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MINI-REVIEW

The ligninolytic peroxidases in the genus *Pleurotus*: divergence in activities, expression, and potential applications

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Abstract Mushrooms of the genus *Pleurotus* are comprised of cultivated edible ligninolytic fungi with medicinal properties and a wide array of biotechnological and environmental applications. Like other white-rot fungi (WRF), they are able to grow on a variety of lignocellulosic biomass substrates and degrade both natural and anthropogenic aromatic compounds. This is due to the presence of the non-specific oxidative enzymatic systems, which are mainly consisted of lacasses, versatile peroxidases (VPs), and short manganese peroxidases (short-MnPs). Additional, less studied, peroxidase are dye-decolorizing peroxidases (DyPs) and heme-thiolate peroxidases (HTPs). During the past two decades, substantial information has accumulated concerning the biochemistry, structure and function of the *Pleurotus* ligninolytic peroxidases, which are considered to play a key role in many biodegradation processes. The production of these enzymes is dependent on growth media composition, pH, and temperature as well as the growth phase of the fungus. Mn^{2+} concentration differentially affects the expression of the different genes. It also severs as a preferred substrate for these preoxidases. Recently, sequencing of the *Pleurotus ostreatus* genome was completed, and a comprehensive picture of the ligninolytic peroxidase gene family, consisting of three VPs and six short-MnPs, has been established. Similar enzymes were also discovered and studied in other *Pleurotus* species. In addition, progress has been made in the development of molecular tools for targeted gene replacement, RNAi-based gene silencing and overexpression of genes of interest. These advances increase the fundamental understanding of the ligninolytic system and provide the opportunity for harnessing the unique attributes of these WRF for applied purposes.

Keywords Diversity · Peroxidases · *Pleurotus* · Manganese peroxidase · Versatile peroxidase · White-rot fungi

Introduction

The genus *Pleurotus* (Fries) Kummer (Basidiomycota, Agaricales) was defined by Paul Kummer in 1871. It is a group of gilled mushrooms comprised of cultivated edible ligninolytic fungi with medicinal properties along with a wide array of biotechnological and environmental applications. This genus is also one of the most diverse groups among cultivated mushrooms and has been a subject of many taxonomic discrepancies due to abundant morphological similarities, which have been used as the main taxonomic criteria in the past (Vilgalys et al. 1993; Zervakis and Balis 1996). In recent years, DNA-based criteria have become a key tool in clarifying the taxonomic status of *Pleurotus* (Avin et al. 2014; Pawlik et al. 2012; Shnyreva et al. 2012). The cultivation of *Pleurotus* spp., which has expanded in the past few years, has become significant for the food industry (Deepalakshmi and Mirunalini 2014). *Pleurotus ostreatus* (the oyster mushroom) is the second most consumed edible mushroom worldwide, with a current production of approximately 3 million metric tons/year (Sanchez 2010) and is grown on a variety of lignocellulosic substrates available locally as agricultural by-products. In addition, several other *Pleurotus* taxa such as *P. pulmonarius* (the Phoenix mushroom), *P. eryngii* (the king oyster), *P. sajor-caju* (Indian oyster), *P. sapidus*, *P. cystidiosus* (Abalone oyster), *P. citrinopieatus* (the golden oyster mushroom), and *P. djamor* (the pink oyster mushroom) are available commercially. As white-rot fungi (WRF), *Pleurotus* spp. are saprotrophic in the wild, where they grow readily on

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woody substrates. Members of this group have a unique ability to selectively mineralize lignin via the production of several types of extracellular oxidizing enzymes, which comprise the lignin degradation system. Due to the non-specific properties of this system, WRF have distinct capabilities to degrade a large range of natural and anthropogenic compounds that have structural and chemical similarities to the lignin substructure. These include dyes, polycyclic aromatic hydrocarbons (PAHs), and various pharmaceuticals (Hadar and Cullen 2013).

One of the major components of the ligninolytic system is the peroxidase enzyme family, which is comprised of several members. Peroxidases are oxidoreductases that utilize hydrogen peroxide to catalyze oxidative reactions of diverse substrates. This process was described as a key reaction in the process of "enzymatic combustion" where enzymatically generated hydrogen peroxide oxidizes the lignin polymer in a reaction catalyzed by ligninolytic peroxidases (Kirk and Farrell 1987). In *Pleurotus* spp., continuous production of hydrogen peroxide is required for ligninolytic peroxidase activity (Gutiérrez et al. 1994). Several peroxide-generating oxidases, such as aryl alcohol oxidase (AAO) (Hernández-Ortega et al. 2012), glyoxal oxidase (Kersten and Kirk 1987), and pyranose oxidase (Daniel et al. 1994), were described in ligninolytic fungi.

All peroxidases are grouped under EC 1.11.x (donor: hydrogen peroxide oxidoreductase) in the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NCJUBMB) system of enzyme classification and nomenclature (Fleischmann et al. 2004). Classification of peroxidases on the basis of protein properties and structure–function relationships is extremely complex. Not only can a single peroxidase have diverse substrates but peroxidases of distinctly different structures may catalyze the same reaction. Furthermore, peroxidases can have different modes of oxidation while catalyzing the same net overall reaction (Hofrichter et al. 2010).

The lignin degradation systems of many WRF are mainly comprised of the triad of laccases (copper-containing oxidases) and two hemeperoxidases; lignin peroxidase (LiP) (EC1.11.1.14) and manganese peroxidase (MnP) (EC 1.11.1.13). A fourth player in the lignin biodegradation system is versatile peroxidase (VP) (1.11.1.16), less common among the WRF and which has mainly been described in *Pleurotus*, *Bjerkandera*, and a few other genera (Carabajal et al. 2013; Fernández-Fueyo et al. 2012). Based on their crystal structure LiP and MnP, together with the *Coprinopsis cinereus* peroxidase (CiP) (EC 1.11.1.7), were classified as class II peroxidases, which are distinct from the class I intracellular peroxidases and class III secretory plant peroxidases (Welinder 1992). VP has been shown to have properties of both LiP and MnP and was classified into the class II peroxidases group. It is assumed that VP has a similar function to LiP, as the latter is not found in *Pleurotus* spp. In addition, two other peroxidases were identified in WRF: heme-thiolate peroxidases (HTPs) and dye-decolorizing peroxidases (DyPs). Both have a clearly distinct sequence and protein structures that diverge them from the class II peroxidases, justifying their classification as unique superfamilies (Hofrichter et al. 2010). To date, only a single report on a DyP enzyme in *Pleurotus* (*ostreatus*) has been published (Faraco et al. 2007), and we are not aware of any HTPs reported in this genus. Hence, the focus of this review is on the diversity of ligninolytic class II peroxidases (PODs) of *Pleurotus*: VPs and MnPs.

