Journal of Biotechnology 168 (2013) 15–23

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

The secretome of *Trametes versicolor* grown on tomato juice medium and purification of the secreted oxidoreductases including a versatile peroxidase

Maira Carabajal[a,b], Harald Kellner[a], Laura Levin[b,*], Nico Jehmlich[c], Martin Hofrichter[a], René Ullrich[a]

[a] *TU Dresden, International Institute Zittau, Department of Bio- and Environmental Sciences, Markt 23, 02763 Zittau, Germany*
[b] *University of Buenos Aires, Faculty of Exact and Natural Sciences, Department of Biodiversity and Experimental Biology, Intendente Güiraldes 2160, Ciudad Universitaria, C1428EGA, Argentina*
[c] *Interfaculty Institute for Genetics and Functional Genomics, Department of Functional Genomics, Ernst Moritz Arndt University of Greifswald, Greifswald, Germany*

ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 30 July 2013
Accepted 5 August 2013
Available online xxx

Keywords:
Laccase
Manganese peroxidase
Secretome
Trametes versicolor
Versatile peroxidase

ABSTRACT

The present work was carried out with the aim to analyze the secretome of *Trametes versicolor* BAFC 2234 grown on tomato juice medium supplemented with copper and manganese. *T. versicolor* BAFC 2234 was selected among diverse wood dwelling agaricomycetes from Argentina by its ability to cause a strong white rot on hardwood and in addition to show high tolerance toward phenolic compounds. A considerable number of the identified proteins were related to the degradation/modification of lignocelluloses. Hydrolases, peroxidases and phenoloxidases were the most abundant enzymes produced under the above-mentioned culture conditions. The lignin-modifying oxidoreductases laccase, manganese peroxidase (MnP) and versatile peroxidase (VP) were successfully purified – the latter for the first time from *T. versicolor*. The native VP protein has a molecular mass of 45 kDa and an isoelectric point of pH 3.7. The study clearly shows that complex plant-based media being rich in phenolics, such as tomato juice, can stimulate the secretion of a broad set of extracellular lignocellulolytic enzymes. Using such natural products as fungal culture media may give the opportunity to investigate plant biomass decomposition as well as the biodegradation of organic pollutants in an environment close to nature.

1. Introduction

White-rot fungi are unique in their ability to degrade all polymeric components of wood and other lignocellulosic substrates. Interest in this group of fungi and their lignocellulolytic enzyme system has increasingly spurred by their biotechnological potential, among others by the degradation of numerous environmental pollutants (Gao et al., 2010). Proteomic analysis is a powerful, modern tool that can provide a systematic understanding of events at the molecular level and clarify complex and divergent physiological mechanisms involved in degradation processes. It is defined as the global assessment of cellular proteins expressed in a particular biological state (Kim et al., 2007); therefore culture conditions might influence protein expression. The analysis of the extracellular proteome can offer new clues either for the general understanding of fungal degradation and/or for potential industrial applications of the secreted proteins (Barreiro et al., 2011).

Phenoloxidases and peroxidases are among the most common extracellular proteins secreted by white-rot fungi. *Trametes versicolor* is an effective lignin-degrader and known to secrete high amounts of the phenoloxidase laccase (EC 1.10.3.2) (Jönsson et al., 1997). In addition, various peroxidases have been shown to be secreted by this fungus, amongst them manganese peroxidase (MnP, EC 1.11.1.13) and lignin peroxidase (LiP, EC1.11.1.14) are the best known (Johansson et al., 1993). Versatile peroxidase (VP, EC 1.11.1.16) is also a ligninolytic peroxidase but with MnP–LiP hybrid properties and thus capable of oxidizing both the typical substrates of MnP (Mn^{2+}) and LiP (veratryl alcohol). Its hybrid molecular structure that provides multiple binding sites is the basis for the oxidation of different substrate types spanning a wide range of redox potentials, including low- and high-redox potential compounds as well as compounds, which other peroxidases are not able to oxidize efficiently (e.g. the dye Reactive Black 5). This peroxidase

* Corresponding author at: University of Buenos Aires, Faculty of Exact and Natural Sciences, Laboratorio de Micología Experimental, Department of Biodiversity and Experimental Biology, Intendente Güiraldes 2160, Ciudad Universitaria, C1428EGA, Argentina. Tel.: +54 11 4576 3300; fax: +54 11 4576 3384.
E-mail addresses: mcarabajal@conicet.gov.ar (M. Carabajal), mail@haraldkellner.com (H. Kellner), lale@bg.fcen.uba.ar (L. Levin), Nico.jehmlich@ufz.de (N. Jehmlich), hofrichter@ihi-zittau.de (M. Hofrichter), ullrich@ihi-zittau.de (R. Ullrich).

0168-1656/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jbiotec.2013.08.007

was first observed in members of the genera *Pleurotus* and *Bjerkandera* in 1999 and then purified and further investigated (Camarero et al., 1999; Martínez, 2002; Ruiz-Dueñas et al., 2001, 2009). VP was later also detected in species from the genus *Panus* (Lisov et al., 2007). Kim et al. (2005) reported VP production by *T. versicolor* but, as far as we know, this *T. versicolor* VP was not purified up to now.

Basidiomycetous white-rot fungi are versatile and robust organisms having an enormous potential for biodegradation of various recalcitrant materials including toxic chemicals (Gao et al., 2010). These fungi are capable of mineralizing even xenobiotic compounds due to the nonspecific nature of their extracellular oxidative enzymatic system that naturally completely degrades complex polymeric materials of phenolic origin such as lignin or humic substances (Asgher et al., 2008). A few strains of the genus *Trametes* were even reported to productively degrade phenol and its derivatives (Carabajal et al., 2012; Chakroun et al., 2012; Grey et al., 1998; Yemendzhiev et al., 2008).

The herein studied strain of *T. versicolor*, BAFC 2234, was selected among a number of white-rot fungi from Argentina owing to its tolerance toward phenolic compounds and its ability to use phenol as sole carbon source (Carabajal et al., 2012). The objectives of this work were (i) to analyze the secretome of *T. versicolor* BAFC 2234 grown on tomato juice medium using a proteomic approach and (ii) to partially purify its secreted lignin-modifying enzymes laccase, MnP and VP.

2. Materials and methods

2.1. Fungal strain

Twenty-five Argentinean basidiomycetous fungi were tested for their tolerance toward phenol during growth on agar plates. At a phenol concentration of 10 mM, *T. versicolor* (strain BAFC 2234), still reached 45–50% of the growth rate observed in the absence of phenol. Fungal strains that were capable of growing in the presence of 10 mM phenol were then cultivated on GA agar plates (Levin and Forchiassin, 2001) supplemented with other phenolic compounds, namely 2-methoxyphenol, 2,6-dimetoxyphenol, or 3,4,5-trihydroxybenzoic acid (gallic acid). Again *T. versicolor* strain BAFC 2234 showed the highest growth rate and was therefore selected for further studies (Carabajal et al., 2012). The fungus is deposited in the Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires (BAFC). Stock cultures were maintained on 2% malt extract agar (MEA) at 4 °C.

