Effects of agroindustrial by-products on wood-dwelling Agaricomycetes: lignocellulolytic enzyme enhancement and residue transformation

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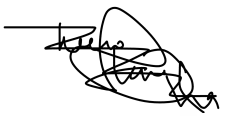

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2. Reina, R., Kellner, H., Jehmlich, N., Ullrich, R., García-Romera, I., Aranda, E., Liers, C., 2014. Differences in the protein expression pattern (secretome) *of Bjerkandera adusta* induced by a phenolic olive mill extract. Fungal Genetics and Biology 72, 99-105.
3. Reina, R., Ullrich, R., García-Romera, I., Liers, C., Aranda, E., 2016. Integral valorization of wine and olive mill by-products using *Trametes versicolor* to produce enzymes of industrial interest and soil amendments (Accepted). Spanish Journal of Agricultural Research.
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3. Reina, R., Aranda, E., Kluge, M., Ullrich, R., Hofrichter, M., García-Romera I., Liers, C., 2011. New tools for enzyme production by the fungus *Bjerkandera adusta*. MICROBIOTEC 2011. Braga (Portugal).
4. Reina, R., Liers, C., Ullrich, R., García-Romera, I., Hofrichter, M., Aranda, E., 2012. Induction of manganese peroxidases of wood and leaf-litter colonizing Agaricomycetes by olive oil mill residues. Annual Conference of the Association for General and Applied Microbiology (VAAM 2012). Tübingen (Germany).
5. Reina, R., Kellner, H., Jehmlich, N., García-Romera, I., Aranda, E., Liers, C., 2013. Olive mill waste “alpeorujo” as eliciting substrate for the production of manganese-oxidizing peroxidases of *Bjerkandera adusta*. 5th Congress of European Microbiologists (FEMS 2013). Leipzig (Germany).
6. Reina, R., Torres, R., García-Romera, I., Liers, C., Aranda, E., 2015. Valorización integral de subproductos del vino y aceite de oliva mediante el hongo *Trametes versicolor*: Obtención de enzimas de interés industrial y enmiendas de suelo. X Congreso de Biotecnología “Chiapas 2015”. Chiapas (México).

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GENERAL

INTRODUCTION

1. Lignocellulose

Lignocellulose, the principal component of the cell wall, represents a class of polymers of considerable structural diversity composed by three polymeric constituents: the recalcitrant lignin, the highly crystalline cellulose and the short and branched hemicellulose (Fig. 1). The amount of each of these components varies depending on the origin of the biomass. The lignin complex forms a matrix surrounding the cellulose in woody cell walls which protects both hemicellulose and cellulose from microbial oxidation. Therefore, the three polymers are strongly integrated by no covalent forces and account for half of the carbons fixed by photosynthesis (Sánchez, 2009).

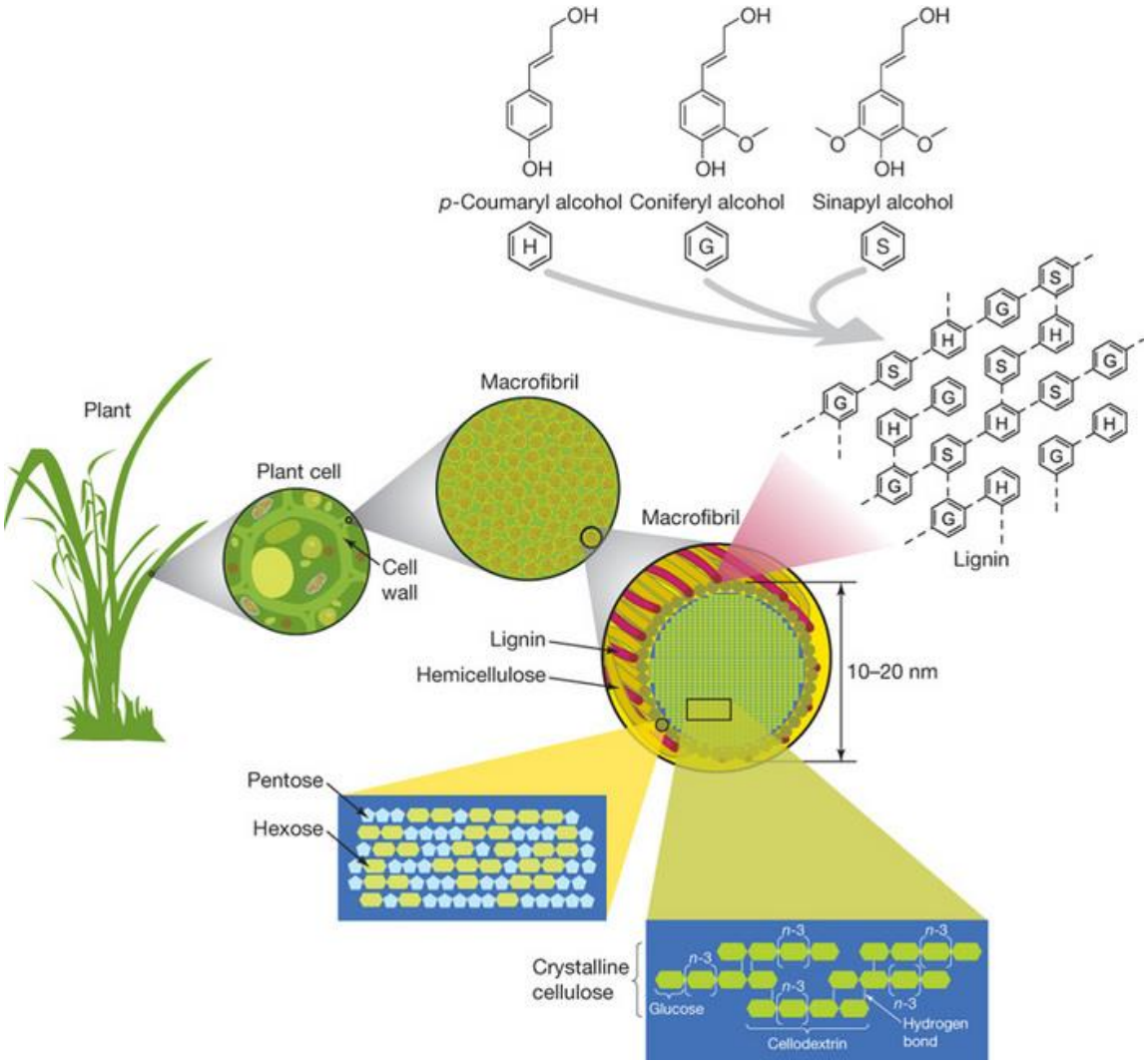

Fig. 1. Plant cell wall structure (Rubin, 2008).

1.1. Lignocellulose composition

1.1.1. Lignin

Lignin is the second main constituent of plant biomass after cellulose. On the whole, it could be regarded as a complex and heterogeneous polymeric structure which is synthetized from the oxidative coupling of p-hydroxycinnamyl alcohol monomers (known as monolignols) and related compounds, which provide rigidity to cell walls, allowing plants to grow upward (Eudes et al., 2014).

The most important primary monomers for lignification are the three p-hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl), which differ in their degree of methoxylation. When the monolignols are incorporated into the lignin polymer, their respective phenylpropanoid subunits are formed: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Fig. 1).

The process of lignification requires reactions that cause the extension of the macromolecule through the coupling of new monomers to the growing polymer. The phenylpropanoid units are attached to one another by characteristic linkages (β–O–4, β–5, β–β, etc.), and branching also occurs (Adler, 1977).

1.1.2. Cellulose

Cellulose is the most abundant compound on Earth since it is the main component of plant biomass. It is a fibrous and water-insoluble substance which is found in the protective cell walls of plants, particularly in stalks, stems, trunks and all woody portions. It consists of an unbranched homopolysaccharide of β-D-glucopyranose units linked by (1→4) glycosidic bonds (O'Sullivan, 1997). The parallel glucans fit perfectly to form a crystalline microfibril that is mechanically strong and highly resistant to enzymatic attack, in other words, a virtually ideal scaffold material (Cosgrove, 2005).

1.1.3. Hemicellulose

Hemicellulose is the second most abundant polysaccharide on Earth. It is a heterogeneous polymer of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and sugar acids (Mosier et al., 2005). Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses principally contain glucomannans. Hemicellulose varies in quantity and composition between the different cell types and between the tissues of an individual organism (stem, branches, roots and bark).

1.2. Lignocellulose recalcitrance

The degradation of lignocellulose constitutes a natural process of organic matter recycling in which different fungi and bacteria are involved (Daniel, 2003). The biological and chemical degradation of lignocellulose is difficult to achieve due to the different composition of the lignocellulosic material and the rigid and compact structure of plant cell wall. The factors that contribute to its recalcitrance are: (a) the epidermal tissue of the plant body (especially cuticular and epicuticular waxes), (b) the arrangement and density of vascular bundles, (c) the amount of sclerenchymatous tissue, (d) the degree of lignification, (e) the structural heterogeneity and complexity of cell wall constituents such as microfibrils, (f) the challenges faced by enzymes acting on an insoluble substrate, and (g) the inhibitors to subsequent fermentations that exist naturally in cell walls or are generated during conversion processes and matrix polymers (Himmel et al., 2007).

Some fungi are among the organisms that can oxidize lignin compounds (Kirk and Farrell, 1987). Their most likely purpose is to gain access to hemicellulose and cellulose in order to use them as carbon sources (Ten Have and Teunissen, 2001). These fungi are known as wood decay fungi.

2. Wood decay fungi

Wood decay fungi have traditionally been classified into three categories: brown, soft and white rot, depending on the morphological and chemical changes produced on the lignocellulose by different degradation mechanisms rather than taxonomic factors. **Brown rot** fungi produce a partial depolymerization of the complex lignin polymer through non-enzymatic mechanisms (Pandey and Pitman, 2003). **Soft rot** fungi degrade cellulose and hemicellulose from the cell wall and slightly alter lignin. In contrast to brown rot fungi, they lightly alter wood hygroscopicity (Papadopoulos, 2012). **White rot** fungi can totally degrade lignocellulose to carbon dioxide and water due to its oxidative enzymatic arsenal, which includes: class II peroxidases (PODs), dye-decolorizing peroxidases (DyPs) and laccases (Kirk and Farrell, 1987; Cullen and Kersten, 2004). In addition, glycoside hydrolases (GHs), polysacharide monoxogenases (PMOs) and additional carbohydrate active enzymes (CAZys) are also implicated (McCartney et al., 2004).

The classification of white/brown rot fungi in terms of their degradation mechanism has been used extensively (Liese, 1970). However, the advent of a large number of new sequenced fungal genomes has demonstrated that this classification does not adequately reflect the different mechanisms by which the fungi perform their

nutrition and, in the words of Floudas et al. (2015) "*this separation could be an oversimplification*". For instance, fungi such as *Botryobasidium botryosum* and *Jaapia argillacea* lack PODs but show similarities to white rot fungi in other predicted carbohydrate- and lignin-active enzymes (Riley et al., 2014). *Fistulina hepatica* and *Cylindrobasidium torrendii* (white and brown rot respectively) do not possess the typical white rot characteristics (Floudas et al., 2015), although they are able to degrade all wood components. Thus, the term 'white rot fungi' should be strictly confined to those fungi which use PODs for wood decay purposes (Floudas et al., 2012).

The principal fungi responsible for lignocellulose degradation belong to the divisions *Ascomycota* and *Basidiomycota*, which are contained in the subkingdom *Dikarya* (Fig. 2), reflecting the putative synapomorphy of dikaryotic hyphae (Hibbett et al., 2007). The fungi within this clade were formerly called "higher fungi" and the most rapid lignin degraders belong to the division *Basidiomycota*, in which high-redox potential peroxidases are widely distributed (Ten Have and Teunissen, 2001).

As DNA sequencing has become faster and more affordable, phylogenetic studies of *Basidiomycota* have proliferated (James et al., 2006; Brandon-Matheny et al., 2007; Floudas et al., 2012; Binder et al., 2013; Drew et al., 2013). In this thesis, we will refer to the classification proposed by Hibbet et al. (2007), in which a comprehensive analysis is proposed as a result of a consensus on the results obtained by phylogenies suggested in various taxonomic studies (Fig. 2).

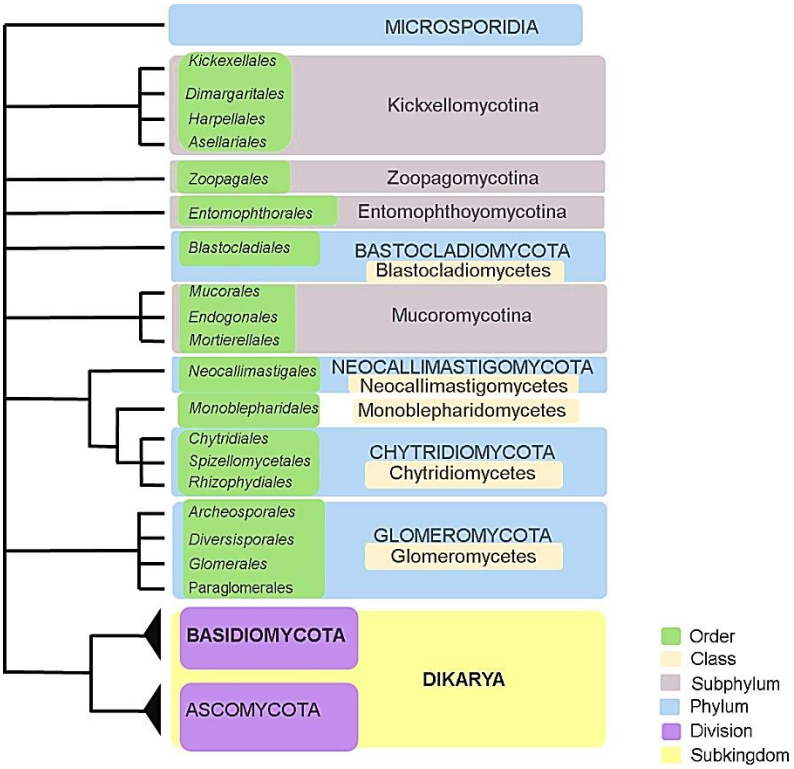

Fig. 2. Phylogeny and classification of fungi (Hibbet et al., 2007). Branch lengths are not proportional to genetic distances.

Although some species of ascomycetes, which include certain soft rot fungi belonging to the family *Xylariaceae*, contribute to lignocellulose biodegradation, the ability to degrade wood is widely found in many species of basidiomycetes, such as in the orders *Agaricales*, *Auriculariales*, *Boletales, Russulales* and *Polyporales* (class *Agaricomycetes*). In most of the polyporal species, lignin and carbohydrates that form cellulose and hemicellulose are simultaneously degraded (Ruiz-Dueñas et al., 2013). In the present thesis, two representative wood-rotting *Polyporales* were studied: *Bjerkandera adusta* and *Trametes versicolor*.

The order *Agaricales* is the largest clade of mushroom-forming fungi. Though ubiquitous, they are all terrestrial and many are capable of degrading lignocellulosic compounds. In this study, the fungi *Coprinellus radians, Agrocybe aegerita, Marasmius alliaceus* and *Chondrostereum purpureum* were used.

The Auriculariales order of fungi, many of which used to be regarded as heterobasidiomycetes ("jelly fungi"), grow in decayed wood and are able to secrete some

class II peroxidases. In this study, the fungus *Auricularia auricula-judae*, a representative of this order, was used.

3. Mechanisms of lignin degradation

The extracellular oxidative system used by fungi to achieve ligninolysis is composed of lignin-modifying enzymes (LME) such as lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) (Lundell et al., 2010), the recently described dye-decolorizing peroxidase (DyP, EC 1.11.1.19) and the unspecific or aromatic peroxygenase (UPO, EC 1.11.2.1). Apart from peroxidases, laccases (Lacs, EC 1.10.3.2), which are copper oxidases, also participate in lignin degradation (Bourbonnais and Paice, 1990, Kellner et al., 2014) (Fig. 3). Additional auxiliary activities, such as peroxide generation, are required to carry out the ligninolytic process.

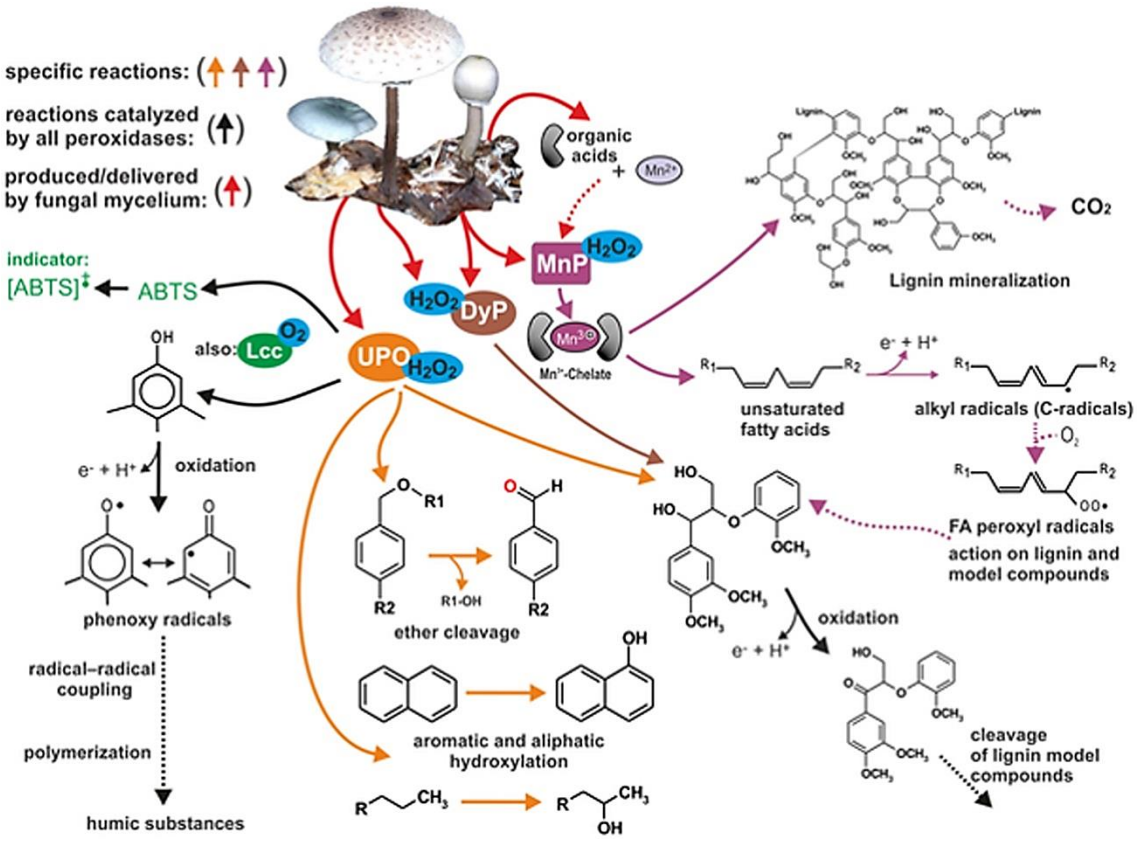

Fig. 3. Schematic summary of enzymatic lignocellulose degradation (Kellner et al., 2014).

3.1. Enzymatic lignin degradation

3.1.1. Laccase (Lac; EC 1.10.3.2)

Lacs (benzenediol: oxygen oxidoreductases) belong to the group of blue oxidases and represent the main subgroup of multicopper oxidases. Lacs, which can be found in almost all fungi that produce wood decay, are the most ubiquitous LME (Fernández-Fernández et al., 2013). Despite being found in several plant species, insects and bacteria, the most common Lac producers are wood-rotting fungi. They are mainly extracellular, although some intracellular lacs have also been found in fungi such as *Lentinula edodes.* Apart from detoxification and lignin degradation, intracellular Lacs are also involved in mycelium growth, fruiting-body formation and pigmentation, concretely in melanin synthesis (Nagai et al., 2003; Giardina et al., 2010). Lacs occur as well in saprophytic ascomycetes such as *Myceliophthora thermophila* or *Xylaria sp.* and are, in those cases, involved in humification processes (Liers et al., 2006; Morozova et al., 2007). They are common in higher basidiomycetes such as *T. versicolor*, *Trametes hirsuta, Trametes multicolor, Trametes villosa, Trametes gallica, Cerrena maxima, Funalia floccosa, Lentinus tigrinus and Pleurotus eryngii* among others (Yaver et al., 1996; Collins and Dobson, 1997; Muñoz et al., 1997; Leitner et al., 2002; Dong and Zhang, 2004; Cadimaliev et al., 2005; Lyashenko et al., 2006; Polyakov et al., 2009; Díaz et al., 2010). They share a certain structural homology with other copper oxidases like ferroxidases that form a monophyletic clade (Appendix I, Fig. A1).

Lac proteins possess two centers. The first one is a mononuclear center (T1). It is responsible for the “blue” colour of Lac and it acts as the first electron acceptor in charge of the oxidation of phenols and amines (Hatakka, 1994; Thurston, 1994). The other is a trinuclear center composed of two T2 and one T3 center (Fig. 4). The molecular mass of the monomer ranges from about 50 to 100 kDa. Lacs have a wide substrate specificity and a variable range of oxidable substrates including mono-, di-, and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate (Madhavi and Lele, 2009).

Lacs are capable of degrading the phenolic subunits present in lignin. They can participate in processes of biodegradation and bioremediation of different compounds such as dyes, agricultural wastes, xenobiotics, pharmaceuticals and phenols (Lu et al., 2007; Gasser et al., 2014; Rahmani et al., 2015; Xu et al., 2015).

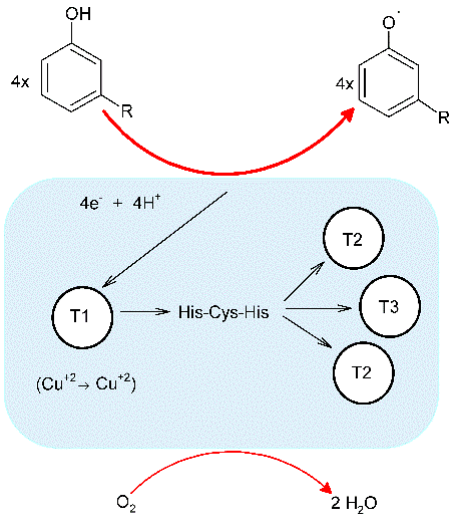

Fig. 4. Simplified catalytic cycle of Lac with depiction of copper coordination centres (Dwivedi et al., 2011). The substrates are oxidized by the Cu-T1 centre, and electrons are transferred by a highly conserved motif: His-Cys-His to the T2 and T3 copper centres where molecular oxygen is reduced to water (Baldrian, 2006).

3.1.2. Heme peroxidases

Heme peroxidases contain an iron protoporphyrin IX (heme) group in the active site (Poulos et al., 1978). They can be found in bacteria, fungi, plants and animals and are therefore omnipresent in all forms of life. Overall, these enzymes use hydrogen peroxide (H_2O_2) as an electron-accepting co-substrate to trigger a number of oxidative reactions involving a wide variety of organic and inorganic substrates (Battistuzzi et al., 2010).

Heme peroxidases can be divided into two superfamilies: one of which includes plant, bacterial and fungal PODs and belongs to the peroxide-catalase superfamily; the other is composed of the vertebrate enzymes which belong to the peroxidase-cyclooxygenase superfamily (Zamocky et al., 2008). The first superfamily is organized into 3 classes: class I was identified as containing mainly yeast cytochrome c peroxidases, ascorbate peroxidases, and bacterial catalase–peroxidases (Fig. 5), class II is represented by the best characterized fungal peroxidases and class III is composed of secretory plant peroxidases related to horseradish peroxidase (HRP) (Welinder, 1992). There are two new groups of heme peroxidases which do not fit the previous classification due to the differences in the primary and tertiary structure and their ability to catalyze unique reactions. The first group consists of the heme-thiolate peroxidases, which includes chloroperoxidases (CPO; EC 1.11.1.10) and unspecific peroxygenase

(UPO). The other group is formed by the dye-decolorizing peroxidases (DyP) (Hofrichter et al., 2010).

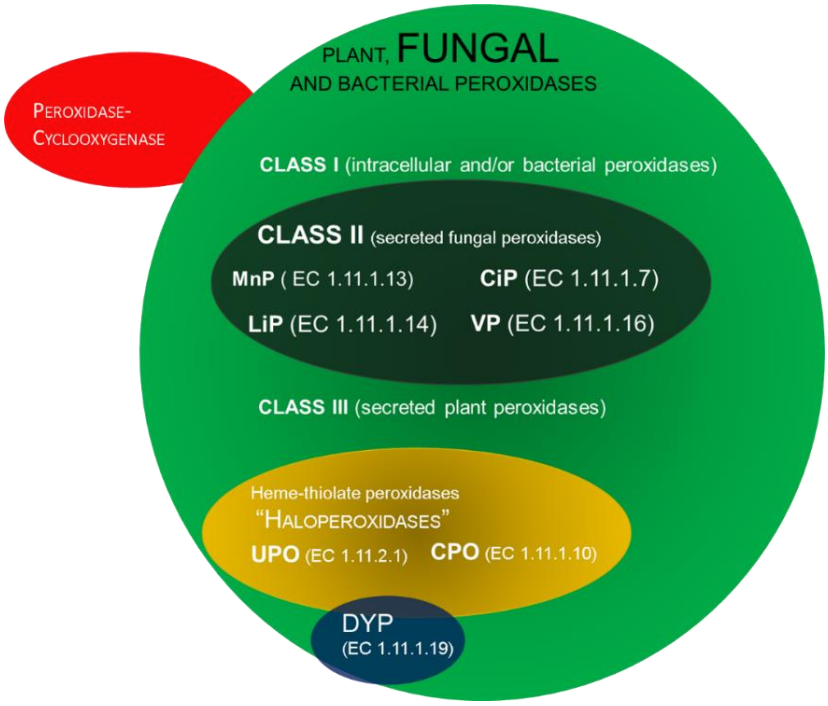

Fig. 5. Heme peroxidase classification (Hofrichter et al., 2010).

3.1.3. Class II Peroxidases

The most studied enzymes within this group are MnPs, LiPs and VPs. However, there are also the so-called "generic peroxidases" such as *Coprinopsis cinerea* peroxidase (CiP; EC 1.11.1.7), whose amino acid sequences are closely related to ascomycete peroxidases (Appendix I, Fig. A2). The catalytic cycle of these peroxidases starts with the oxidation of the native enzyme by H_2O_2 (organic peroxide) to form compound I (C-I). C-I is an Fe^{4+}-oxo-porphyrin radical which undergoes two consecutive electron reductions, thus changing the state of the enzyme from C-I to compound II (C-II) and from C-II back to the native enzyme (Fig. 6). This cycle is identical for the three above-mentioned PODs, except for the electron donor, which differ depending on the enzyme: for MnPs, they are Mn^{2+} ions, for LiPs, they are aromatic compounds (AH), and for VPs either Mn^{2+} ions, phenolics or non-phenolic aromatic compounds (Camarero et al., 1999).

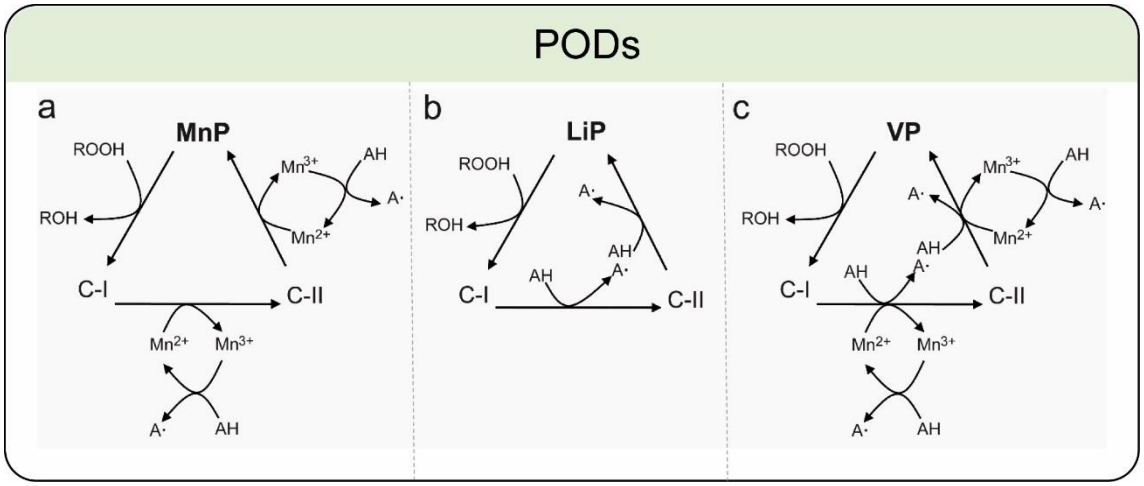

Fig. 6. Scheme of catalytic cycle for class II peroxidases: (a) MnP, (b) LiP and (c) VP (Camarero et al., 1999).