Structure–function relationship

CiP as well as LiP and MnP of *Phanerochaete chrysosporium* were the first peroxidases whose crystal structures were solved, in the early 1990s (Petersen et al. 1994; Piontek et al. 1993; Poulos et al. 1993; Sundaramoorthy et al. 1994). The crystal structures present an overall compact protein comprised of a helical fold with heme tightly located between two domains, both of which contain one stabilizing Ca^{2+} ion and two conserved His residues (proximal and distal). Data concerning the 3D protein structure of class II fungal peroxidases are mainly based on these structures.

MnP and VP, along with laccases, are the major part of the *Pleurotus* ligninolytic system. So far, several isoenzymes in four *Pleurotus* species have been identified, isolated, and characterized at the protein level (Table 1). MnPs are the most common ligninolytic enzymes, first discovered in the WRF *P. chrysosporium* (Gold et al. 2000; Kuwahara et al. 1984). They contain Mn^{2+}-binding catalytic site that is formed by three acidic residues (two Glu and one Asp). This site has been determined as the site where the enzyme oxidizes Mn^{2+} to Mn^{3+} and then Mn^{3+} chelates complexes are formed with dicarboxylic acid anions. The Mn^{3+}-dicarboxylic acid complex is thought to dissociate from the enzyme and functions as a diffusible oxidant that can degrade phenolic substrates including dyes and phenolic lignin structures (Hammel and Cullen 2008; Hofrichter 2002).

Recently, phylogenetic analysis has led to a new clustering of MnPs to two subgroups long and short MnPs (Hofrichter et al. 2010; Ruiz-Dueñas et al. 2011). The first have been shown to depolymerize lignin in vitro. They include an additional C-terminal extension in their amino acid sequence from the model ligninolytic fungus *P. chrysosporium*. These enzymes are specific and require Mn^{2+} to complete their catalytic cycle. The short MnPs were initially described in *Phlebia radiata* (Hildén et al. 2005) and are able to directly oxidize phenols, amines, and small dye compounds, in the absence of Mn^{2+}, through an additional active site that contains an exposed heme edge (Hofrichter et al. 2010; Morales et al. 2012). This site is typical to some other peroxidases, such as LiP, CiP,

Table 1 Isolated and identifed ligninolytic peroxidases from *Pleurotus* spp.

Pleurotus spp.	Protein name	Culture	GenBank entry[a]	References	p*I*	MW	Notes
P. eryngii	VPL1	Liquid (GP)	AF007223 (Q9UR19.1)	Martínez et al. 1996a,b Ruiz-Dueñas et al. 1999	3.70	43	
	VPL2	Liquid (GP)	AF007222/AF007224 (O94753.1)	Martínez et al. 1996a,b Ruiz-Dueñas et al. 1999	3.65	43	
	VPL3	Liquid (GP)	DQ056374.1 (Q8J154)	Ruiz-Duenas et al. 2007	–	–	
	VPS1	SSF (straw)	AF177510 (Q9UVP6)	Camarero et al. 1999 Martínez et al. 1996b	3.67	45	
	VPS2	SSF (straw)	–	Camarero et al. 1999 Martínez et al. 1996b	3.65	45	
	MnP-PS3	SSF (straw)	–	Camarero et al. 1999 Martínez et al. 1996b	3.80	42	This enzyme was identified as MnP, since it unable to direct oxidizes VA
P. ostreatus[b]	MnP1	Liquid (GP)	AAA84396 (Q12332)	Kofujita et al. 1991 Asada et al. 1995		42	
	MnP3	Liquid (GP with wheat bran extracted)	BAA33449/BAA33009 (O74640/O74179)	Irie et al. 2000	3.60	42	At the amino acids sequence level 100 % homology (pI 4.06 and MW 42 kDa) was found with an enzyme
		SSF (poplar sawdust/perlite)		Giardina et al. 2000			produced in PGY medium (Kamitsuji et al. 2004)
				Cohen et al. 2001			In liquid Mn^{2+}-amendment GP, MnP3 peptides sequences confirmed that it is the predominante peroxidase under this culture conditions (Salame et al. 2013)
	VP1	Liquid (GP, not-amendment Mn^{2+}) Wheat straw mixed with poplar chips	Q96V56[c]	Sarkar et al. 1997 Cohen et al. 2001 Fernández-Fueyo et al. 2014b	3.75	43	
	VP2	SSF using sawdust	AJ243977 (Q9UVY8)	Giardina et al. 2000	3.70	41	At the amino acids sequence level 100 % homology (pI 3.77 and MW 42 kDa) was found with an enzyme produced in GY medium (Kamitsuji et al. 2004), but this enzyme has higher catalytic activity to VA
P. pulmonarius	VPL VPS	Liquid (GP) SSF (straw)	– –	Martínez et al. 1996a	3.55	43	An additional two enzymes of *P. pulmonarius* were founded in UniProtKB database with no published reference. I6TLM2 and Q2VT17 that are corresponded to VP and MnP5 respectively
P. sapidus	VP	Liquid with residue of a biogas plant as nitrogen and carbon sources	AM039632.1 (Q4QZ27)	Schüttmann et al. 2014	3.50 5.00	45	Genome sequencing of *P. sapidus* reveled at least one MnP and one VP (Zorn et al. 2005)

[a] The databases were used in this study are NCBI and UniProtKB (in brackets)

[b] These enzymes were identified before the genome sequenced. Unique peptides of MnP2 and MnP6 were also identified in both liquid GP and lignocellulos medium (Fernández-Fueyo et al. 2014a; Salame et al. 2013)

