showed the highest number of interactions and was co-expressed with 36 genes. The second gene with the highest number of interactions was the Nitroreductase-encoding gene (GENE 6498).

Cytochrome P450 is a family of monooxygenases involved in secondary metabolite biosynthetic pathways, fungal adaptation, ecological roles, and organic matter decomposition [53–56]. Regarding lignin breakdown, for this polymer to be oxidatively cleaved into ring-opened compounds, it must first be O-demethylated to diols [55]. Cytochrome P450 are enzymes already reported catalyzing the O-demethylation reaction and also acting in the degradation of organic pollutants and xenobiotics [51,56–59]. Thus, the CYP450 encoding genes significantly expressed in the AL medium may together be acting in the O-demethylation of lignin and formation of central intermediate compounds. This is an important and limiting step in lignin catabolism and to obtain bioproducts [51,58].

Nitroreductases are enzymes acting in the mineralization of nitroaromatic compounds and can be used in the degradation of organic pollutants, such as 2,4,6-trinitrotoluene (TNT), which is a highly explosive compound used in the manufacture of bombs [59,

Table 3
Trametes villosa CCMB561 transcriptomes sequencing and mapping statistics from each culture condition tested.

Libraries	Number of reads	Mapping ratio (%)
AL1 (RF1)	41,686,793	84.9
AL2 (RF2)	40,526,137	88.1
AL3 (RF4)	43,947,185	86.1
BG1 (RF7)	38,570,847	84.8
BG2 (RF8)	36,609,047	85.2
BG3 (RF9)	43,335,879	84.8
MEA1 (RF12)	41,096,239	86.1
MEA2 (RF13)	40,709,103	85.6
MEA3 (RF14)	41,498,105	86
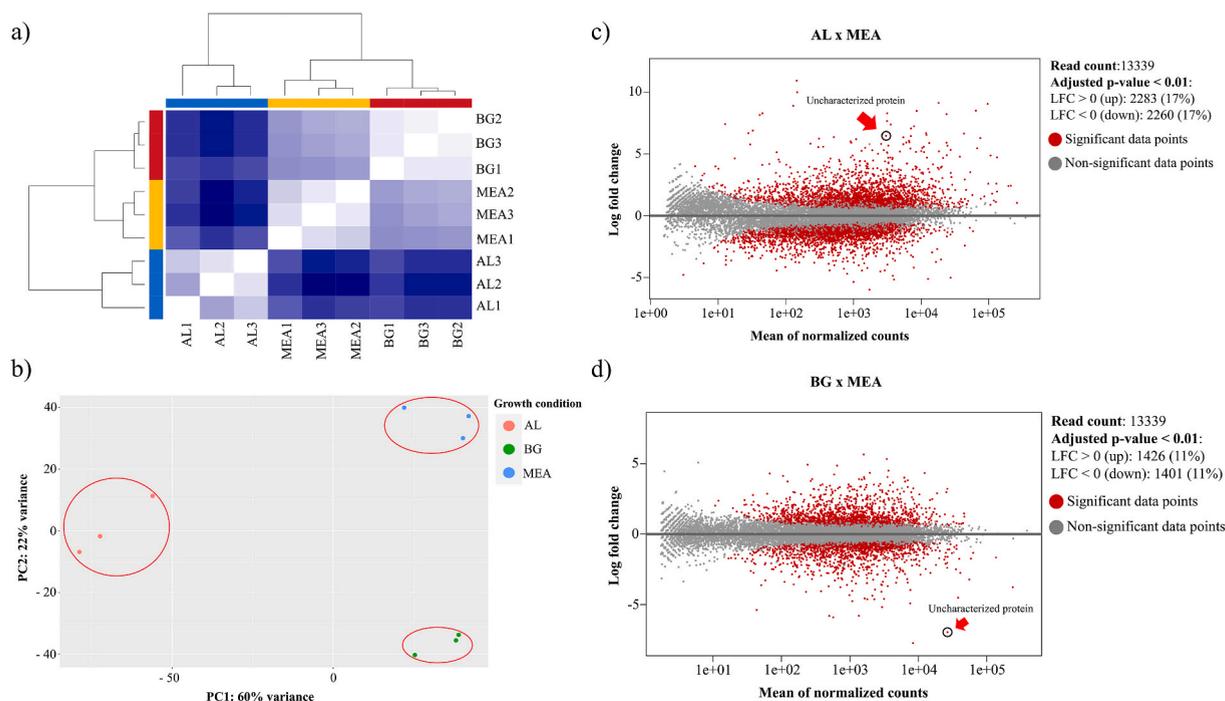
Fig. 2. – Visualization of transcriptomes samples clustering and global gene expression. Heatmap clustering (a) and PCA (principal component analysis) plot (b) based on the expression profiles of the nine sequenced samples. MA plot showing genes differentially expressed in AL medium compared to MEA (c), and BG compared to MEA (d). In both MA plots (c–d) the most differentially expressed genes are highlighted (black circle) considering an adjusted P-value <0.01.