3.1.3.1. Manganese peroxidase (MnP; EC 1.11.1.13)

MnPs were first discovered in the 1980s in *Phanerochaete chrysosporium* (Glenn and Gold, 1985). They are considered to be the key enzymes in lignin degradation as they are known to be secreted by nearly all white rot wood decay and soil-litter decomposing fungi (Steffen et al., 2000; Hofrichter et al. 2001; Hofrichter, 2002).

MnPs are often produced in multiple forms. Their molecular weight ranges from 38 to 62.5 kDa, although most purified enzymes have molecular weights of around 45 kDa (Hatakka, 1994).

The Mn^{3+} ions formed at the end of the catalytic cycle are stabilized by organic acids (e.g. oxalate, malonate). These chelates of Mn^{3+} can oxidize different phenolic substrates in lignin-forming phenoxy radicals. These diffusible radicals are highly reactive and can be subject to an efficient enzymatic attack of the persistent lignin molecule (Fig. 3), eventually producing carbon dioxide (“enzymatic combustion”) and low molecular mass organic acids (Hofrichter, 2002). In *Phlebia radiata*, it has been shown how the enzymatic attack from one of its MnP isoforms resulted in the polymerization of lower molecular mass soluble wood components and in the partial depolymerization of the insoluble bulk of pinewood (Hofrichter et al., 2001).

MnP is specific to Mn^{2+} ions oxidation (Wariishi et al., 1992) due to a conserved solvent-exposed Mn^{2+}- binding site in the vicinity of two heme propionates and consisting of three acidic amino acid residues: two glutamine E35 and E39 and one aspartate D179. These residues are essential for the hexa-coordination of the Mn^{2+} ion, promoting the

rapid electrons transfer to the heme (from C-I to C-II and from C-II to the native form) (Hofrichter et al., 2010).

MnPs can be classified into two different groups: one is formed by the typical long MnP and the other by a short–type MnP, containing the conserved Mn^{2+}-binding site but lacking an exposed tryptophan (Hofrichter et al., 2010). These short MnPs have been found in fungi like *P. radiata* or *Agrocybe praecox* (Hildén et al., 2005; Hildén et al., 2014) (Appendix I, Fig. A2). Short MnPs are not able to oxidize veratryl alcohol but share with the VPs the Mn^{2+}-independent ability to oxidize phenols and small dye compounds (Lundell et al., 2010).

3.1.3.2. Lignin peroxidase (LiP; EC 1.11.1.14)

LiPs were first described 27 years ago in *P. chrysosporium* (Tien and Kirk, 1988). They are mainly secreted by higher white rot fungi, for example *T. versicolor* (Johansson and Nyman, 1993), *P. radiata* (Lundell, 1993), *Phlebia tremellosa* (Vares et al., 1994), *B. adusta* (Ten Have et al., 1998) and *Phanerochaete sordida* (Sugiura et al., 2003) (Appendix I, Fig. A2).

These biocatalysts are secreted as sets of multiple isoforms that differ in terms of physical characteristics, substrate specificity and stability (Hofrichter et al., 2010). The molecular mass of these glycoproteins ranges from 38 to 46 kDa and their pI value from 3.2 to 4.0. LiPs have an uncommon low pH optimum of 3.

The oxidation site in LiP is located on the surface, in a catalytically active tryptophan: Trp171 in the case of *P. chrysosporium* (Choinowski et al., 1999). This site is the substrate-intermediate protein radical centre upon LiP catalysis and the initiation location of the long-range electron transfer (LRET) (Hofrichter et al., 2010). LiPs can be easily turned into an inactive form with high concentrations of H_2O_2. Veratryl alcohol plays a protective role, as its presence helps to ensure that C-II will be turned into a native enzyme (Fig. 6), instead of reacting with the excess of H_2O_2 to form Compound III (Wariishi and Gold, 1989).

LiPs are the strongest class II fungal peroxidases (they have a redox potential of approx. 1.5 eV, Muheim et al., 1990). So they can cleave the aromatic rings of complex polymers and they are able to oxidize directly synthetic lignins (e.g. dehydropolymer, DHP; Hammel and Moen, 1991), dimeric lignin model compounds, (such as the non-phenolic β-O-4 linkage type aryl glycerol-aryl ethers), organo-pollutants and xenobiotics such as polycyclic aromatic hydrocarbons (Haritash and Kaushik, 2009), as well as high-redox potential dyes (e.g. methylene blue, Ferreira-Leitão et al., 2006) by producing the bleaching of the coloured compounds (Young and Yu, 1997). This decolorizing property

is also present in DyPs and VPs (Ruiz-Dueñas et al., 2008; Liers et al., 2013; Linde et al., 2015).

3.1.3.3. Versatile peroxidase (VP; EC 1.11.1.16)

VPs combine the catalytic properties of MnPs and LiPs (Camarero et al., 1999; Salvachúa et al., 2013a) since they are able to either oxidize Mn^{2+} ions like MnP (Fig. 6b), that leads to an indirect Mn^{3+}-chelates-driven oxidation of phenols, non-phenolics and dyes. However, like LiPs, they are also able to directly convert phenolics, high-redox potential methoxylated aromatics and dyes. Therefore, VPs have a wide range of substrates to be oxidized. To date, these enzymes have only been described in a limited number of *Basidiomycota* species from the genera *Bjerkandera* and *Pleurotus* (Ruiz-Dueñas et al*.,* 1999; Moreira et al., 2005).

VPs contain Trp164 (equivalent to Trp171 residue in LiPs). They also contain MnP-characteristic acidic amino acid residues: the Mn^{2+}-binding site, which, in VP, is formed by the side-chains of Glu35, Glu39, and Asp175, located in front of the internal (i.e. further from the main heme-access channel) (Ruiz-Dueñas et al., 2008). VPs have a molecular mass of around 43-45 kDa with the isoelectric point ranging from 3.4 to 3.9.

3.1.4. Unspecific peroxygenase (UPO; EC 1.11.2.1)

UPOs are heme-thiolate proteins that can act as functional hybrids of peroxidases and cytochrome P450 monooxygenases (CytP450) (Ullrich et al., 2004). UPO genes are widely found in various orders of *Agaricomycetes* and also in *Ascomycetes*, although they do not form a monophyletic clade (Appendix I, Fig. A3). To date, the purification of wild-type UPO representatives has only been achieved with six agarical fungi: *C. radians*, *Coprinus verticillatus* (Anh et al., 2007), *Marasmius rotula* (Gröbe et al., 2011), *Agrocybe parasitica* (Ullrich, unpublished) and *Agrocybe pediades* (Dolge, unpublished) where it was first described for *A. aegerita* (Ullrich et al., 2004).

The representative UPOs identified so far are produced in complex plant-based liquid media such as soybean meal and alfalfa (Ullrich et al., 2004; Gröbe et al., 2011). It is therefore likely that certain leguminose-typical ingredients induce UPO activity.

These enzymes can act as real heme peroxidases, by catalyzing the H_2O_2-dependent oxidation of typical peroxidase substrates (e.g. ABTS), in which case they follow the proposed catalytic cycle of peroxidases (Fig. 6). Besides, they also present functional similarities with CytP450 by mediating selective hydroxylation of different substrates. The catalytic pathway in this case would be similar to the peroxidase “shunt” pathway of CytP450s (Ullrich and Hofrichter, 2007), in which these enzymes can use an

oxygen atom from a peroxide to catalyze oxygen insertion without electron transport proteins or the NAD(P)H cofactor (Joo et al., 1999).

These proteins are highly glycosylated (10-40%) with up to seven N-glycosylation sites. Their molecular masses and isoelectric points vary from 32 to 46 kDa and 3.8 to 6.1, respectively (Hofrichter and Ullrich, 2014). UPOs contain heme as prosthetic group, which is linked via the iron to an exposed axial ligand that contains a cysteine (PCP motif), as occurs in CytP450s and CPO proteins.

The so far characterized UPOs (e.g. *Aae*UPO, *Cra*UPO, *Mro*UPO) are stable enzymes, which are capable of efficiently catalyzing multiple reactions, acting over multiple substrates without additional cofactors, rendering them appealing enzymes for chemical synthesis. The enzyme is able to oxygenate (epoxidize, hydroxylate), brominate and oxidize many aromatic and aliphatic substrates such as human drug metabolites (HDM), organosulfur compounds, polycyclic aromatic hydrocarbons (PAHs) and phenols, which are transformed into free phenoxy radicals that undergo to coupling and polymerization reactions (Ullrich and Hofrichter, 2005; Aranda et al., 2009; Poraj-Kobielska et al., 2011). Thanks to this wide range of substrates, UPOs are considered to be the most versatile oxidoreductases known to date.

3.1.5. Dye decolorizing peroxidase (DyP; EC 1.11.1.19)

DyPs were first described in 1999 after being found in an arthroconidial culture of the fungus *B. adusta* (formerly identified as *Geotrichum candidum*) (Kim and Shoda, 1999). Since then, DyPs have been described not only in fungi, but also in prokaryotes (Santos et al., 2014). According to the InterPro database (http://www.ebi.ac.uk/interpro/), his superfamily currently comprises 5019 enzymes, of which 4886 are produced in bacteria, 122 in eukaryotes, and 11 in archaea (Mitchell et al., 2015). The molecular mass of DyPs is approximately 58 kDa and its pl value is 3.8 (Hofrichter et al., 2010). In fungi, they have been so far isolated from *A. auricula-judae, B. adusta, Exidia glandulosa, Irpex lacteus, Marasmius scorodonius, Mycena epipterygia* and *Termitomyces albuminosus* cultures (Kim and Shoda, 1999; Johjima et al., 2003; Scheibner et al., 2008; Liers et al., 2010; Liers et al., 2013; Salvachúa et al., 2013b), all of them have been purified as wild-type proteins from culture supernatants of the original organisms, indicating that these proteins are secreted outside the cell.

According to the amino acid sequence, they are very different to class II peroxidases and lack the typical heme-binding region. All DyPs contain the so-called GXXDG motif, which is part of the heme-binding region. This motif is important for

peroxidase activity and the replacement of the conserved aspartate produces the inactivation of the enzyme (Colpa et al., 2014).

On the basis of primary structural similarity, DyP-type peroxidases were initially subdivided into classes A, B, C and D. The DyPs of basidiomycetes were assigned to class D. However, high degrees of similarity between members of any two different classes were not found, and Yoshida and Sugano (2015) have recently proposed a new classification based on structure sequence alignments where the DyP-type peroxidase family is reclassified into 3 classes: P (primitive), I (intermediate) and V (advanced). Class V includes the former classes C and D since the many additional sequences in both classes are located in the same regions (Yoshida and Sugano, 2015) (Appendix I, Fig. A4).

Despite small differences with respect to the formation of C-I, the catalytic cycle of DyPs is similar to that of class II peroxidases, in which the enzyme reacts with hydrogen peroxide to form oxo-ferryl intermediates (Fig. 6b) (Yoshida et al., 2011).

DyPs are able to directly oxidize hardly convertible phenols (e.g. *p*-nitrophenol), azo dyes (e.g. Reactive Black 5), ascorbic acid, veratryl alcohol and even lignin model dimers (Kim and Shoda, 1999; Scheibner et al., 2008; Liers et al., 2010; Yoshida et al., 2012; Liers et al., 2013; Salvachúa et al., 2013b; Linde et al., 2015).

To date, the role of DyPs in ligninolysis has not been definitely evidenced; it remains unclear how these enzymes participate in the transformation of plant biomass and/or soil organic matter, despite their extended presence in forest soils (which has also been shown at the transcript level) was recently proofed (Kellner et al., 2014).

3.1.6. Peroxide producing enzymes

Peroxide generation plays a key role in lignocellulose degradation and is necessary e.g. for the ligninolytic PODs activity. Hydrogen peroxide can be also part of Fe (II) and Mn (IV) reduction, leading to the production of oxy-radicals that can be responsible for subsequently cleaveage of lignin and cellulose units (Roy et al., 1994). Many fungal enzymes can produce peroxides but only a limited number of them are verifiable secreted. These enzymes belong to the auxiliary activity (AA3 and AA5) families and are included in the CAZy database (http://www.cazy.org/) together with the LME (Levasseur et al., 2013), which covers redox enzymes that act in conjunction with CAZymes. These groups contain enzymes such as glyoxal oxidases (GLXs), alcohol oxidases (AOs), glucose oxidase (GOXs), oxalate oxidase (OXOs) and cellobiose dehydrogenases (CDHs). ***Glyoxal oxidase*** (EC 1.1.3) catalyzes the oxidation of a variety of simple dicarbonyl and β-hydroxycarbonyls, especially glyoxal and

methylglyoxal, to carboxylic acids (Leuthner et al., 2005). This enzyme belongs to the AA5 family and is secreted in several white rot fungi (Orth et al., 1993). GLX is transcriptionally regulated, and appears to follow the same regulation pattern as Lip genes in *P. chrysosporium* (Stewart et al., 1992). ***Aryl alcohol oxidase*** (alcohol: oxygen oxidoreductase; EC 1.1.3.7) is a flavoenzyme belonging to the AA3 family. It catalyzes the oxidation of alcohols to the corresponding carbonyl compounds, releasing hydrogen peroxide. Depending on their substrate specificity, they can be classified into four groups: short chain alcohol oxidase (SCAO), long chain alcohol oxidase (LCAO), aromatic alcohol oxidase (AAO), and secondary alcohol oxidase (SAO) (Goswami et al., 2013). These enzymes are, to a certain extent, homologous with glucose oxidases (Ferreira et al., 2009). ***Glucose oxidase*** (β-D-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to gluconic acid by using molecular oxygen as an electron acceptor with the concomitant production of hydrogen peroxide (Bankar et al., 2009). GOXs belong to the AA3 family and have been purified from different fungi, mostly from the species *Aspergillus* and *Penicillum* (Hatzinikolaou et al., 1996; Kalisz et al., 1997). ***Oxalate oxidase*** (EC 1.2.3.4) catalyzes the conversion of oxalate and dioxygen to carbon dioxide and hydrogen peroxide. Although the best-characterized OXOs are found in cereal plants, they are also present in bacteria and filamentous fungi like *Ceriporiopsis subvermispora* and *Abortiporus biennis* (Escutia et al., 2005; Grąz et al., 2009). ***Cellobiose dehydrogenase*** (EC 1.1.99.18) is the only extracellular fungal flavocytochrome known to date. It is secreted by wood degrading and phytopathogenic fungi (Zamocky et al., 2006). It is a monomeric protein consisting of two domains which oxidizes several carbohydrates at the flavodehydrogenase domain carrying an FAD cofactor. This enzyme enhances cellulose degradation by coupling the oxidation of cellobiose to the reductive activation of copper-dependent lytic polysaccharide monooxygenases (LPMOs) (Sygmund et al., 2013).

3.2. Non enzymatic lignin degradation

The ability to decompose the aromatic lignin polymers in wood is mostly restricted to white rot basidiomycetes. However, brown rot fungi use an alternative strategy, based on Fenton chemistry, to overcome the lignin barrier in wood decay (Cameron and Aust, 1999). These fungi synthetize enzymes that are able to generate oxidizing species via a Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$) and quinone redox cycling (Cameron and Aust, 1999; Guillén et al., 2000). In the quinone redox cycling, which can be mediated by ligninolityc enzymes, a quinone is reduced to semiquinone or hydroquinone with the concomitant reduction of O_2 to superoxide anion radical ($O_2^{\bullet}$), the subsequent disproportion to H_2O_2 and the beginning of the Fenton reaction. This process

generates continuously hydroxyl radicals. This strategy is also been described for white rot fungi *in vivo* systems (Gomez-Toribio et al., 2009; Guillen et al., 2000). The hydroxyl radical (•OH) is a diffusible oxidant that can oxidize and depolymerize cellulose and lignin. Although it is also able to attack lignin, the transformation of the lignin polymer by this non-enzymatic mechanism is always more limited (Martínez et al., 2011). Although most research to date has focused on hydroxyl radicals (•OH), other reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{\bullet}$), peroxyl radicals (ROO•) or hydroperoxyl radicals (•OOH), might also be produced by fungi to attack wood polymers, although they are less reactive than (•OH). This mechanism is also shared by some white rot fungi, such as *P. chrysosporium* (Hammel et al., 2002).

4. Cellulose, hemicellulose and pectin degradation mechanisms

The enzymatic degradation of plant polysaccharides is a crucial step in the carbon cycle. To accomplish cellulolysis, fungi have developed a batch of hydrolases and oxidoreductases enzymes, the synergistic action of which is required for the enzymatic degradation of lignocellulose. Enzymes with cellulase, hemicellulase and pectinase activities, cooperate to break down cellulose and its associated cell wall components (Medie et al., 2012). In recent years, a parallel oxidoreductive cellulolytic mechanism, aside from the cellulolytic traditional system, involved in the oxidative degradation of cellulose has been described. This mechanism involves lytic polysaccharide monooxygenase enzymes (Hemsworth et al., 2014). All these enzymes have potential uses in biorefinery processes where lignocellulosic biomass is converted to platform chemicals such as aromatic compounds, fermentable sugars and later to biofuels and other renewable energy carriers (Kuhad et al., 2011).

Carbohydrate active enzymes of basidiomycetes have been studied considerably less than those of ascomycetes. This group of enzymes includes different sequence-based families of GHs, polysaccharide lyases (PLs), glycosyltransferases (GTs) and carbohydrate esterases (CEs) (Fig. 7). These enzymes, together with those which may exhibit a carbohydrate-binding module (CBMs) and those which present AAs are included within the CAZy database (Fig. 7). The variety of CAZyme differs depending on the fungus and often reflects its habitat requirements. An updated classification of these enzymes can be found in the CAZy database, as well as information concerning auxiliary activities such as oxidases (Lombard et al., 2014)

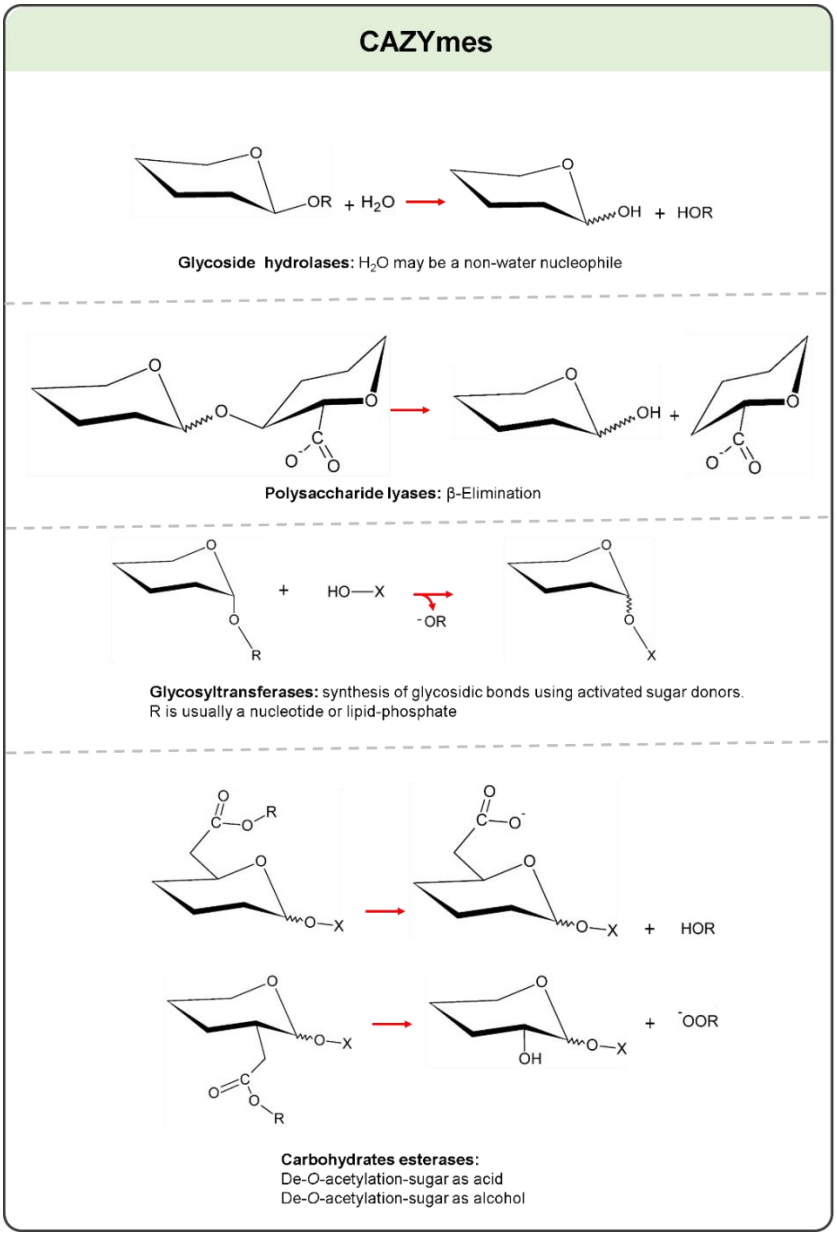

Fig. 7. CAZymes scheme of catalytic activity according to Davies et al. (2005).

4.1. Glycoside hydrolase (GH; EC 3.2.1)

Glycoside hydrolases are responsible for catalysing the glycolytic cleavage of O-glycosidic bond (Fig. 7). The hydrolytic activities of some GHs are crucial for the hydrolysis of celluloses and hemicelluloses from lignocellulosic material: endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), and endo-β-1,4-xylanase (EC 3.2.1.8). The first one hydrolyzes glycosidic bonds randomly within the polymers, creating new reducing and non-reducing ends for the action of exo-β-1,4-glucanases or cellobiohydrolase, which cleaves cellobiose from cellulose or their derived oligosaccharides. While β-glucosidases liberate D-glucose from

the non-reducing ends of cellulose-derived oligosaccharides, especially cellobiose, are β-1,4-xylanases responsible for the hydrolysis of β-D-xylosidic linkages in xylans (Jovanovic et al., 2009).

The traditional GH classification according to the European Nomenclature (EC), which is based on catalytic similarities and substrate specificities, does not accurately reflect the nature of enzymes, which are able to act on several substrates; it is therefore not appropriate for many glycoside hydrolases which share substrate similarities or act on several substrates (Henrissat and Davies, 1997). Notwithstanding, the CAZy classification system provides genomic, structural and biochemical information concerning these enzymes. Some GH families contain enzymes characterized by different hydrolytic activities, with GH1, for instance, having 17 different enzyme activities (Jovanovic et al., 2009). Moreover, the number of gene models for each GH family varies in white rot fungi (Várnai et al., 2014). The essential GH families responsible for hemicellulose degradation are described in Table 1.

Table 1. Principal cellulose and hemicellulose enzymes: GH family and corresponding activity.

Activities related to cellulose and hemicellulose degradation	GH family
endo-β-1,4-glucanase	**5**, **6**, 7, **8**, **9**, **10**, **12**, **26**, **44**, 45, 48, **51**, 74, 124
cellobiohydrolase	**5**, **6**, **9**
β-glucosidase	1, 2, 3, **5**, **9**, 30, 116
endo-β-1,4-xylanase	**5**, **8**, **9**, **10**, 11, **12**, 16, **26**, 30, 43, **44**, **51**, 62, 98

4.2. Carbohydrate-binding modules (CBMs)

Enzymes that degrade cellulose and hemicellulose can be modular, with a variable number of additional modules appended to the catalytic module. In this complex and modular structure, an accessory component can bind complex carbohydrates, in which case the ancillary module is called the CBM (Medie et al., 2012). The majority of the GHs carry a CBM attached to the catalytic core domain by a flexible linker (Teeri et al., 1992). These modules were thought to potentiate the activity of the catalytic modules by targeting the enzyme to a specific component of the cell wall. This explains why CBMs were commonly considered to be essential components of cellulases, especially in

cellobiohydrolases. Nevertheless, current genome data indicate that many cellulases lack the binding domains in cellulose-degrading organisms. CBMs can also be found in non-cellulolytic enzymes, suggesting that they play other complex roles (Várnai et al., 2014). CBMs are classified into 71 CBM families based on their sequence similarity and predicted structure-function relationship.

4.3. Polysaccharide lyase (PL; EC 4.2.2)

Polysaccharide lyases (EC 4.2.2) are enzymes that cleave uronic acid-containing polysaccharides via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end at the point of cleavage. Eliminative cleavage complements the GH hydrolysis strategy. PLs can be found in a wide range of different microorganisms and are involved in processes such as biomass degradation, tissue matrix recycling and pathogenesis (Lombard et al., 2010).

4.4. Glycosyltransferase (GT; EC 2.4)

Glycosyltransferases (EC 2.4) are implicated in the biological synthesis of complex carbohydrates and polysaccharides (Bertozzi and Kiessling, 2001). GTs transfer monosaccharide fractions from activated donor molecules to biological molecules. They can be classified as either retaining or inverting enzymes according to the stereochemistry of the substrates and reaction products.

GTs are ubiquitous enzymes that can be found in a wide range of organisms. The CAZy database currently contains more than 90 families of GTs. Fungi possess many polysaccharide structures, especially in their cell walls (e.g. chitin, glucans). Their proteins may be extensively glycosylated, bearing hundreds of sugar residues, and their glycolipids are also quite complex (Klutts et al., 2006).

4.5. Carbohydrate esterase (CE; EC 3.1)

Carbohydrate esterases (EC 3.1) are enzymes that catalyze the O- or N-deacylation of substituted saccharides, e.g. esters or amides in which sugars play the role of alcohol and amine. These enzymes exhibit a great substrate specificity. The CAZy database, for example, contains 26 CEs families, within which we can find acetylxylan esterases, acetyl esterases, chitin deacetylases, peptidoglycan deacetylases, feruloyl esterases, pectin acetyl esterases, pectin methylesterases and glucuronoyl esterases, among others (Biely, 2012).

4.6. Auxiliary Activities (AAs)

Since ligninis together with polysaccharides found in the plant cell wall and due to the newly acquired knowledge about the oxidative mechanism of the polysaccharide degradation, a short while ago, a new class, named “Auxiliary Activities”, were incorpporated in the CAZy database, including ligninolytic enzymes (e.g. peroxidases), peroxide producing enzymes and four families of LPMOs (AA9, 10, 11 and 13, Levasseur et al., 2013).

Lytic polysaccharide monooxygenases (LPMOs): oxidative decomposition of cellulose

Lytic polysaccharide monooxygenase is a novel class of oxidative enzymes, (formerly GH61), which participates in cellulose degradation via oxidative mechanisms (Jung et al., 2015). LPMOs are widely distributed in the genome of most ascomycetes and basidiomycetes fungi. Their expression levels of most LPMO genes increase considerably during growth on lignocellulosic substrates. LPMOs have an unusual surface-exposed active site with a tightly bound Cu (II) whose role is to reduce O_2. This requires electrons from an external electron donor ion, which catalyzes the hydroxylation of crystalline cellulose, leading to glycosidic bond cleavage. The electrons can be derived from CDH or small-molecule reductants present in lignocellulosic biomass (Baldrian and Valášková, 2008) (Fig. 8).