[c] Partial sequence

and horseradish peroxidase (HRP) (EC 1.11.1.7) (Doyle et al. 1998; Hofrichter et al. 2010; Morales et al. 2012; Smith and Veitch 1998). It is assumed that phenolic compounds are oxidized through this channel, in direct contact with the heme group as in CiP and HRP (Smith and Veitch 1998), and the same site has been suggested to be critical for oxidation of anionic dyes by LiP (Doyle et al. 1998). The second group of peroxidases, VPs, is capable of oxidizing Mn^{2+} via a mechanism similar to that described for MnPs (Ruiz-Dueñas et al. 2007). Due to the VPs Mn^{2+}-oxidizing activity, these enzymes were first described as MnP isoenzymes (Giardina et al. 2000; Martínez et al. 1996b) but later recognized as representing a new peroxidase type. VPs have a third active site on the enzyme surface, the exposed tryptophanyl radical (Trp-164), as found in LiP (Pérez-Boada et al. 2005). This site is responsible for direct oxidation of low and high redox potential compounds, including those that LiP can oxidize only in the presence of redox mediators (Pérez-Boada et al. 2005; Ruiz-Dueñas et al. 2008). It was suggested that this site acts as a substrate intermediate protein radical center and initiates of long-rang electron transfer (LRET) pathway leading to the heme (Doyle et al. 1998; Blodig et al. 2001; Smith et al. 2009). The mechanistic conclusions described above have all been confirmed by genetic analyses utilizing site-directed mutagenesis to alter the various functional residues/sites (Morales et al. 2012; Pérez-Boada et al. 2005; Ruiz-Dueñas et al. 2007, 2008). Taken together, it appears that the high number of VP and MnP isoenzymes can compensate for the lack of LiP in the genus *Pleurotus*, since VPs can function (along with additional properties) as LiPs. In addition, the distinctive function of efficient oxidation of Mn^{2+} has been shown to encompass the entire MnP/VP family members, while the direct oxidation of aromatic compounds is specific for each isoenzyme group and may be unique for each isoenzyme.

DyP is an additional peroxidase that was first discovered in the fungus *Bjerkandera adusta* (previously designated as *Thanatephorus cucumerie* Dec 1) (Kim et al. 1995). This enzyme appears to be distinct from other known peroxidases. So far, hundreds of homologous proteins have been identified as DyP/DyP-type via PSI-BLAST analyses. However, only several reports on the functional and structural characterization of these proteins have been published (Sugano 2009), among them, a single study on DyP in *Pleurotus* (Faraco et al. 2007). The crystal structure of DyP from the fungus *B. adusta* was solved, and it was shown that the region surrounding the heme group in this protein is unique. While a proximal histidine is present, the distal histidine is lacking, indicating that heme binding occurs normally, while the H_2O_2 reaction proceeds by a unique mechanism (Liers et al. 2013; Sugano et al. 2007). HTPs are extracellular peroxidases, which also differ from the class II group, as they have a cysteine-ligated heme group at their active site. The most interesting catalytic property of secreted HTPs is the capability of transferring peroxide-oxygen from H_2O_2 (or other ROOHs) to substrate molecules, conferring peroxygenase activity to this group of enzymes. The *Leptoxyphium fumago* chloroperoxidase (CPO) was the first HTP described (Shaw and Hager 1959). The main evident activity of this enzyme is the oxidation of chloride into hypochlorous acid. A second group of HTPs was discovered, including the aromatic peroxigenase (APO) from the wood and litter dwelling black poplar mushroom *Agrocybe aegerita* (Ullrich et al. 2004). These enzymes have since been classified and grouped as unspecific peroxygenases (UPO) (EC 1.11.2.1) (Hofrichter and Ullrich 2014). To date, the genes encoding for these enzymes were identified in the *Pleurotus* genome, but the corresponding proteins have not been isolated, identified, or otherwise characterized.

Molecular characterization of PODs

All the heme peroxidases in *Pleurotus* are acidic, glycosylated, and secreted, with a high Reinheitszahl value (Rz; A_{409}/A_{280} ratio) (Hofrichter et al. 2010). *Pleurotus*' mature PODs molecular mass (MW) and isoelectric point (p*I*), estimated by their amino acid sequences, are 35–37 kDa and 4.5–5.7, respectively. Experimental data, based on gel-based separation, demonstrated a MW ranging 41–45 kDa and p*I*s of 3.55–3.8 (Table 1) and Rz value of above 4. The single isolated *Pleurotus* DyP had a molecular mass of about 73 kDa and a large glycosylation moiety (38 % by weight) (Faraco et al. 2007).

The catalytic cycle of PODs: MnP versus VP

All enzymes that catalyze the reaction: $2AH_2+H_2O_2$—$2H_2O+2AH^{\cdot}$ are defined as peroxidases, even if the catalytic cycle or the intermediates obtained are different from those seen in the well-studied general heme peroxidase cycle, as was described for horse radish peroxidase (Hofrichter et al. 2010). In this section, we focus on specific aspects of *Pleurotus* PODs with an emphasis on the differences between short-MnP and VP. The data concerning the catalytic cycle of short MnP and VP are schematically described in Fig. 1, based on integration of data from Ertan et al. (2012), Hofrichter et al. (2010), Morales et al. (2012), and Ruiz-Dueñas et al. (2008). This cycle can support various reactions that are dependent on the nature of the substrate to be oxidized. When the substrate is solely Mn^{2+}, only the basic catalytic cycle, common to both enzyme classes, occurs. As a next step, Mn^{3+} is coupled with organic acids to form the chelated Mn^{3+}, which is a small reactive diffusible compound that able to attack phenols, amines, and even lignin model compounds (Fig. 1b). In the case of other aromatic (Ar) substrates, mostly of low potential redox, the compound gains access to the heme active site and