2.2. Production of ligninolytic enzymes

Ligninolytic enzymes of *T. versicolor* were produced in a 30-L stirred-tank bioreactor (Biostat B; Braun Biotech International GmbH, Melsungen, Germany). Twenty liter of the complex liquid medium TJM (tomato juice medium) consisting of eco-tomato juice (Albi & Co., Germany) and distilled water (50:50, v/v) was used as growth medium. The inoculum consisted of 1 L of a homogenized fungal suspension pre-cultured in 500-mL Erlenmeyer flasks containing 200 mL of TJM on a rotary shaker at 100 rpm and 24 °C for 10 days. To ensure the stimulation of laccase and MnP production by the fungus, $CuSO_4$ and $MnCl_2$ were added 60 h after inoculation (final concentration 250 μM and 500 μM, respectively). Samples (1 mL of the culture liquid) were taken one or two times per day, and the ligninolytic enzyme activities and pH were measured. Fermentation was carried out under following conditions: 120 rpm, 4 L min^{-1} aeration rate and 28 °C; the pH was not regulated. Whole cultures were harvested after 7 days, filtrated and used for subsequent purification and secretome studies.

2.3. Enzyme purification

Fungal biomass was removed by filtration (filter GF6; Whatman, Dassel, Germany) and the cell-free culture liquid obtained was concentrated and dialyzed by repeated ultrafiltration at 11 °C (10-kDa cutoff; Pall-Filtron, Dreieich, Germany). A minor part of this concentrated extracellular enzyme mix was analyzed using proteomic methods (see below). To obtain laccase and peroxidases, the major part of the crude extract was further purified by up to four steps of fast protein liquid chromatography (FPLC) using anion exchange separation media. In the first step, extracellular proteins were separated on Q-sepharose® (strong anion exchanger, column: 16 mm × 100 mm, GE Healthcare, Freiburg, Germany) and eluted with a linear gradient of 0–0.8 M NaCl in 10 mM sodium acetate buffer (pH 5.5) at a flow rate of 5 mL min^{-1}. Fractions containing laccase/peroxidase activities were pooled, concentrated, dialyzed against 10 mM sodium acetate (pH 5.5–7.0) with 10 kDa Vivaspin concentrators (Sartorius Stedim Biotech; Göttingen, Germany) and loaded onto a Mono-Q® column (10 mm × 100 mm, GE Healthcare, Freiburg, Germany).

Bound proteins were eluted with 10 mM sodium acetate buffer containing 2 M NaCl at a flow rate of 6 mL min^{-1} (2nd step, sufficient for laccase separation). Fractions containing peroxidase activity were further purified by loading them onto a Mono-S column (10 mm × 100 mm, GE Healthcare, Freiburg, Germany) that was eluted with 10 mM sodium acetate buffer containing 2 M NaCl at pH 4.5. After the respectively last purification step, the final fractions of laccase, MnP and VP were pooled, concentrated and stored at −20 °C. The elution of proteins was monitored at 280 nm (total protein) and 407 nm (heme).

Part of the VP fraction obtained was further purified by semi-preparative HPLC-SEC with an HPLC system (Agilent 1200 series, Waldbronn, Germany) equipped with a Biosep-SEC-S-2000 column (300 mm × 7.8 mm, Phenomenex) under isocratic conditions (flow rate, 1 mL min^{-1} at 25 °C; solvent: aqueous mixture of 50 mM sodium acetate and 100 mM sodium chloride, pH 6.5). SDS-PAGE was performed to verify the purity of the enzyme preparations (12% NuPAGE Bis-Tris gel; Invitrogen, Karlsruhe, Germany). Analytical isoelectric focusing (IEF) was carried out using IEF precast gels (3–10 pH; Invitrogen). After electrophoretical separation, the gel was stained and protein bands were visualized with the Colloidal Blue Staining Kit (Invitrogen). Single protein bands of interest were gel-excised and further analyzed by nano LC–MS/MS (see below).

Protein concentration was determined by the method of Bradford using the Roti®-Nanoquant Protein Assay Kit (Roth, Karlsruhe, Germany) with serum albumin as the standard.

2.4. Secretome and peptide analysis by nano LC–MS/MS

The secretome of *T. versicolor* as well as single purified enzymes (gel excised) were investigated by shotgun LC–MS/MS. In total, 4 μg of protein lysates were diluted with 20 mM ammonium bicarbonate (ABC) to a final concentration of less than 1 M urea. Samples were reduced (2.5 mM DTT for 1 h at 60 °C) and alkylated (10 mM iodacetamide for 30 min at 37 °C). Proteolysis was performed overnight using trypsin (Promega, Madison, WI) with a ratio of 1:25 at 37 °C. The tryptic digestion was stopped by adding acetic acid at a final concentration of 1%, followed by desalting and purification using ZipTip-μC 18 tips (Millipore, Billerica, MA). Proteolytically cleaved peptides (400 ng) were separated prior to mass spectrometric analyses by reverse phase nano HPLC on a 15 cm Acclaim PepMap100-column (C18 3 μm, 100 Å) using an EASY-nLCProxeon system (Thermo Scientific, Waltham, MA) at a constant flow rate of 300 nl min^{-1}. Separation was achieved using a non-linear gradient of 70 min with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated

peptides were monitored using either a LTQ Orbitrap or an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) equipped with a nano electrospray ion source. Both MS-instruments were operated in data-dependent acquisition (DDA) mode. MS/MS scan events were repeated for top 6 peaks using the collision induced dissociation (CID) at normalized collision induced energy of 35%.

Raw data from the MS instrument were processed using Proteome Discoverer (Thermo Scientific, v1.3.0.339). MS-spectral data were searched against forward/reverse databases of (i) the genome-sequenced *T. versicolor* v1.0 (14,296 sequence entries) downloaded from the JGI database (http://genome.jgi.doe.gov/programs/fungi/index.jsf) (Floudas et al., 2012) and (ii) fungal phenoloxidases and peroxidases (43 sequence entries) using the SEQUEST algorithm. Database searches were performed with carbamidomethyl on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion and 0.8 Da MS/MS tolerances. Only peptide hits with an XCorrelation score >2 (SEQUEST and only proteins with ≥2 peptides were considered as confidentially identified. The proteins were further classified according to the Enzyme Commission numbers (EC number). The relative abundance of the selected proteins was calculated based on the normalized spectral abundance factor (NSAF). The NSAF for a protein k is the number of spectral counts (SpC) identifying a protein, k, divided by the protein's length (L), divided by the sum of SpC.L^{-1} for all N proteins in the experiment (Zybailov et al., 2006). The phylogeny of class II peroxidases (Morgenstern et al., 2008) was calculated using the algorithms included in Geneious 6.0 (Biomatters Ltd, Auckland, New Zealand).