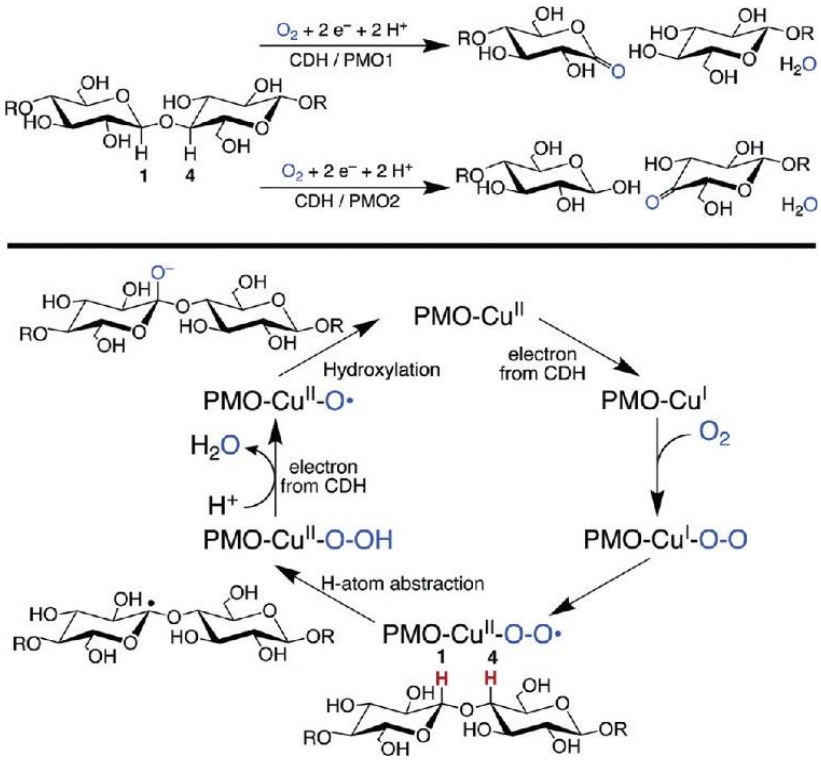

Fig. 8. LPMO reactions and proposed mechanism according to Phillips et al. (2011).

5. Induction of lignocellulosic-degrading enzymes

The previously described oxidative and hydrolytic fungal catalysts are of considerable biotechnological interest. The improvement of industrial processes such as biopulping, biobleaching and bioremediation as well as the development of alternative biofuels, instead of traditional fossil fuels, by converting plant cell walls into fermentable sugars require the production of high concentrations of stable fungal lignocellulolytic enzymes using cost-effective methods.

The presence of ligninolytic and cellulolytic-encoding genes in the genome of a fungus does not necessarily mean that an organism is able to produce the proteins efficiently, since the transcription and secretion mechanisms of a specific enzyme upon exposure to a specific substrate are gene-regulated. For instance, the recently discovered bifunctional GH78 of *Xylaria polymorpha*, which is capable of hydrolyzing ester and glycosidic bonds, is only secreted in wheat straw (Nghi et al., 2012) and in liquid orange peel-containing media (Liers, unpublished, oral communication). *A. auricula-judae* DyP has only been purified from tomato juice media, and *A. aegerita* UPO from soybean meal suspensions (Ullrich et al., 2004; Liers et al., 2010). Some carbon sources can dramatically up-regulate the expression and secretion of a wide variety of lignocellulolytic enzymes, especially plant-based media. For instance, Lacs from both basidio- and ascomycetes have been produced in complex plant-based media or are inducible by addition of aromatic substances (e.g. veratryl alcohol, xylidine) or copper salts (e.g. copper sulfate) (Ullrich et al., 2005; Liers et al., 2006; Michniewicz et al., 2006)

It is clear that the production of ligninolytic enzymes occurs during the second metabolism and is usually triggered by nitrogen or carbon depletion. When the secretion is regulated by carbon limitation, it is usually done via the cAMP mechanism: significant increases in intracellular cAMP concentrations precede the production of MnPs and LiPs in *P. chrysosporium* (MacDonald et al., 1984). However, the means by which filamentous fungi respond to the presence of different carbon sources in the environment remains unclear. For example, LiP and MnP activities can be suppressed in *P. chrysosporium* when nitrogen concentration in the medium is high (Van der Woude et al., 1993). On the other hand, high concentrations of nitrogen do not affect Lac and MnP secretion in species like *Pleurotus ostreatus* and *Trametes trogii* (Janusz et al., 2013).

Low molecular weight compounds, (e.g. monosaccharides or disaccharides), trigger cellulases and hemicellulase production as polysaccharides are too large to enter the cells. These inducers are produced by hydrolysis of cellulose and hemicellulose by small amounts of constitutively expressed cellulases and hemicellulases. The regulation

mechanisms of the CAZy genes involved in cellulosic biomass degradation have been mainly studied in industrial strains such as *Aspergillus sp.*, *Trichoderma reesei* and *Neurospora crassa*, showing differences concerning the number of genes encoding the CAZy enzymes and transcription factors. Since regulation varies depending on the species, the carbon source and other environmental conditions, it is not possible to define other general rules governing cellulolytic enzyme regulation (Tani et al., 2014).

6. Lignocellulosic agricultural by-products

Approximately 90% of the global plant biomass production (200 x 10^9 tons/year) consists of lignocelluloses, with approximately 8-20 x 10^9 tons of this primary biomass being potentially accessible (Kuhad and Singh, 1993). In addition, agro-industrial activity represents a serious environmental problem in producing countries, resulting in the generation of enormous amounts of waste that, in some cases, is either left in fields or burned. Nowadays, one of the main challenges of green biotechnology is the need to develop solutions to manage the waste generated by industrial agriculture by regarding this lignocellulose material as a feedstock and energy resource given the large quantities available and its renewable nature.

In Spain, the most produced agricultural commodities are wine, barley beer and olive oil (http://faostat3.fao.org/home/E) (FAOSTAT, 2013). The production of these commodities generates large amounts of lignocelluloses residues. In this doctoral thesis, we will focus on the study of dry olive and winery residues as marketable products (Fig. 9).

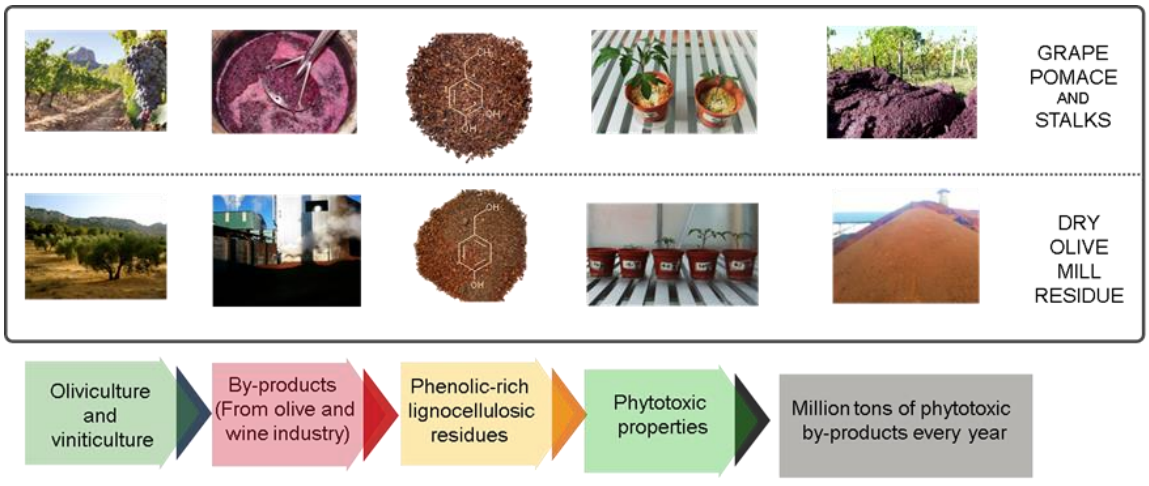

Fig. 9. Diagram showing dry olive mill residue (DOR) and grape pomace and stalks (GPS) residues.

6.1. "Alpeorujo" Dry Olive Residue (DOR)

Approximately one million tons of olive oil is produced annually in southern Spain, making it the leading producer of this commodity in the world, while, at the same time, generating 4 million tons of waste during the extraction process (FAOSTAT, 2013).

In the early 1990s, the two phase olive oil extraction system was introduced in Spain, replacing the traditional three-phase system, which required higher water consumption levels. This technology yields 200 kg of oil per 1000 kg of olives and generates 800 kg of a two-phase olive-mill waste (TPOMW) also known as "alpeorujo". TPOMW undergoes a drying process and subsequent extraction of residual oil using organic solvents such as hexane which generates a low quality oil, also called "orujo" (Fig. 10). It also generates a dry olive residue, known as "alpeorujo" (Roig et al., 2006).

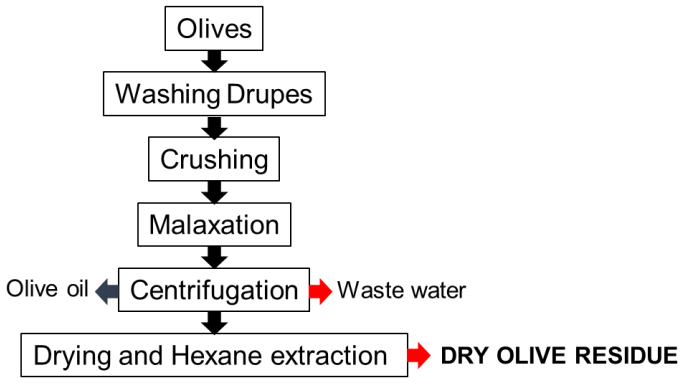

Fig. 10. Diagram of the olive oil extraction process.

DOR has a high moisture content, slightly acidic pH values and a very high organic matter content, mainly composed of lignin, hemicellulose and cellulose as well as a considerable amount of fats, proteins, water-soluble carbohydrates and a small but active fraction of hydrosoluble phenolic substances (Alburquerque et al., 2004). The phenolic composition can vary depending on the campaign, extraction and analytical methods employed, but the major phenolic acids that are present in DOR include tyrosol, oleuropein, catechin, trans-cinnamic acid, caffeic acid and 3,4-dimethoxybenzoic acid (Priego-Capote et al., 2004). The harmful effects of DOR have been attributed to the phenols. The olive residues are phytotoxic, and they induce mutagenic, genotoxic and cytotoxic effects at low concentrations in a wide range of microorganisms. They also produce short-term negative alterations in the physic-chemical properties of soil (Aranda et al., 2006; Justino et al., 2012; Di Bene et al., 2013).

6.2. Grape pomace and stalks (GPS)

In recent years, wine production has varied from 30.9 to 50 million of hectolitres (Hl) per year (OIV, 2013). This activity generates huge amounts of residues. It is estimated that, in Europe alone, 14.5 million tons of grape by-products are produced every year (Chouchouli et al., 2013).

Wine industry generates wine lees, wastewater, greenhouse gas emisions (e.g. CO_2, volatile organic compounds), inorganic wastes (e.g. diatomaceous earth, bentonite clay, and perlite) and organic wastes (e.g. grape pomace, containing seeds, pulp and skins, grape stems and grape leaves), which contain high contents of biodegradable compounds and suspended solids (Mateo and Maicas, 2015).

Grape pomace is produced during the production of grape juice after the pressing process and grape stalks, which constitute between 1.4 and 7.0% of the raw matter processed and are usually removed before the vinification process (Fig. 11).

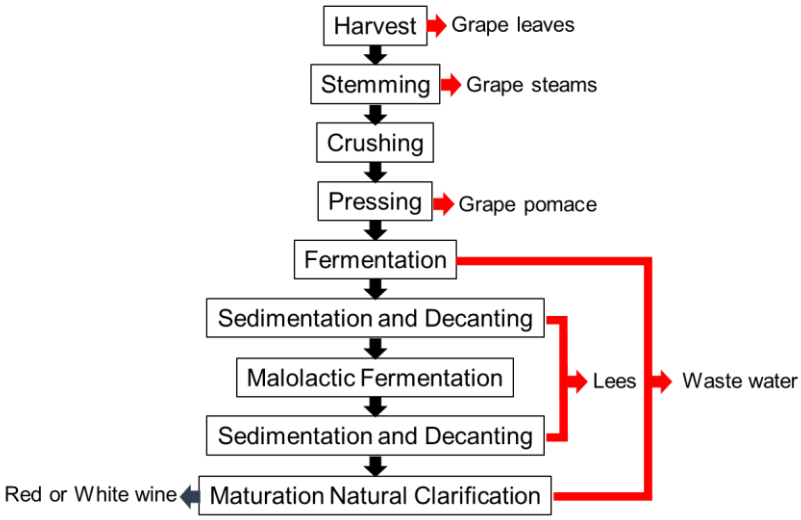

Fig. 11. Diagram of the vinification process.

The moisture of grape pomace varies from 50 to 72% and for grape stalks from 55 to 80% depending on the grape variety considered and it ripening state. Wine wastes have a low pH, and high organic matter content (879-940 g kg^{-1}). GPS also contain high concentrations of macronutrients, especially K, as well as a large amounts of water-soluble polyphenols and a low content of micronutrients and heavy metals (Bustamante et al., 2008). In grape pomace, the lignin content of cell wall polysaccharides varies from 16.8 to 24.2%, pectic substances from 37 to 54% and cellulose from 27 to 37% (Mateo and Maicas, 2015).

6.3. Valorization of olive mill and winery wastes

Recent research studies highlight on the treatment and valorization of these phytotoxic by-products: DOR and GPS, regarding them as renewable resources instead of residues. The valorization strategies consist in the recovery of valuable natural components such as phenolic compounds, dietary fibres and polymers (Roig et al., 2006; Moumen et al., 2008; Paradelo et al., 2013; Monetta et al., 2014). Additionally, the aforementioned by-products are suitable substrates for obtaining biofuels (e.g. bioethanol, biomethane) and relevant biocatalysts (e.g. LME and cellulases), prior to adequate microbiological treatment (Dermeche et al., 2013). All these alternatives could be regarded as involving biorefinery processes, which can be defined, according to the International Energy Agency (IEA), as "*the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, and chemicals) and energy (fuels, power, heat)*".

The establishment of cost-effective fermentation methods in biorefineries is necessary for the utilization of lignocellulosic material. Thus, these methods require microorganisms with the abilities to produce high lignocellulolytic activity and titers in this material, to use many carbon sources, and to be resistant to the toxic compounds released (Hasunuma et al., 2013).

There are several advantages of using fungi as microorganisms and DOR and GPS as biomass for biorefinery processes: the reduction of waste to landfill and the cost to obtain these materials, the generation of enzyme rich extracts and the possibility to use the treated wastes for further applications such as their use in agriculture.

The alternatives for the agronomic valorization of these treated residues consist in their application to soil as an organic amendment. This could be beneficial to soil due to their high organic matter content, the absence of heavy metals and their content in some cations such as K, P and Ca (Bustamante et al., 2008; López-Piñeiro et al., 2008). Some studies suggest that the potential phytotoxicity of these residues can be amended by fungal treatment, then these toxic wastes can be transform into natural fertilizers (Aranda et al., 2006; Aranda et al., 2012).

7. OMICS in fungal research

To date, hundreds of fungal genomes have been sequenced, providing new directions to study fungal biodiversity and functionality. Nonetheless, there is a breach between the gene content and the expressed gene catalogue of an individual organism in a particular media. In order to better understand the biological mechanisms involved in fungal life styles and the interrelation between fungi and their surroundings, functional

studies beyond the genome are required. The development of new high-throughput "*omics"* techniques and software, the availability of annotated fungal genomes, as well as the improvement and expansion of databases, have made possible to obtain large amounts of functional data, such as proteomes and transcriptomes. A transcriptome is defined as the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition (Wang et al., 2009). Whereas a proteome is defined as the systematic analysis of the proteome, the protein complement expressed by a genome, cell, tissue or organism (Bhadauria et al., 2007).

These advanced analytical methods combined with traditional physiological research will make it possible to highlight the connections between the activity of fungal communities and their single organisms on the one hand and biochemical and metabolic processes on the other in combination with macromolecules and proteins and their complex natural environment.

7.1. Fungal proteomics

The aim of proteomics is to study proteins as the cellular building blocks that directly asserts the potential function of genes via enzymatic catalysis, molecular signalling and physical interactions (Yates et al., 2009). Generally, the secretome can be understood as the combination of native secreted proteins and the cellular machinery involved in their secretion (Kim et al., 2007).

Few intracellular proteomic studies of white rot fungi such as *P. chrysosporium*, *L. edodes* (Hernández-Macedo et al., 2002) and certain ascomycetes (*Aspergillus sp*. for example; Grinyer et al., 2004; Ström et al., 2005; Heitman, 2006) have been carried out. However, extensive detailed analyses of the secreted proteins (secretome) of ascomycetes such as *Trichoderma harzianum*, *Botrytis cinerea, Aspergillus oryzae and Fusarium graminearum* have been made (Oda et al., 2006; Paper et al., 2007; Do Vale et al., 2012; González-Fernández et al., 2014). Following the advances in software, algorithms and experimental methods, the secretomes of white rot fungi have recently been gaining in importance. Secretomes of fungi, such as *P. chrysosporium, Postia placenta, Laccaria bicolor, Trichoderma sp., T. versicolor* and *Coprinus cinereus,* have been published in recent years (Wymelenberg et al., 2005; Wymelenberg et al., 2010; Druzhinina et al., 2012; Vincent et al., 2012; Carabajal et al., 2013). The advantage of secretomic approaches, is that they provide valuable information of the complex interactions of the enzymatic set that fungi secrete to adapt their metabolism to various nitrogenous and carbonaceous sources. It is therefore especially important to understand lignocellulolytic processes in wood-degrading fungi.

The current tendency in fungal studies is to combine different types of genome-scale data. For example, Liu et al. (2013) have carried out genomic studies of *Penicillium decumbens* that focus on the evolution of cell wall degradation mechanisms. In addition, complementary proteomic studies have revealed that carbon complex media are more suited to LME secretion than glucose-supplemented media (Liu et al., 2013). Floudas et al. (2012) used an integrated comparative genomic, secretomic and transcriptomic approach to reconstruct the Paleozoic origin of enzymatic lignin degradation using 31 fungal genomes and different transcriptomic and secretomic analyses (Floudas et al., 2012). Secretomic and transcriptomic studies of *C. subvermispora* have provided strong biological evidence for sequential degradation of lignocellulosic material, by first selectively removing lignin in advance of cellulose which undergoes hydrolytic and oxidative attacks (Hori et al., 2014).

High-throughput methods in fungal proteomic studies

Immense advances in electrophoretic, chromatographic, and mass spectrometric techniques have been made in recent years to increase throughput and improve the identification of proteins. In particular, mass spectrometry (MS) is the most comprehensive and versatile tool used in large-scale proteomics due to the introduction of new ionization techniques that transfer analytes to the gas phase without extensive degradation (Beck et al., 2011). Currently, the soft ionization techniques of matrix-assisted laser desorption ionization (MALDI) (Hillenkamp et al., 1991) and electrospray ionization (ESI), are the most effective and frequently methods used (Fenn et al., 1989).

The MALDI matrix absorbs laser energy which is transferred to the acidified analyte, while rapid laser heating causes desorption of the matrix and $[M+H]^+$ ions of the analyte into the gas phase (Liao and Allison, 1995). Mass analyzers separate ions according to mass-to-charge ratios (m/z). Ion trap (IT), Orbitrap and Fourier transform ion cyclotron resonance (FTICR) mass analyzers separate ions according to their m/z resonance frequency, while quadrupoles (Q) use m/z stability and time-of-flight (TOF) analyzers use flight time. TOF analyzers are usually interfaced with MALDI to carry out pulsed analysis, while ion-beam and trapping instruments are frequently coupled to a continuous ESI source.

The most widely used instruments in proteomics are 3D ion trap (QIT) and linear ion trap (LIT). Like the triple quadrupoles LTQ-Orbitrap or LTQ-FTIRC and the TQ-FTICR hybrid instruments Q-TOF and IT-TOF (Yates et al., 2009). Apart from the linear ion trap (LTQ Orbitrap family of instruments), the Orbitrap analyzer can be interfaced to a quadrupole mass filter (Q Exactive family) or directly to the ion source (Exactive family).

One of the advantages of a quadrupole Exactive instrument or “Q Exactive” is its ability to select ions virtually instantaneously, unlike Exactive instruments, because of the rapid switching times of quadrupoles. Furthermore, it is able to fragment peptides in a Collisional Dissociation (HCD) mode. These characteristics make the instrument a worthwhile addition to the proteomics toolbox (Michalski et al., 2011).

To correlate mass spectral data of peptides with amino acid sequences in a protein database, peptide identifications are made by comparing the experimental MS/MS spectra with the predicted MS/MS spectra generated from a set of “possible” proteins using automated database searching software such as SEQUEST (Eng et al., 1994), Mascot (Perkins et al., 1999) or Sonar (Field et al., 2002).

7.2. Fungal Genomics

P. chrysosporium was the first white rot fungus to be sequenced and its publication was a milestone in fungal genomics studies (Martínez et al., 2004). One hundred and fifteen fungal genomes of the phylum *Basidiomycota,* 102 of which belong to the class *Agaricomycetes* according to the DOE Joint Genome Institute (JGI http://jgi.doe.gov/), have been sequenced and published (Nordberg et al., 2014). Large-scale community sequencing programs such as the 1000 Fungal Genomes Project (http://1000.fungalgenomes.org) and the Fungal Genome Initiative (http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative) will provide an even broader picture of genome data from all major fungal groups.

7.2.1. Overview of the history of sequencing technology

In the 1970s, the first methods to sequence DNA using chain termination and fragmentation techniques were developed (Sanger et al., 1977; Maxam and Gilbert, 1977). These methods, together with the development of automated sequencers (Smith et al., 1986), enabled a range of filamentous fungi such as *P. chrysosporium, T. reesei, Agaricus bisporus, Schizophyllum commune, P. placenta* and *C. cinereus* to be sequenced three decades later (Martínez et al., 2004; Martínez et al., 2008; Martínez et al., 2009; Ohm et al., 2010; Stajich et al., 2010; Morin et al., 2012).

Sanger sequencing is faced with some limitations, such as its small sample handling capacity. This has been overcome with the emergence of next-generation sequencing (NGS) methods which make it possible to obtain a larger number of long DNA sequences per cycle in shorter. In terms of usage, NGS platforms can be classified into two categories: high-end (e.g. Illumina-HiSeq) and bench-top (Ion PGM, Illumina-

MiSeq) which are less costly and more appropriate for common microbiology laboratories. In this thesis, we work with the Ion Torrent Semiconductor.

The sequencing method for each of these platforms has a common procedure that can be summarized in three steps: library preparation, template amplification and sequencing. Library preparation begins with the extraction and purification of genomic DNA. The extracted DNA is then broken up into overlapping fragments. During the amplification step the DNA molecules are cloned by PCR in the library and are prepared for sequencing. The last step involves sequencing, which varies across platforms (Appendix II).

Although Ion Torrent technology has mostly been used to study fungal communities in recent years, it can equally be employed to decipher individual fungal genomes such as *G. candidum*, *Pyrenophora teres* and *Sphaerulina musiva* (Polev et al., 2014; Leboldus et al., 2015).

In addition to genomic sequencing, some of the NGS techniques can also be used to analyze exomes, large gene panels, complete RNA transcriptomes and chromatin inmunoprecipitation (Kim and Mitchell, 2011; Boedi et al., 2012).

7.2.2 Genome annotation

Gene annotation in eukaryotic genomes, which have multi-exon gene structures, is challenging and requires a combined approach. Gene structures are predicted using three general kinds of methods: *de-novo*, homology-based, and transcriptome-based (Ohm et al., 2014). A complete genome annotation consists of a structural and a functional process. The structural annotation of the genome is a process that identifies genes, transcripts, and intron–exon structures. It starts with the assembly of the genome and concludes with the gene prediction. The functional genome annotation is the process of attaching meta-data such as gene ontology terms to structural annotations (Fig. 12).

The first step towards the successful annotation is the *Assembly*. NGS technologies generate short sequences with high error rates from relatively short insert libraries. The assembly of longer repeats and duplications is hampered by this short read length since short reads are based on de Bruijn graph and Eulerian path approaches, which have difficulty in assembling complex regions of the genome and long reads. This problem can be minimized by obtaining longer inserts and using hybrid assemblers (Alkan et al., 2011).

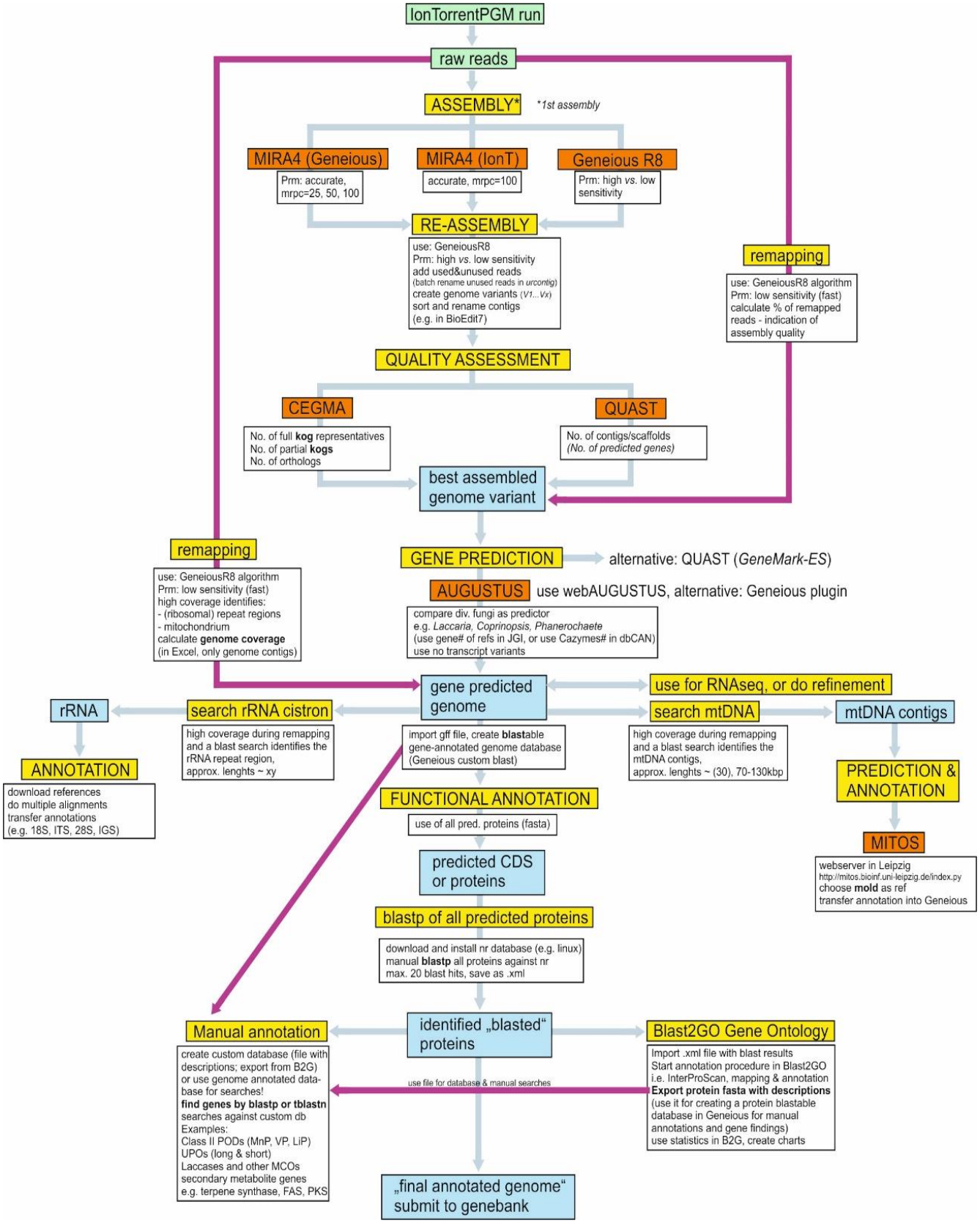

Fig. 12. Flowchart describing general annotation procedure.

To determine whether an assembly is ready for annotation, several summary statistics are used to describe the completeness and contiguity of a genome assembly. The most important statistic, by far, is the N50. It is the contig length for which the collection of all contigs of that length or longer, contains at least half (50%) of the basis of the assembly. To assess the completeness of the eukaryotic genome, one can also determine how many of the Eukaryotic Orthologous Groups (KOG) genes are present in an assembled genome. The CEGMA platform, for instance, is able to estimate the number of highly conserved KOGs genes which are present in an assembled genome, and thus provides an indication of the completeness of the genome (Parra et al., 2007). Other useful statistic is genome coverage. Genome coverage is the percentage of the genome that is contained in the assembly. This is based on size estimations and most genomes contain a considerable fraction of repetitive regions that are difficult to sequence (Goel et al., 2013).

The next step in genome annotation is *Gene prediction*. The main challenges this process are the prediction of short exons, especially those bordered by large introns determination of the precise boundaries of exons and their assembly into complete genes, alternative splicing, the reliance on known sequences, the presence of overlapping genes, the possibility of sequences stored in databases containing errors, the prediction of partial and multiple genes, non-canonical splice sites (splice sites other than those based on GT–AG dinucleotides) and the prediction of genes in newly sequenced genome (Goel et al., 2013). Soft computing programs (e.g. Augustus, Stanke and Morgenstern, 2005), are suitable to cope with these problems.