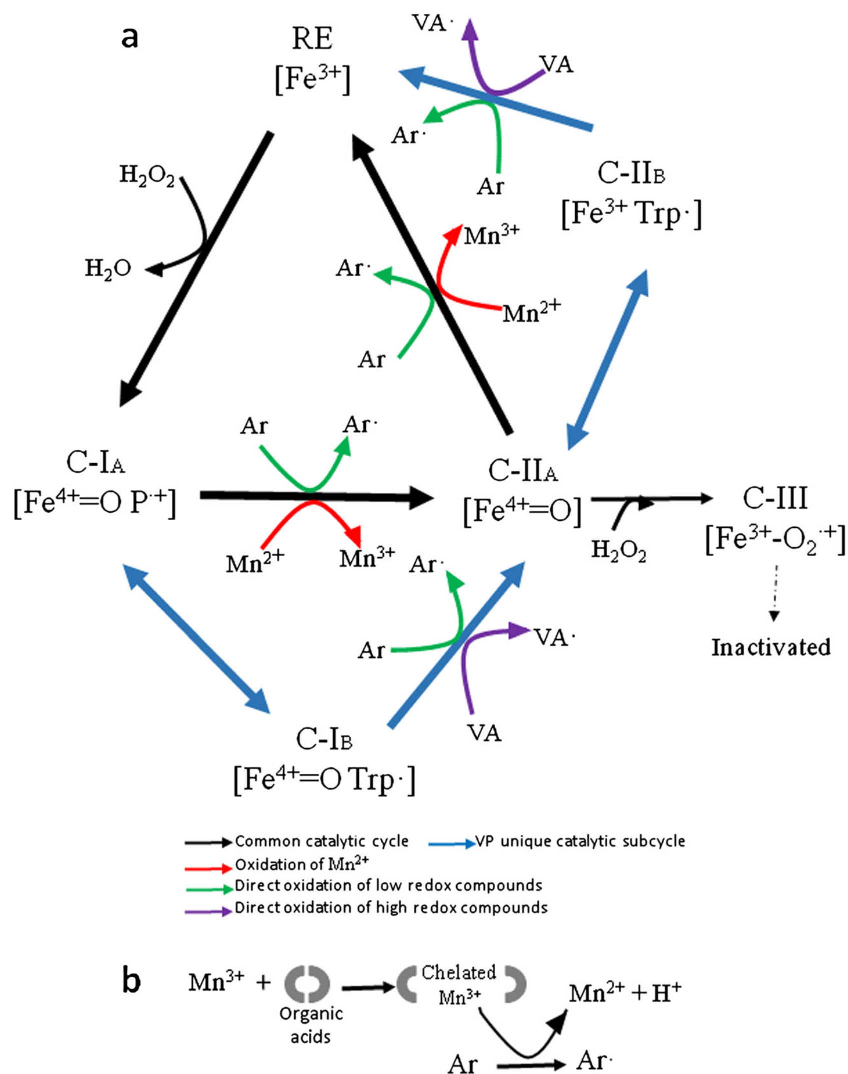

Fig. 1 **a** An integrated scheme of the *Pleurotus* PODs catalytic cycle (based on Ertan et al. 2012; Hofrichter et al. 2010; Morales et al. 2012; Ruiz-Dueñas et al. 2008). **b** Oxidation of aromatic compounds by Mn^{3+}. In these cycles, the first step, in which the enzyme oxidizes the substrate by hydroperoxide, is pH independent, whereas the subsequent steps are pH dependent (Ertan et al. 2012). Due to the chemical properties of the active sites, the optimal pH of Mn^{2+} oxidation and Mn^{2+}-independent (direct oxidation of substrates) reactions are 5 and 3, respectively. The higher optimal pH of the Mn^{2+} oxidation reaction, compared to direct oxidation of substrates, is likely to be caused by the three acidic residues and the heme propionate, which dissociates at a higher pH and binds Mn^{2+} (Camarero et al. 1999)

is oxidized via indirect contact with the δ-position of the porphyrin macrocycle by compounds I and II. In VPs, the protein radical centered on Trp-164 exposed to the solvent is responsible for direct oxidation of high redox potential compounds such as veratryl alcohol (VA). This site was found to be involved in direct oxidation of low redox potential compounds depending on their concentration. At high concentrations (mM), substrates gain access to the heme active site in a mechanism similar to that occurring in MnPs. At low concentrations (μM), they are preferentially oxidized at a protein radical centered on Trp-164 exposed to the solvent by compound I and compound II in this subcycle (Fe^{4+}=O $Trp^{·}$ and Fe^{3+}=Trp, respectively) (Morales et al. 2012). Although this cycle is H_2O_2 (or other ROOH) depended, an excess of H_2O_2 to the heme pocket in the absence of a reducing substrate at low pH (3.0–3.5) inactivates the enzyme by converting compound II to compound III (Fe^{3+}–$O_2^{·+}$) (Ertan et al. 2012; Wariishi and Gold 1990).

On the basis of the Trp-164 active site, compound I has a high-redox potential (E'o, ~1.2 V at pH 3) compared to other peroxidases (horseradish; E'o, ~0.95 at pH 6.3) and oxidases (laccase; E'o, ~0.79 at pH 5.5). This enables the oxidation of nonphenolic aromatic substrates with high redox potentials. In contrast, the Mn^{3+}-dicarboxylic acid complex that is produced as a result of Mn^{2+}-binding-oxidation site of MnP or VP has an E'o value of only 0.8 V at pH 4.5.

Genome organization in *Pleurotus ostreatus*

The study of the lignin degradation system in *Pleurotus* spp. (mainly *P. ostreatus* and *P. eryngii*) has been ongoing for over two decades. In 2009, the genome sequence of two monokaryons of *P. ostreatus* (PC9 and PC15) was completed (http://genome.jgi-psf.org/PleosPC9_1/PleosPC9_1.home.html and http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_

1.home.html). Among other data, the results obtained confirmed the absence of LiP in *P. ostreatus*. Furthermore, in addition to the four members previously described (three MnPs and one VP), the presence of five additional family members was established. Comparing the wood decay machinery of 22 basidiomycete genomes, Riley et al. (2014) concluded that *P. ostreatus* ligninolytic POD gene families resemble typical white-rot fungus.