60]. Nitroreductases are available for the degradation of nitroaromatics even under nitrogen-limited conditions [60]. In AL culture media, two genes encoding Nitroreductases (GENE 6498 and 864) are among the most differentially expressed (up-regulated) (Fig. 3), and the gene 6498 was connected to other 22 genes in the co-expression network analysis (Fig. 4).

Interestingly, the genes encoding both, CYP450 and Nitroreductase (GENE 6498) are being co-expressed with an RNA polymerase II transcription factor B (GENE 12516), which is a component of the initiation complex for RNA polymerase II and mRNA transcription [61]. The CYP450 (GENE 5632) is still co-expressed with a DNA helicase encoding gene (GENE 4528), which acts opening the DNA double strand and in the maintenance of nuclear and mitochondrial DNA (Fig. 4) [62].

One homogentisate 1,2-dioxygenase (HGT) encoding gene is also among the most expressed in the AL medium (*up-regulated*) (Fig. 3). HGT was up-regulated in *Phanerochaete chrysosporium* in the presence of vanillin, one of the main intermediates found during lignin depolymerization [63,64]. Another study demonstrated that *Exophiala lecanii-corni* expressed homogentisate-1,2-dioxygenase in a culture medium containing ethylbenzene as a carbon source (volatile organic compounds, VOCs) [65]. Thus, the high-expression of HGT in *T. villosa* CCMB561 may be an indication that the isolate is acting in the lignin depolymerization and forming bioproducts, such as vanillin.

In the AL medium, two genes encoding Heat-shock proteins (HSPs, GENE 8034 and 11244) were co-expressed with CYP450 and nitroreductase genes (GENE 5632 and 6498) (Fig. 4). The 8046 gene, which encodes an HSP, is among the 20 most significantly expressed genes in AL (Fig. 3). Heat-shock proteins play a key role in the physiological regulation of fungi in biotic and/or abiotic stress. HSPs are also involved in different biological functions such as transcription, translation, and post-translational modifications [66].