There are almost as many strategies for creating *Automated Annotation* as there are annotation pipelines, but the common theme is to use evidence to improve the accuracy of gene models, usually through a combination of pre- and post-processing of the gene predictions. One of the most used software platforms for genome annotation is Blast2Go: it is able to create, manage and use genome annotations, including the analysis, visualization and redistribution of annotation data (Götz et al., 2008).

Even the annotated genomes obtained from the most accurate gene predictors and genome annotation pipelines contain missannotated exons. Therefore, manual corrections are still required. As long as tools and sequencing technologies continue to develop, periodic updates to previously obtained genome annotations will be necessary. Incorrect and incomplete annotations poison every experiment that makes use of them.

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OBJECTIVES

OF THE THESIS

*Agaricomycete*s are one of the most efficient lignin modifying enzyme (LME) producers on Earth. LME secretion varies according to different factors, such as the presence of certain elicitors in the medium like lignocellulosic materials. Olive oil and winery by-products are phytotoxic phenol-rich lignocellulosic residues produced in large amounts in Mediterranean countries, for which various valorization processes have been proposed to deal with their management and disposal. Given these premises, the principal aim of this PhD dissertation is to investigate the effect of the agricultural dry olive mill residue (DOR) and grape pomace and stalks (GPS) wastes on the enzymatic secretion profile of selected *Agaricomycetes*. We will focus on the promising role of DOR as a LME enhancer and at the same time, to study its degradation and potential use as an organic amendment to improve plant growth. The improved valorization of DOR and GPS would facilitate the development of a multiproduct industry that utilizes various components in biomass and their intermediates thus maximizing the value derived from biomass feedstock ("biorefinery" concept). This overall goal was achieved by carrying out the following specific key objectives:

1. Characterization and comparison of the lignocellulosic by-products DOR and GPS as well as a study of their suitability as media for fungal growth and elicitors for LME secretion.

2. Study of the differential secretion patterns of lignin-modifying enzymes during solid state fermentation of different media supplemented with DOR by *Agrocybe aegerita, Auricularia auricula-judae, Bjerkandera adusta, Chondrostereum purpureum, Coprinellus radians, Marasmius alliaceus* and *Trametes versicolor* and the effects of fungi on the residue phenolic content, polymerization and plant toxicity.

3. Study of the regulation of enzymes involved in lignocellulolysis by means of a shot-gun secretomic approach of the wood decay fungus *Bjerkandera adusta* in the presence of DOR, characterization of the most representative peroxidases secreted in DOR-supplemented media and quantification of the secreted proteins by label free proteomics.

4. Description of new insights into lignocellulolytic mechanisms of the fungus *Chondrostereum purpureum* by a genome sequencing shot-gun strategy and a proteomic study in different lignocellulosic media.

MATERIALS AND METHODS

1. By-products

1.1. Dry Olive Mill Residue (DOR)

DOR was obtained from the Sierra Sur olive oil company in Granada, Spain (2009-2010 harvest). It was sieved, autoclaved in three cycles and stored at 4 ºC before use. The aqueous extract of DOR (ADOR) was produced by a 1:2 (w:v) DOR:water extraction process lasting 8 h under orbital shaking (170 rpm) and subsequent centrifugation and filtration through Whatman no. 5 filter paper.

1.2. Grape Pomace and Stalks (GPS)

GPS from a cultivar in La Herradura (Granada, Spain) were kindly provided by wineries in Southern Andalucía during the vintage 2012. GPS was generated from a mixture of grapevine varieties (Moscatel, Garnacha, Garnacha Tintorera, Monastrel, Airen, Jaen black and Jaen white). The samples were collected immediately after the pressing operation and dried for 1 week at 40 ºC. The dried stalks and pomace were then milled, autoclaved in three cycles and stored at 4 ºC. The aqueous extract of GPS (AGPS) was obtained in a similar way to ADOR, using a 1:5 (w:v) GPS:water ratio.

2. Organisms and fungal cultures

DOR degradation studies require the pre-culture of the wood-dwelling fungi *T. versicolor* (JAO-EEZ 13), *A. auricula-judae* (DSMZ, accession no. 11326), *B. adusta* (DSMZ, accession no. 23426), *C. radians* (DSMZ, accession no. 888) *M. alliaceus* (Zi06), *A. aegerita* (DSMZ, accession no 22459) and *C. pupureum* (DSMZ, accession no 4894). *T. versicolor* was sequence-based identified by amplifying and further sequencing the fragments corresponding to the ITS4-ITS5 rRNA (Internal Transcribe Spacer ITS) (White et al., 1990) (Data S1). All the fungi were cultivated at 24-25 ºC on 2% MEA plates for 1-2 weeks to obtain fresh inoculum. To produce the inoculum for the experiment, an agar plate containing fungal mycelia was homogenized in 80 ml of a sterile NaCl solution (0.9%) and the suspension was added to the liquid medium (5% v:v) and solid medium (0.5% v:w).

2.1. Submerged Fermentation (SF)

Submerged fermentation was performed in three complex liquid media: soy flour, barley flour and eco-tomato juice (ETJ) and a defined synthetic media: Kirk (Kirk et al., 1978). The soy and barley were prepared with distilled water at 2% (v:v) and ETJ was mixed with distilled water (50:50 v:v). ADOR and AGPS were added after 5 days of incubation. Cultures were agitated (100 rpm) at 25 ºC and 1.5 ml of culture supernatants were harvested every 2-3 days until the end of the experiments. Samples were

centrifuged at 9000 *g* for 5 min and stored at -20 ºC. Enzyme activity, phenol content and pH were measured in the culture supernatants after centrifugation.

All experiments were carried out in triplicate for each treatment, and all the data reported are mean values with standard deviation.

2.2. Solid State Fermentation (SSF)

Solid state fermentation was performed as previously described in Reina et al. (2013) using barley and/or soy as organic support. Half of the replicates were mixed with DOR (50% w:w) and GPS (28% w:w) in the case of *T. versicolor* and the remaining flasks were kept as barley and/or soy controls. The same treatments were carried out on the heat-inactivated mycelium as control in order to detect any possible phenol adsorption. The inoculated flasks were harvested after 0, 1, 2, 3, 4, 5 and 6 week(s) after the addition of DOR (and GPS for *T. versicolor* cultures) until the end of the experiment. DOR (and GPS for *T. versicolor* cultures) were manually separated from barley and soy and dried for 3 days in an oven at 60 ºC for phytotoxicity experiments (Reina et al., 2013). Before drying, an aliquot was separated to obtain an aqueous extract using distilled water (1:5 w:v) by shaking on a rotary shaker for 2 h. The extracts were centrifuged, filtered and used to measure extracellular enzyme activity, pH, total phenol content and the molecular mass distribution of water-soluble aromatic fragments released from DOR and GPS by fungal treatment.

3. Enzyme assays

3.1. Heme peroxidases, laccase and aryl alcohol oxidase

MnP was measured by monitoring the formation of Mn^{3+}-malonate complexes at 270 nm (ε_{270nm}: 11.59 mM^{-1} cm^{-1}) using Na-malonate buffer at pH 4.5 (Wariishi et al., 1992). LiP was monitored using veratryl alcohol at 310 nm (ε_{310nm}: 9.3 mM^{-1} cm^{-1}) using tartrate buffer at pH 3.0 and H_2O_2 (final concentration 0.1 mM) (Liers et al., 2011). Laccase was determined by monitoring the oxidation of 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (ε_{420nm}: 36 mM^{-1} cm^{-1}) using a 50 mM Na-malonate buffer at pH 4.5 (Wolfenden and Willson, 1982). DyP-type peroxidase was also determined by monitoring the oxidation of ABTS ($\varepsilon_{420\ nm}$: 36 mM^{-1} cm^{-1}) using a combination assay in 50 mM Na-malonate buffer at pH 4.5, and the peroxidase activities were adjusted for laccase activity (Eggert et al., 1996; Liers et al., 2010). Veratryl alcohol oxidizing activities (peroxidases and oxidases) were monitored by using veratryl alcohol at 310 nm ($\varepsilon_{310\ nm}$: 9.3 mM^{-1} cm^{-1}) with the aid of potassium phosphate at pH 7.0 for UPO (Ullrich et al., 2004) and at pH 6.0 for AAO (Muheim et al., 1990) with and without H_2O_2 (final concentration 1 mM), respectively.

Enzyme activities were expressed as U g^{-1} of DOR or GPS in SSF experiments and U L^{-1} for SF experiments. A unit of activity is defined as the amount of enzyme that catalyses the conversion of 1 µmol of substrate min^{-1}. Mean values of triplicate measurements were calculated and error bars represent standard deviation.

3.2. Glycoside hydrolases

The hydrolytic activities endoglucanase (CMC-ase, EC 3.2.1.4), xyloglucanase (Xyl-ase, EC 3.2.1.151) and endopolymethylgalacturonase (PO-ase, EC 3.2.1.15) were determined with the aid of the viscosimetry method (Rejon-Palomares et al., 1996). The substrates used were carboxymethylcellulose, xyloglucan extracted from *Tropaeolum majus* seeds and citrus pectin, respectively (McDougall and Fry, 1989). The reaction mixture contained 1 ml of substrate (0.3% carboxymethylcellulose, 0.3% xyloglucan and 1% pectin for each enzyme in a 50 mM citrate–phosphate buffer (pH 5)) and 0.1 ml of sample. The reduction in viscosity was determined at intervals of 0-30 min by the changes in drainage time using a calibrated 0.1 ml syringe. Reactions were performed at 37 ^{o}C. Enzyme activity was expressed as relative activity (AR) calculated as the inverse of the time needed to reduce viscosity by 50% according to the following formula ($AR=1/T_{50}$ x1000) ($T_{50}=50\ T_{30}/\%X$); $\%X=(T_0-T_{30}/T_0)x100$ (Rejón-Palomares et al., 1996).

3.3. Esterase

Esterase activity was determined spectrophotometrically using a *p*-nitrophenyl acetate (*p*NPH) substrate (Sumby et al., 2009). 50 µL of sample was prepared in 400 µL of 50 mM sodium phosphate buffer (pH 6.5) and pre-warmed for 2 min at 45 ^{o}C. 50 µL of 20 mM pNPH substrate in isopropanol was then added. The reaction mixture was incubated for 10 min at 45 ^{o}C and 500 µL of Na-phosphate buffer (300 mM, pH 7.0) containing 5% (w:v) SDS was then added. The release of *p*-nitrophenol (*p*NP) was measured spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of *p*NP per min.

3.4. Intracellular enzymes

3.4.1. Experiment settings

In order to obtain mycelia for *C. purpureum* intracellular enzymes determination, a submerged fermentation (SF) was performed in a soy complex liquid medium (2% of soy flour) and an aqueous extract of DOR (ADOR) (Aranda et al., 2006). Non-inoculated soy and ADOR-soy media were used as controls. Each flask was inoculated using 4 mL of a homogenized solution obtained as described before. ADOR was added after 7 days of incubation. Aminobenzotriazole (ABT) was added to the ADOR-soy media in a 1 mM concentration at the same time as ADOR. The *C. purpureum* cultures were agitated (100

rpm) at 25 ºC during 5 and 15 days. Samples were filtrated and fungal biomass was used for intracellular determination of CytP450s.

3.4.2. Preparation of free cell extracts

The cytosolic and microsomal fractions were isolated from *C. purpureum* cultures after 5 and 15 days of ADOR addition using the modified method of Lehner et al, (2003). Biomass was filtered, washed with sterile water and frozen in liquid nitrogen. The lysis buffer was added to 0.5 g of frozen biomass (1:2 w:v). This buffer contained 20 mM of Tris-HCl at pH 7.6, 7 mM NaCl, 1 mM EDTA, 4% SDS, 30% glycerol and 5 mM and 200 mM of freshly prepared phenylmethylsulfonyl fluoride (PMSF) in absolute ethanol and dithiothreitol (DTT) respectively. Finally, 1 µL of benzonase per 10 mL of sample was added. Samples were disrupted and homogenized in an ultrasonic homogenizer Virtis 45 Blender at 375 Hz according to the following sequence: 3 x 10 s separated by 20 s cooling, 1 min cooling and 2 x 15 s separated by 20 s cooling. Afterwards, samples were centrifuged for 10 min at 10.000 rpm and 4 ºC. Supernatants were kept as *cytosolic fraction* and the pellets were suspended in a new buffer which contained 100 mM of Tris-HCl at pH 7.4, 0.5% v/v of Triton X-100, 300 mM of sucrose, 100 mM of NaCl, 3 mM of $MgCl_2$, 5 mM EDTA, 10 µL $mL^{-1}$of aprotinin. The mixture was incubated for 30 min at 4 ºC and then ultracentrifuged at 40.000 rpm for 90 min at 4 ºC. The supernatants were kept again as *microsomal fraction*. Both fractions were stored at -80 ºC until use.

3.4.3. CytP450 activity

CytP450 was measured spectrophotometrically in microsomal and cytosolic fractions obtained from *C. purpureum* mycelia, according to the modified protocol described by Zazueta-Sandoval et al. (2003). Enzyme activity was calculated subtracting reference slope (absorbance at 460 nm *vs* time) from sample slope and interpolating this value on a peroxidase activity calibration curve made with different concentrations of H_2O_2 as substrate of the enzyme in the presence of *o*–dianisidine (Zazueta–Sandoval et al., 2003). The CytP450 activity was expressed as U mL^{-1}. One unit of enzyme (U) was defined as the amount of enzyme that will catalyze the consumption of 1 nmol O_2 per second.

4. Total phenol content

Total phenol content of the aqueous extracts was determined spectrophotometrically by using the method described by Ribéreau-Gayon (Ribéreau-Gayon et al., 1968) using tannic acid for the standard curve.

5. Total sugar content

Total sugar content was assessed using an anthrone-modified method described by Dubois et al. (1956). 1 mL of the diluted sample was added to 5 mL of anthrone reagent, mixed and immediately heated at 30 ºC for 10 min and rapidly cooled in iced water. Absorbance was measured at 620 nm, and total sugar content was calculated based on a glucose standard curve.

6. Mineral composition of DOR and GPS

Mineral quantitative determination of DOR and GPS was carried out using inductively coupled plasma-optical emission spectrometry (Varian ICP 720-ES model), and nitrogen and carbon were determined by the Dumas method using a Leco TruSpec CN analyzer. These mineral determinations were carried out by the Instrumental Technical Services of the Estación Experimental del Zaidín and Instituto de Nutrición Animal (CSIC, Granada, Spain) respectively.

7. Chromatographic analyses

7.1. Phenolic composition of DOR and GPS by UPLC

DOR and GPS were milled to powder. Samples (0.5 g) were extracted with methanol/water according to the method described by Sampedro et al. (2004). Phenols in DOR and GPS were analyzed by UPLC-MS (Acquity UPLC System, Waters) by the Scientific Instrumentation Centre of the University of Granada (CIC, Granada, Spain) using a Waters ACQUITY UPLC™ HSS T3 column (2.1 x 100 mm, 1.8 µm). Acetonitrile and acetic acid (0.5%) were used in a gradient of 5 to 95% (until min 15) and from 95 to 5% (from 15 to 15.1 min). The detector used was a high definition spectrophotometer (SYNAPT G2 HDMS Q-TOF, Waters). Measurements were performed by using negative electrospray ionization (ESI). Column temperature, flow and injection volume were 40 ºC, 0.4 mL min^{-1} and 10 µL, respectively. Phenol concentrations were expressed as the mean value of the four biological replicates and errors were expressed as standard deviation.

7.2. Phenolic composition of DOR and GPS by GC-MS

Phenols in the solid residues of the fermented cultures after incubation with *A. auricula-judae*, *B. adusta* and *C. radians* for 0, 2 and 4 week and in the corresponding controls were analysed by GC-MS. The analyses were carried out by the Instrumental Technical Service of the Estación Experimental del Zaidín (CSIC), Granada, Spain. Sample preparation was carried out according to a modified version of the method described by Zafra et al. (2006) using 0.1 g of the fermented DOR. 1-naphthol was used as the internal standard. DOR was extracted 3 times with ethyl acetate and 3 times with

methanol. Subsequent purification procedures were carried out using a C18 solid phase extraction cartridge (Waters).

The samples were finally eluted in 0.5 mL methanol. They were then evaporated under N until almost dry and were dissolved in 0.2 mL ethyl acetate. The samples were derivatized with N,O-Bis(trimethylsilyl) acetamide-trimethylchlorosilane (9:1 v:v). Gas chromatographic analysis was carried out using a Varian 450-GC gas chromatograph fitted with a splitless injector for a Factour VF-5ms fused silica capillary column (30 m × 0.25 mm id x 0.25 µm). A silanized split/splitless liner (2 mm i.d.) was used. GC–MS analysis was performed using a method previously described by Juárez et al. (2008) slightly modified. Helium (purity 99.9%) was used as carrier gas at a flow rate of 1.0 mL min^{-1}. The samples were injected in splitless mode. All reagents were of analytical reagent grade. For calibration, stock solutions of phenols (Sigma-Aldrich) containing 1000 µg mL^{-1} were prepared in absolute ethanol 99% (v/v).

7.3. Determination of ergosterol content

Total ergosterol content in the samples was used to evaluate the quantity of fungal biomass in barley-based media and DOR-barley media incubated with *A. auricula-judae*, *B. adusta* and *C. radians* after 0, 2 and 4 week of incubation. A modified version of the method described by Davis and Lamar (1992) was used for ergosterol extraction. The samples were analysed using isocratic elution with the aid of an Agilent HP 1090 (LC; Hewlett-Packard) system, equipped with a diode array detector (HP 1090) and a Nova Pack C18 column (14 µm, 3.9x150 mm, Waters). The mobile phase used was methanol with a flow rate of 0.8 mL min^{-1}. Ergosterol was identified by UV detection at 282 nm.

7.4. Analysis of aromatic water-soluble DOR and GPS fragments by HPSEC

Size exclusion chromatography (SEC) was performed by HPLC-SEC using a HP 1090 LC, Hewlett–Packard, (Waldbronn, Germany) equipped with a diode array detector (HP 1100) according to Liers protocol (Liers et al., 2011). Column used was a HEMA-Bio linear (8 x 300 mm, 10 µm) from Polymer Standard Service (Mainz, Germany). Mobile phase was a mixture of a filtered 80% salt buffer (3.44 g L^{-1} sodium chloride, 2 g L^{-1} dipotassium hydrogen phosphate at pH 10) with 20% acetonitrile (prepared for its direct used in HPLC). Column temperature was set a 30 ºC. Sample volume, flow rate and time for runs were 10 µL, 1 mL min^{-1} and 25 min respectively. For the calibration curve, sodium polystyrene sulfonates (0.208-356 kDa, Polymer Standard Service),

lignosulfonate (Biotech Lignosulfonate GesmbH, St. Valentin, Austria) and alkaline lignin (Sigma–Aldrich) were used.

8. Determination of phytotoxic effect of agro-industrial by-products in *Solanum lycopersicum* plants

In order to improve the application possibilities of the fungi-fermented DOR (and also GPS in *T. versicolor* experiments), a phytotoxicity test of the residues incubated for 0, 1, 2, 3 and 4 week was carried out to analyse the effect of the fungal incubation time on the decrease in phytotoxicity. Tomato (*Solanum lycopersicum*) was used as the test plant. The experiments were carried out in 0.3 L pots using a mixture of soil: vermiculite (1:7). The soil used was obtained from Finca Peinado (Fuente Vaqueros, Granada, Spain) and had a pH of 8.1 in a 1:1 w:v ratio. The P, Ca, K, Fe, Mn, Mg, Na, Cu and Zn contents were P 1.04 mg kg^{-1}, Ca 63.5 mg kg^{-1}, K 9.1 mg kg^{-1}, Fe 26.6 g kg^{-1}, Mn 344.9 mg g^{-1}, Mg 21.1 mg kg^{-1}, Na 1.9 mg kg^{-1}, Cu 22.6 mg kg^{-1} and Zn 68.9 mg kg^{-1}, respectively. Total C was 0.04 mg kg^{-1} and total N was 0.001 mg kg^{-1}.

Tomato seeds were superficially sterilized. After pre-germination, prior to transplanting, the plants were uniformly selected according to their size. Fermented DOR (after different incubation periods) and the uninoculated negative control (DOR 5%) were applied to the soil pots at 25 g kg^{-1}. Plants without residue were used as positive control.

Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cold-white lamps (400 nmol $m^{-2}s^{-1}$ at 400-700 nm, with a 16/8 h day night cycle at 25/19 ºC and 50% relative humidity). Plants were watered from below.

A completely randomized design was used for the experiments, with 4 replicates per treatment. After 4 week, the plants were harvested. The shoot and root dry weight of tomato plants was estimated after being kept for 48 h in a dried oven.

For *T. versicolor*, phytotoxicity experiments with the transformed GPS were also performed using the same procedure described for DOR.

9. Enzyme purification

9.1. *B. adusta* Manganese-oxidizing peroxidase (VP) purification

9.1.1. Enzyme purification

B. adusta was cultured according to the above mentioned conditions in a modified Kirk media in the presence of ADOR. After reaching maximal activities of MnoP, culture liquid was harvested at day 8 after ADOR addition (MnoP: ~220 U L^{-1}) and enzyme-containing extract was filtrated and concentrated by ultrafiltration. Purification was performed by two steps of a fast protein liquid chromatography (FPLC) procedure using

an ÄKTA system (GE Healthcare, Freiburg, Germany) with sodium acetate buffer (10 mM) and Q Sepharose (26/100; pH 5.5) and Mono Q (10/100; pH 5.0) columns, respectively. Elution of the target protein was done with an increasing sodium chloride gradient (0 to 0.8 or 0.3 M for Q Sepharose and Mono Q, respectively) and followed at 407 and 280 nm. Two MnoP-active fractions were pooled, concentrated and dialyzed against Na-acetate buffer (pH 6.0), stored at -80 °C and subsequently used for substrate specificities and degradation studies.

9.1.2. Enzyme identification

The molecular mass of the two purified MnoP active fractions were determined by SDS-PAGE (12% Bis-Tris gel; Invitrogen, Karlsruhe, Germany) according to manufactures protocols. After electrophoretic separation, peptides were analyzed by the above described method.

9.1.3. DHP conversion

Enzymatic conversion for DHP (dehydrogenated polymer) by MnoP of *B. adusta* were carried out in 1.5-mL Eppendorf reaction tubes. DHP of coniferyl alcohol was prepared by Paula Nousiainen (Laboratories of Organic Chemistry, University of Helsinki, Finland) according to methods described previously (Sipilä and Syrjänen, 1995). The reaction mixture (0.5 mL) contained 100 mM Na-malonate buffer (pH 4.5), 5 mM $MnCl_2$, 5 mM linoleic acid, 2 units of the purified MnoP and 0.1 mg mL^{-1} DHP in 5% DMF (DHP stock solution 1 mg mL^{-1} dissolved in 50% DMF). For a continuous supply of cosubstrate, 30 mM glucose and 0.1 U glucose oxidase were added to the reaction that was incubated on a magnetic stirrer at 24 °C for 12, 24 and 48 h. The controls either contained no DHP, no MnoP or no glucose/glucose-oxidase system, respectively. Resulting reaction products were analyzed by HPSEC as described previously (Liers et al., 2011).

9.2. T. versicolor MnP purification

9.2.1. Enzyme purification

T. versicolor was cultivated according to the above mentioned conditions in a soy medium supplemented with 5% ADOR. Cultures were harvested at day 10 of incubation (day 5 following addition of ADOR) after reaching maximal activity (~420 U L^{-1}), filtrated with glass fiber filters and then concentrated by ultrafiltration. A three-phase fast protein liquid chromatography procedure using an ÄKTA system (GE Healthcare, Freiburg, Germany) was required for MnP purification, with one step being run on a Q Sepharose (26/100) column and two on a MonoQ (10/100) column. Buffers used during the runs were a 10 mM Na-acetate at pH 5 (buffer A) and the same buffer with the addition of 2

M of NaCl (buffer B). Gradients, flow rates and fraction sizes were 50% in 15 column volumes, 13 mL min^{-1} and 7 mL with Q sepharose and 20% in 15 column volumes, 6 mL min^{-1} and 2 mL in MonoQ columns. Elution was monitored at 280 and 407 nm. A further HPSEC step was necessary to select the 45 kDa peak and to achieve complete purification.

9.2.2. Enzyme identification

The molecular mass of the two purified MnP active fractions was determined by SDS-PAGE (10% NuPAGE MOPS-buffer gel; Invitrogen, 50 min of colloidal blue stain) according to the manufacturers' protocols. After electrophoretic separation, the bands were excised and analysed by Protagen AG (Dortmund, Germany) using LC-ESI-MS and MS/MS. Joint Genome Institute (JGI) enzyme names and accession numbers were used to identify the proteins (Nordberg et al., 2014).

The substrate specificity of purified enzymes was tested toward veratryl alcohol, Mn^{2+}, reactive blue 5, reactive black 5, ABTS and 2,6 dimetoxyphenol at pH 5.0 in Na-acetate buffer (50 mM) at 310 nm.

10. Statistics

The statistics were analyzed using the IBM SPSS Statistics 21 program. The Shapiro-Wilk and Levene tests were used to check normality and variance homogeneity, respectively. Depending on the Levene test results, different posthoc tests were carried out. ANOVA and Tukey test was used for homogeneous variances and the Dunnet test for non-homogeneous variances.

11. Secretome analysis of *B. adusta*

11.1. Experiment settings

B. adusta liquid cultivation was performed in a 500 mL-flask containing 200 mL modified Kirk medium (10 g L^{-1} glucose, 0.5 g L^{-1} ammonium tartrate, 2 g L^{-1} KH_2PO_4, 0.5 g L^{1} $MgSO_4$, 0.1 g L^{1} $CaCl_2$, 0.3 g L^{-1} yeast, 2 g L^{-1} sodium acetate and 2.5 mM $MnCl_2$). The medium was inoculated with 9% (v/v) of a homogenized mycelia suspension (one MEA plate in 80 mL of sterile water) and the fungal cultures were agitated on a rotary shaker at 100 rpm and 24 ºC.

To study the differences between protein secretion profiles 5% of an aqueous extract of dry olive mill residue (ADOR) was added to certain cultures after one week of pre-cultivation (ADOR supplemented cultures = ASC). In controls, no ADOR was supplemented (Kirk medium culture = KM). ADOR was made from an extracted two-phase olive mill waste DOR (García-Sánchez et al., 2012) by orbital shaking (170 rpm)

with distilled water in a 1:2 (w:v) ratio for 8 h. The phenol-rich ADOR liquid was then filtered and used for the subsequent protein induction studies.

For enzyme measurementsASC and KM were each grown in six parallel culture series. Samples were taken every two days. Manganese-oxidizing peroxidase (MnoP) was measured photometrically by following the formation of Mn^{3+}-malonate complexes (ε_{270}: 11.95 mM^{-1} cm^{-1}). Reactions were performed in 1 mL-cuvettes in the presence of $MnCl_2$ (0.5 mM) in sodium malonate buffer (50 mM, pH 4.5) and were started by adding the cosubstrate H_2O_2 (0.1 mM) (Wariishi et al., 1992). AAO was followed by the oxidation of veratryl alcohol (5 mM, ε_{310}: 9.3 mM^{-1} cm^{-1}) in sodium phosphate buffer (50 mM, pH 6.0) (Muheim et al., 1990).

The activities of the purified MnoP (VP) were determined by the oxidation of Mn^{2+}-ions, veratryl alcohol, 2,6-dimethoxyphenol (DMP, ε_{469}: 49.6 mM^{-1} cm^{-1}), Reactive Black 5 (ε_{598}: 47.6 mM^{-1} cm^{-1}) and Reactive Blue 5 (ε_{598}: 8.0 mM^{-1} cm^{-1}). The reactions were performed in sodium malonate buffer (50 mM, pH 4.5) in the presence of H_2O_2. The mean values of triplicate determinations were calculated and expressed in international units (U) defined as the amount of enzyme that forms or converts 1 µmol of product or substrate, respectively, per minute under assay conditions.