Grouping of the nine ligninolytic peroxidases can be performed either on the basis of their molecular structure (defined by the presence of a putative catalytic tryptophan, Trp-164) (Hofrichter et al. 2010; Ruiz-Dueñas et al. 2011) or according to their activity (defined by the ability to directly oxidize high redox potential substrates) (Fernández-Fueyo et al. 2014a). In this review, we chose to adopt the nomenclature suggested by Fernández-Fueyo et al. (2014a) where the classification is according to the activity (Table 2). As a result, the peroxidase encoded by the *mnp1* gene, which was first classified as a VP due to the presence of Trp-164 (Hofrichter et al. 2010; Ruiz-Dueñas et al. 2011), is now defined as MnP, as it is unable to oxidize high redox potential substrates directly (Fernández-Fueyo et al. 2014a). To summarize, on the basis of enzymatic activity, *P. ostreatus* has three VPs and six MnPs. The high number of the PODs in *Pleurotus* suggests differential regulation, potential redundancy, and/or diversity, in their properties. In addition, four DyPs and three HTPs/CPOs genes were identified in the *P. ostreatus* genome (Ruiz-Dueñas et al. 2011). Based on changes introduced to gene/protein nomenclature and groupings over time, we suggest the use of JGI/Broad or other genome sequencing resource locus designation numbers/protein ID, in association with gene names used by the different members of the research community, to provide a common baseline that will assist in reducing discrepancies.

The availability of the genome sequence has proven invaluable, especially when added to the constant accumulation of tools involving the genetic manipulation of *P. ostreatus*. An improved transformation system, coupled with an efficient homologous recombination system based on the deletion of the *ku80* gene has increased the amenability of *P. ostreatus* to the introduction of genetic changes and has advanced the value of this fungus as a model for comprehensive analysis of the ligninolytic system (Salame et al. 2012a). As part of this process, a series of ligninolytic enzyme mutants was produced; among them are Δ*vp1*, Δ*vp2*, Δ*mnp2*, and Δ*mnp3* (Salame et al. 2012a,b, 2013). In addition, studies on the factors involved in the expression of these genes along with heterologous expression of the nine PODs coupled with analyses of their kinetics has become possible (Fernández-Fueyo et al. 2014a,b; Knop et al. 2014; Salame et al. 2010, 2012b, 2013, 2014).

Table 2 Current and previous nomenclature of ligninolytic peroxidases present in *P. ostreatus*

Current designations		Protein ID		Previouse designations	
Gene	Protein	PC9	PC15	Protein	References
vp1	VP1	137757	137757	MnP	Sarkar et al. 1997
				MnP4	Cohen et al. 2001
				VP4	Salame et al. 2012b-2014
					Knop et al. 2014
vp2	VP2	137766	1113241	MnP2	Giardina et al. 2000
					Cohen et al. 2001
					Kamitsuji et al. 2004-2005a, b
					Tsukihara et al. 2006a,b, 2008
vp3	VP3	123383	156336	MnP5	
mnp1	MnP1	137760	1096331	MnP1[a]	
mnp2	MnP2	137764	199510	MnP9	Salame et al. 2012b, 2013, 2014
mnp3	MnP3	137740	1089546	MnP3[a]	
mnp4	MnP4	121638	1099081	MnP7	Salame et al. 2013
mnp5	MnP5	137765	199511	MnP6	Knop et al. 2014
mnp6	MnP6	51713	1041740	MnP8	Salame et al. 2014

[a] Under the same designation in the literature

Evolutionary relationships of PODs

Evolutionary analysis of PODs revealed that the general peroxidase (GP) ancestor likely lacked the Mn^{2+} binding and the Trp residues present in MnPs, LiPs, and VPs, suggesting that it was non-ligninolytic (Floudas et al. 2012). The evolutionary relationships among *Pleurotus* spp. PODs (Fig. 2) indicate the presence of three main subgroups (A–C). The *P. ostreatus* genes appear in all groups, along with representatives from other species. It should be noted that *P. ostreatus* is the only *Pleurotus* sp. sequenced so far, and POD genes from other *Pleurotus* species are likely to be discovered. Interestingly, *P. ostreatus* VP1 groups together with VPs from *P. sapidus*, *P. pulmonarius*, and *P. eryngii* VPLs (group B). This may imply that this type of VP is the most abundant POD among *Pleurotus* spp. In addition, *P. eryngii* VPS1 and *P. ostreatus* VP2 cluster together, and both exhibit highly abundant transcripts in solid state fermentation (SSF) cultures (Camarero et al. 1999; Salame et al. 2014). The classification of ligninolytic enzymes from different genera by enzyme type, rather than by genus, was also observed by when genomes of over 30 basidiomycetes were analyzed (Floudas et al. 2012; Ruiz-Dueñas et al. 2013).

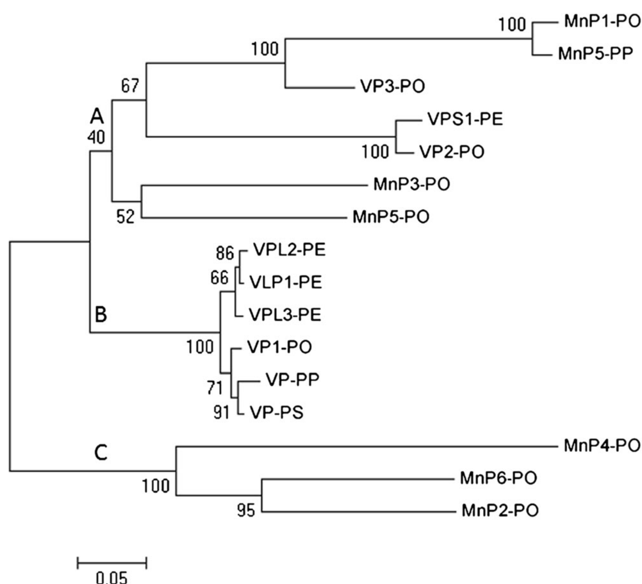

Fig. 2 Molecular phylogenetic analysis of PODs from different *Pleurotus* species. The protein sequences (protein designations are identical to those in Table 1) found in *P. ostreatus* (PO), *P. eryngii* (PE), *P. pulmonarius* (PP), and *P. sapidus* (PS) were aligned by MUSCLE and evolutionary history was inferred using the maximum likelihood method. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

Heterologous expression

There have been extensive studies on MnP and VP enzymes from various WRF heterologously expressed in *Escherichia coli*. VPL2 of *P. eryngii* has been the most intensively studied ligninolytic enzyme and was the first *Pleurotus* ligninolytic enzyme that was heterologously expressed (Martínez 2002). Optimization of the refolding process enabled the production of active ligninolytic enzymes (Pérez-Boada et al. 2002). This also provided the basis for studying structure–function relationships by site-directed mutagenesis. Heterologous expression of the nine *P. ostrestus* MnP/VP isoenzymes, followed by solving the crystal structure of two enzymes (one VP and one short MnP) were instrumental in elucidating the molecular differences between the two groups (Fernández-Fueyo et al. 2014a). However, in spite of the advantages and the success accompanying the use of the bacterial expression systems, the catalytic properties of the recombinant enzymes were found to be slightly different from the native enzymes. One reason proposed to be involved in the mentioned differences is the lack of glycosylation in these systems. This was reflected in lower molecular weights of the recombinant proteins and lower affinity to Mn^{2+}, as the Mn^{2+} binding-catalytic site requires glycosylation. Heterologous expression of *P. eryngii* VP in *P. chrysosporium* was demonstrated by Coconi-Linares et al. (2014). Now, this fungus having endogenous LiP and MnP could produce also the third type of ligninolytic POD, VP. This approach may lead to development of more effective white rot strain.