2.5. Enzymatic assays

Laccase activity was determined by following the oxidation of ABTS (2,2-azino-*bis*-3-ethylbenzo-thiazoline-6-sulfonate) in 50 mM citrate-phosphate buffer (pH 4.5, 25 °C) at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) (Bourbonnais et al., 1995). The activity of manganese peroxidase (MnP) was measured at 270 nm (ε_{270} = 11,590 M^{-1} cm^{-1}) by following the formation of Mn^{3+}–malonate complexes in 50 mM sodium malonate buffer (pH 4.5) containing 0.5 mM $MnCl_2$ and 0.2 mM H_2O_2 (Wariishi et al., 1992). LiP activity was measured with veratryl alcohol (Kirk et al., 1986) by following the formation of veratraldehyde at 310 nm (ε_{310} = 9300 M^{-1} cm^{-1}) in 50 mM sodium tartrate buffer (pH 3.0), 5 mM veratryl alcohol, 0.2 mM H_2O_2, and appropriate amounts of enzyme samples. Versatile peroxidase was measured at 598 nm

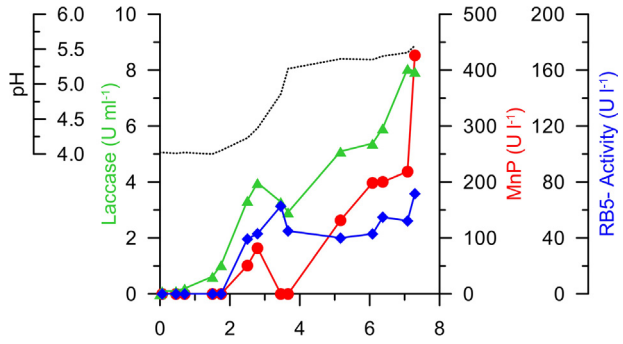

Fig. 1. Time course of laccase (triangles), VP (diamonds) and MnP (circles) production during a 30-L fermentation of *T. versicolor* in tomato juice medium. VP activity was measured with RB5 as substrate. The dotted line shows the time course of pH.

(ε_{598} = 47,600 M^{-1} cm^{-1}) by following the oxidation of the azo dye Reactive Black 5 in 50 mM sodium tartrate buffer (pH 3.0) containing 0.1 mM Reactive Black 5 and 0.2 mM H_2O_2 (Heinfling et al., 1998). The peroxidase reactions were initiated by adding H_2O_2. All enzymatic activities were expressed in International Units (U) defined as the amount of enzyme required to produce 1 μmol of product or to convert 1 μmol of substrate per minute (μmol min^{-1}). They were measured as initial velocities taking linear increments (decrease in the case of the azo dye Reactive Black 5) using a UV–visible spectrophotometer (Varian Cary 50, Darmstadt, Germany).

2.6. Statistical analysis

Experiments were carried out in triplicate. The results are expressed as mean values with standard deviation (SD); variance analysis was carried out using the ANOVA program followed by Tukey's Test using the Statistica 7.0 program. Differences at $p < 0.05$ were considered to be significant.

3. Results

3.1. Enzyme production and purification

To obtain a sufficient total amount of protein for subsequent purification of laccase and peroxidases (MnP, VP), *T. versicolor* BAFC 2234 was cultured in a 30-L bioreactor in TJM for 7 days. The time course of laccase, VP and MnP production during fermentation is depicted in Fig. 1. Visually, the fungus grew relatively fast

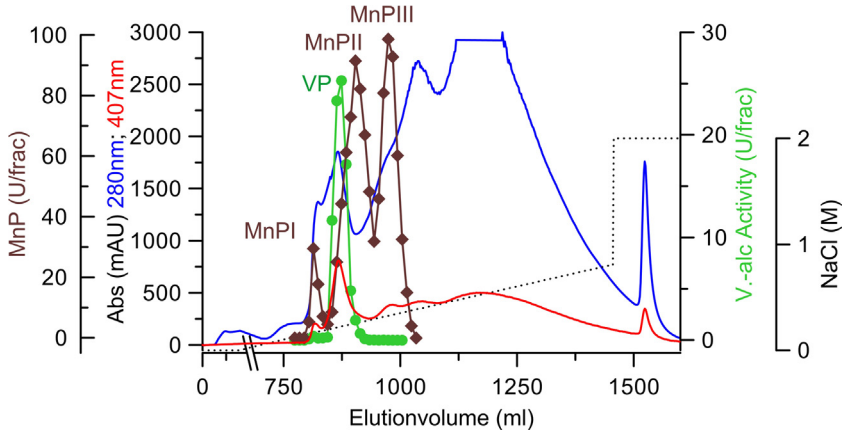

Fig. 2. FPLC elution profile of the 1st purification step performed on Q sepharose at pH 5.5: solid lines without symbols: absorbance at 407 nm (lower red line) and at 280 nm (upper blue line), MnP activity (brown diamonds), VP activity (green circles) and NaCl gradient (dashed line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

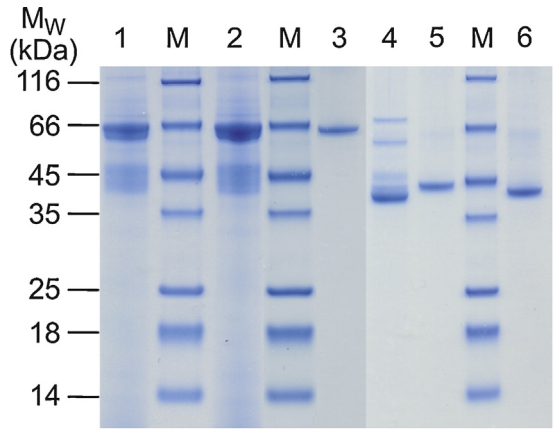

Fig. 3. SDS-PAGE illustrating the success of different purification steps for the oxidoreductases produced by *T. versicolor*: lane 1, culture liquid; 2, ultra-filtrate (10 kDa); 3, laccase; 4, MnP I; 5, MnP II; 6, MnP III, M, protein markers.

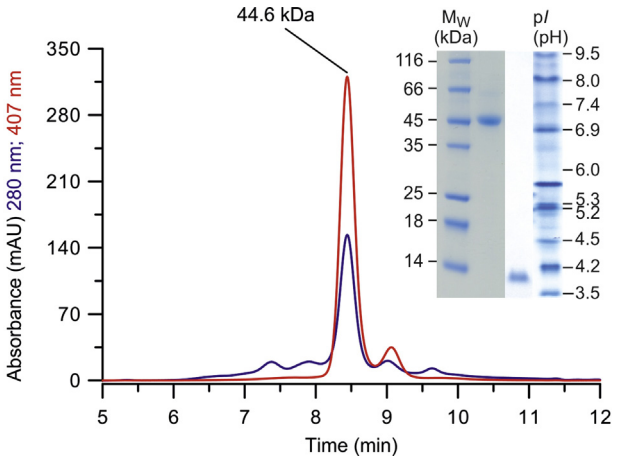

Fig. 4. HPLC-SEC elution profile of a pre-purified VP of *T. versicolor*: absorbance at 407 nm (upper red line) and at 280 nm (lower blue line). The insets show the SDS-PAGE (left) and isoelectric focusing gels (right) of the isolated VP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in TJM in form of abundant pellets. Since this type of medium is a suspension that contains small solid particles, the gravimetric determination of biomass was not possible. Mn^{2+} and Cu^{2+} supplementation evidently ensured the production of lignin-modifying oxidoreductases; the enzymatic activities peaked first on day 3 of culturing, then formed a shoulder/plateau around day 5 and 6, and increased again until harvesting on day 7 yielding following final activities in the culture liquid: laccase – 7947 U L^{-1}, MnP – 427 U L^{-1} and VP (RB5) – 72 U L^{-1}. The increase of enzymatic activities was accompanied by an increase in pH from 4 to 5.5 accomplished between days 2 and 4.