In BG medium, genes-encoding Lactamase_B domain-containing protein (n = 1), Oligopeptide transporter 1 (n = 1), Lignin Peroxidase (n = 1), Aspartic proteinase (n = 1), and Cysteine proteinase (n = 1) are among the most differentially expressed (up-regulated) (Fig. 5a). The gene encoding one beta-lactamase had the highest log2fold change value (5.65) (Fig. 5b). Interestingly, among the differentially expressed genes, 11 were down-regulated in BG medium, such as the Catalase and Pyranose 2-oxidase (P2O) that presented a log2fold change value of -7.73 and -3.21, respectively. They displayed significantly higher TPM values and number of counts in the MEA culture medium (Fig. 5b and c). P2O acts on the oxidation of carbohydrates such as D-glucose to generate 2-keto-sugars and H_2O_2 [24,67]. Catalase acts on the decomposition of H_2O_2, a compound that, in excess, may be responsible for the inactivation of Pyranose-2-oxidase [68]. Furthermore, one lignin peroxidase encoding gene (LiP) was identified among the most significantly expressed genes in BG (Fig. 5). LiPs are H_2O_2-dependent peroxidases that act in the oxidation of phenolic aromatic compounds [24]. The other genes differentially expressed in this condition are part of the normal fungi metabolism and are not directly related to biomass depolymerization.
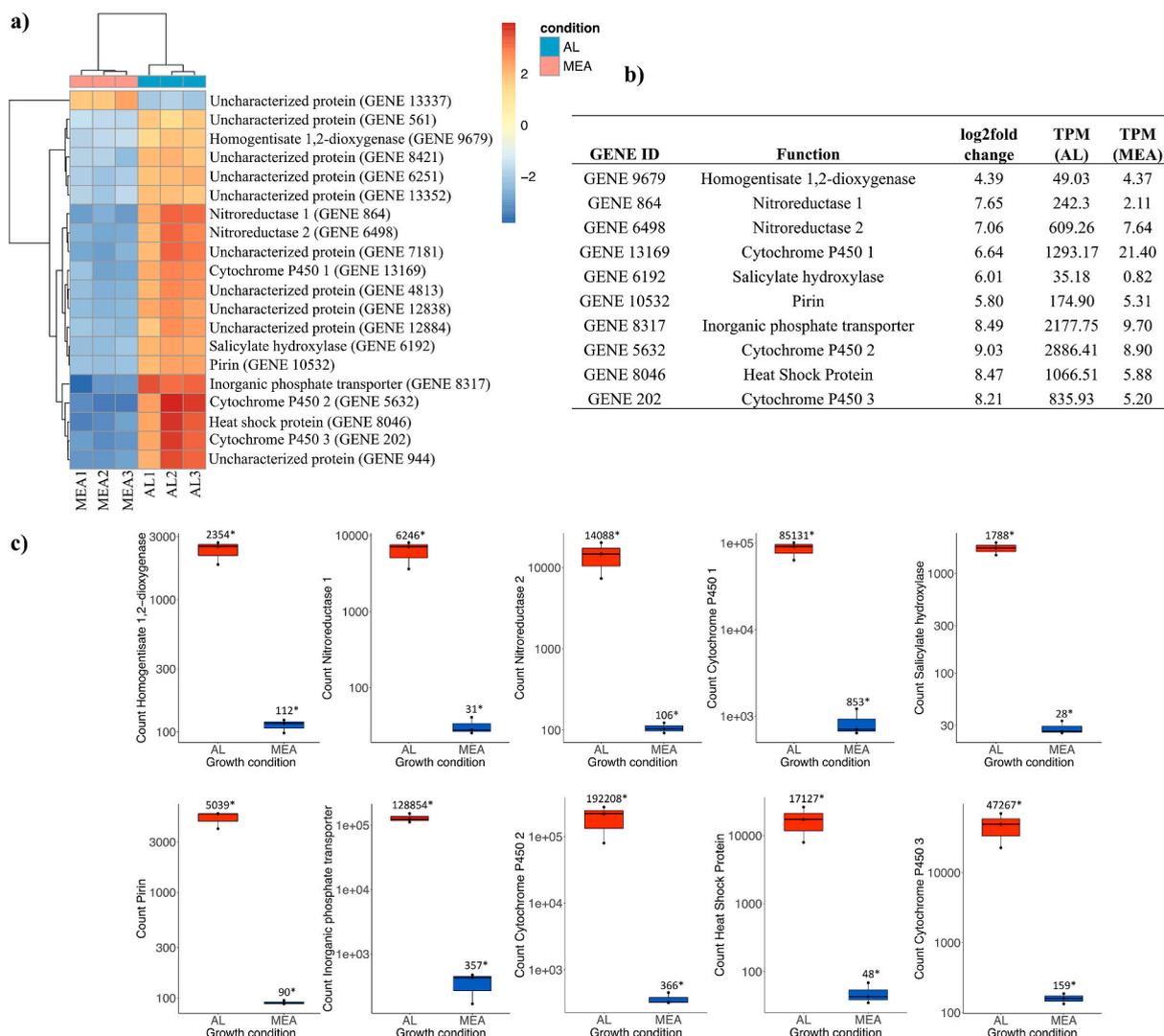
Fig. 3. Genes differentially expressed comparing AL x MEA culture media considering the adjusted P-value <0.01. a) Heatmap based on the normalized count data (log2 scale/rlogTransformation) considering the amount in which each gene deviates in a specific sample from the average of the gene in all samples. b) Table containing the value of log2fold change and Transcripts Per Million (TPM) for genes that had an assigned function based on the GoFeat and blastp analyses using the NCBI and UniProt databases. c) Graphs of the absolute count number of the differentially expressed genes. The average number of reads mapped in each gene is highlighted with an asterisk.

3.6. Expression of genes encoding Carbohydrate-Active enzymes

Based on the study of Tomé et al. [33], 186 genes encoding Carbohydrate-Active enzymes were selected from the genome of *T. villosa* CCMB561, and their expression in the different carbon sources (AL, BG, and MEA) was evaluated. Fig. 6 shows the differential expression heatmaps of the CAZymes-encoding genes as mentioned.