11.2. Proteomic analyses

ASC and KM were harvested with increasing MnoP activity (day 6 after ADOR addition) and a volume of 100 mL was concentrated to 10 mL by centrifugation (Vivaspin20, 10 kDa cut-off, Sartorius, Göttingen, Germany). Induced cultures were run in six parallels (ASC 1-6) and controls were done in duplicates (KM 1-2). The secretomes of *B. adusta* were measured by LC–MS analysis. Therefore, the protein lysates were reduced (2.5 mM DTT for 1 h at 60 °C) and alkylated (10 mM iodoacetamide for 30 min at 37 ºC). Proteolytic cleavage was performed overnight using trypsin (Promega, Madison, WI) in a ratio of 1:20 at 37 °C. Peptides were desalted and purified using ZipTip-C18 tips (Millipore, Billerica, MA). Proteolytically cleaved peptides were separated by a linear gradient of solvent B (100% acetonitrile) from 2% up to 25% for 60 min with a flow rate of 300 nL min^{-1}. The liquid chromatography was coupled to a Q Exactive mass spectrometer (Thermo Scientific) via the TriVersa NanoMate (Advion Biosciences, Norwich, UK). The Q Exactive was operated in data-dependent mode with MS scans acquired at a resolution of 70,000, an ion- target value of $3e^6$ and maximum ion-injection time for the MS scan was set to 120 ms. Up to 10 most abundant isotope patterns with charge ≥2 from the survey scan were selected for MS/MS. An isolation window of 3 m/z and higher energy collisional dissociation (HCD) with normalized collision energies of

27.5% was applied. The maximum ion-injection time for the MS/MS scans was set to 120 ms with an ion- target value of $2e^5$. Repeated sequencing of peptides was kept to a minimum by dynamic exclusion of 30 s. Raw data from the MS instrument were processed using a Proteome Discoverer (Thermo Scientific, v1.3.0.339). MS data were searched against a concatenated database of (i) the genome-sequenced *B. adusta* v1.0 (14,296 protein sequence entries, downloaded from the JGI database (http://genome.jgi.doe.gov/programs/fungi/index.jsf)) (Binder et al., 2013; Ruiz-Dueñas et al., 2013), and (ii) *B. adusta* peroxidases (43 protein sequence entries) using the SEQUEST algorithm. Enzyme specificity selected to trypsin with up to two missed cleavages allowed using 10 ppm precursor mass tolerance and 20 mmu MS/MS tolerances. Only peptide hits with an XCorrelation score >2 and FDR <1% were assigned as identified. Only proteins with ≥2 peptides per protein were considered for abundance comparison. 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 proteins were further classified according to Enzyme Commission numbers (EC number). The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium via the PRIDE partner repository with the dataset identifier PXD000962 (Vizcaíno et al., 2014).

Principal component analysis (PCA) was performed using non-linear iterative partial least squares algorithms (NIPALS) to follow the main trends in the protein data set and as a first approach to data classification (Wold et al., 1987). Software used for statistical analyses were SPSS Statistics 21 and Unscrambler X10.2.

12. *C. purpureum* Genomics

12.1. Fungal cultivation

Solid state fermentation (SFF) was performed in 250 mL flasks. Each flask contained 4 g of beech wood and 14 mL of distilled water (added prior to autoclaving). These media were inoculated with 9 mL of a homogenized suspension of 4 fungal agar plates in 80 mL sterile water (55% v/w). After 1 week of incubation at 25 ºC, half of the flasks were mixed with DOR (50% w/w). Sampling was carried out at distinct steps of fermentation: 1, 2, 3, 4, 5, 6 and 7 weeks. Water extractions of the fermented solids (1:5 w/v) were performed. The mixtures were shaken during 2 h on a rotatory shaker, centrifuged and filtered. Extracts were used to measure extracellular enzyme activities and 25 mL of the samples harvested at week 7 of incubation (when activity was submaximal), was lyophilized and used for label-free proteome analysis.

Submerged fermentation (SF) was performed in 500 mL spherical Erlenmeyer flasks in a complex liquid media: soy and a defined synthetic media: Kirk (Kirk et al., 1978) as previously described. ADOR and birch wood were added after 4 days of incubation in 5% (v:v) and 1% (w:v) respectively. Cultures were agitated (100 rpm) at 25 ºC. 1.5 mL of culture supernatants were harvested every 2-3 days until the end of the experiment on day 16 (or day 12 after ADOR and birch wood supplementation). 40 mL of the harvested samples were then filtered and lyophilized to analyze the secreted protein profile. All treatments were carried out in triplicate.

12.2. DNA isolation and genome sequencing

Genomic DNA was purified from a dikaryotic strain of *C. purpureum*. High-quality RNA-free DNA was obtained using DNeasy Plant Maxi Kit (QIAGEN). 1 µg of gDNA was fragmented with Ion Shear™ Plus Reagent to obtain a 200-basepair-read library. The reaction was performed at 37 ºC during 4 min. Fragmented gDNA was purified with the Agencourt® AMPure® XP Kit and the fragment size was checked with the Agilent 2100 Bioanalyzer. Adapters were ligated and blunt-end was nick-repaired with the Ion Plus Fragment Library Kit, then the ligated DNA was again purified. Subsequently, fragments of 250 bp were size selected on an E-Gel® Size-Select agarose gel. No barcodes were used. Fragment size was again checked. PCR amplification was not required. Library was diluted to a final concentration of 26 pm with the aid of Bioanalyzer to calculate the dilution factor. Template-positive ion sphere particles (ISP) containing clonally amplified DNA fragments were obtained using the Ion OneTouch™ 200 Template Kit v2 according to the described protocol. The quality of the unenriched template-positive ISPs was assessed using a Qubit 2.0 fluorometer and the Quality Control of the Ion Sphere™ kit. The percentage of Templated ISPs was 16%. ISP enrichment was performed with the aid of Ion OneTouch™ ES. The enriched template-positive ISPs were sequenced using an Ion Torrent Personal Genome Machine (PGM) (Life Technologies; Grand Island, NY, USA) according to the manufacturer's protocols provided for a 318 v2 chip.

12.3. Assembly and genome annotation

Only raw-reads with a read length between 120-250 bp were considered for the assembly. They were assembled using the MIRA 4 - Whole Genome Shot-gun and EST Sequence Assembler with an accurate sensibility and a minimum of 50 reads per contig. The obtained contigs were reassembled with a Geneious *de novo* assembler and the new contigs obtained were gathered with the unused contigs. To assess the completeness of the assembled genome, we used CEGMA v2.5 (Core Eukaryotic Genes Mapping Approach) (Parra et al., 2007) and the quality of the assembly was calculated

using QUAST (Quality Assessment Tool for Genome Assemblies) (Gurevich et al., 2013).

Ab initio gene prediction was performed with Augustus (Stanke and Morgenstern, 2005), using *L. bicolor* as a reference organism. No transcript variants were selected. We did a remap of the initial raw reads against the assembled genome using the Geneious R8 algorithm and calculated the coverage of the whole genome as well as the individual contigs. The contigs with higher coverage were chosen for searching rRNA cistron and mitochondrial genes. Once founds, they were extracted and annotated either manually or using MITOS (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013) for ribosomal and mitochondrial genes respectively. The functional annotation was carried out by a manual blastp search of all the predicted proteins against a non-redundant database (nr) obtained from GenBank. The output file (xml) with the blast results was imported into the Blast2GO platform, which was used for mapping the blast results and displaying gene ontology terms (GO). A comprehensive analysis of CAZy genes was performed by custom blast searches and dbCAN webserver (Yin et al., 2012). A phylogenetic analysis of 100 basidiomycetes was performed using the combined 18S, 28S, 5.8S, *rpb2* and *tef1* nucleotide sequences. *C. purpureum* gene sequences were obtained by manual blast against the sequenced genome, the rest of the sequences were obtained from Matheny et al. (2007) and downloaded from the AFTOL database (http://aftol.biology. duke.edu/pub/alignments/download_alignments). Sequences were concatenated and aligned by ClustalW alignments using a blocks substitution matrix. A neighbour joining (NJ) distance phylogenetic tree was performed with the Geneious 8.0 software.

12.4. Proteome analysis of C. purpureum strain

Lyophilized cell pellets (3 mg) were resuspended in 4 mL SDS buffer (1.25% SDS, 0.1 M Tris, 0.3% DTT), a spatula of glass beads were added and the solution was incubated for 1 h at room temperature under shaking. Afterwards, the FASTPrep (5.5 m/s, 45 sec, 3 cycles) protocol was applied followed by further steps including three cycles of freeze and thaw (freeze in liquid nitrogen, thaw in 60 °C water bath), the addition of 0.6 mL of 10% (w:v) SDS solution and two cycles of ultrasonic treatment using an ultrasonic disintegrator (ultrasonic processor UP50H equipped with ultrasonic probe MS7, Hilscher Inc., Germany) (4 °C, 2 min/80% amplitude/80% power, break of 2.5 min between cycles). Phenol extraction was applied to the solution by adding 4 mL phenol solution (10 g mL^{-1} in ddH_2O) to the supernatant and incubated under shaking at 500 rpm and room temperature for 1 h. The mixture was centrifuged at 4 °C and 12,000 x *g* for 10 min for phase separation (Sorvall RC 6 plus, Thermo Fisher Scientific, Waltham, MA,

USA). The lower phase was mixed with a five-fold volume of ice cold 100 mM ammonium acetate in methanol. Precipitation was performed overnight at -20 °C followed by centrifugation at 12.000 x *g*. The supernatant was removed and the pellet was air-dried. The resulting protein pellets were subjected to SDS-PAGE with a 15 µL sample buffer. Each sample lane was then cut into one gel band and prepared for proteolytic cleavage. Protein lysate was 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, USA) with an enzyme/substrate ratio of 1:25 at 37 °C. Peptide lysates were extracted from the gel and desalted using SOLAµ (Thermo Scientific).

The peptide lysates were separated on a UHPLC system (Ultimate 3000, Dionex/Thermo Fisher Scientific, Idstein, Germany). 5 µL were first loaded for 5 min on the precolumn (µ-precolumn, cartridge column, 3 µm particle size, 75 µm inner diameter, 2 cm, C18, Thermo Scientific) at 4% mobile phase B (80% acetonitrile in nanopure water with 0.08% formic acid), 96% mobile phase A (nanopure water with 0.1% formic acid), then eluted from the analytical column (PepMap Acclaim C18 LC Column, 25 cm, 3 µm particle size, Thermo Scientific) over a 120 min gradient of mobile phase B (4-55% B).

Mass spectrometry was performed on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC chip coupling mode. The MS was set on top speed with a cycle time of 3 s, using the Orbitrap analyzer for MS and MS/MS scans with higher energy collision dissociation (HCD) fragmentation at normalized collision energy of 28%. MS scans were measured at a resolution of 120,000 in the scan range of 400-1600 *m/z*. MS ion count target was set to $4x10^5$ at an injection time of 60 ms. Ions for MS/MS scans were isolated in the quadrupole with an isolation window of 1.6 Da and were measured with a resolution of 15,000 in the Iontrap. The dynamic exclusion duration was set to 30 s with a 10 ppm tolerance around the selected precursor and its isotopes. Automatic gain control target was set to $1x10^4$ with an injection time of 35 ms.

Proteome Discoverer (v1.4.1.14, Thermo Scientific) was used for protein identification and the acquired MS/MS spectra were searched with the Sequest HT algorithm against the protein-coding database of *C. purpureum* (containing 13,739 sequence entries). Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion tolerance and 0.1 Da MS/MS tolerances. Oxidation (methionine) and carbamylation (lysine and arginine) were selected as variable modifications and carbamidomethylation (cysteine) as a static modification. Only peptides with a false discovery rate (FDR) <0.01 calculated by Percolator and peptide

rank =1 were considered as identified. Protein abundances were calculated based on the normalized spectral abundance factor (NSAF) (Zybailov et al., 2006).

12.5. Statistical analysis

Secretome samples were triplicated for each treatment. Hotelling's T2 was performed to find statistically significant differences between treatments and principal component analysis (PCA) using non-linear iterative partial least squares algorithms (NIPALS) was used to determine the main trend in the data set and to compare the samples replicates and treatments. The software used for statistical analyses were SPSS Statistics 21, Unscrambler X10.2 and R (Team, 2014).

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CHAPTER 1

Integral valorization of wine and olive mill by-products using *Trametes versicolor* to produce enzymes of industrial interest and soil amendments

Abstract

An integral and affordable strategy for the simultaneous production of lignin-modifying and carbohydrate active enzymes with the aid of *Trametes versicolor* was developed by using olive oil and wine extraction by-products.

We cultivated the polyporal fungus in complex plant-based media supplemented with dry olive mill residue (DOR) and grape pomace and stalks (GPS) in submerged fermentation (SF) and solid state fermentation (SSF). This strategy led to a 4-fold increase in the activity of laccase, the principal enzyme produced by SFF. Manganese peroxidase was moderately secreted and slightly stimulated by adding aqueous extract of DOR (ADOR) to soy cultures. This MnP fraction was purified and characterized in order to study one of the less well known peroxidase groups induced by natural elicitors. In SF, high levels of carbohydrate active enzymes were found when ADOR was added to the cultures and through the use of soy as a support medium. *T. versicolor* managed to secrete lignin-modifying enzymes in GPS, although no stimulative effect was observed. Finally, the reuse of exhausted solid by-products as amendments after fermentation was also investigated.

Different sets of enzymes can be stimulated by *T. versicolor* using DOR and GPS. Additionally, the exhausted residue can increase plant growth allowing an integral valorization.

This study provides a basis for understanding the stimulation and repression of two groups of enzymes of industrial interest in the presence of different by-products, possible enzyme recovery and the final reuse as soil amendments.

Keywords: *Polyporales*, lignin-modifying enzyme, carbohydrate active enzymes, agricultural by-products, *Trametes versicolor*, phytotoxicity.

Introduction

The development of agro-industrial activity over the last 100 years has increased the amount of lignocellulosic by-products, including residues from wood, herbaceous, agricultural and forestry (Sánchez, 2009). These residues can be bio-converted into valuable products by a mechanical or chemical pre-treatment followed by biological fungal processes and further separation and purification if required. Once treated, these by-products can be used as raw material for ethanol production, paper manufacturing, mushroom cultivation and even animal feed (Vlyssides et al., 2004).

According to FAO data, the most commonly produced commodities in Spain are beer of barley, wine as well as olive and soybean oil (FAOSTAT 2012, OIV 2013). As a result of these agro-industrial activities, millions of tonnes of waste are produced every year, which need to be managed appropriately in order to avoid adverse environmental effects. “Alpeorujo” or Dry Olive Residue (DOR) is a phytotoxic by-product resulting from the process of olive oil production which involves a second hexane extraction to reduce oil content (Alburquerque et al., 2004), leading to a low-humidity phenol-rich material. DOR also contains celluloses and hemicelluloses rich in xylans and xyloglucans (Jiménez et al., 2000) as well as soluble carbohydrates like mannitol, sucrose and fructose (Alburquerque et al., 2004) which make it a suitable substrate for fungal growth.

Grape pomace and stalks (GPS) are the main by-products generated by the wine industry. Though not toxic residue themselves, their high organic matter content and the seasonally high production levels can potentially lead to environmental problems (Spigno et al., 2013). Some studies have proposed that grape stalks be used as bio-sorbent material for the removal of toxic compounds, as composting or to obtain high added-value compounds such as phenolic compounds with antioxidant activity, lignin and cellulose (Spigno et al., 2008). GPS have a high tannin content, thus making it a potentially rich source of antioxidants, and quite high hemi-cellulose content mainly composed of xyloglucans.

Some fungi are among the known organisms that can oxidize lignin compounds (Kirk and Farrell, 1987), although their purpose is probably to gain access to hemicellulose and cellulose in order to use them as carbon sources (Ten Have and Teunissen, 2001). According to their capacity to degrade and their effects on this material, these fungi have been traditionally classified into brown, soft and white rot fungi groups. Although this classification is becoming obsolete, as it does not accurately reflect the diversity of mechanisms by which wood-decay fungi obtain their nutrition (Riley et al., 2014), it is still generally used. White rot basidiomycetes are considered to be the most extensive degraders of lignin which they are able to completely break down to

carbon dioxide and water due to their oxidative enzymatic mechanisms (Papadopoulos, 2012). These fungi are able to secrete a set of oxidative enzymes which present broad substrate specificity, allowing them to gain access to the 3D phenylpropanoid network. Oxidases and peroxidases (PODs) are more efficient degraders than hydrolases due to their ability to cleave different carbon-carbon bonds such as C_α-C_β, β-aryl and C_1-C_α including aromatic bonds present in the polymer (Janusz et al., 2013).

The enzymatic weapons in the oxidative arsenal include: the extracellular heme-containing class II peroxidases such as lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16); the recently discovered dye-decolorizing peroxidases (DyPs, EC 1.11.1.19) and the unspecific peroxygenases (UPOs, EC 1.11.2.1); laccases (Lacs, EC 1.10.3.2) and auxiliary enzymes such as copper radical oxidases (EC 1.1.3). Not only do PODs play an important role during lignin degradation, Lacs are also copper-containing polyphenol oxidases (Gianfreda et al., 1999) which are able to oxidize different phenolic compounds to simultaneously reduce molecular oxygen to water. These enzymes include glucose oxidase (GOx, EC 1.1.3.4) and aryl alcohol oxidases (AAOs, EC 1.1.3.7). Lignin modifying enzymes (LME) are valuable, ubiquitous oxidative biocatalysts which are able to cause a variety of enantioselective oxygen-transfer reactions with H_2O_2, thereby converting harmful compounds such as PAHs and xenobiotics and thus with high industrial interest (Paszczynski and Crawford, 1995; Aranda et al., 2009).

In addition, carbohydrate active enzymes such as hydrolytic enzymes play an important role in the conversion of lignocelluloses among other functions. Thus, glycoside hydrolases such as endoglucanases, xyloglucanases and endopolymethylgalacturonase (EC 3.2.1) participate in the hydrolysis of cellulose and hemicellulose breaking the non-covalent interactions present in the amorphous cellulose polymer, the most abundant polymer in plants (Coughlan, 1985). They are members of the glycoside hydrolase (GH) super family on the basis of their amino-acid sequence similarity (Henrissat, 1991; Cantarel et al., 2009), with more than 100 GH families having been recognized. Proteomics analysis of various ligninolytic fungi has shown the different expression pattern of cellulolytic glycoside hydrolases such as xylanases and endoglucanases grown in cellulose and wood substrates (Martínez et al., 2009; Wymelenberg et al., 2009). Carbohydrate esterases are also members of this super family of carbohydrate active enzymes which catalyse the hydrolysis of carbohydrate esters. The diversity of these carbohydrate-degrading enzymes is described in the Carbohydrate-Active Enzymes (CAZy) database (http://www.cazy.org) and is being continuously evaluated. These enzymes have demonstrated their biotechnological

potential in various industries, including the food, animal feed, textile, pulp and paper, agricultural sectors as well as research and development (Bhat, 2000).

Ligninolytic and hydrolytic enzymes seem to be a promising tool for solving numerous environmental problems including its application to the modern phenomenon of integrated biorefineries. However, their industrial application is limited by the high cost, limited operational stability and low output of these enzymes when synthetic media are used. Thus, the utilization of natural substrates, such as agro industrial by-products, could be an alternative means of obtaining higher yields for purification purposes.

Although it is difficult to comprehend the gene regulation of ligninolytic, cellulolytic and hemicellulolytic enzymes in filamentous fungi, the enhancement of hydrolytic enzymes by small inducer molecules liberated from cellulosic biomass is clearly understood (Tani et al., 2014). Nevertheless, the effect of complex polymers on the induction or repression of lignocellulolytic enzymes and, most importantly, their high yields for industrial application require further explanation (Kapich et al., 2004; Amore et al., 2013).

The conspicuous polyporal *T. versicolor* produces several Lacs, class II PODs and dye-decolorizing peroxidases, information concerning the simultaneous production of groups of GH and oxidative enzymes is scarce (Bourbonnais et al., 1995; Johansson and Nyman, 1996; Elisashvili and Kachlishvili, 2009; Carabajal et al., 2013). *T. versicolor* is a well-known fungus on which genomic and proteomic studies have already been carried out (Floudas et al., 2012) enabling us to gain a rough understanding of the protein set capable of secretion. This has provided us with a starting point to determine which components trigger these activities as one of the biggest challenges facing the biotechnology industry is to find a suitable expression medium to activate enzymatic production and subsequently to improve their purification and marketing of the fungal biocatalysts.

In a second approach, comparative use of the applicability of both residues has been investigated. In the case of DOR, the exhausted residues, according to different fungal treatment, usually presents less phytotoxicity that the original one (Reina et al., 2014), being suitable for a second valorization using them as soil amendment. However, little information about fungal bioremediated GPS is available, and its potential use as soil amendment.

The aim of this study was to find a suitable method to valorise the agricultural by-products DOR and GPS and to possibly use them as natural enhancers for simultaneous LME and hydrolytic enzyme production in the well-known fungus *T. versicolor*. We also investigated similarities within the peroxidase group obtained in the presence of natural

inductors. Finally, the importance of exhausted residue for post application as a soil amendment was investigated.

Results

Chemical characterization of DOR and GPS

Supplementary material Table S2 provides information on the mineral composition of DOR and GPS. The analyses performed by ICP show variations in the composition of minerals such as Al, B, Ca, Cr, Cd, Cu, Fe, K, Li, Ni, Ti, V and Zn, which were present in DOR in considerably higher concentrations. On the other hand, concentrations of Mg and Na were much greater in GPS than in DOR. Total phenolic content in both these by-products differs considerably, being nearly 3-fold higher in DOR (16.42 mg g^{-1}) as compared to GPS (6 mg g^{-1}). The main phenols present in DOR (Table S2) were *p*-tyrosol, hydroxytyrosol and protocatechuic acid (105, 128, 140 µg g^{-1} DOR, respectively) whereas, in GPS, only three simple phenols were detected and quantified: gallic acid, protocatechuic acid and hydroxytyrosol (133, 18 and 17 µg g^{-1} GPS, respectively). Gallic acid was not detected in DOR samples (Table S3).

Total sugar content was 174 mg g^{-1} in DOR and 260 mg g^{-1} in GPS at the beginning of fermentation (Table S2).

Submerged fermentation

Lignin-modifying enzyme secretion

Data obtained from SF demonstrated that Lac was the predominant enzyme in *T. versicolor* culture supernatants (Fig. 1). In the four media tested, there was a noteworthy induction of Lac activity in ADOR-supplemented cultures. 30-fold in barley, 3-fold in soy, 1.5-fold in ETJ and 8-fold in Kirk medium, as compared to maximum activity in both ADOR and control cultures. In contrast to Lac hyper secretion, moderate POD activity was observed. MnP peaked at day 9 in ADOR-soy (89.48 U l^{-1}), but no significant differences were found with respect to control soy cultures. Lac activity was determined at day 7 in ADOR-Kirk media (537 U l^{-1}), which was 100-fold higher than control Kirk media. The increase in LiP activity was insignificant in most of the media tested. However, when the fungus was grown in ETJ, there was a gradual increment in this activity, which reached a maximum level at day 13 (54.49 U l^{-1}) in ADOR-ETJ, a 5-fold increase with respect to its ETJ control at day 13 (10.89 U l^{-1}). In AGPS supplemented cultures, we found LME production patterns similar to those for its respective control cultures, as this by-product does not enhance Lac secretion except when soy was used as a support medium. At day 13 of incubation, Lac activity in AGPS-soy cultures was 2635 U l^{-1} whereas it was only 194.44 U l^{-1} in the respective soy controls.

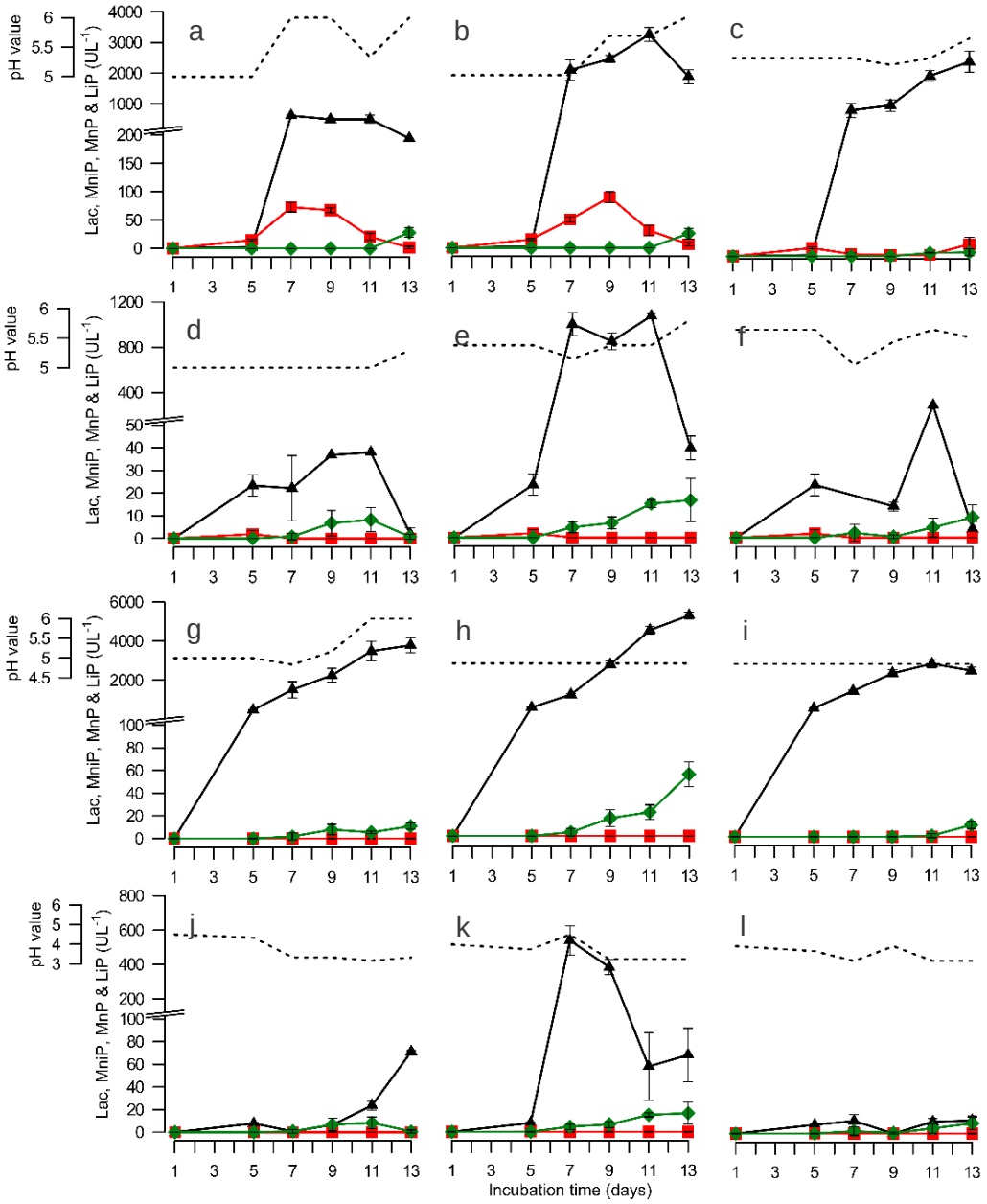

Fig. 1. Time course of Mn-dependent peroxidase (MnP) (*squares*), laccase (Lac) (*triangles*), lignin peroxidase (LiP) (*diamonds*) and pH value (*dotted line*) activity of the polyporal fungus *T. versicolor* during SF in soy, barley, eco-tomato juice (ETJ) and Kirk media (a, d, g and j, respectively). DOR-supplemented cultures in soy, barley, ETJ and Kirk (b, e, h and k, respectively) and AGPS-supplemented cultures in soy, barley, ETJ and Kirk media (c, f, i and l, respectively).

Glycoside hydrolases

Soybean meal, in which higher activity levels were recorded, was a suitable medium for *T. versicolor* GH secretion (Table 1). Maximal CMC-ase and PO-ase production were determined at day 5 of cultivation, with relative activity levels being 68 and 55, respectively. However, the highest Xyl-ase activity was found in ADOR-soy

cultures at day 7 after 2 days of ADOR supplementation. After the addition of ADOR, activity measured in soy-based cultures increased. This boost caused by ADOR was also observed in CMC-ase and Xyl-ase activity after 13 days of incubation in ETJ support media. No significant differences were found among the different treatments in barley or Kirk media. A GH induction effect mediated by AGPS was not detected.

Esterase

Esterase activity was detected in all the media used (Table 2). The highest secretion levels of this biocatalyst were detected in ADOR-soy medium, reaching 610 U l^{-1}, or 1.5-fold higher than in the respective soy-controls and 13-fold higher than in the ADOR-Kirk medium. In the case of the ADOR-ETJ medium, activity increased 3-fold with respect to ETJ and 5.5-fold with respect to the artificial medium. AGPS did not significantly increase the secretion of esterase in any of the media tested.