Factors involved in expression and activity: Are *Pleurotus* PODs redundant?

The dual function of Mn^{2+}

The chemical nature and abundance of manganese provides a basis for its involvement in lignin biodegradation (Blanchette 1984; Kerem and Hadar 1995; Perez and Jeffries 1992; Rüttimann-Johnson et al. 1993). Manganese oxide deposits are usually associated with preferentially delignified wood, and the quantity of manganese found in delignified regions has been reported to by far exceed the quantity present in sound wood or surrounding decayed wood. Therefore, it has been suggested that manganese may be an important component in the degradative processes resulting in the selective removal of lignin by WRF and that an external source of manganese is essential for the process to take place (Blanchette 1984). Thus, manganese transport, accumulation, and immobilization in wood decayed by WRF are important components of the lignin biodegradation system. Lignin degradation by *Pleurotus* has also been shown to be enhanced by the addition of Mn^{2+} to the lignocellulosic substrate (Kerem

and Hadar 1995; Cohen et al. 2001, 2002a; Camarero et al. 1996; Giardina et al. 2000; Salame et al. 2014). Hence, the study of the effect of manganese on the ligninolytic system is well documented. It was established that Mn^{2+} has a dual role in this system: first, as an inducer or repressor of MnP/VP transcription (Salame et al. 2010), and second, it is considered as the best known substrate for both MnP and VP in *Pleurotus*. The effect of Mn^{2+} on isoenzyme expression level varies in the presence of different carbon and nitrogen sources. Due to the fact that natural substrates contain a basal level of Mn^{2+}, the study of the effect of Mn^{2+} on relevant gene expression level was performed in cultures grown in synthetic medium, mainly in GP, where the Mn^{2+} level was found to be negligible. In GP medium, all nine *P. ostreatus* PODs were found to be expressed. Mn^{2+} amendment to GP-grown cultures resulted in dramatic differences in the transcript abundance of the various *mnps* (Cohen et al. 2002c; Salame et al. 2010). The *mnp3*, *mnp2* (short-MnPs), and *vp1* (VP) genes were found to be the predominant genes expressed during the active growth phase (tropophase) (Salame et al. 2010). In addition, a redundancy phenomenon in MnP/VP isoenzymes was identified under these growth conditions (Salame et al. 2010). However, under Mn^{2+} deficiency, *vp1* was found to be the predominantly expressed gene in both tropophase and the idiophase and was found to play a key role in oxidation of different substrates (Salame et al. 2010; Knop et al. 2014). It was also determined that when VP1 was inactive, both degradation of dyes and oxidation of aromatic substrates, as well as Mn^{2+}, are impaired (Knop et al. 2014).

Physical conditions

In addition to manganese, other factors were found to influence transcript abundance and MnP/VP protein expression levels in *Pleurotus*. In general, it was found that different isoenzymes are preferentially expressed in liquid or SSF cultures (Table 2). In some cases, the same enzyme was produced in both culture types. Interestingly, when *P. ostreatus* VP2 was produced in liquid culture, its capability to oxidize VA was much higher than under SSF conditions (Kamitsuji et al. 2004). Static or shake conditions have also been shown to affect the expression of these isoenzymes. Static cultures are considered to better reflect the natural conditions in which these fungi grow. To date, no differences were found in isoenzyme characteristics between shake and static cultures, excluding the fact that, in stationary cultures, maximum activity was registered at the idiophase instead of the tropophase stage of growth (Martínez et al. 1996a). The effects of pH and temperature on the expression level of the nine *P. ostreatus* PODs were also studied. Under standard conditions (25 °C, pH 5.5), peroxidase transcript levels were the highest (mainly *mnp3* and *vp1*) and, accordingly, both Mn^{2+}-oxidation and Mn^{2+}-independent activity. Changing the temperature or pH to extreme conditions (10 °C or 37 °C and pH3 or pH8, respectively) led to the conclusion that the *vp* genes represented the majority of the total ligninolytic peroxidases (excluding *mnp3*) expressed (Fernández-Fueyo et al. 2014b). In addition, at pH 8, the most highly expressed genes were *mnp4* and *mnp5* (Fernández-Fueyo et al. 2014b).

The stability of the heterologously expressed *P. ostreatus* peroxidases at various pH levels (2–9) and temperatures (25–70 °C) was investigated. MnP4 appears to be the most stable at both acidic and alkaline pHs (in correlation with the high expression of its corresponding gene under alkaline conditions). In contrast, MnP2 and MnP3 were the most unstable peroxidases under both acidic and alkaline conditions. Spectroscopic analyses confirmed that acidic/alkaline conditions caused significant modifications of the MnP3 spectra and only slight changes in those of MnP4. Overall, VP1 was found to be the most stable peroxidase at different pHs and temperatures (Fernández-Fueyo et al. 2014a).

Carbon and nitrogen sources

Synthetic medium In *Pleurotus*, the ligninolytic activity was found to be higher in nitrogen-rich medium, such as glucose-peptone (GP) (C/N ratio, 38:1) and negligible when other organic sources of nitrogen were used even at the same concentration of nitrogen (Cohen et al. 2002c; Kamitsuji et al. 2004; Martínez et al. 1996a; Ruiz-Dueñas et al. 1999; Stajić et al. 2006). This is in contrast to that described in *P. chrysosporium* (Capdevila et al. 1990). To date, there is no appropriate defined synthetic culture medium that supports proper culturing of *Pleurotus*. This fact imposes difficulties in the process of examining the effect of components in the medium, especially carbon and nitrogen sources, on the induction of MnP/VP expression. Ruiz-Dueñas et al. (1999) investigated which components of peptone were involved in the altering MnP/VP expression, on the basis peptone fractionation by gel filtration. They showed that only the highest molecular weight fraction, comprised of a comparatively low content of aromatic amino acids, can function as a potential peroxidase expression inducer.