Fungal biomass was removed by filtration and the cell-free culture liquid obtained was concentrated and dialyzed by repeated ultrafiltration. After ultrafiltration, an aliquot of the 10-times concentrated cell-free culture liquid was fractionated by anion exchange chromatography (Q-sepharose). FPLC elution profile of the 1st purification step performed on Q sepharose is shown in Fig. 2. Most of the laccase activity (30 kU) did not bind under the conditions used, indicating an isoelectric point above pH 5.5. The elution profile showed four different activity peaks consistent with three Mn^{2+}-oxidizing and one veratryl alcohol-oxidizing peroxidase activity as well as a low diffuse ABTS-oxidizing activity without clear activity maximum (data not shown). The laccase containing fraction (unbound) was further purified by anion exchange chromatography on Mono Q at pH 7.0 leading to a final specific activity of 712 U mg^{-1} (Table 1). The molecular mass was estimated at 63 kDa by SDS-PAGE (line 3, Fig. 3).

MnP activities (MnP I, MnP II and MnP III) were also subjected to further purification by Mono Q (Table 1) followed by a Mono S purification step. Eventually, the purified MnP fractions formed single bands in the SDS-PAGE except for MnP I (line 4, Fig. 3) and had been purified 8-, 27- and 45-fold with overall yields of 5.8%, 6.4% and 21.5%, respectively. A summary of the different purification steps is given in Table 1. The molecular masses of the MnPs (Fig. 3) were calculated in the range from 40 to 44 kDa. As mentioned before, MnP I could not be purified to homogeneity, which becomes visible by four additional weak bands in the SDS-PAGE.

After subjecting the TJM crude extract to two steps of anion exchange chromatography VP specific activity increased almost 20-fold, up to 10.6 U mg^{-1} (Table 1). The Mono Q separation, however, was not satisfactory, as indicated by several bands in the SDS gel (data not shown). Therefore two additional purification steps (Fig. 4) were performed using Mono S and HPLC-SEC columns. The combination of these chromatographic steps did not lead to higher specific activities (Table 1) but resulted in single bands in the electrophoretic analyses (insets of Fig. 4). Purified VP had a molecular mass of 44.6 kDa determined by HPLC-SEC and of 45.5 kDa according to reducing SDS-PAGE as well as an isoelectric point of pH 4.0.

3.2. Secretome analysis

The database search resulted in the identification of 190 redundant peptides using the XCorr score >2 threshold for matching. Peptides were assigned to 121 proteins (with equal or more than two high confidence peptides) in ultra-filtrated cell-free medium (Fig. 5, Supplementary Material). The relative abundance of the selected proteins was calculated; the most abundant enzyme was laccase ID 68023 (11.01%) and MnP ID 74595 was positioned in the second place (4.38%). This MnP together with MnP ID 51375 (3.49%), MnP ID 44897 (4.49%) and MnP ID 131350 (0.49%) were the second most abundant group of lignin-modifying enzymes secreted by the fungus.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.08.007.

We present the phylogenetic relationships of class II peroxidases for *T. versicolor* in Fig. 6. As expected, MnPs, VPs and LiPs encompasses a clade in the phylogenetic tree of class II peroxidases. Five of the secreted proteins were placed in the MnP clade and one in the small VP clade. Among the extracellular class II peroxidases (MnPs, LiPs and VPs), only LiP was not expressed by this strain under the cultivation conditions in TJM.

Fig. 7 shows the protein alignment of our expressed class II peroxidases from *T. versicolor*. All characteristic amino acid ligands that define the Mn^{2+}-binding site (D: aspartate residue and E: glutamate residue) of MnPs and VP were present. The expressed VP (ID 26239) showed also the characteristic tryptophan (W) residue that is crucial for the oxidation of high-redox potential substrates and starting point of the long range electron transfer (LRET) (Fig. 7); furthermore, we found residues required for the LRET I, II and III pathway present in gene sequences of *T. versicolor* (Morgenstern et al., 2008).

From the five different MnPs *T. versicolor* produced in TJM, three – called MnP I (score: 32.42), MnP II (score: 111.88) and MnP III (score: 191.51) – were matched to MnP I ID: 131080, MnP II ID: 44897 and MnP III ID: 74595 in the *T. versicolor* genome. Moreover, the VP purified was identified as VP ID 26239.

Table 1
Purification of oxidoreductases secreted by *T. versicolor* in tomato juice medium.

Purification step	Total activity (U)	Total protein (mg)	Spec. act. (U mg^{-1})	Yield (%)	Purification (fold)
Laccase					
Ultrafiltr.	47,966	846.5	57.1	100.0	
Q-seph	39,523	64.0	615.3	82.1	11.0
Mono Q	19,935	28.0	712.0	42.2	13.1
MnP I					
Ultrafiltr.	3817	846.5	4.5	100.0	
Q-seph	510	56.0	9.1	13.4	2.0
Mono Q	427	19.0	22.5	11.2	5.0
Mono S	220	6.0	36.7	5.8	8.1
MnP II					
Ultrafiltr.	3817	846.5	4.5	100.0	
Q-seph	1619	115.0	14.1	42.4	3.1
Mono Q	1536	13.0	118.2	40.2	26.2
Mono S	245	2.0	122.5	6.4	27.2
MnP III					
Ultrafiltr.	3817	846.5	4.5	100.0	
Q-seph	2465	80.0	30.8	64.6	6.8
Mono Q	1172	27.0	43.4	30.7	9.6
Mono S	822	4.0	205.5	21.5	45.6
VP					
Ultrafiltr.	540	846.5	0.6	100.0	
Q-seph	411	75.3	5.5	76.1	9.1
Mono Q	130	12.3	10.6	24.1	17.7
Mono S	37	7.7	4.8	6.9	8.0
HPLC-SEC	22	6.2	3.6	4.1	6.0

4. Discussion

The first step in generating a fungal biodegradation/bioremediation process is the identification of suitable organisms and the optimization of the culture conditions facilitating rapid growth and the production of relevant enzymes (Ryan et al., 2007). With this aim, 25 Argentinean basidiomycetous fungi were screened for their tolerance toward phenolic compounds and the most promising strain *T. versicolor* BAFC 2234 was selected for further investigations (Carabajal et al., 2012). To get insight into the physiology of this fungal strain, we explored its secretome and purified oxidative key enzymes. It is a potent member of the ecophysiological group of white-rot fungi with recognized biodegradation and bioremediation potential (Marco-Urrea et al., 2009). The fungus was grown on a complex tomato juice medium and a proteomic approach was followed to analyze the secreted enzymes. A considerable number of the identified proteins could be related to the degradation of lignocellulosic biomass, i.e. polysaccharide hydrolases, peroxidases and oxidases were the most abundant enzymes.