Enzyme purification

A summary of the purification steps of MnP is shown in Table S4. Two runs with a Q sepharose column, one with a MonoQ column and one with HPLC-SEC were required to separate MnP thoroughly from other proteins, polysaccharides and phenols. Starting from a total of 2200 units, many were lost during the ultrafiltration stage, with 380 units remaining. Two MnP fractions were purified. The final specific activity achieved by MnP fractions I and II were 427 and 471 U mg^{-1}, respectively (Table S4). A strong ~45 kDa band appeared in both fractions in a SDS-PAGE gel, while a very pale ~30 kDa band was also observed (Fig. S5). For sequence analysis, the protein bands on SDS–PAGE gels were excised manually, trypsin-digested and the peptides obtained were subjected to proteomic analyses for De-Novo-Sequencing Peptide Mapping by LC-ESI-MS and MS/MS by Protagen AG (Dortmund, Germany). Proteins identified in the strong band in both fractions (MnP I and MnP II) corresponded to isoforms MnP2s (ID 112835), MnP1s (ID 51375) and VP2 (ID 26239). The faint lower bands in both fractions corresponded to MnP2s (ID 112835) and MnP1s (ID 51375). Substrate specificities were calculated for both isoforms (Table 3). Purified MnPI and MnPII reached 297.8 and 368.27 U mg^{-1}, respectively, the highest levels of specific activity, with Mn^{2+} (calculated as V max/protein amount). In addition, the specific activity for both isoforms show that they have activity toward reactive blue 5, reactive black 5 and ABTS but no activity toward veratryl alcohol pH 3 and 2,6 dimethoxyphenol.

Table 1. Carboxymethylcellulase, xyloglucanase and endopolymethylgalacturonase relative activity in 30 min of *T. versicolor* reaction during SF in soy, ADOR-soy, AGPS-soy, barley, ADOR-barley, AGPS-barley, ETJ, ADOR-ETJ, AGPS-ETJ, Kirk, ADOR-Kirk and AGPS-Kirk.

	Day	Soy	ADOR-soy	AGPS-soy	Barley	ADOR-barley	AGPS-barley	ETJ	ADOR-etj	AGPS-etj	Kirk	ADOR-Kirk	AGPS-Kirk
CMC-ase	5	68 ± 11	68 ± 11	68 ± 11	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	3 ± 2	3 ± 2	3 ± 2	2.9 ± 0.3	2.9 ± 0.3	2.9 ± 0.3
	7	23 ± 4	48 ± 5	23 ± 8	2 ± 1	6 ± 1	3 ± 1	2.4 ± 0.6	1.7 ± 0.3	3 ± 2	2.4 ± 0.4	4.4 ± 0.8	4.4 ± 0.9
	9	15 ± 4	40 ± 8	7 ± 3	3.4 ± 0.5	5 ± 1	1.1 ± 0.5	4 ± 2	10.2 ± 0.9	2.0 ± 0.1	3.3 ± 0.4	3 ± 1	4.1 ± 0.9
	11	16 ± 1	29 ± 4	18 ± 2	4.9 ± 0.5	6 ± 2	0.9 ± 0.3	5.0 ± 0.7	7 ± 4	3.4 ± 0.8	2.7 ± 0.4	1.7 ± 0.3	2.06 ± 0.08
	13	18 ± 6	18 ± 6	23 ± 3	1.7 ± 0.4	3.9 ± 0.3	0.3 ± 0.1	19 ± 1	71 ± 12	5 ± 2	9 ± 5	2.5 ± 0.8	0.8 ± 0.3
Xyl-ase	5	65 ± 3	65 ± 3	65 ± 3	3.1 ± 0.8	3.1 ± 0.8	3.1 ± 0.8	0.58 ± 0.02	0.58 ± 0.01	0.58 ± 0.01	2.4 ± 0.8	2.4 ± 0.8	2.4 ± 0.8
	7	45 ± 7	150 ± 13	36 ± 7	0.5 ± 0.1	0.7 ± 0.3	0.4 ± 0.1	1.03 ± 0.07	1.0 ± 0.1	0.73 ± 0.07	0.7 ± 0.6	7 ± 1	18 ± 2
	9	4.4 ± 0.8	16 ± 3	5 ± 1	0.52 ± 0.03	0.53 ± 0.08	0.56 ± 0.01	0.49 ± 0.02	0.6 ± 0.2	1.2 ± 0.8	3 ± 1	0.8 ± 0.2	0.61 ± 0.08
	11	5.3 ± 0.7	21 ± 2	5 ± 1	0.42 ± 0.05	1.25 ± 0.04	0.77 ± 0.01	1.02 ± 0.01	2.6 ± 0.4	1.15 ± 0.06	3.04 ± 0.05	0.7 ± 0.1	1.60 ± 0.7
	13	17 ± 7	32 ± 4	7 ± 1	0.56 ± 0	4 ± 1	1.13 ± 0.02	6.3 ± 0.7	55 ± 2	1.7 ± 0.5	3.2 ± 0.1	2.5 ± 0.7	3.40 ± 0.08
PO-ase	5	55 ± 1	55 ± 1	55 ± 1	2.0 ± 0.6	2.0 ± 0.6	2 ± 0.6	2.0 ± 0.6	2.0 ± 0.6	2.0 ± 0.6	1.8 ± 0.6	1.8 ± 0.6	1.8 ± 0.6
	7	37 ± 11	46 ± 7	45 ± 1	6 ± 2	5 ± 2	9 ± 3	5 ± 1	8 ± 2	6.9 ± 0.9	4 ± 1	8.2 ± 0.9	10 ± 1
	9	22 ± 4	40 ± 3	36 ± 6	13 ±2	7 ± 1	4 ± 1	0.50 ± 0.01	0.44 ± 0.04	1.2 ± 0.8	9 ± 4	3.4 ± 0.1	2.9 ± 0.9
	11	20 ± 1	32 ± 5	33 ± 4	10 ± 2	5 ± 1	4.2 ± 0.5	1.2 ± 0.7	0.6 ± 0.1	2.2 ± 0.7	7.7 ± 0.4	3.3 ± 0.1	3.0 ± 0.9
	13	29.9 ± 0.8	25 ± 7	30 ± 7	8.96 ± 0	2.4 ± 0.7	3.2 ± 0.3	16 ± 3	13 ± 2	9 ± 2	6 ± 2	3 ± 1	5.6 ± 0.7

Table 2. Esterase activity measured in SF of *T. versicolor* in different media. Activities are expressed in U L^{-1}.

	Soy	ADOR-soy	AGPS-soy	Barley	ADOR-barley	AGPS-barley	ETJ	ADOR-ETJ	AGPS-ETJ	Kirk	ADOR-Kirk	AGPS-Kirk
5	400 ± 100	400 ± 100	400 ± 100	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	200 ± 40	400 ± 100	6 ± 2	nd	1.0 ± 0.2	nd	nd	0.02 ± 0.01	nd	nd	nd	nd
9	460 ± 80	410 ± 70	nd	2 ± 1	nd	nd	35 ± 7	nd	nd	nd	nd	nd
11	530 ± 90	320 ± 60	nd	nd	130 ± 30	nd	nd	nd	nd	nd	6 ± 2	nd
13	420 ± 30	600 ± 100	130 ± 60	140 ± 20	520 ± 60	8 ± 1	80 ± 30	250 ± 40	90 ± 20	nd	50 ± 30	30 ± 10

nd: Non detected

Table 3. Substrate specificities of two manganese-*dependent T. versicolor* peroxidases purified from ADOR-soy medium (*Tv* MnP I and II).

	Specific activity U mg^{-1} protein	
Substrate	***Tv* MnP I**	***Tv* MnP II**
Veratryl alcohol (pH 3)	0	0.48
Mn^{2+}	297.80	368.27
Rblue 5	1.82	0.53
Rblack 5	0.32	0.42
ABTS	4.98	4.06
2,6-DMP	0	0

Solid state fermentation

Lignin-modifying enzyme secretion

T. versicolor showed a noteworthy capacity to secrete Lac, especially in soy-based cultures supplemented with DOR, peaking at 42.6 U g^{-1} in SSF cultures after 5 weeks (Fig. 2). When grown in a DOR-barley medium, maximum Lac activity was found to be 2.4 U g^{-1} in the second week of incubation. A non-stimulating effect was observed for GPS-soy and barley. The highest MnP activity detected was 1.3 U g^{-1} in DOR-soy cultures at week 3 of fermentation, with no significant differences being found with respect to cultures to which GPS had been added. However, MnP activity was moderate, with the maximum MnP activity being found after 2 and 3 weeks of cultivation in DOR-barley cultures (1.9 U g^{-1}) which showed a similar pattern to that of Lac. The activity levels found in GPS-barley cultures were unremarkable. 80% of phenol conversion occurred within two weeks of incubation in both cases. pH remained stable for 5 weeks in DOR-barley cultures and recorded a slight increase in barley-control cultures. However, pH in soy based cultures rose more sharply from 4.8 to 7.2.

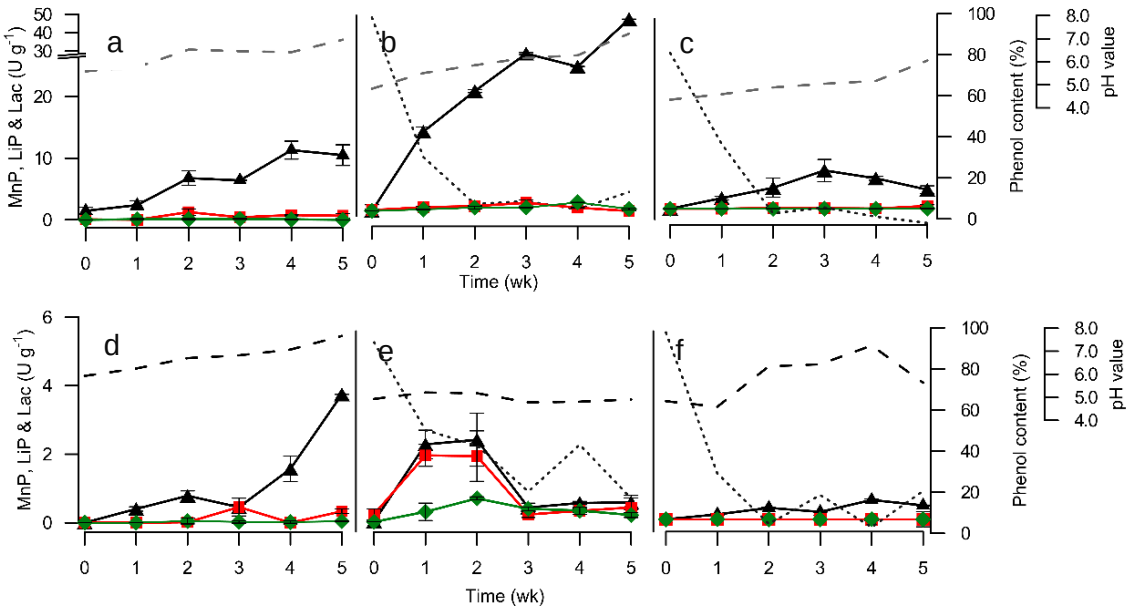

Fig. 2. Time course of manganese peroxidase (MnP) (*squares*), laccase (Lac) (*triangles*), lignin peroxidase (LiP) (*diamonds*) and pH value (*dotted line*) of the polyporal fungus *T. versicolor* during SSF in soy (a), DOR-soy (b), GPS-soy (c), barley (d), DOR-barley (e) and GPS-barley (f). Total percentage of phenol content (*dotted line*), pH value (*dashed line*).

Glycoside hydrolases

There was no significant difference in the CMC-ase activity of *T. versicolor* during SSF culture between soy and DOR-soy cultures (Table 4). Although activity was found to increase after 2 weeks of incubation in both cases, CMC-ase activity in GPS-supplemented soy cultures was lower. The CMC-ase secretion profile was different in the three barley-based media used. Although maximum activity levels in barley and soy did not differ substantially, maximum activity in GPS-supplemented barley cultures at week 4 of fermentation was 3-fold higher than in barley control cultures where activity peaked during the first week of incubation (Table 4).

Table 4. Carboxymethylcellulase (CMC-ase), xiloglucanase (Xyl-ase) and endopolymethylgalacturonase (PO-ase) relative activity in 30 min of *T. versicolor* reaction during SSF in soy, DOR-soy, GPS-soy, barley, DOR-barley and GPS-barley.

	wk	Soy	DOR-soy	GPS-soy	Barley	DOR-barley	GPS-barley
CMC-ase	0	7 ± 1	1 ± 3	3 ± 1	37 ± 9	0	0
	1	6 ± 2	9 ± 2	1.4 ± 0.6	25 ± 4	50 ± 7	3.2 ± 0.4
	2	38 ± 10	31 ± 10	5 ± 2	50 ± 10	74.9 ± 0.4	16 ± 5
	3	38 ± 10	45 ± 5	4 ± 1	40 ± 10	25 ± 5	52 ± 6
	4	38 ± 1	34 ± 5	5 ± 2	52 ± 5	18 ± 7	96 ± 6
	5	29 ± 10	27 ± 4	40 ± 10	53 ± 6	6 ± 3	91 ± 5
Xyl-ase	0	42 ± 8	2 ± 1	5.1 ± 0.9	52 ± 3	2.3 ± 0.7	0
	1	34 ± 6	122 ± 6	5.1 ± 07	40 ± 10	96 ± 4	4 ± 2
	2	150 ± 10	120 ± 30	44 ± 8	20 ± 6	64 ± 7	53 ± 1
	3	79 ± 8	134 ± 8	58 ± 6	14 ± 3	37 ± 2	80.3 ± 0.9
	4	90 ± 30	115 ± 4	21 ± 7	31 ± 6	14 ± 7	91 ± 10
	5	48 ± 1	100 ± 20	14.5 ± 0.9	13 ± 3	10 ± 4	50 ± 3
PO-ase	0	7 ± 1	1 ± 1	0	7 ± 3	0	0
	1	6 ± 2	9 ± 6	2.9 ± 0.7	7 ± 1	14 ± 7	3 ± 1
	2	40 ± 10	30 ± 3	4.2 ± 0.4	6 ± 1	3.2 ± 0.8	5 ±1
	3	40 ± 10	44 ± 8	17 ± 8	2.3 ± 0.1	5 ± 2	10 ± 2
	4	37 ± 4	34 ± 4	5 ± 3	6 ± 1	3 ± 1	28.2 ± 0.9
	5	28.6 ± 0.5	27 ± 1	7 ± 3	7 ± 5	5 ± 1	31.4 ± 0.9

Among the three GH activities measured, Xyl-ase showed the highest values, especially in DOR-soy and soy cultures where maximal activity levels were reached during weeks 2 and 3 of fungal growth, peaking at 150 and 134, respectively. Xyl-ase activity measured in GPS-soy cultures was considerably lower than that in soy control

and DOR-soy cultures. As in the case of CMC-ase activity in barley-based cultures, no reduction in Xyl-ase activity occurred in GPS-barley media. Activity peaked at 52, 96 and 91 at week 0, 1 and 4 in barley, DOR-barley and GPS-barley media, respectively.

PO-ase activity was notably lower than that of CMC-ase and Xyl-ase in GPS-soy media whose activity was lower than that of DOR-soy and soy cultures, as was the case with CMC-ase and Xyl-ase activities. Again, this did not occur in barley-based media, where activity in *T. versicolor* SSF culture measured during week 4 and 5 in GPS-barley cultures was more than 6-fold higher than in DOR-barley and barley cultures. PO-ase activity was lower than that of the other hydrolases measured when comparing equal treatments. Peak activity in all the soy-based media was reached in week 3 of incubation. There were no differences in PO-ase secretion between soy and DOR-soy media, although PO-ase tittles in the GPS-soy medium were considerably smaller than in the other media.

Water soluble aromatic fragment distribution

There was a clear shift in the polymerization profile of DOR treated with *T. versicolor* using soy as a support medium (Fig. 3a). In unfermented control samples, the highest peak contained fragments of 1.12 kDa after 5 weeks of DOR incubation, while there was a noticeable increase in water soluble aromatic compounds to 73.4 kDa. This trend was not observed when DOR was fermented using barley as a support medium (Fig. 3c), with fragment size distribution remaining stable throughout the incubation process, although a decrease in peak heights was observed.

In contrast to HPSEC DOR profiles, GPS control profiles showed a lower signal, with the highest peak containing fragments ~1 kDa. As the weeks went by, HPSEC GPS profiles were lower and more irregular when soy was used as a support medium (Fig. 3b). A decrease in fragment size occurred, peaking at ~0.65 kDa in the control sample with unfermented DOR and at ~0.28 kDa after 5 weeks of incubation. On the other hand, in fermented GPS in barley support media, the highest peak shifted from ~0.99 kDa in control samples to ~2.82 kDa after 5 days of incubation.

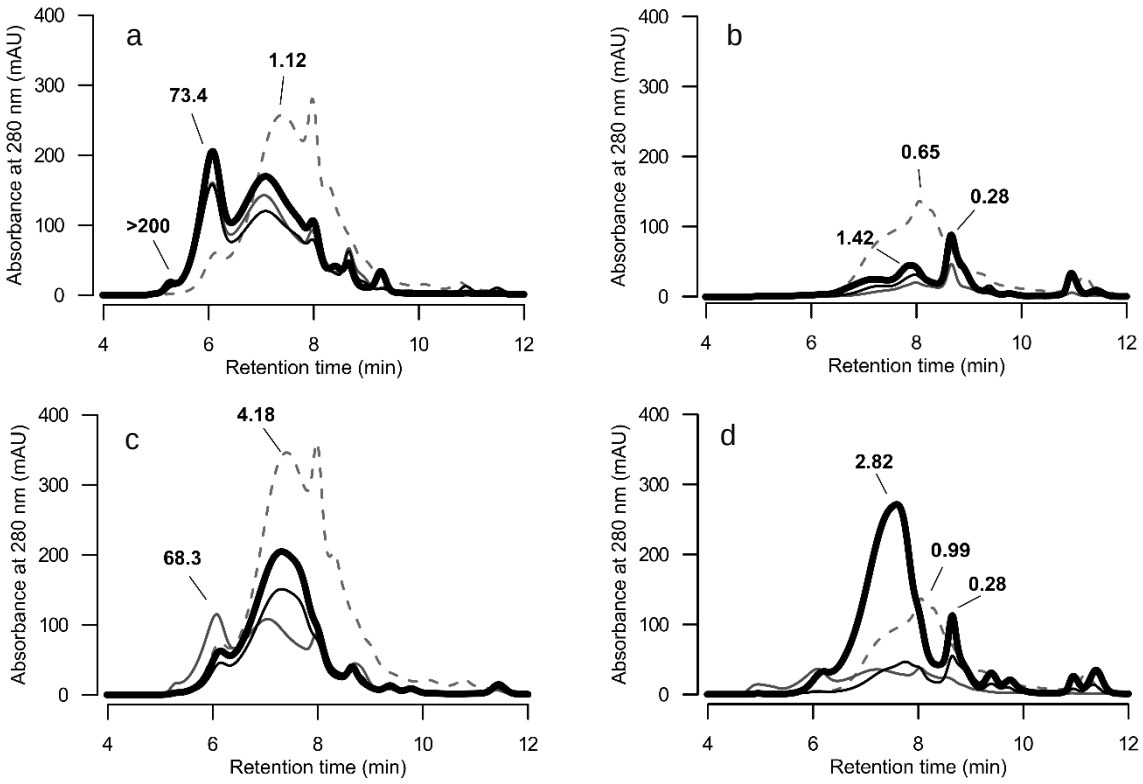

Fig. 3. Changes in the molecular mass distribution of water-soluble aromatic fragments formed by *T. versicolor* during SSF in DOR-soy (a), GPS-soy (b), DOR-barley (c) and GPS-barley (d) media. Unfermented DOR and GPS (*dashed line*) and residues after 1 (*thin grey line*), 3 (*thin black line*) and 5 (*black thick line*) weeks of fermentation. Numbers represent peak fragment size in kDa.

Plant phytotoxicity

Data obtained from *Solanum lycopersicum* phytotoxicity experiments followed a normal distribution pattern. Variances were homogenous according to the Levene test (0.05 significance). The reduction in DOR phytotoxicity began at week 1 in DOR-soy based media as no significant differences in the shoot dry weight (SDW) and root dry weight (RDW) of the DOR-soy treatment at this time with control samples were observed (Fig. 4a), whereas the decrease in GPS phytotoxicity started at week 4 even though the reduction in phenols was similar to that in DOR (Fig. 4b). In DOR-barley media, this phytotoxic effect was dissipated at week 3 of incubation in both SDW and RDW (Fig. 4c), as there were no significant differences between dry weights of plants treated with DOR fermented for 3 and 5 weeks with *T. versicolor* in barley based media and tomato controls (Fig. 4d).

The weights for tomato plants, which were treated with fermented GPS in a barley support media (Fig. 4d) gradually grew as incubation time increased. At week 4 of

incubation with *T. versicolor* in a barley-based media, the phytotoxicity of GPS was totally eliminated.

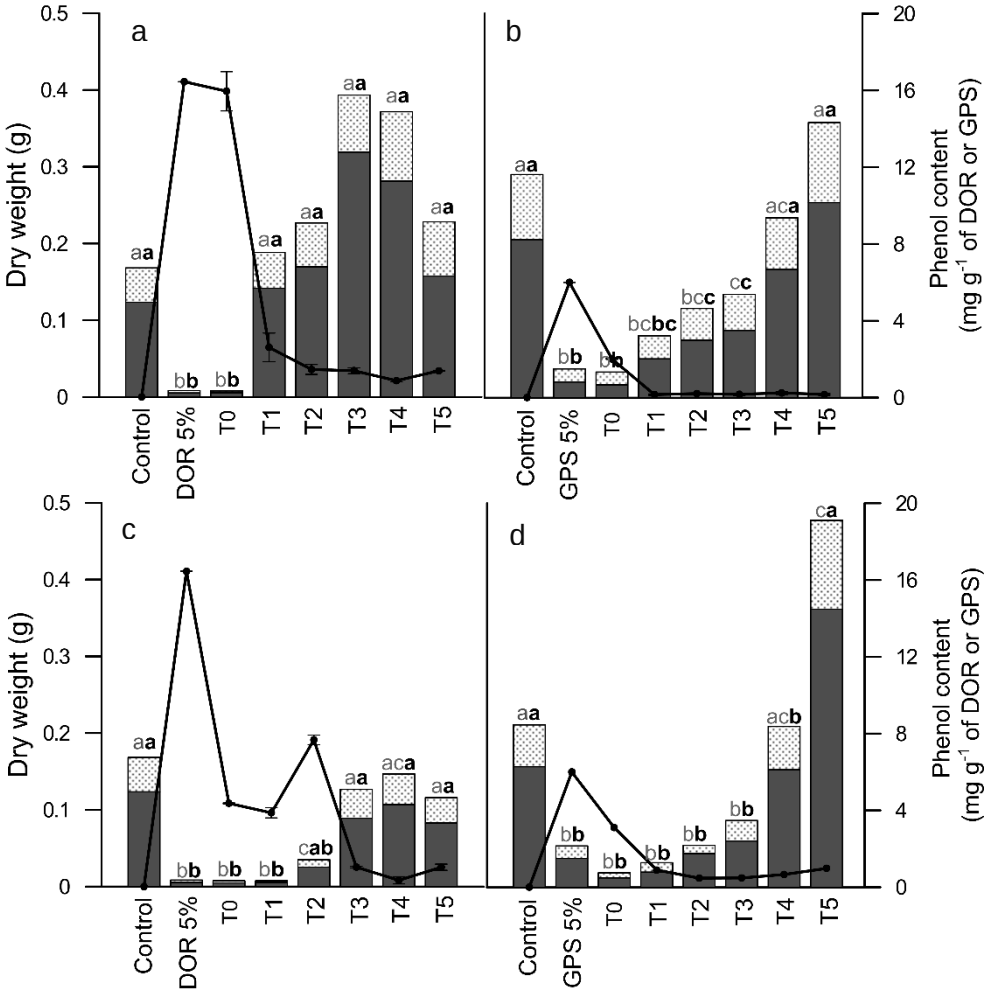

Fig. 4. Shoot (*upper bar*) and root (*lower bar*) dry weight of tomato plants (control) cultivated in the presence of 5% dry olive mill residue (DOR), grape pomace and stalks (GPS) non-fermented and fermented by *T. versicolor* during 0, 1, 2, 3 ,4 and 5 week(s) (T0–T5). DOR-soy (a), GPS-soy (b), DOR-barley (c) and GPS-barley (d). Lower case letters distinguish between statistically different groups for each treatment, bold letters are used for shoot dry weight and normal letters for root dry weight (p <0.05 Tukey test). The solid line represents total phenol content (mg g^{-1} DOR or GPS).

Discussion

Different white rot fungi have been used to valorise agro-waste residues from the olive oil industry and wine by-products, such as stalks and seeds. In most cases, these studies mainly focus on LME production disregarding the analysis of those by-products that are further obtained from the originally exhausted ones. We have optimized an integral valorization process, in which not only we have studied the secretion of enzymes

in SSF and SF in depth, but also we have analysed the final by-products that were obtained to measure their possible impact in the natural environment.

To this end *T. versicolor* was used. It is a well-known polyporal fungus with a remarkable ability to secrete an ample set of LME (Schlosser et al., 1997) as well as some valuable GH (Lahjouji et al., 2007). Recent studies of secretome (Floudas *et* al., 2012; Carabajal et al., 2013) have provided relevant information about *T. versicolor*'s adaptation to different carbon and nitrogen sources. Laccases, peroxidases, hydrogen peroxide-producing enzymes and carbohydrate-active enzymes were identified in these studies, although information regarding comparisons of SSF and SF with respect to LME and carbohydrate enzyme production and nutrient consumption is limited. Despite this scarcity of information, some studies have pointed out that SSF produces higher titers of biocatalysts than SF (Viniegra-González et al., 2003). SSF is more appealing from an ecological point of view given that natural substrates are better emulated as ligninolytic fungi grow in terrestrial habitats on wet substrates. We detected considerable amounts of secreted LME, specifically Lac in both SSF and SF cultures. Major differences were found between the support media used and the residue added, thus demonstrating a variable response to carbon sources and their concentrations in the nutrient medium.

Our results have shown that there is a need to use different natural N and C sources as supplement for by-product conversion. That is the main reason behind the support media that we selected for optimizing the valorization process. In the case of soy, either in SSF and SF, it provides the appropriate support and ingredients for POD and hydrolytic enzyme production, since higher activity was recorded in these media among all control cultures. This is aligned with the extensive use of soybean cake for fungal cultivation in SSF, which not only guarantees all nutritional requirements for fungal growth but also stimulates cellulolytic enzyme production and is an appropriate medium for LME secretion such as Lac and UPOs (Ullrich et al., 2009; Zeng et al., 2011; Delabona et al., 2012). Barley has been also used for fungal cultivation to enhance certain fungi like *Phanerochaete chrysosporium* MnP and LiP (Moredo et al., 2003). However, we found that the titles of hydrolytic and ligninolytic enzymes produced by *T. versicolor* cultivated in this lignocellulosic material were modest in size in comparison with those obtained in soy.

ETJ has also been used as a natural inductor of different LME such as DyPs and MnPs (Liers et al., 2010). Carabajal et al. (2013) found that MnP proteins accounted for 14% of the total in *T. versicolor* supernatants when cultivated in ETJ, which differs from our findings due to the low secretion of MnP being eclipsed by the hypersecretion of Lac in ETJ. Nonetheless, ETJ has already been used to induce Lac production in *Polyporales*

such as *Cerrena unicolor* (Michniewicz et al., 2006), meaning that this complex medium possibly contains enhancer ingredients that stimulate Lac secretion.

DOR and GPS, due to their lignocellulosic nature, appear to be appropriate substrates to emulate natural conditions; however their potential as enzyme elicitors was significantly different. DOR in SSF and ADOR in SF strongly stimulated Lac production and slightly altered MnP and LiP secretion. Olive oil residues have been reported to stimulate Lac, MnP and LiP in polyporal fungi (Díaz et al., 2010; Reina et al., 2013; Reina et al., 2014). No previous evidence of GPS induction on LME has been reported, although it is capable of stimulating lipase secretion in *Aspergillus sp*. (Salgado et al., 2014). Our results have shown that GPS can stimulate Lac secretion. However, despite the ingredients presents in GPS, the induction of GH enzymes has not been detected.