Natural substrates Recently, Schüttmann et al. (2014) studied the effect of different natural substrates on VP activity in *P. sabidus*. They showed that the highest VP activity was measured when the fungus was cultured on residues of biogas plant material, where the C/N ratio was 10:1. This finding supports previous findings (see above), repeatedly demonstrating that ligninolytic activity is induced in nitrogen-rich medium. The effect of different natural substrates on the expression level of the nine *P. ostreatus* peroxidase isoenzymes was examined. *mnp3*, *vp1*, and *vp2* genes were found to be the most highly expressed genes when the fungus was grown in the presence of different natural substrates (Salame

et al. 2010, 2013, 2014; Fernández-Fueyo et al. 2014b). When grown on cotton stalks, *vp2* was the predominantly expressed gene, and there was a correlation with the abundance of its corresponding protein in the secretome (Salame et al. 2014). However, when grown on poplar chips, under standard conditions, *mnp3* and *vp1* were the most highly transcribed, yet the VP1 enzyme was the most abundant protein in the secretome. Fernández-Fueyo et al. (2014b) suggested that, since comparable levels of MnP3 were found in the intra- and extracellular fractions, this is most likely due to impaired secretion of MnP3. Another possibility is that MnP3 secretion is regulated in a substrate or environmental condition-dependent manner.

Regulation at the transcriptional level

The presence of a high number of different ligninolytic genes in *Pleurotus* spp. raises the question concerning common versus differential transcriptional regulation in this gene family. The *Pleurotus* POD-encoding genes have been found to have different responsive elements at the 5′-upstream sequences. These include putative heat-shock elements (HSE), metal-response elements (MRE), xenobiotic-response elements (XRE), and cAMP response elements (CRE) (Asada et al. 1995; Fernández-Fueyo et al. 2014b; Giardina et al. 2000). All of these have the potential to play a role in the environmental regulation of POD gene expression.

Lignin and other aromatic compounds degradation as well as many other degradation phenomena have been described to occur during the idiophase of the fungal life cycle. Accordingly, it was suggested that production of PODs is a secondary metabolic event, as shown in *P. chrysosporium* (Kamitsuji et al. 2004).

Our understanding of the transcriptional regulation of POD gene expression is lacking. The fact the transcriptional regulatory elements have been identified is an important initial step towards identification of activators/repressors and further elucidation of the regulatory signals and networks involved.

Biotechnological applications

Due to the presence of the non-specific oxidative systems, *Pleurotus* spp., like other WRF, are able to grow on a variety of lignocellulosic biomass substrates and degrade both natural and anthropogenic aromatic compounds. The use of *Pleurotus* has been suggested for many potential applications such as cultivation of edible and medicinal mushrooms, bio-bleaching and bio-pulping, bio-remediation, upgrade, and pretreatment of biomass for ruminants feed and biofuel production (Cohen et al. 2002b; Haas et al. 2004; Singh and Singh 2014; Stajić et al. 2009). In many cases, these applications require the utilization of the fungus as whole-cell biocatalysts and involve the concerted activity of several enzymatic systems, many of which are extracellular peroxidases and oxidases. Here, we will describe suggestions for practical utilization of cell-free or purified forms of *Pleurotus* peroxidases. The use of these enzymes is aimed towards more specific applications such as degradation of persistent organic pollutant, such as textile dyes and humic substances and synthesis and modifications of organic molecules (Rao et al. 2014). Several approaches for the production and use of enzymatic preparation were suggested.

(a) Enhanced PODs production: applications of enzymes require large amounts of the protein and their production from native sources (wild-type *Pleurotus* grown in liquid or solid media) may not provide sufficient, economically justified, yields. Hence, heterologous expression of PODs seems to be a promising alternative, as it enables rapid, high-yield, protein production (Martínez 2002). However, quantitative data that can enable comparison between different approaches are not yet available. Here, we describe several examples. Schüttmann et al. (2014) produced a recombinant VP from *P. sapidus* in cultures of *Hansenula polymorpha* showing carotenoid degrading activity. The nine *mnps* of *P. ostreatus* were successfully expressed in *E. coli* (Fernández-Fueyo et al. 2014a). In addition, studies to increase stability and improve the kinetics of the transgenic enzymes are part of the efforts conducted with biotechnological applications in mind (Fernández-Fueyo et al. 2014a). Arunkumara and Sheik Abdullaa (2014) described a mutant of *P. ostreatus* able to hyper-produce MnP. The strain was obtained following the performance of a random mutagenesis procedure in which the wild strain (*P. ostreatus* MTCC 142) was exposed first to UV light and then to ethidium bromide. The stable strain obtained following the dual mutagenesis procedure showed an 80 % increase in MnP activity when compared with the parental strain (Arunkumara and Sheik Abdullaa 2014). Many reports suggest the cultivation of *Pleurotus* for enzyme production on agricultural and food industry by-products (Stajić et al. 2006; Songulashvili et al. 2007). Arunkumara and Sheik Abdullaa (2014) used rice and wheat brans to grow the *Pleurotus* mutant described above. In another study, Bazanella et al. (2013) investigated the effect of initial moisture levels of MnP production from *P. pulmonarius* using agricultural and food wastes as substrates. They showed that cultures grown on corn cobs at low initial moisture of 50–70 % obtained higher level of MnP activities.