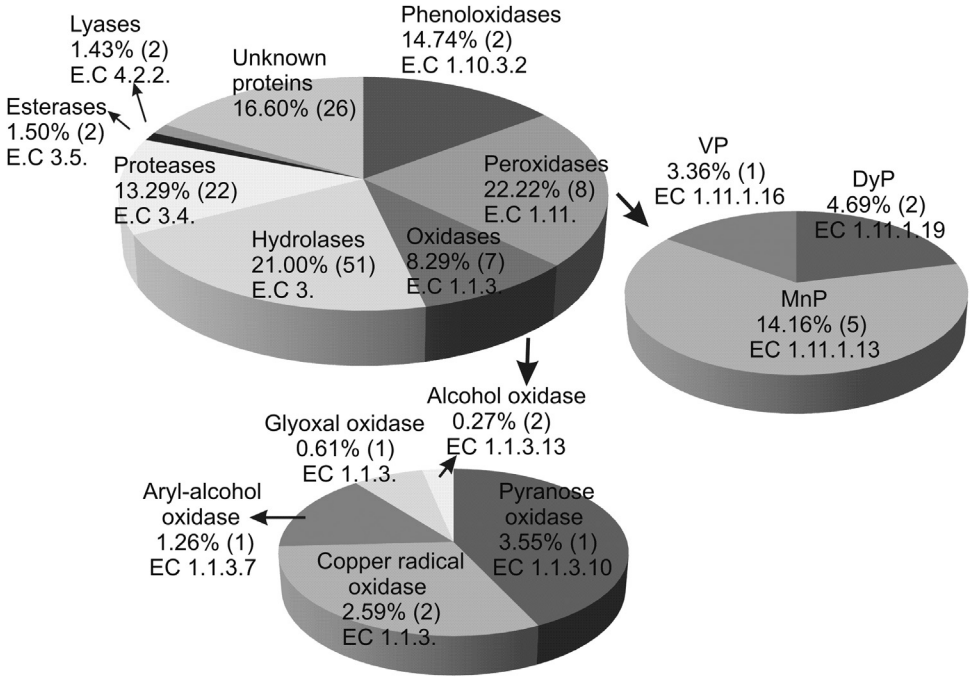

Fig. 5. A total of 121 secretory proteins were identified and sorted into 8 categories according to their EC number. Their relative abundance was calculated on the basis of the normalized spectral abundance factor (NSAF, values given in percentage). The number of enzymes which corresponds to each category is given in brackets.

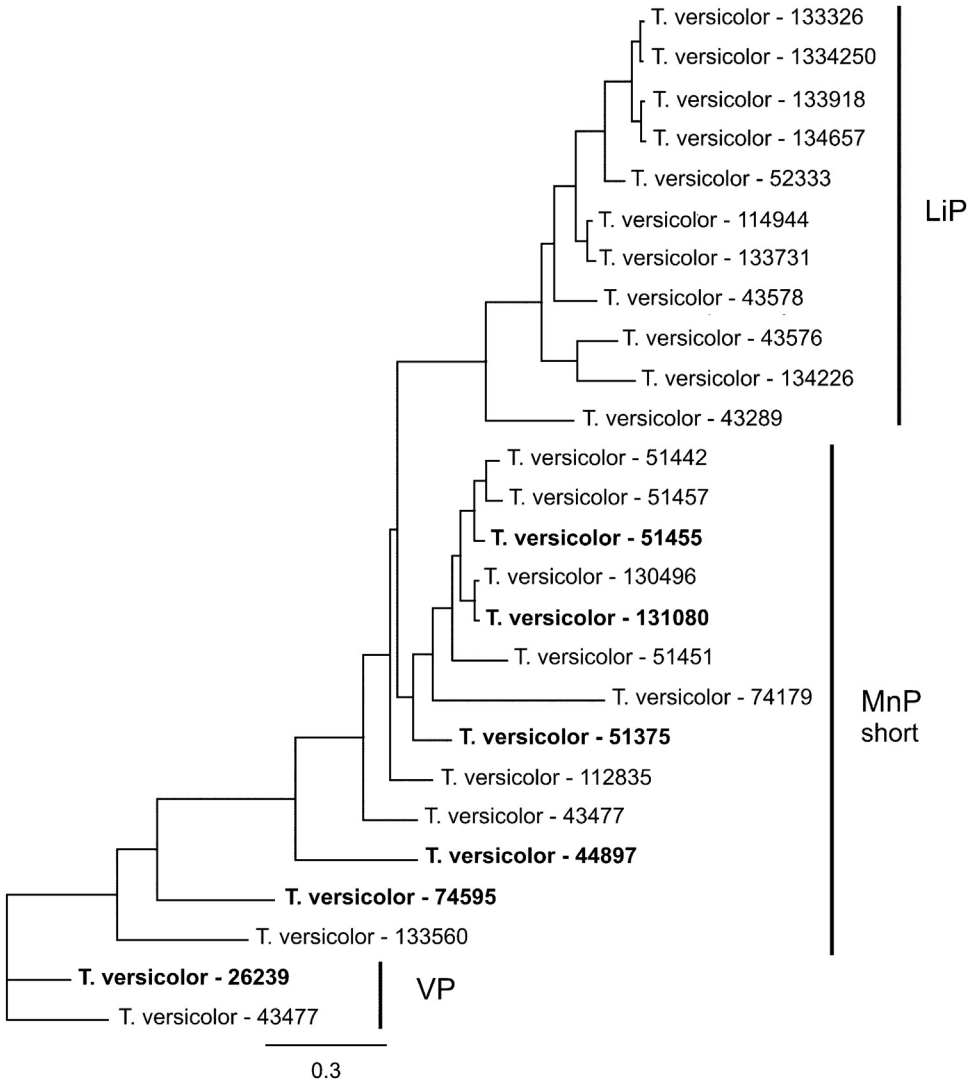

Fig. 6. Phylogenetic relationships among *T. versicolor* class II peroxidases. In bold, enzymes expressed in TJM during fungal growth (detected in the 10-fold concentrated culture liquid). The analysis was performed using the neighbor-joining method.

The production of oxidoreductases can be stimulated by heavy metals, phenols or by culture media naturally containing high amounts of low-molecular mass plant ingredients (Hatakka, 2001; Liers et al., 2007; Ullrich et al., 2004). Tomato juice media are nature friendly culture broths that are rich in phenolic compounds like flavonoids, tannins and tocopherols (Hertog et al., 1992) as well as in unsaturated hydrocarbons like carotenoids and phytoenes (Khachik et al., 1995). They have been in use as culture media since the 1920s, firstly to cultivate acidophilic lactobacilli but later also for other aciduric eukaryotes like yeasts (Kulp, 1927). Seven decades later, different working groups showed that diluted tomato juice as such or in combination with natural solids could stimulate the production of extracellular oxidoreductases such as MnP and laccase in agaric fungi (Rohene-Soustrade et al., 1992; Ullrich et al., 2004).

Considering these facts, *T. versicolor* was cultivated in a 30-L stirred-tank bioreactor in a TJM supplemented with copper (Cu^{2+}) and manganese (Mn^{2+}) to obtain high amounts of lignin-modifying enzymes for further purification and secretome studies. One copper containing polyphenol oxidase (laccase), and four class II peroxidases, MnP (3) and VP (1), were successfully purified.