Although DOR is a complex mixture of ingredients that include salts, fatty acids, soluble carbohydrates, proteins and phenols (Díaz et al., 2010), the latter are likely to be responsible for the stimulating effect of Lac on *T. versicolor*, as has been demonstrated with the addition of phenolic extracts from corn steep liquor (Wang et al., 2014). However, the induction levels achieved by the addition of DOR has not so far been reported in relation to additional agro-industrial wastes. No previous studies have been reported using GPS as a culture medium for fungal growth and the production of enzymes involved in lignocellulose degradation. The individual aromatic phenol compounds that lead to Lac secretion enhancement are difficult to determine, with some studies suggesting that vanillic and ferulic acid are capable of stimulating its production in *T. versicolor* and *Pleurotus pulmonarius* (Pazarlioğlu et al., 2005; Wang et al., 2014). The majority of phenols found in our residues were protocatechuic acid, p-tyrosol and hydroxytyrosol, which could mediate this high level of stimulation.

The addition of Mn induces the secretion of MnP in polyporal fungi (Hamman et al., 1999; Swamy and Ramsay, 1999). Apart from Mn regulation, MnP stimulation depends on nitrogen-limitation in most cases; when vegetative growth comes to an end and secondary metabolism begins, the fungi need to continue to mineralize lignin due to nutrient depletion (Buswell, 1991). Although this has been the generally accepted model, this pattern is not followed by all the fungi (Kaal et al., 1995). MnP was not significantly stimulated by GPS or by DOR. In both cases, nitrogen content was lower than in other urban wastes, but total sugar levels were much higher in GPS, which could make the fungus lethargic, rendering the production of LME to obtain nutrients unnecessary. When DOR or ADOR were added to soy and barley liquid or solid media, there was a slight increment of MnP activity. However, this did not occur in the other media used, meaning that the increment could be attributed to additional DOR ingredients, the form nitrogen

takes or the C/N ratio rather than limited or sufficient amounts of nitrogen. In fungi such as *Bjerkandera adusta*, ADOR leads to an extraordinarily level of enhancement of MnP secretion (Reina et al., 2014). Despite MnP's lower rate of induction than that of Lac in the presence of ADOR, we focused on its purification, not only because of its importance in the ligninolytic process and its biotechnological potential but also because *Tv*MnP, unlike Lac, had not previously been purified from ADOR-containing media. The two MnP isoforms isolated in this inductor medium were MnP2s and MP1s. The high affinity for the substrates tested, especially azo dyes such as Reactive blue 5 and Reactive black 5, suggests that *T. versicolor* MnP would be suitable for industrial applications (Champagne and Ramsay, 2005).

T. versicolor has 11 LiP gene copies even though LiP production did not reach significant levels during SSF and SF in control cultures, which is consistent with the results reported by Carabajal et al. (2013) who did not detect LiP proteins when *T. versicolor* was grown in ETJ media. However, in DOR-supplemented cultures, there was a noteworthy LiP secretion. DOR's low nitrogen content, mainly in an organic form (Sampedro et al., 2009), could explain the slight LiP stimulation that occurred, which is in line with previous studies where LiP proteins were purified when *T. versicolor* was grown under carbon and nitrogen starvation conditions (Johansson and Nyman, 1993). As white rot fungi are unable to degrade lignin as a unique energy source, they depend on co-substrates for LME production. Since these co-substrates are obtained through polysaccharide degradation (Kirk and Farrell, 1987), in the current study, higher hydrolytic activity was observed in the second week of incubation when activities were triggered, and participated not only in cellulose degradation but also in LME regulation. Soy turned out to be the appropriate medium for LME, glycosidase and esterase secretion in both SSF and SF. Despite being widely distributed in diverse groups of naturally occurring fungi, hydrolases are frequently produced by Ascomycetes. Our studies show that ADOR stimulated esterase production in the basidiomycete *T. versicolor*, mainly when cultivated in the SF ADOR-soy medium. This is probably due to the presence of feruloyl-polysaccharide from the plant cell wall in the olive pulp and peel. Although these enzymes are of major importance in industrial processes, esterases in basidiomycetes have not been thoroughly characterized. As a result we can conclude that olive by-products show a greater capability than grape wine by-products to elicit lignin modifying enzymes and GH enzymes.

Once the eliciting potential of the studied by-products was analysed, a further step was performed in order to reuse them as soil amendments. DOR is phytotoxic when added in agronomic doses, although once proper fungal treatment is provided,

phytotoxicity is eliminated (Aranda et al., 2006). The incubation of DOR with *T. versicolor* in a soy-support medium not only reduced its phytotoxicity but also appears to stimulate plant growth. Phenols are the main cause of DOR phytotoxicity (Reina et al., 2013). However, their polymerization, mediated by fungal Lacs and PODs as shown by the shift in the polymerization profile to 73.4 KDa, made these compounds inaccessible for the plant, thus leading to a reduction in phytotoxicity. Furthermore, DOR contains mineral salts that can be beneficial. Information concerning the effect of phytotoxicity on GPS is scarce. Our data show that, despite having smaller amounts of phenols, the effects of phytotoxicity are comparable to those of DOR. This could be explained by the presence of high levels of gallic acid in GPS, which is not present in DOR samples. After 5 weeks of incubation, when evidence of phenol polymerization was greatest, there were observable signs of plant growth caused by GPS residue when grown in a barley-based media.

Our findings show the potential use of agro-residues as enzyme elicitors, specifically DOR for the production of Lac, MnP, CMC-ase and esterase. Next-generation sequencing methods and comparative genomics are a valuable tool to obtain in-depth knowledge of lignin mineralization in *Agaricomycetes*. However, there are still many questions which cannot be answered by genome sequencing alone. With respect to biotechnological applications, it is still necessary to find effective low-cost media to obtain high yield enzyme production and to identify possible inducers. Among the already existing transformation options for DOR and GPS, we propose a dual approach in which these by-products are used as fungal LME-carbohydrate-active enzyme inducers and, once transformed, as organic fertilizers.

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CHAPTER 2

Solid state fermentation of olive mill residues by wood- and dung-dwelling *Agaricomycetes*: effects on peroxidase production, biomass development and phenol phytotoxicity

Abstract

The *in-vivo* conversion of dry olive mill residue (DOR) by wood- and dung-dwelling fungi −*Auricularia auricula-judae*, *Bjerkandera adusta* and *Coprinellus radians* − increases peroxidase secretion up to 3.2-3.5-fold (~ 1.3, 3.5 and 7.0 U g^{-1} DOR for dye-decolorizing peroxidase, manganese peroxidase and unspecific peroxygenases, respectively). The incubation of DOR with these fungi produced a sharp decrease in total phenolic content (100% within 4 weeks), a reduction in phytotoxicity as well as a certain degree of plant growth caused by the stimulating effect of fungal-treated DOR. These findings correlate with a characteristic shift in the fragmentation pattern of water-soluble aromatics (detected at 280 nm) from low (0.2, 1.5 and 2.2 kDa, respectively) to high molecular mass (35 to >200 kDa), which demonstrates the presence of a polymerization process. Phenol-rich agricultural residues are a useful tool for enzyme expression and production studies of peroxidase-producing *Agaricomycetes* which could make DOR a valuable organic fertilizer.

Keywords: Dry Olive Mill Residue; *Agaricomycetes*; phenols; DyP-type peroxidase; unspecific peroxygenase

Introduction

Considerable work has been done on filamentous fungal biotechnology in recent years in order to obtain worthwhile products such as enzymes, secondary metabolites and spores. Basidiomycetes secrete a set of extracellular oxidoreductases such as class II peroxidases for example, (manganese peroxidase – (MnP, EC 1.11.1.13), lignin peroxidase – (LiP, EC 1.11.1.14), versatile peroxidase – (VP, EC.1.11.1.16) and laccases – (Lacs, EC 1.10.3.2)), that have enormous potential for industrial and biotechnological applications such as bioremediation and bleaching (Hofrichter et al., 2010). These biocatalysts are known to be key enzymes in lignin degradation due to their stability and wide substrate spectrum. Apart from the above-mentioned classic peroxidases, new fungal heme-containing peroxidases like DyP-type peroxidases (DyP, EC 1.11.1.19) and unspecific peroxygenases (UPO, EC 1.11.2.1) have been described and studied during the last few years (Hofrichter et al., 2010). They are produced by jelly fungi, such as *Auricularia auricula-judae,* and some representatives of dung-dwelling (coprophilic) mushrooms, such us the inky cap *Coprinellus radians*. Both have been described as efficient peroxidase and peroxygenase producers (Anh et al., 2007; Liers et al., 2010). These fungi seem to play an important role in progressive decay and the specific role played by their enzymes in nature is as yet unclear. There is evidence to show that DyPs and UPOs are secreted in the solid state fermentation of beech wood material under near-natural conditions (Liers et al., 2011). Furthermore, both peroxidases are able to oxidize methoxylated aromatics which can be useful in the conversion and degradation of lignin-derived compounds, plant constituents and recalcitrant compounds (Hofrichter et al., 2010).

In recent decades, several enzyme production methods have been developed and optimized using solid state fermentation (SSF) and submerged fermentation. The main focus has been on the higher production rates of more stable enzymes (Barrios-Gonzalez, 2012). Several solid renewable substrates have been used to enhance enzyme production, including lignocellulosic residue, molasses (Singh and Satyanarayana, 2008), dry olive mill residue (DOR) (Sampedro et al., 2004; Sampedro et al., 2012) and olive mill wastewater (Fenice et al., 2003).

DOR is a by-product of olive oil extraction with large quantities produced in the Mediterranean basin (Owen et al., 2000). It is mainly composed of semi-solid sludge obtained after a two-phase extraction process followed by a second extraction using solvents in order to obtaining the residual oil. Thousands of tons of the said two-phase mill waste are produced annually, which constitutes, in both raw and post extraction states, a source of biomass, animal feed and organic amendments following

fermentation and microbiological pre-treatment (Morillo et al., 2009). It contains polymeric components such as cellulose, hemicellulose, lignin, residual oil and complex sugars (oligosaccharides) as well as a number of low-molecular weight compounds such as phenolics, terpenoids and oleuropein (Aranda et al., 2007). These compounds are present in DOR and in its extracts, whose quantity varies according to the efficiency of the extraction process and the particular characteristics of the residue. Such combinations of nutrients, carbohydrates, polymers and partially toxic ingredients such as methoxylated and phenolic aromatics can be suitable additives to the culture media of ligninolytic fungi in order to stimulate or induce industrially appropriate oxidoreductases (Morillo et al., 2006). In recent studies, DOR and its components have been pointed to as a source of phenols and stimulators of fungal growth that induce several enzymes such as MnP and Lac (Díaz et al., 2010; Sampedro et al., 2012).

In this study, we analysed the production of MnP, DyP and UPO by the white rot basidiomycetes *A. auricula-judae* and the coprophilic fungus *C. radians* in SSF using a barley-based media supplemented with solid DOR. In addition, the enzymatic production of the well-known fungus *Bjerkandera adusta* was also analysed to be used as control. The effects on the polymeric phenol fraction and the free phenolic fraction, which are mainly responsible for the phytotoxicity caused, were evaluated in order to determine the transformations and the potential agronomic applications of the fermented DOR after fungal treatment.

Results and discussion

Production of fungal oxidoreductases during growth in DOR-supplemented SSF

DOR tested in our study in a barley-based medium stimulated the biomass development of two wood- and one dung-dwelling *Agaricomycetes* as well as the production of peroxidases such as MnP, DyP and UPO by the respective fungi (Fig. 1). The addition of the phenol-rich DOR led to an earlier activity shift in the time course as compared to the corresponding controls and was followed by a reduction in phenol in the fermented DOR substrate.

The fungus *A. auricula-judae* secreted MnP and DyP as the principal peroxidases and a moderate level of Lac during growth in DOR-barley (Fig. 1a). By using phenol-rich DOR, the activities of these enzymes increased during an earlier cultivation phase, starting in week 1 and reaching maxima in week 3 (0.9, 1.3 and 1.8 U g^{-1} for Lac, DyP and MnP, respectively). This is in contrast to the control, where activity reached a maximum 1 weeks later, and DyP levels (0.4 U g^{-1}) were approximately 3-times lower

than those observed with DOR (Fig. 1a). While Lac activity decreased due to the presence of DOR (from 2.0 U g^{-1} in the barley-control media to 0.9 U g^{-1}), the amount of MnP (1.8 U g^{-1}) secreted by *A. auricula-judae* was identical for both media and was clearly not stimulated by the addition of DOR. This is in contrast to the MnP and AAO secretion of *B. adusta* that was enhanced by the phenol-rich DOR (Fig. 1b). Activity reached maximum levels of 3.5 and 1.2 U g^{-1} for MnP and AAO, respectively, as compared with the highest enzyme levels in the control media (~1.1 and 0.3 U g^{-1}, respectively). Since no veratryl alcohol oxidation was observed at pH 3, clearly no LiP or VP was produced by *B. adusta* under the culture conditions in the experiment. Interestingly, veratryl alcohol oxidation at pH 7 was observed in the cultures of *C. radians* that showed a 20-fold increase in UPO activity in DOR-supplemented cultures (maximum ~7 U g^{-1} = 1400 U L^{-1}) as compared with the controls (1.9 U g^{-1}) (Fig. 1c). For all three fungi, the increase in peroxidase activity was accompanied by a noticeable decrease in phenol content down to 0% and up to 19% within the first 2 week and an increase in pH from 4 and 5 to 5.5 and 6.0 for *A. auricula-judae* and *B. adusta*, respectively, and to 7.2 for *C. radians*.

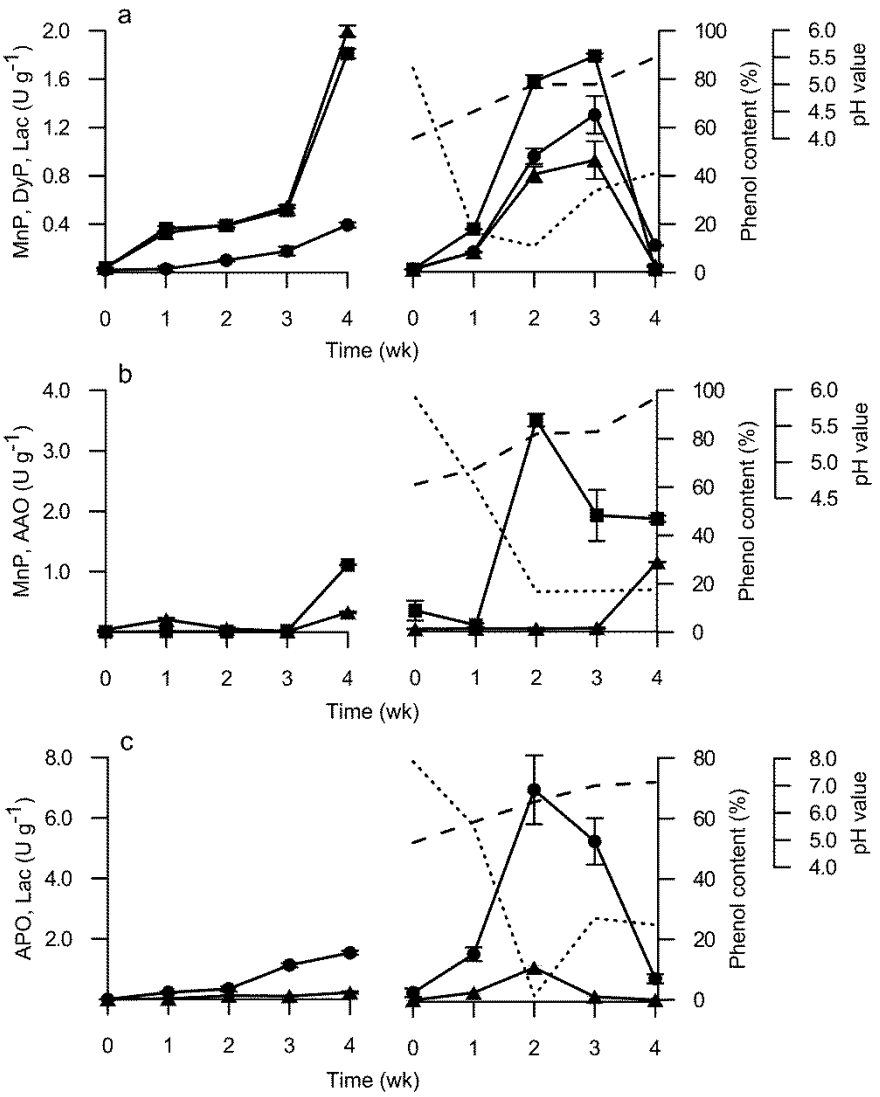

Fig. 1. Time course of extracellular oxidoreductase production by wood- and dung-dwelling *Agaricomycetes*: *Auricularia auricula-judae* (a), *Bjerkandera adusta* (b) and *Coprinellus radians* (c) during growth on solid state cultures containing barley (*left*) and barley supplemented with olive mill residue (DOR-barley, *right*). Enzyme activities (*solid line*) - MnP (*squares*), DyP and UPO (*circles*), AAO and Lac (*triangles*), total phenol content (*dotted line*) and pH value (*dashed line*).

Several studies have reported enhanced production of fungal oxidoreductases (MnP by Mn^{2+}-ions, Wariishi et al., 1992; Li et al., 1995; Manubens et al., 2003; LiP by reactive oxygen species, Belinky et al., 2003; Lacs by guaiacol, flavonoides, tannins and xylidine, Carbajo et al., 2002). The induction of fungal enzymes by agricultural residues has been extensively studied (Hölker et al., 2004; Rodríguez-Couto and Sanromán, 2005); however, there is little evidence that complex plant-based materials, such as polymeric lignocelluloses or phenolics, show a direct stimulation of DyPs (Liers et al. 2013), UPOs (Hofrichter et al., 2010) or classic ligninolytic fungal peroxidases. In

addition, almost nothing is known about natural 'elicitors' of MnPs, DyPs and UPOs (Hofrichter et al., 2010). Poerschmann et al. (2013) have described components of DOR such as lipids, proteins, carbohydrates, lignin and metal ions that would be capable of enhancing the biomass development of plants and microorganisms. However, other compounds such as polyphenols and monophenols can also induce industrially important oxidoreductases such as Lac and MnP (Camarero et al., 1999, Fenice et al., 2003). DOR and its components can therefore be useful for studying the expression of oxidoreductase fungal enzymes.

Fungal growth

All three fungi studied showed enhanced biomass growth (estimated in terms of ergosterol content in µg g^{-1}, Table 1) during solid state cultivation in the presence of DOR. In the case of *A. auricula-judae* and *C. radians,* the highest ergosterol content (505 and 344 µg g^{-1}, respectively) was achieved after 2 weeks of incubation in the DOR-barley medium. This is in contrast to the maximum values of the corresponding controls in which just 2 and 213 µg g^{-1} of ergosterol were detected. After the incubation period, DOR-supplemented culture substrates led to an increase in ergosterol content, and thus in biomass, of 86, 21 and 65% for *A. auricula-judae*, *B. adusta* and *C. radians*, respectively, as compared to the barley cultures. The increase in ergosterol appears to coincide with the peroxidase maxima for the fungi, whereas *B. adusta* and *C. radians* recorded optimal biomass levels when the highest peroxidase levels were reached after 4 weeks. This finding bolsters the notion that the application of DOR would be an appropriate growth-stimulating supplement to the solid culture substrates for wood-decaying and coprophilous fungi probably due to the presence of the above-mentioned ingredients, phenols and polymeric components such as lignin (Sampedro et al., 2012).

Table 1. Ergosterol concentrations of cultures from *Auricularia auricula-judae*, *Bjerkandera adusta* and *Coprinellus radians* during growth in DOR-barley and barley medium (control) for 2 and 4 weeks. The values represent means and standard deviations from three replicates.

Incubation time (wk)	Growth medium	Ergosterol (µg g^{-1} dry weight)		
		A. auricula-judae	***B. adusta***	***C. radians***
0	Barley	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
	DOR-Barley	8.7 ± 1.5	33 ± 0.4	32 ± 5
2	Barley	1.9 ± 0.2	1.2 ± 0.1	212 ± 21
	DOR-Barley	505 ± 19	85 ± 1	343 ± 10
4	Barley	71 ± 7	104 ± 21	125 ± 4
	DOR-Barley	370 ± 3	131 ± 3	354 ± 31

Changes in the free and polymeric phenolic fraction

Changes in water-soluble aromatic fragments and phenols caused by the fungal treatment of DOR-barley medium were monitored using HPSEC and GC-MS, respectively. Table 2 gives an overview of the phenolic compounds detected in the residue and after 2 and 4 weeks of incubation with *A. auricula-judae*, *B. adusta* and *C. radians*. The principal phenols present in DOR were *p*-tyrosol, hydroxytyrosol, protocatechuic and vanillic acid (62; 119; 55 and 17 µg g^{-1} DOR, respectively). Although there is no distinct tendency observable in the degradation effect, most of the phenolic compounds were almost completely oxidized and removed by all three fungi tested within the first 2 weeks (ferulic acid up to 100%, pyrogallol up to 67-100% and vanillic acid up to 72-86%) and after the overall incubation period (protocatechuic acid up to 91-100% and *p*-tyrosol to 82-96%). The corresponding phenols can be assumed to have been oxidized by the secreted peroxidases of *A. auricula-judae*, *B. adusta* and *C. radians* and also by the Lac detected. These emerging phenoxyl radicals undergo further non-enzymatic polymerization processes which we monitored using size exclusion chromatography (Liers et al., 2006).

Table 2. Relative content and abundance of simple phenols in the exhausted residue analysed by GC-MS after incubation with *A. auricula-judae*, *B. adusta* and *C. radians* during 0, 2 and 4 weeks. The values represent means and standard deviations from three replicates.

Phenolic compounds (µg.g^{-1} DOR)	*Auricularia auricula-judae*			*Bjerkandera adusta*			*Coprinellus radians*		
	0 wk	2 wk	4 wk	0 wk	2 wk	4 wk	0 wk	2wk	4 wk
pyrogalol	0.7 ± 0.1	0.04 ± 0.01	nd	0.9 ± 0.2	0.3 ± 0.1	nd	0.5 ± 0.02	nd	nd
p-tyrosol	63 ± 6	45 ± 3	11 ± 0.3	54 ± 2	2.5 ± 0.3	0.5 ± 0.2	64 ± 13	39 ± 8	2.4 ± 0.5
vanillic acid	13 ± 1	3.7 ± 0.6	2.4 ± 0.7	13 ± 1	2.5 ± 0.2	0.7 ± 0.03	14 ± 3	1.9 ± 0.2	1.1 ± 0.1
protocatechuic acid	53 ± 2	8.5 ± 0.8	5.1 ± 0.2	55 ± 1	26 ± 1	4.4 ± 0.9	54 ± 8	2.8 ± 0.4	nd
syringic acid	0.9 ± 0.03	0.7 ± 0.1	0.4 ± 0.02	0.6 ± 0.1	0.5 ± 0.2	0.04 ± 0.02	0.6 ± 0.3	0.04 ± 0.02	nd
caffeic acid	0.8 ± 0.2	0.6 ± 0.1	0.3 ± 0.03	0.8 ± 0.03	nd	nd	1.1 ± 0.3	0.4 ± 0.05	nd
p-coumaric acid	3.7 ± 0.02	1.8 ± 0.1	nd	3.6 ± 0.3	1.1 ± 0.4	1.2 ± 0.1	5.5 ± 1.4	4.4 ± 0.6	1.6 ± 0.02
ferulic acid	3.9 ± 0.7	nd	nd	4.1 ± 0.9	nd	nd	4.2 ± 0.6	nd	nd
gallic acid	0.7 ± 0.02	0.4 ± 0.1	nd	0.9 ± 0.3	1.8 ± 0.02	0.8 ± 0.03	1.1 ± 0.2	0.3 ± 0.05	0.3 ± 0.05
hydroxytyrosol	117 ± 1	7.6 ± 1.3	1.8 ± 0.2	120 ± 6	4.4 ± 1.2	1 ± 0.2	118 ± 15	17.5 ± 0.2	1.8 ± 0.3

nd= not detected

In all three cases, a characteristic shift in water-soluble aromatics, detected at 280 nm, was observed (Fig. 2). In the control (non-inoculated DOR-barley), a release of low-molecular mass fragments of between 0.2 and 2.2 kDa occurred. After 2 weeks of incubation with all three fungi, a removal of these small constituents of 2.2 to 1.5 kDa was clearly observed, as shown by a decrease in absorbance at 7.4 to 8.0 min. The subsequent polymerization effects were evident, with the high-molecular mass fragments increasing from > 200 Da to 35 kDa and eluting between 5.2 and 6.2 min. These oxidation and polymerization effects were observed when the fungi studied also generated their maximum levels of peroxidase activity (MnP, DyP and UPO) and biomass growth.

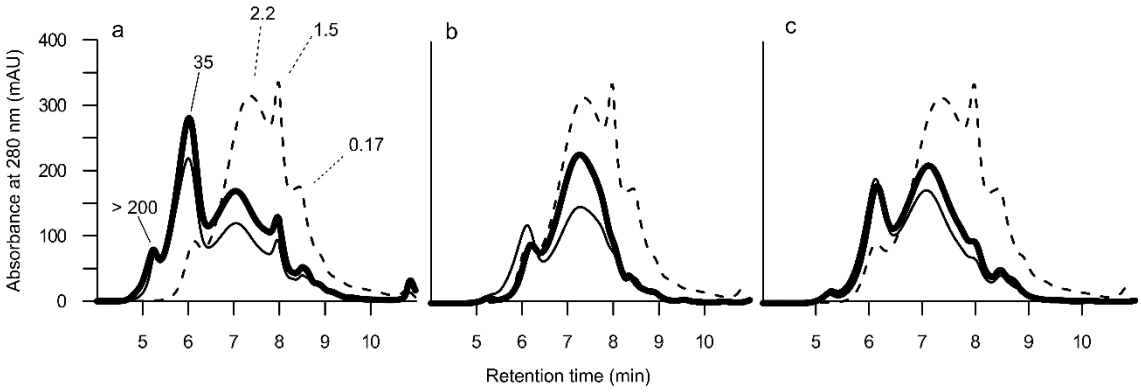

Fig. 2. Changes in the molecular mass distribution of water-soluble aromatic fragments formed by *Auricularia auricula-judae* (a), *Bjerkandera adusta* (b) and *Coprinellus radians* (c) during growth on DOR-barley solid media after 0 (*dashed line*), 2 (*thin line*) and 4 (*solid line*) weeks of cultivation.

The most pronounced polymerization effect was observed for *A. auricula-judae,* probably due to the presence of large quantities of the three phenol-oxidizing oxidoreductases (MnP, DyP and Lac). In the case of *B. adusta* and *C. radians,* which only secreted MnP and UPO respectively, the fragmentation patterns were very similar.

The effect of oxidoreductases (such as that for ascomyceteous Lac, MnP and the independent peroxidase Mn^{2+}) on DOR as well as their aqueous and organic extractives has to some extent been described *in vivo* and *in vitro* (Jaouani et al., 2005; Iamarino et al., 2009; Díaz et al., 2010; Sampedro et al., 2012). However, the enzymatic influence of DyPs and UPOs on DOR biodegradation has so far not been studied. In addition, most DOR degradation and treatment studies have been carried out in relation to long incubation periods such as 20 weeks (Sampedro et al., 2004). By contrast, in our study, effective phenol degradation of DOR was achieved after 4 weeks of fungal treatment.

Phytotoxicity experiments

It has previously been reported that the phytotoxicity effects of DOR are caused by the presence of numerous phenolic compounds (Capasso et al., 1992). Consequently, there is a decrease in phytotoxicity following the removal of phenolic compounds through enzymatic oxidation and polymerization, as polymers of a certain size may not be able to access the plant via the cell membrane (Hulzebos et al., 1991).

The positive relationship we have observed between oxidoreductase enzyme induction (Fig. 1) and phenol depletion (Fig. 1) caused by polymerization processes (Fig. 2) may make it possible to use residues for agronomic purposes. Our results show a progressive decrease or removal of phytotoxicity involved in tomato plant growth in the presence of DOR bio-remediated by the three wood- and dung-dwelling fungi (Fig. 3). This finding is related to the increase in oxidoreductase activity and phenol depletion from ~10 up to 2 mg g^{-1} DOR (Fig. 1), with slight differences being observed between the fungi when DOR was applied in agronomic doses.