In pure lignin (AL), genes encoding enzymes that act in the depolymerization of lignin (Glyoxal oxidase, Laccase, Lignin Peroxidase, and Manganese Peroxidase), cellulose (β-1,4-endoglucanases and Exoglucanases), hemicellulose (Endo-1,4-β-xylanases) and pectin (Endo-polygalacturonase) had its expression significantly increased (Table 4) and formed a cluster of 15 genes (Fig. 6a). Among them, ten act on lignin depolymerization, and of these, five are LiP-encoding genes. Fourteen (14) genes displayed signal peptide and were identified as extracellular protein-coding ones. Two genes, one encoding for the endo-1,4-β-xylanases and the other encoding for exoglucanases (GH6), exhibited the CBM1 domain (Table 4). Although the AL medium does not contain cellulose, hemicellulose, and pectin, it induces the expression of genes that act in the degradation of all lignocellulose polymers.

In the BG and MEA media, the CAZymes-encoding genes had more homogeneous expression compared to the AL versus MEA media (Fig. 6). In BG medium, a cluster of nine genes had statistically increased expression (adjusted P-value <0.01) (Fig. 6b). Among these, genes encoding proteins that act in the degradation of all lignocellulose constituents (Table 5), such as LiP, MnP, Lytic polysaccharide monooxygenases, Alcohol oxidase, β-1,4-endoglucanases, β-glucosidases, and Endo-polygalacturonase were identified. Seven genes
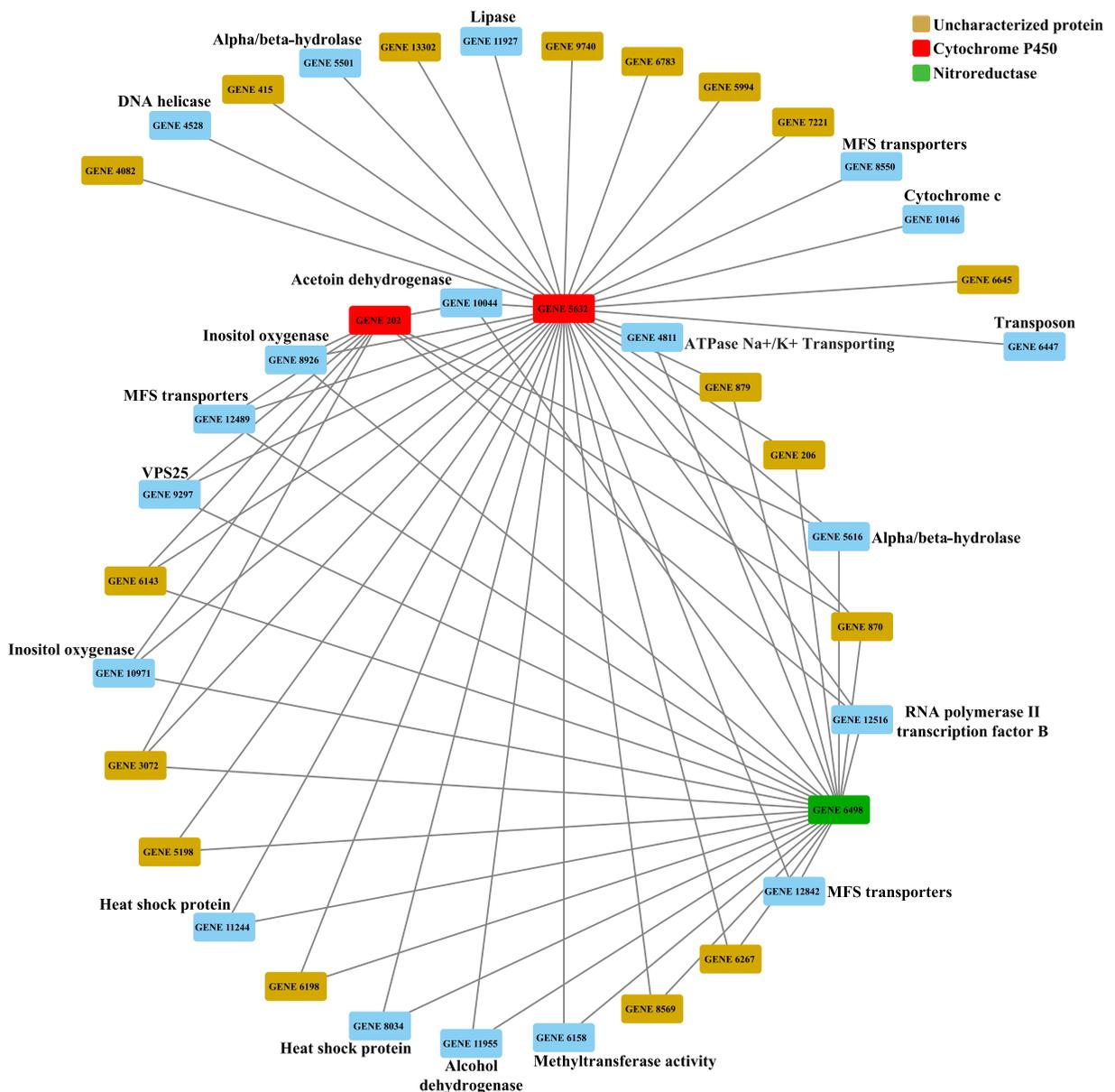
Fig. 4. Co-expression gene network based on transcriptomes of *Trametes villosa* CCMB561 cultivated in AL and MEA media. A minimum correlation value of 0.8 was considered.