From the two laccases secreted by *T. versicolor*, laccase (ID 68293) was three times more abundant than the other one at the secretomic level. The calculated p*I* of this isoenzyme is 6.44 (Suppl. Data), which fits very well with the failed binding of the major laccase fraction in the first anion exchange step at pH 5.5 and its successful binding and separation in the second step at pH 7.0. The second laccase has a calculated p*I* of 5.08, which may explain the "smearing" of ABTS-oxidizing activity at pH 5.5 near its isoelectric point. Similar p*I*s and molecular masses were observed for laccases of another member of the genus *Trametes*; the three purified laccases from *Trametes villosa* showed molecular masses of 60–70 kDa by SDS-PAGE and different p*I*s (3.5, 5–6 and 6–6.5 corresponding to laccases 1, 2 and 3, respectively) (Yaver et al., 1996). The calculated molecular mass of laccase (ID 68293) is with 55.8 kDa about 89% of the determined molecular weight of the purified laccase (63 kDa), which may refer to a typical amount of glycosylation of 11% (Baldrian, 2006).

In lignocellulose-degrading basidiomycetous fungi, MnPs are so far the most commonly occurring class II peroxidases (Hofrichter, 2002; Wesenberg et al., 2003). *T. versicolor* secreted five MnPs in TJM and MnP III was the enzyme with highest purity grade and yield. The molecular masses of the purified MnPs were in the range of typical MnPs with 40–45 kDa (Lundell et al., 2010) including a recently purified 43-kDa MnP from another strain of *T. versicolor* (Asgher and Iqbal, 2011). Hofrichter (2002) listed five members of

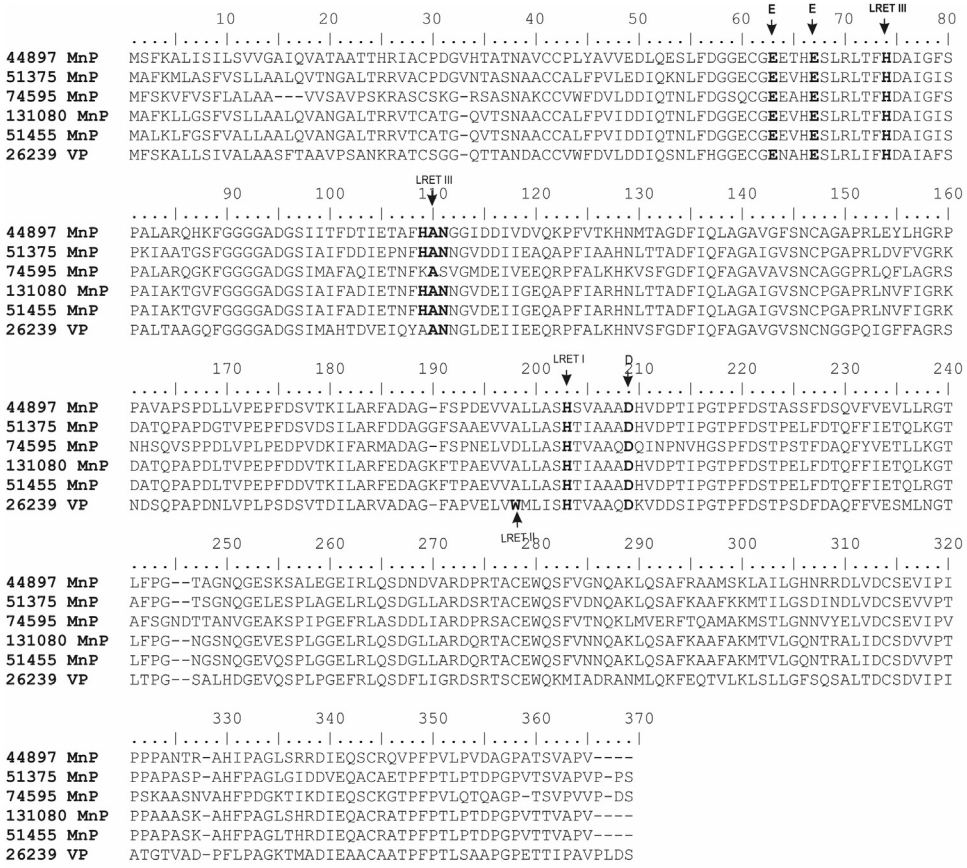

Fig. 7. Protein alignment of class II peroxidase sequences of *T. versicolor* expressed in TJM. Arrows indicate glutamic acid (E) and aspartate (D) as characteristic MnP binding residues, and residues proposed to be active in the LRET I, II and III pathway.

the genus *Trametes* producing 38–45 kDa MnPs as well as one *T. versicolor* MnP with a higher mass of 49 kDa. The molecular masses calculated here slightly differ from the detected values indicating a relative low degree of glycosylation (<5%). In contrast, for the purified VP, a higher glycosylation degree of about 16% was calculated based on its sequence-calculated mass of 38.1 kDa (Suppl. Data). Furthermore, the determined p*I* differs from the theoretical one, probably also due to posttranslational glycosylation (Gianazza, 1995).

T. versicolor is able to produce different lignin-modifying enzymes but each one may contribute in different ways to the final degradation of lignin. Among these enzymes are different MnPs, LiP, laccase and peroxide-generating oxidases (Hatakka, 1994). Laccase and MnP were the major lignin-modifying enzymes detected in TJM cultures of *T. versicolor* BAFC 2234, as well as in a previous study with the same strain in cultures supplemented with phenol (Carabajal et al., 2012). LiP was not detectable in both studies, although its successful purification from C- and N-limited cultures of *T. versicolor* was reported in an earlier publication (Johansson and Nyman, 1993). LiP is not-easy to detect enzyme, since its activity can be masked or repressed in complex media as reported by Paice et al. (1993) during pulp bleaching with *T. versicolor*. Repression of LiP expression by Mn^{2+} – that was used in the present study as elicitor – was observed in *Phanerochaete chrysosporium* (Pérez and Jeffries, 1992).

On the other hand, DyPs – a just recently discovered group of heme peroxidases (EC 1.11.1.19) representing a superfamily evolutionary independent of class II peroxidases (Hofrichter et al., 2010) – were also found to be secreted by *T. versicolor* 2234. DyPs decolorize synthetic dyes (Sugano, 2009), catalyze remarkable reactions such as the cleavage of anthraquinone derivatives and may be part of an "alternative" biocatalytic system for the direct oxidation of recalcitrant methoxylated aromatics like lignin (Liers et al., 2010). However, the catalytic mechanism of the DyP-catalyzed oxidation of lignin, that unlike VP does not oxidize Mn^{2+}, remains unclear. Up to now, DyPs have been found in several bacterial and fungal species, such as *Pleurotus ostreatus*, *Termitomyces albuminosus*, *Marasmius scorodonius*, *Auricularia auricula-judae, Irpex lacteus* and *Thanatephorus cucumeris*, but not *in T. versicolor* strains (Liers et al., 2010; Salvachúa et al., 2013; Shimokawa et al., 2009; Sugano, 2009).

T. versicolor secreted also auxiliary enzymes of lignin degradation such as glyoxal oxidase, glucose oxidase and aryl alcohol oxidase, which play an important role in the production of H_2O_2 as co-substrate for peroxidases and are involved in feedback circuits linking ligninolysis with cellulose and hemicellulose degradation in nature (Cullen and Kersten, 2004; Leonowicz et al., 1999).