(b) Enzyme technology: several approaches have been proposed to improve the functionality of fungal peroxidases

by methods such as enzyme immobilization, cross-linking with other enzymes and combination of bio- and inorganic catalysts (Rao et al. 2014; Vásquez et al. 2014). For example, Gasser et al. (2012) reviewed new perspectives for the combination of bio- and inorganic catalysts in one-pot reactions, having the potential to be used for lignin depolymerization for production of added-value aromatic chemicals. VP is one candidate that can be used as a biocatalyst in such systems. In addition, VP from *P. eryngii* has been suggested to be a source for generating new biomolecules by homogeneous and heterogeneous cross-linking of different substrates (Salvachúa et al. 2013). They demonstrated that VP can catalyze the polymerization of low molecular mass compounds, such as lignans and peptides, as well as larger macromolecules, such as proteins and complex polysaccharides. It was concluded that the presence of Mn^{2+} improved the stability of VP in the presence of organic co-solvents, which are essential in most reactions involving lignans (Salvachúa et al. 2013). In another study, a wide range of pharmaceutically active compounds were transformed by three cross-linked oxidative enzymes: laccase from *Trametes versicolor*, VP from *B. adusta*, and glucose oxidase from *Aspergillus niger*. These cross-linked aggregates lead to the formation of insoluble and versatile biocatalysts with expanded substrate ranges as well as versatile operating conditions (Touahar et al. 2014). *Pleurotus* MnPs and VPs were also studied for use in specific applications, mainly for decolorization of textile dyes (Heinfling et al. 1998; Zhao et al. 2007; Solís et al. 2012). Asgher et al. (2013) showed that MnP produced by partially purified *P. ostreatus* pregrown on wheat straw can be immobilized using hydrophobic sol–gel entrapment comprising tetramethoxysilane and propyltrimethoxysilane. After 24 h of incubation at varyious pH levels and temperatures, the MnP fraction immobilized in the sol–gel retained 75–82 % of its original activity. The immobilized MnP was able to decolorize four different effluents collected from textile plants (Asgher et al. 2013).

In summary, these examples suggest that large-scale production of PODs can provide efficient catalysts for bioremediation, purification of contaminated water, as well as for synthetic chemistry.

Prospects

VPs and MnPs are components of the complex ligninolytic system found in different *Pleurotus* spp. as well as other WRF. As shown in this review, a significant amount of information concerning the biochemistry, structure, and production of *Pleurotus* PODs has been accumulated. Much of the biochemical and structural analysis has been performed in heterologous expression systems, yielding exciting results. Determining the implications of some of these findings, and others, in the fungus and on natural/ artificial substrates is becoming more feasible. During the past few years, progress has been made in the development of molecular techniques for targeted gene replacement, RNAi-based gene silencing, and overexpression of genes in *P. ostreatus* (Salame et al. 2011, 2012a,b, 2013; Knop et al. 2014; Yao et al. 2013; Amore et al. 2012). This molecular genetic “toolbox” has been successfully used for better understanding VP and MnP function. Further analysis, coupled with additional methods such as transcriptomics and metabolite analysis will further elucidate the function of other relevant genes. Such studies can provide a better mechanistic understanding of the ligninolytic systems in fungi as well as a basis for strain improvement and mushroom breeding.

To enhance the understanding and enable the exploitation and implementation of the potential of this group of fungi and the enzymes they produce, several lines of research can prove to be advantageous. First, full genome sequence and analysis of additional members of the *Pleurotus* genus is required. Together with the many genomes of other wood decay fungi recently made available (Floudas et al. 2012; Levasseur et al. 2014; Ohm et al. 2014), these data will not only shed more light on the evolution of the ligninolyic fungi but will also provide the tools for more genetic-based experimental advances. Second are studies at the protein level to evaluate the functionality/specificity of the enzymes. Third are transcriptional and proteomic analyses of *P. ostreatus* under different conditions while degrading different aromatic compounds for discovery of additional mechanisms, pathways, and regulatory elements involved in the degradation process.

PODs are not the sole components of the ligninolytic system, and other enzymes are required for their proper function (Riley et al. 2014). Perhaps, the most prominent ones are the H_2O_2-producing enzymes such as aryl alcohol oxidases (AAOs). The AAO gene family of *P. ostreatus* is composed of 36 members; yet, the specific role(s) of each member is not known. AAOs are simultaneously expressed with class II peroxidases in *Pleurotus* cultures (Camarero et al. 1996; Hernández-Ortega et al. 2012). In addition to AAOs, the *P. ostreatus* genome contains 4 glyoxal oxidase genes and 12 copper radical oxidases, all of which are capable of producing H_2O_2 (Kersten and Cullen 2014).

An additional family of enzymes involved in the fungal ligninolytic capacity are the laccases, which have been intensively studied and have been suggested to have many applications (Pezzella et al. 2009). This *P. ostreatus* gene family is composed of 11 putative coding genes. Laccase expression was found to be regulated by culture conditions and developmental stages of the fungus, suggesting diverse functions of

the different iso-enymes in vivo (Pezzella et al. 2013). The cytochrome P450 family also plays important roles in WRF (Ichinose 2013). It has been suggested that members of this family are involved in the degradation of PAHs (Bezalel et al. 1997), carbamazepine (Golan-Rozen et al. 2011), and lignin. However, as the *P. ostreatus* genome contains 153 members of this gene family, detailed studies are required to understand the common and differential role of these enzymes.

The expression level of the different genes families comprising the ligninolytic system has been shown to respond to the physiological state of the fungus, as well as fungal nutrition and environmental conditions. These include the different phases of growth, different growth media or induction by specific substrates, as well as substrate specificity of the enzymes. Recently, Gaskell et al. (2014) demonstrated the sensitivity to changes in lignin composition (syringyl/guaiacyl monomers) in transgenic poplar trees, as found in *P. chrysosporium* gene transcript and protein abundance. Peptides corresponding to oxidoreductases were identified in media consisting of biomass from transgenic lines (containing 85 mol.% syringyl units), but not in the parental clone (65 mol.% Syringyl). They concluded that *P. chrysosporium* gene expression patterns are substantially influenced by these changes in lignin composition (Gaskell et al. 2014).

The interest in *Pleurotus* PODs, oxidases, and other components of the ligninolytic system is steadily increasing but the function and regulation of the various components and, more so, the concerted system, are not completely understood. The practical relevance of these activities is also clear but has the potential of being much more widely exploited. On the one hand, it is important to progress our understanding of each component, yet on the other, obtaining a more holistic view of the contributions and interactions between the various constituents of the ligninolytic system is warranted. In addition to the basic data obtained, this may also pave the way to optimization of the unique attributes of WRF for applied purposes, including the adaptation to novel substrate transformation.

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