displayed signal peptide and were classified as coding for extracellular proteins. In this cluster, the CBM domain was not identified.

In lignin, the results revealed that the fungus *T. villosa* CCMB561 simultaneously and differentially expressed genes encoding for enzymes that act in the depolymerization of all lignocellulose polymers (Table 4). Five genes encoding LiP were significantly increased in the AL medium and two in BG (Tables 4 and 5). This finding corroborates those obtained in the analysis of relative expression by qPCR, which demonstrated that the highest LiP expression was achieved in the AL medium. Interestingly, two genes encoding MnP had increased expression in both, AL and BG media, a result that also corroborates the qPCR data.

In the AL medium (Table 4), one Glyoxal oxidase encoding gene (AA5_1) exhibited significantly increased expression. This enzyme plays a central role in the generation of H_2O_2 [24,69]. In BG (Table 5), the gene with significantly increased expression was the Alcohol oxidase encoding gene (AA3), which is also responsible for hydrogen peroxide generation and activation of peroxidases (LiP, MnP, and VP) [24].

Regarding hemicellulose degradation in the AL medium (Table 4), one gene encoding Endo-1,4-β-xylanase (GH10) had significantly increased expression. Endo-β-1,4-endoxylanases act on the degradation of linear chains of β-1,4-linked D-xylose residues [7,11]. In addition, the CMB1 domain was identified in this gene, which promotes the association of enzymes with the substrate and increases the enzymatic hydrolysis and degradation of polysaccharides [11,70]. In BG (Table 5), a gene coding for Lytic polysaccharide
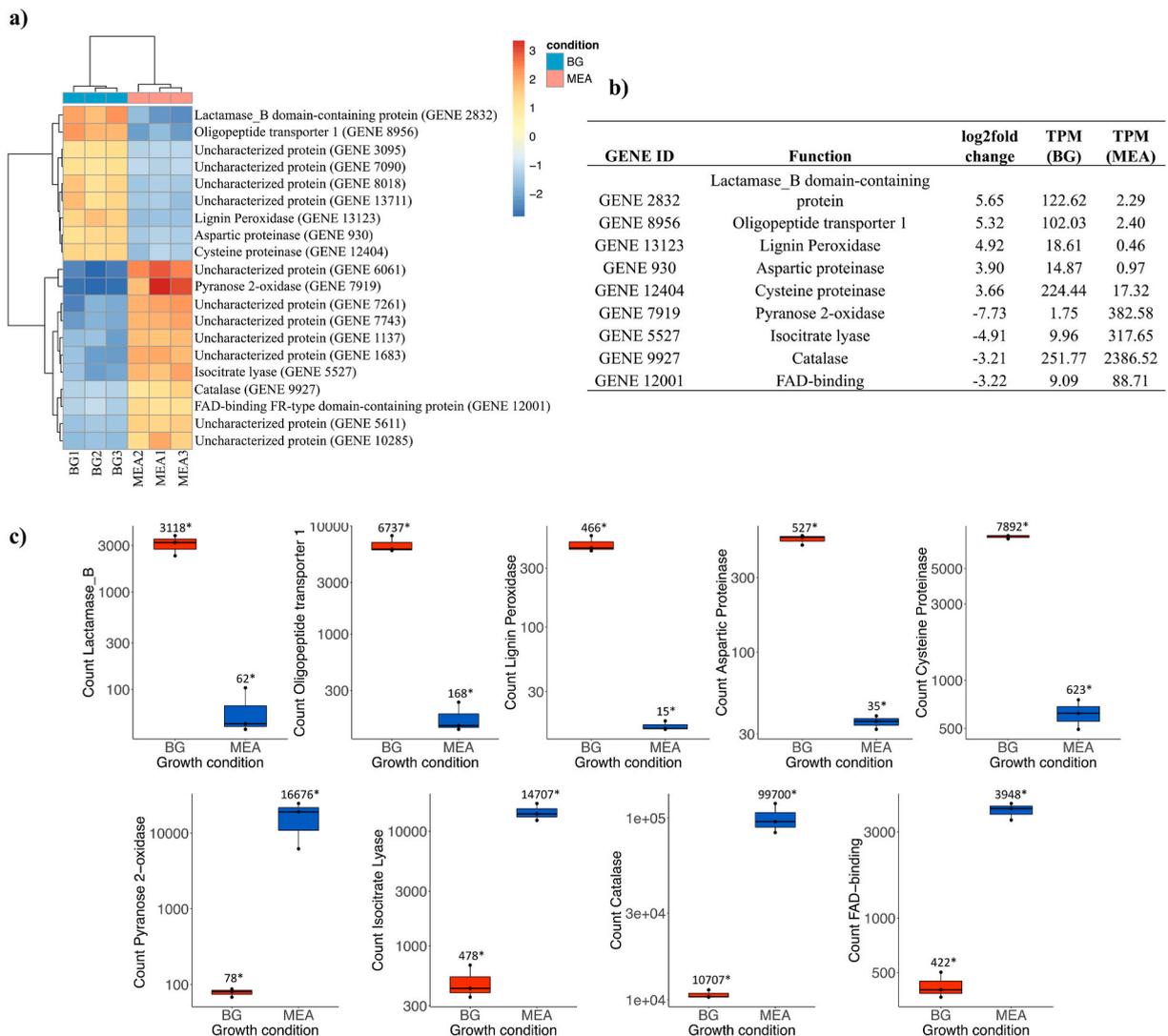
Fig. 5. Genes differentially expressed comparing BG x MEA culture media considering the adjusted P-value <0.01. a) Heatmap based on the normalized count data (log2 scale/rlogTransformation) considering the amount in which each gene deviates in a specific sample from the average of the gene in all samples. b) Table containing the value of log2fold change and Transcripts Per Million (TPM) for the genes that had an assigned function based on the GoFeat and blastp analyses using the NCBI and UniProt databases. c) Graphs of the absolute count number of the differentially expressed genes. The average number of reads mapped in each gene is highlighted with an asterisk.