The results of this study may help to expand the knowledge on the enzymatic tools of the widespread fungus *T. versicolor* and generally on this diverse fungal genus. Recently, several secretomes have been described for different species of the genus *Trametes*. The secretome of *Trametes trogii* grown in a minimal medium with glucose and ammonium tartrate as carbon and nitrogen sources, respectively, was analyzed by Ji et al. (2012). The fungus was found to secrete several enzymes that are thought to be involved in lignin degradation (Hatakka, 2001), among them LiP, MnP, laccases, aryl-alcohol oxidase, pyranose 2-oxidase, and cellobiose dehydrogenase. *Trametes gibbosa* grown on maize bran secreted cellulose, xylan and pectin-degrading enzymes, but only laccase as representative of the lignin-modifying enzymes (Berrin et al., 2012). On the other hand, MnP, VP and LiP as well as laccase genes were found in

the genome of another *T. versicolor* strain grown on milled aspen wood, but neither LiP nor VP proteins were detectable in the culture filtrates by LC–MS/MS analysis (Floudas et al., 2012).

A wild-type VP from *T. versicolor* has been purified for the first time. The catalytic cycle of the well-studied *Pleurotus eryngii* VP is a combination of the LiP- and MnP-specific activities; the LiP-characteristic exposed tryptophan residue is found in VP (Trp164) together with three MnP-characteristic acidic amino-acid residues (Asp179, Glu35, and Glu39) involved in Mn^{2+} binding (Martínez, 2002; Ruiz-Dueñas et al., 2009) and in the subsequent oxidation of Mn^{2+} into Mn^{3+}, a key step in the initial phases of lignin biodegradation (Lundell et al., 2010; Martínez et al., 2005). The latter residues are conserved as in all class II peroxidases with Mn-oxidizing activity (Kusters-van Someren et al., 1995). All these residues were also found in the MnP and VP sequences of the peroxidases secreted by *T. versicolor* 2234.

5. Conclusion

For the evaluation of the degradative potential of a fungus and its lignin-modifying system, the characterization of the secretome has proven to be a useful tool. We provide here a catalog of relevant enzyme proteins secreted by *T. versicolor* BAFC 2234 during growth in a simple complex medium (tomato juice). By proteomics, enzymes related to the degradation of lignocellulosic biomass, i.e. peroxidases, oxidases and hydrolases can easily be identified. The proteomic approach has suitably been combined with protein production and purification (laccase, MnP and VP), which additionally verified the secretome data. The observed diversity of secreted lignocellulolytic enzymes on a natural product such as tomato juice may encourage further investigations on fungal degradation processes in complex plant-based media representing simple but effective microcosms.

Acknowledgments

We gratefully acknowledge the support from the German Academic Exchange Service (DAAD), the European Union (integrated project PEROXICATS, KBBE-2010-4-265397) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). We thank also our co-workers M. Kluge, L. Papinutti, M. Pecyna, M. Poraj-Kobielska, T. Arnstadt, C. Liers, F. Hahn, R. Reina Prego and A. Kluttig, for scientific support, useful comments and discussions, as well as U. Schneider and M. Brandt for their technical assistance.

References

Asgher, M., Bhatti, H.N., Ashraf, M., Legge, R.L., 2008. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19, 771–783.

Asgher, M., Nasir Iqbal, H.M., 2011. Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04. Bioresources 6, 4317–4330.

Baldrian, P., 2006. Fungal laccases – occurrence and properties. FEMS Microbiol. Rev. 30, 215–242.

Barreiro, C., García-Estrada, C., Martín, J.F., 2011. Proteomics methodology applied to the analysis of filamentous fungi – new trends for an impressive diverse group of organisms. In: Prasain, J.K. (Ed.), Tandem Mass Spectrometry – Applications and Principles. InTech, Rijeka, Croatia, pp. 127–160.

Berrin, J.-G., Navarro, D., Couturier, M., Olivé, C., Grisel, S., Haon, M., Taussac, S., Lechat, C., Courtecuisse, R., Favel, A., Coutinho, P., Lesage-Meessen, L., 2012. Exploring the natural fungal biodiversity of tropical and temperate forests toward improvement of biomass conversion. Appl. Environ. Microbiol. 78, 6483–6490.

Bourbonnais, R., Paice, M.G., Reid, I.D., Lanthier, P., Yaguchi, M., 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl. Environ. Microbiol. 61, 1876–1880.

Camarero, S., Sarkar, S., Ruiz-Dueñas, F.J., Martinez, M.J., Martinez, A.T., 1999. Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J. Biol. Chem. 274, 10324–10330.

Carabajal, M., Ullrich, R., Levin, L., Kluge, M., Hofrichter, M., 2012. Degradation of phenol and *p*-nitrophenol by the white-rot polypore *Trametes versicolor*. In: Proceedings Book of the 5th International Symposium on Biosorption and Bioremediation, Prague, pp. 69–72.

Chakroun, H., Bouaziz, M., Dhouib, A., Sayadi, S., 2012. Enzymatic oxidative transformation of phenols by *Trametes trogii* laccases. Environ. Technol. 33, 1977–1985.

Cullen, D., Kersten, P.J., 2004. Enzymology and molecular biology of lignin degradation. In: Brambl, R., Marzluf, G.A. (Eds.), The Mycota III: Biochemistry and Molecular Biology. , 2nd ed. Springer-Verlag, Berlin, Heidelberg, pp. 249–273.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martinez, A.T., Otillar, R., Spatafora, J.W., Yadav, J.S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P.M., de Vries, R.P., Ferreira, P., Findley, K., Foster, B., Gaskell, J., Glotzer, D., Gorecki, P., Heitman, J., Hesse, C., Hori, C., Igarashi, K., Jurgens, J.A., Kallen, N., Kersten, P., Kohler, A., Kües, U., Kumar, T.K., Kuo, A., LaButti, K., Larrondo, L.F., Lindquist, E., Ling, A., Lombard, V., Lucas, S., Lundell, T., Martin, R., McLaughlin, D.J., Morgenstern, I., Morin, E., Murat, C., Nagy, L.G., Nolan, M., Ohm, R.A., Patyshakuliyeva, A., Rokas, A., Ruiz-Dueñas, F.J., Sabat, G., Salamov, A., Samejima, M., Schmutz, J., Slot, J.C., St John, F., Stenlid, J., Sun, H., Sun, S., Syed, K., Tsang, A., Wiebenga, A., Young, D., Pisabarro, A., Eastwood, D.C., Martin, F., Cullen, D., Grigoriev, I.V., Hibbett, D.S., 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336, 1715–1719.

Gao, D., Du, L., Yang, J., Wu, W.M., Liang, H., 2010. A critical review of the application of white rot fungus to environmental pollution control. Crit. Rev. Biotechnol. 30, 70–77.

Gianazza, E., 1995. Isoelectric focusing as a tool for the investigation of posttranslational processing and chemical modifications of proteins. J. Chromatogr. 705, 67–87.

Grey, R., Höfer, C., Schlosser, D., 1998. Degradation of 2-chlorophenol and formation of 2-chloro-1,4-benzoquinone by mycelia and cell-free crude culture liquids of *Trametes versicolor* in relation to extracellular laccase activity. J. Basic Microbiol. 38, 371–382.

Hatakka, A., 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. 13, 125–135.