monooxygenases (LPMOs) displayed increased expression. LPMOs oxidatively degrade xylan-type hemicelluloses [71]. In AL and BG media (Tables 4 and 5), one Endo-polygalacturonase encoding gene (GH28), that acts on pectin hydrolysis, had its expression significantly increased in a similar way.

Regarding cellulose depolymerization, a significantly increased expression of β-1,4-endoglucanases (GH12) and Exoglucanases (GH6) was identified in pure lignin. In sugarcane bagasse, a greater expression of β-1,4-endoglucanases and β-glucosidases were detected. β-1,4-endoglucanases act randomly in the internal amorphous region of the cellulose fiber, generating oligosaccharides. Exoglucanases act on reducing and non-reducing ends of oligosaccharides and release glucose and/or cellobiose units. Finally, β-glucosidases act by hydrolyzing the bond between the two glucose units of cellobiose, generating glucose monomers [7,10,11].

4. Conclusions

Our findings demonstrated that *Trametes villosa* CCMB561 is able to grow using lignin as the sole carbon and energy source, and such condition induces significative changes in the overall gene expression when compared to the other culture conditions evaluated. The overexpression of different genes encoding Cytochrome P450 monooxygenases was remarkable, demonstrating their putative key role in the O-demethylation of lignin for the subsequent depolymerization by oxidases (Lac) and peroxidases (LiP and MnP). Genes
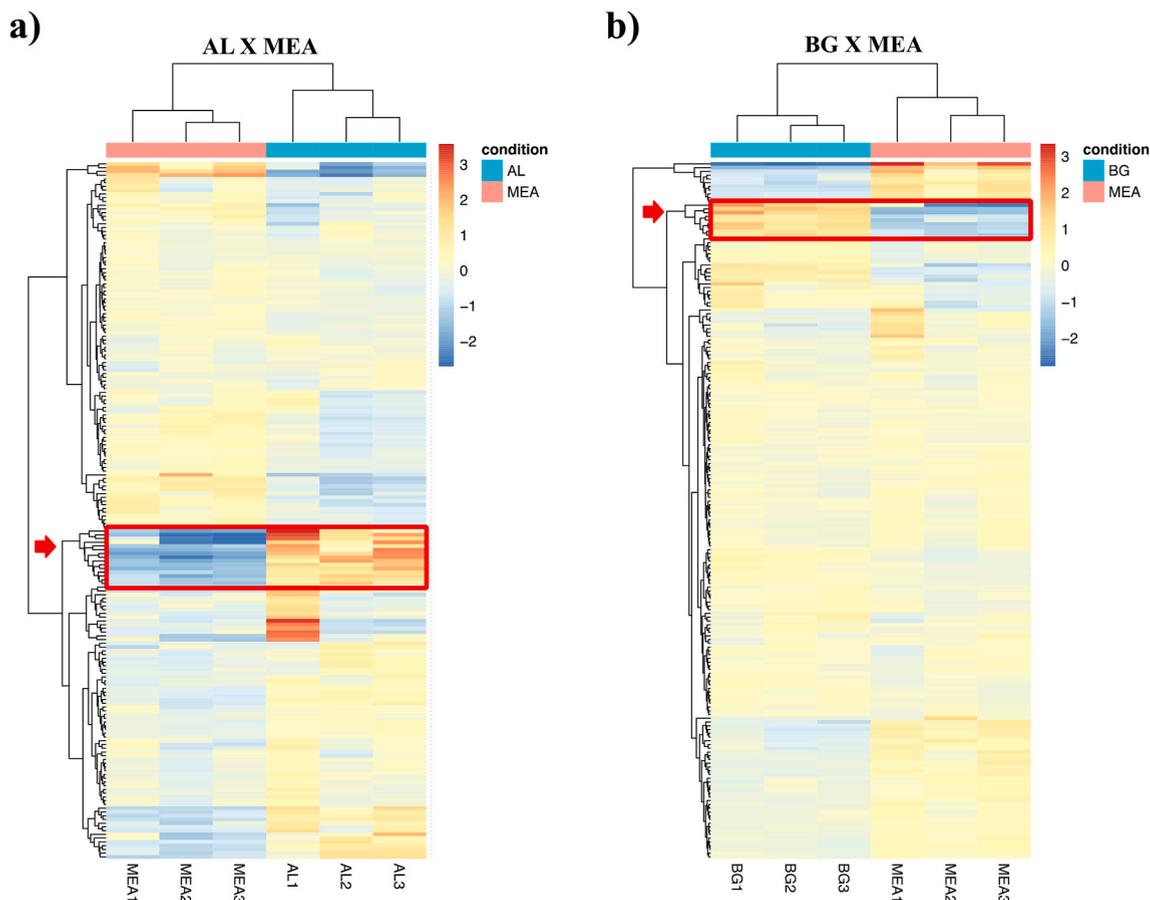
Fig. 6. Heatmap clustering of the differential expression of 186 genes encoding for enzymes belonging to the families of Auxiliary Activity oxidoreductases (AA) Carbohydrate Esterases (CE), Glycosyl Hydrolases (GH), Pectate Lyases (PL) of the *Trametes villosa* CCMB561 grown on different carbon sources. a) Differentially expressed genes comparing AL and MEA. b) Differentially expressed genes comparing BG and MEA.

encoding nitroreductases and homogentisate-1,2-dioxygenase (HGT), which act in the detoxification of organic pollutants were also differentially expressed (up-regulated). Lignin and sugarcane bagasse also induced the expression of a specific cluster of CAZymes encoding genes, which revealed the ability of *T. villosa* CCMB561 to simultaneously act in the degradation of all lignocellulose polymers. Finally, our findings contribute to the comprehension of the complex genetic mechanisms of lignocellulose breakdown by *T. villosa* CCMB561. In agreement with other studies, we demonstrate here the great potential of this fungal strain to be used for the production of enzymes with wide biotechnological applications.

Funding statement

Data availability statement

Data associated with this study will be made available on request to the corresponding author.

CRediT authorship contribution statement

Luiz Marcelo Ribeiro Tomé: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mariana Teixeira Dornelles Parise:** Formal analysis. **Doglas Parise:** Formal analysis. **Vascov Ariston de Carvalho Azevedo:** Resources, Funding acquisition. **Bertram Brenig:** Resources, Investigation, Funding acquisition. **Fernanda Badotti:** Writing – review & editing, Writing – original draft, Supervision,

Table 4

The more differentially expressed CAZymes-encoding genes (cluster highlighted in red in Fig. 6) of *Trametes villosa* CCMB561 cultivated in AL versus MEA.