Hatakka, A., 2001. Biodegradation of lignin. In: Hofrichter, M., Steinbuchel, A. (Eds.), Biopolymers, Volume 1: Lignin, Humic Substances and Coal. Wiley-VCH, Weinheim, pp. 129–180.

Heinfling, A., Ruiz-Dueñas, F.J., Martinez, M.J., Bergbauer, M., Szewzyk, U., Martinez, A.T., 1998. A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*. FEBS Lett. 428, 141–146.

Hertog, M.G.L., Hollman, P.C.H., Katan, M.B., 1992. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. J. Agric. Food Chem. 40, 2379–2383.

Hofrichter, M., 2002. Lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. 30, 454–466.

Hofrichter, M., Ullrich, R., Pecyna, M., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. 87, 871–897.

Ji, X.-L., Zhang, W.-T., Gai, Y.-P., Lu, B.-Y., Yuan, C.-Z., Liu, Q.-X., Mu, Z.-M., 2012. Patterns of lignocellulose degradation and secretome analysis of *Trametes trogii* MT. Int. Biodeter. Biodegrad. 75, 55–62.

Johansson, T., Nyman, P.O., 1993. Isozymes of lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. I. Isolation of enzyme forms and characterization of physical and catalytic properties. Arch. Biochem. Biophys. 300, 49–56.

Johansson, T., Welinder, K.G., Nyman, P.O., 1993. Isozymes of lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. II. Partial sequences, peptide maps, and amino acid and carbohydrate compositions. Arch. Biochem. Biophys. 300, 57–62.

Jönsson, L., Saloheimo, M., Penttilä, M., 1997. Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of lcc1 and expression in *Pichia pastoris*. Curr. Genet. 32, 425–430.

Khachik, F., Beecher, G.R., Smith, J.C., 1995. Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer. J. Cell. Biochem. 22, 236–246.

Kirk, T.K., Croan, S., Tien, M., Murtagh, E., Farrell, R.L., 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of mutant strain. Enzyme Microb. Technol. 8, 27–32.

Kim, Y., Yeo, S., Kum, J., Song, H., Choi, H., 2005. Cloning of a manganese peroxidase cDNA gene repressed by manganese in *Trametes versicolor*. J. Microbiol. 43, 569–571.

Kim, Y., Nandakumar, M.P., Marten, M.R., 2007. Proteomics of filamentous fungi. Trends Biotechnol. 25, 395–400.

Kulp, W.L., 1927. Scientific apparatus and laboratory methods an agar medium for plating *L. acidophilus* and *L. bulgaricus*. Science 25, 512–513.

Kusters-van Someren, M., Kishi, K., Lundel, T., Gold, M.H., 1995. The manganese binding site of manganese peroxidase: characterization of an Asp 179Asn site-directed mutant protein. Biochemistry 34, 10620–10627.

Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N.S., Hofrichter, M., Rogalski, J., 1999. Biodegradation of lignin by white-rot fungi. Fungal Genet. Biol. 27, 175–185.

Levin, L., Forchiassin, F., 2001. Ligninolytic enzymes of the white rot basidiomycete *Trametes trogii*. Acta Biotechnol. 21, 179–186.

Liers, C., Ullrich, R., Pecyna, M., Schlosser, D., Hofrichter, M., 2007. Production, purification and partial enzymatic and molecular characterization of a laccase from

the wood-rotting ascomycete *Xylaria polymorpha*. Enzyme Microb. Technol. 41, 785–793.
Liers, C., Bobeth, C., Pecyna, M., Ullrich, R., Hofrichter, M., 2010. DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl. Microb. Biotechnol. 85, 1869–1879.
Lisov, A., Leontievsky, A., Golovleva, L., 2007. Hybrid Mn peroxidases from basidiomycetes. Appl. Biochem. Microbiol. 43, 536–543.
Lundell, T.K., Mäkelä, M.R., Hildén, K., 2010. Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. J. Basic Microbiol. 50, 5–20.
Marco-Urrea, E., Perez-Trujillo, M., Caminal, G., Vicent, T., 2009. Dechlorination of 1,2,3 and 1,2,4-trichlorobenzene by the white-rot fungus *Trametes versicolor*. J. Hazard. Mater. 166, 1141–1147.
Martínez, A.T., 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb. Technol. 30, 425–444.
Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A., del Río, J.C., 2005. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. 8, 195–204.
Morgenstern, I., Klopman, S., Hibbett, D., 2008. Molecular evolution and diversity of lignin degrading heme peroxidases in the agaricomycetes. J. Mol. Evol. 66, 243–257.
Paice, M.G., Reid, I.D., Bourbonnais, R., Archibald, F.S., Jurasek, L., 1993. Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies kraft pulp. Appl. Environ. Microbiol. 59, 260–265.
Pérez, J., Jeffries, T.W., 1992. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58, 2402–2409.
Rohene-Soustrade, I., Lung-Escarmant, B., Bono, J.J., Taris, B., 1992. Identification and partial characterization of an extracellular manganese-dependent peroxidase in *Armillaria ostoyae* and *Armillaria mellea*. Eur. J. Forest Pathol. 22, 227–236.
Ruiz-Dueñas, F.J., Camarero, S., Perez-Boada, M., Martinez, M.J., Martinez, A.T., 2001. A new versatile peroxidase from *Pleurotus*. Biochem. Soc. Trans. 29, 116–122.
Ruiz-Dueñas, F., Morales, M., García, E., Miki, Y., Martínez, M., Martínez, A., 2009. Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J. Exp. Bot. 60, 441–452.
Ryan, D., Leukes, W., Burton, S., 2007. Improving the bioremediation of phenolic wastewaters by *Trametes versicolor*. Bioresour. Technol. 98, 579–587.
Salvachúa, D., Prieto, A., Martínez, A.T., Martínez, M.J., 2013. Characterization of a novel dye-decolorizing peroxidase (DyP)-type enzyme from *Irpex lacteus* and its application in enzymatic hydrolysis of wheat straw. Appl. Environ. Microbiol. 14, 4316–4324.
Shimokawa, T., Shoda, M., Sugano, Y., 2009. Purification and characterization of two DyP isozymes from *Thanatephorus cucumeris* Dec 1 specifically expressed in an air-membrane surface bioreactor. J. Biosci. Bioeng. 107, 113–115.
Sugano, Y., 2009. DyP-type peroxidases comprise a novel heme peroxidase family. Cell. Mol. Life Sci. 66, 1387–1403.
Ullrich, R., Huong, L.M., Hofrichter, M., 2004. Laccase from the medicinal mushroom *Agaricus blazei*: production, purification and characterization. Appl. Microbiol. Biotechnol. 67, 357–363.
Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelator. J. Biol. Chem. 267, 23688–23695.
Wesenberg, D., Kyriakides, I., Agathos, S., 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22, 161–187.
Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K., Dalbøge, H., 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl. Environ. Microbiol. 62, 834–841.
Yemendzhiev, H., Gerginova, M., Krastanov, A., Stoilova, I., Alexieva, Z., 2008. Growth of *Trametes versicolor* on phenol. J. Ind. Microbiol. Biotechnol. 35, 1309–1312.
Zybailov, B., Mosley, A.L., Sardiu, M.A., Coleman, M.K., Florens, L., Washburn, M.P., 2006. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. J. Proteome Res. 5, 2339–2347.