GENE ID	Activity	Log2Fold change	padj	TPM AL (mean)	TPM MEA (mean)	Counts AL (mean)	Counts MEA (mean)	Signal Peptide (Sec/SPI)	Subcellular Localization	CBM Domain
GENE 7246	Lignin Peroxidase (AA2)	5.58	8.72e-06	23.27	0.82	698	15	YES (21/22)	Extracellular	NO
GENE 10648	Exoglucanases (GH6)	5.91	2.69e-20	242.26	6.02	11863	197	YES (20/21)	Extracellular	CBM1
GENE 5143	Lignin Peroxidase (AA2)	4.43	0.0007	55.73	3.95	2388	110	YES (21/22)	Extracellular	NO
GENE 4057	Manganese peroxidase (AA2)	3.28	0.03	224.62	35.20	8704	892	YES (21/22)	Extracellular	NO
GENE 882	Lignin Peroxidase (AA2)	4.12	1.36e-10	4.26	0.43	192	11	YES (21/22)	Extracellular	NO
GENE 7258	Lignin Peroxidase (AA2)	4.72	1.83e-13	41.62	2.32	1760	67	YES (26/27)	Extracellular	NO
GENE 13123	Lignin Peroxidase (AA2)	5.54	9.41e-35	88.08	0.46	590	13	YES (21/22)	Extracellular	NO
GENE 13714	Glyoxal oxidase (AA5_1)	5.29	2.93e-32	53.45	2.28	3689	94	YES (18/19)	Extracellular	NO
GENE 9985	β-1,4-endoglucanases (GH12)	4.39	7.58e-17	39.69	3.07	1192	57	YES (27/28)	Extracellular	NO
GENE 8352	Endo-1,4-β-xylanases (GH10)	3.74	1.40e-26	36.82	4.79	1548	115	YES (19/20)	Extracellular	CBM1
GENE 13331	Laccase (AA1)	4.07	3.61e-25	40.48	3.60	2344	139	NO	Cytoplasmic	NO
GENE 6618	Manganese peroxidase (AA2)	3.04	1.83e-23	13.86	2.53	509	62	YES (21/22)	Extracellular	NO
GENE 3414	Laccase (AA1)	3.51	1.73e-16	329.58	48.50	24375	2130	YES (17/18)	Extracellular	NO
GENE 12198	β-1,4-endoglucanases (GH12)	2.85	1.32e-15	161.79	36.94	6591	908	YES (22/23)	Extracellular	NO
GENE 8952	Endo-polygalacturonase (GH28)	3.19	1.75e-12	12.76	2.00	534	58	YES (19/20)	Extracellular	NO

Table 5
CAZymes-encoding genes more differentially expressed (cluster highlighted in red in Fig. 6) of *Trametes villosa* CCMB561 cultivated in BG versus MEA.

GENE ID	Activity	Log2fold change	padj	TPM BG (mean)	TPM MEA (mean)	Counts BG (mean)	Counts MEA (mean)	Signal Peptide (Sec/SPI)	Subcellular Localization	CBM Domain
GENE 4057	Manganese peroxidase (AA2)	3.25	0.03	352.72	35.20	10388	1088	YES (21/22)	Extracellular	NO
GENE 13123	Lignin peroxidase (AA2)	4.92	8.37e-57	18.61	0.46	466	15	YES (21/22)	Extracellular	NO
GENE 14059	Manganese peroxidase (AA2)	4.50	2.69e-45	220.60	10.09	7024	310	YES (21/22)	Extracellular	NO
GENE 8313	Lytic polysaccharide monooxygenases (AA14)	2.61	1.11e-20	26.26	4.02	527	86	YES (19/20)	Extracellular	NO
GENE 11636	Alcohol oxidase (AA3)	2.16	1.08e-08	130.25	29.79	7154	1592	NO	Cytoplasmic	NO
GENE 907	β-glucosidases (GH1)	3.49	4.97e-45	76.46	7.35	3218	286	NO	Cytoplasmic	NO
GENE 12452	Lignin peroxidase (AA2)	3.17	5.05e-30	25.79	2.5	2086	231	YES (21/22)	Extracellular	NO
GENE 9985	β-1,4-endoglucanases (GH12)	2.75	1.71e-27	21.77	3.07	464	69	YES (27/28)	Extracellular	NO
GENE 8952	Endo-polygalacturonase (GH28)	3.15	8.08e-25	20.62	2.00	627	71	YES (19/20)	Extracellular	NO

Resources, Project administration, Funding acquisition, Conceptualization. **Aristóteles Góes-Neto:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28449.

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