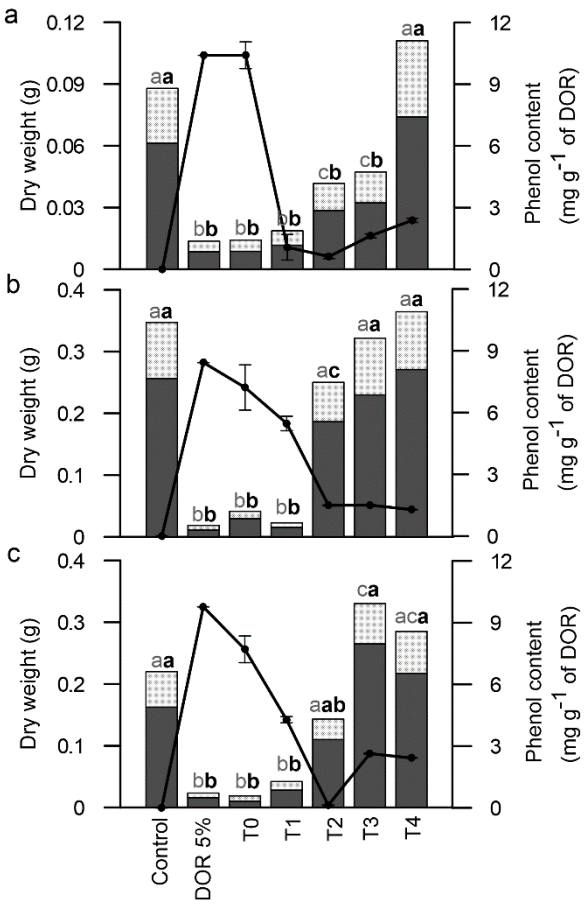

Fig. 3. Shoot (*upper bar*) and root (*lower bar*) dry weight (mg) of tomato plants (control) cultivated in the presence of dry olive mill residue (DOR), non-fermented (DOR 5%) and fermented by *Auricularia auricula-judae* (a), *Bjerkandera adusta* (b) and *Coprinellus radians* (c) for 0, 1, 2, 3 and 4 weeks (T0-T4). Lower case letters distinguish between statistically different groups for each treatment, bold letters are used for shoot dry weight and normal letters for root dry weight ($p < 0.05$ Tukey test). The solid line represents total phenol content (mg g^{-1} DOR).

DOR bio-remediated with *A. auricula-judae* for 3- and 4 weeks periods, unlike other fungi, exhibited a phytotoxic effect despite the complete depletion in total phenol content and high MnP and DyP production (Fig. 1a and 3a). However, we observed incomplete polymerization processes during these periods (data not shown), which indicates that the phytotoxic effect depends on the size of the polymer. On the other hand, bio-remediated DOR with this fungus contains the largest amount of syringic and caffeic acid, which has a phytotoxic effect on plant physiology (Vaughau and Ord, 1991). *A. auricula-judae* only managed to remove DOR phytotoxicity after 4 weeks of incubation. After the incubation of DOR with *B. adusta* and *C. radians* for 2 weeks, the residue also had a phytotoxic effect on tomato plant growth (Fig. 3b, 3c). However, in

the presence of DOR incubated with *B. adusta* for 3 and 4 weeks and with *C. radians* for 4 weeks, the shoot and root dry weight of tomato was similar to that for tomato grown without the residue. It is important to note that the incubation of DOR with *C. radians* not only leads to a decrease in its phytotoxicity but also to its elimination (Fig. 3c). The total transformation of monocyclic phenols into polymeric products, with the exception of *p*-coumaric and gallic acid, over a period of 4 weeks indicates that polymerization constitutes a means of reducing the phytotoxicity of the waste. As *C. radians* was capable of transforming DOR, the dry weight of the tomato plant to which this bio-remediated DOR was applied increased significantly as compared with the plant that was not treated with the residue. *C. radians* appears to have a certain stimulative effect on the fermented residue, with root dry weight and shoot dry weight increasing by 67% and 44%, respectively.

The effective transformation of phenolic ingredients into polymeric fractions makes olive mill and other agricultural phenolic-rich residues candidates as valuable and renewable organic fertilizers. In the case of DOR, this transformation requires the *in-vivo* fermentation of the solid substrate by wood- and dung-dwelling fungi, followed by the effective removal of its phytotoxicity.

Conclusions

Our findings demonstrate for the first time the optimized production of classic ligninolytic peroxidases like MnP and also the stimulation of UPO and DyP by *Agaricomycetes* when DOR is used during SSF. We therefore regard DOR as an effective and appropriate medium for the study of enzyme expression and secretion. Furthermore, the decrease in DOR's phytotoxicity results in a plant-growth-enhancing substrate which makes *Agaricomycetes* and their biocatalysts an appropriate tool for transforming DOR into an organic fertilizer. Further work is planned in order to scale up these results and to develop application strategies in the future.

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CHAPTER 3

Differences in the secretion pattern of oxidoreductases from *Bjerkandera adusta* induced by a phenolic olive mill extract

Abstract

The secretome of the white rot fungus *Bjerkandera adusta* produced in synthetic Kirk medium was compared to that supplemented with an aqueous phenol-rich extract of dry olive mill residues (ADOR). Distinct changes in the protein composition of oxidoreductases, namely diverse class II peroxidases and aryl alcohol oxidases were found.

In the ADOR-supplemented medium (ASC), 157 distinct proteins were identified by the secretome analysis, whereas only 59 of them were identified without ADOR supplementation (Kirk medium culture; KM). Proteome analysis indicated that the number of peroxidases produced in ASC was more than doubled (from 4 to 11) compared to KM. Two short manganese peroxidases (MnP1 & MnP6) and one versatile peroxidase (VP1) represented 29% of the relative abundance (NSAF) in ASC. Two of them (MnP1 & VP1) were also detected in KM at a NSAF of only 3%. Further peroxidases present in ASC were one lignin peroxidase (LiP2), one generic peroxidase (GP) and three dye-decolorizing peroxidases (DyPs). The relative abundance of DyPs and aryl alcohol oxidases (AAO) were lower in ASC in comparison to KM. In addition to peptide sequence analysis, the secretion of Mn^{2+}-oxidizing peroxidases as well as AAOs were followed by enzyme measurement. The Mn^{2+}-oxidizing activity increased nearly 30-fold (from 10 to 281 U L^{-1}) after ADOR addition. Two enzymes responsible for that activity were successfully purified (*Bad*VPI and *Bad*VPII). To prove a potential involvement of these enzymes in the degradation of aromatic compounds, *Bad*VPI was tested for its ability to degrade the recalcitrant dehydrogenated polymer (DHP, synthetic lignin). These results show that natural phenol-rich materials act as secretome-stimulating additives. Applying these substances enables us to investigate fungal degradation and detoxification processes and gives more insight into the complexity of fungal secretomes, e.g. of white rot fungi.

Keywords: Secretome, white rot, olive mill residue, heme-peroxidase, aryl-alcohol oxidase, dehydrogenated lignin polymer.

Introduction

Saprotrophic basidiomycetes have developed complex mechanisms to transform natural polymers such as lignocelluloses, leaf litter and humic materials (Ten Have and Teunissen, 2001). Lignin, the persistent major component of wood, accounts for the wood's structural rigidity and protects cellulose and hemicellulose from hydrolytic attack. The biodegradation of lignin is difficult to achieve due to aromatic non-phenolic molecules and a heteropolymeric network of phenylpropanoid units (Martínez et al., 2005). The fungal degradation of lignocelluloses and humic material depends on the synergistic-cooperative action of certain enzymes, including ligninolytic class II peroxidases (PODs; EC 1.11.1.x). They are crucial for an oxidative attack of the lignin barrier and thus the exposure of cellulose and hemicellulose to the fungal hydrolytic system (Hatakka, 1994; Martínez et al., 2009; Hofrichter et al., 2010).

Numerous fungi belonging to the order *Agaricomycetes* secrete a variable set of these class II PODs, i.e. manganese peroxidase – (MnP, EC 1.11.1.13), lignin peroxidase – (LiP, EC 1.11.1.14) and versatile peroxidase - VP (EC.1.11.1.16) but also certain DyP-type peroxidase – (DyPs, EC 1.11.1.19) or unspecific peroxygenases – (UPOs, EC 1.11.2.1) (Wariishi et al., 1992; Orth et al., 1993; Camarero et al., 1999; Hofrichter et al., 2010). Further lignin breakdown requires the cooperative action of auxiliary enzymes like peroxide-generating oxidases (e.g. aryl alcohol oxidases – (AAO, EC 1.1.3.7), glyoxal oxidases – (GLX, EC 1.1.3) or pyranose 2-oxidases – P2Ox (EC 1.1.3.10) (Hammel et al., 2002; Wymelenberg et al., 2006a) or esterases (e.g. feruloyl esterases – (FAE, EC 3.1.1.73)) that hydrolyze ester bonds between hemicelluloses and lignin moieties (Wong, 2006). Depending on the type and composition of carbon and nitrogen sources diverse profiles of polysaccharide-degrading hydrolases and peptidases can be found in fungal secretomes (Marx et al., 2013).

Besides solid-state fermentation, several studies describe the influence of lignocelluloses on fungal transcriptomes and secretomes during liquid cultivation (Martinez et al., 2009; Wymelenberg et al., 2010; Salvachúa et al., 2013). To the best of our knowledge not much is known how natural phenolics and humic acid-rich material like leaf-litter, soil or compost material exert an influence on the protein expression profile of fungal organisms (Morin et al., 2012; Carabajal et al., 2013). The addition of agricultural by-products to fungal cultures may reflect complex growth conditions close to nature and could stimulate the secretion of various enzymes required for degradation or detoxification processes (Girard et al., 2013). Indeed, not much is known about the nature of substances responsible for the expression and secretion of ligninolytic fungal peroxidases like MnP, VP and LiP (Carabajal et al., 2013; Salvachúa et al., 2013).

Fungal oxidoreductases and hydrolases are promising tools for application in the bioconversion of natural polymers, e.g. lignocelluloses, into aromatic molecules and fermentable sugars, important to the second-generation bioethanol production (Kuhad et al., 2011). Therefore, the fermentation of agricultural by-products or their extractives offers a biological process to convert cheap, underutilized materials into high-value end products (e.g. aromatic compounds, secondary metabolites) and is a resurgent culturing method for the production of technologically relevant enzymes (Pandey, 2003; Hölker et al., 2004).

Phenolic olive mill residues (DOR) and their extractives (e.g. water-soluble ADOR) were studied mainly to achieve soil melioration, phytotoxicity reduction of the residues and plant growth promotion by using various fungi, e.g. *Coriolopsis rigida* (currently *Funalia floccosa)*, *Pycnoporus cinnabarinus* and *Paecilomyces farinosus*, (Aranda et al., 2006; Sampedro et al., 2009; Siles et al., 2013). Whereas in previous studies the DOR-stimulating effect on the production of fungal enzymes (MnP, laccases, lipases) was demonstrated for *Auricularia auricula-judae*, *F. floccosa* or *Aspergillus* sp. and *Rhizomucor* sp. (Cordova et al., 1998; Díaz et al., 2010; Reina et al., 2013; Salgado et al., 2013), the entire set of proteins expressed and secreted under DOR or ADOR influence has not been described yet. Water-soluble ADOR contains numerous protein-inducing compounds (e.g. flavonoids, pectins, polymerins, sugar alcohols like mannitol) (Laufenberg et al., 2003) and therefore might be a suitable compound for inducing fungal enzyme production and secretion. Here, we investigated the *Bjerkandera adusta* secretome in the presence of water-soluble olive mill extractives and the influence of the latter on the oxidoreductase expression pattern. Our results revealed the presence of class II peroxidases that are functionally important in fungal degradation and detoxification processes.

Results

The maximum Mn^{2+}-oxidizing peroxidase activity (MnoP) achieved by a 5% addition of ADOR to the KM of *B. adusta* was at 281 U l^{-1}, hence an activity that was 20-fold higher than in unsupplemented KM (10 U l^{-1}; Fig. 1), and appeared 5 days after ADOR addition. Corresponding cultures were harvested (day 6 after ADOR addition) and the culture liquid was directly used for secretome analyses. The AAO activity did not increase after ADOR addition (~40-50 U l^{-1} at day 4 after supplementation, respectively) in contrast to the KM where the maximum AAO activity reached approx. 150 U l^{-1}. The

pH-value of the KM did not markedly change, whereas a slight increase by 0.4 pH units was observed in ASC during peroxidase secretion.

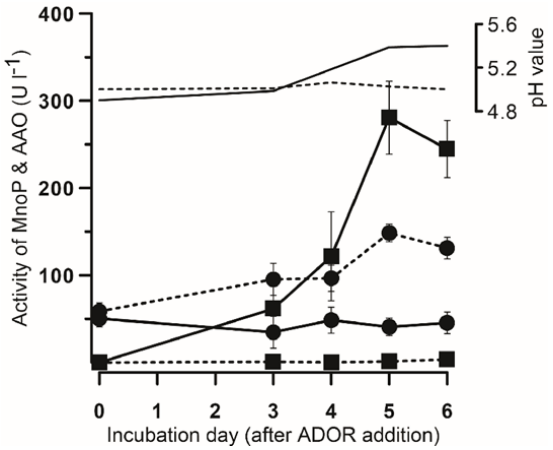

Fig. 1. Activities of manganese-oxidizing peroxidase (MnoP, squares) and aryl alcohol oxidase (AAO, circles) of *B. adusta* during cultivation in Kirk media, with (solid line) and without (dashed line) addition of 5% ADOR; lines without symbols - pH values.

Differences in the abundance pattern of secreted *B. adusta* proteins, mainly oxidoreductases and some hydrolases, were found in ASC in comparison to KM (Fig. 2). In the overall secretome, the calculated molecular weights and p*I*s of protein fragments detected ranged from 11.3 to 113 kDa (e.g. for lectin or a glycoside hydrolase 35), and from 4.3 (for a glycoside hydrolase 18) to 9.5 (for a transferase/helicase; data not shown, Table. S1). Most of these proteins have a definite function in the fungus, and some oxidoreductases but also hydrolases can be related to a lignocellulose breakdown system in *B. adusta* (e.g. MnPs, VPs, LiPs as well as glyoxal oxidases and various glycoside hydrolases). A comparison between the secretome profiles of both culture media revealed a notably different set of proteins. A total of 157 extracellular proteins were found in ASC, 70 in KM and 59 were detectable in both media (Table S1).

Production of proteins belonging to the class II secretory fungal peroxidase family, mainly Mn^{2+}-oxidizing peroxidases (MnoP: MnP and VP), were significantly increased in the secretome of ASC. Especially two short MnPs (MnP1 (43329) and MnP6 (175536)) as well as one VP1 (43095) represented 29% of the relative abundance in ASC (Fig. 2b). However, two of them were detected (MnP1 & VP1) in KM, with a relative abundance of 3% only (Fig. 2b). Further low relative abundances (~0.1%) were detected for LiP2 (41982) and MnP3 (306404) as potentially ligninolytic peroxidases in ASC, which were not detectable in KM. Indeed, three DyPs (DyP2 (72253), DyP5 (172310) and DyP

(169535)) were found in ASC, their abundance of 0.8% relative to the NSAF is lower than that of KM, in which two DyPs (DyP2 and DyP8 (287732)) were detected displaying an abundance of 5%.

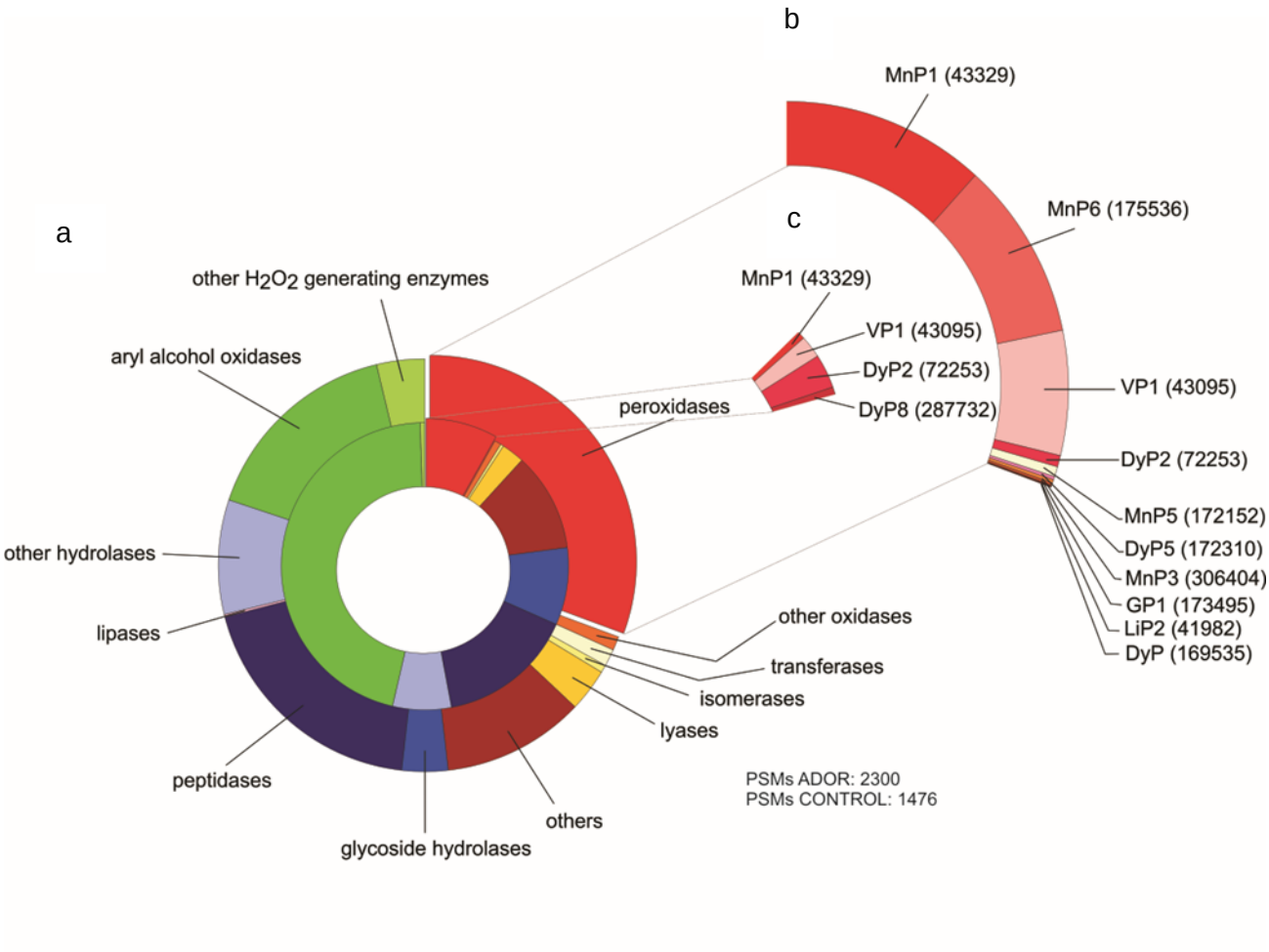

Fig. 2. Relative abundance (% PSM) of identified proteins secreted by *B. adusta* (a), mainly peroxidases (JGI accession numbers) in cultures (Kirk media) with (b, outer ring) and without (c, inner ring) 5% ADOR.

According to the time course of enzymatic activity (Fig. 1), the abundance of AAO in the secretome of KM (46% of NSAF) was higher than in ASC (16% of NSAF; Fig. 2a). Besides an overall of six AAO proteins (171002, 245049, 114902, 171059, 245297, 52991; Table S1) detected, considerable differences were found between both *B. adusta* secretomes particularly for 171002, 245049 and 171059 (Fig. 3). The former was the most abundant protein in KM (26%), followed by 171059 (9%) and 245049 (8%) in comparison to ASC, where they reached a 3.3-fold lower relative abundance in total (13%; Table S1).

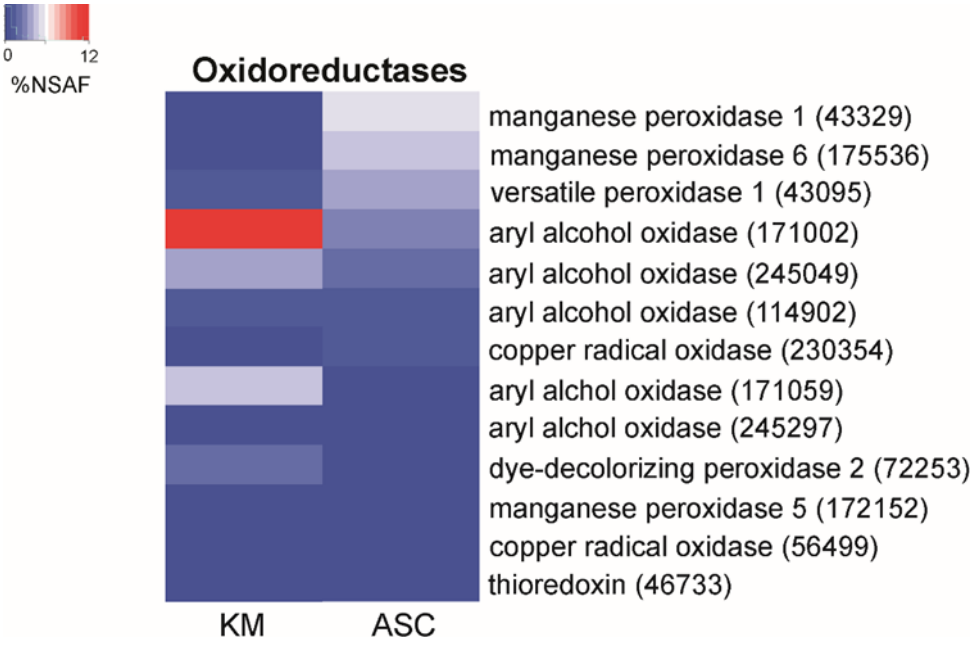

Fig. 3. Heat maps of secreted oxidoreductases from *B. adusta* cultured in Kirk media, with (ASC) and without (KM) ADOR addition. A significant change of protein abundance was proved by Hotelling´s T2 test and is represented by NSAFs transformed with natural logarithms.

Hydrolases (about 31% NSAF in both secretomes) showed slight differences in abundance or richness. Only the glycoside hydrolase GH13 (52811; 1% NSAF), a chitinase (126796; 1% NSAF) and few peptidases (29930, 24229; 1-2% NSAF) are more abundant in ASC and probably correspond in some respects to nitrogen metabolism. In KM, an 8- to 12-fold higher abundance factor as in ASC (overall of about 6% NSAF) was found for GH15 (38562), GH16 (164740) and GH128 (355947). These hydrolases display broad substrate specificities and are therefore not easily relatable to distinct enzyme classes. They can be dedicated to glucosidases, galactosidases (e.g. glucoamylase, 1,4-β-galactosidase and transglucosidase) or glucanases that are obviously involved in starch and polysaccharide degradation.

Due to the low protein content of KM, we were able to measure the respective peptides only in two samples in LC–MS analysis, thus hampering comprehensive statistical analysis. However, PCA clearly indicated differences between KM and ASC, especially with regard to MnoPs and the AAOs (Fig. 4), approx. 98% variability can be explained by the first two principal components: PC-1 (69%) and PC-2 (29%). KM culture samples (KM 1 & 2) remained distinguishable from ASC samples (ASC 1-6) which formed a distinct separate cluster. ASC arose in the lower quadrant in contrast to the KM that was placed in the upper quadrant. The three different AAOs (171002, 171059 and 245059) were more related to KM, whereas the MnoPs (MnP1, MnP6 and VP1) were

particularly related to ASC. The dense cluster of all other enzymes, specifically of the hydrolases, underlines their low abundance and low regulation pattern in dependence of ADOR addition.

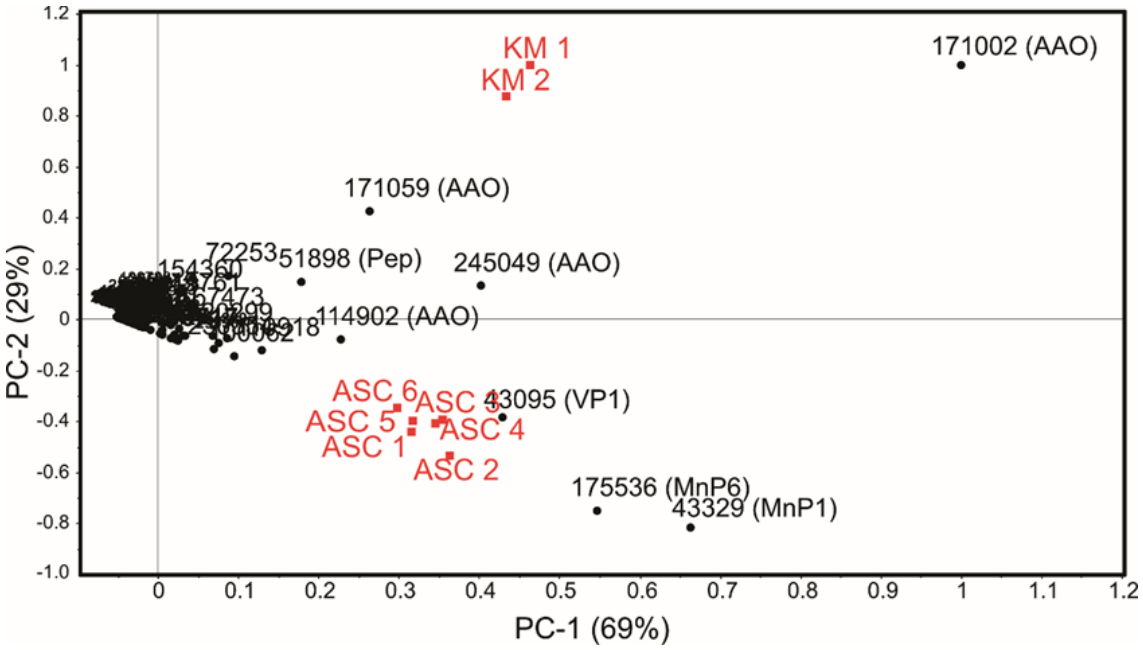

Fig. 4. Principal component analysis (PCA) of *B. adusta* secretome using NIPALS algorithms.

Two peroxidase fractions were purified from ASC fluid (harvested at day 8 after ADOR addition; MnoP activity: ca. 220 U l^{-1}). The dialyzed concentrate was applied to two chromatographic steps using an FPLC system. In the first step, brownish-colored phenols and aromatics were removed from the target peroxidase by a passage through a Q Sepharose column to which the enzyme was bound. Thereby, the specific activity for Mn^{2+}-oxidizing activity increased from 12.1 to 45.1 U mg^{-1} and 75% of the initial activity still remained (data not shown). In a second purification step (Mono Q), two distinct MnoP fractions (allocated as single homogenous SDS bands) were separated with specific activities of 38.9 and 83.1 U mg^{-1} The specific activities of both fractions toward Mn^{2+}-ions, veratryl alcohol, 2,6-DMP and one azo and one anthraquinone dye (RBlack 5and RBlue) are displayed in Table 1. The results indicate that these peroxidases belong to the versatile peroxidase group (VP) (Table 1).

Table 1. Substrate specificities of two manganese-dependent *B. adusta* peroxidases purified from ADOR-supplemented Kirk media (*Bad*VPI and II).

	Specific activity (U mg^{-1})		
	***Bad*VPI**	***Bad*VPII**	***Bad*VP***
Mn^{2+}	38.9	83.1	81.0
Veratryl alcohol	1.3	6.3	4.3
RBlack 5	0.02	8.1	3.0
RBlue 5	0.01	3.5	15.0
2,6-DMP	0.3	4.2	1.0

* Liers et al. (2013)

Mass spectrometric analyses of peptide fragments obtained from SDS gel bands verified this affiliation, precisely the affiliation of both enzymes to VP1 (43095). Therefore *Bad*VPI and *Bad*VPII obviously represent two isoforms of one VP1 gene (Fig. S1). HPLC analyses demonstrated the ability of both *Bad*VPI & II to convert and solubilize the synthetic lignin model compound DHP in the presence of Mn^{2+}-ions and linoleic acid acting as a redox mediator. Water-soluble low molecular mass fragments (~3.5 – 0.7 kDa) with an aromatic character (absorbance at 280 nm) increased within 48 hours in comparison to the corresponding controls (e.g. without Mn^{2+}-ions) (Fig. 5a & b).

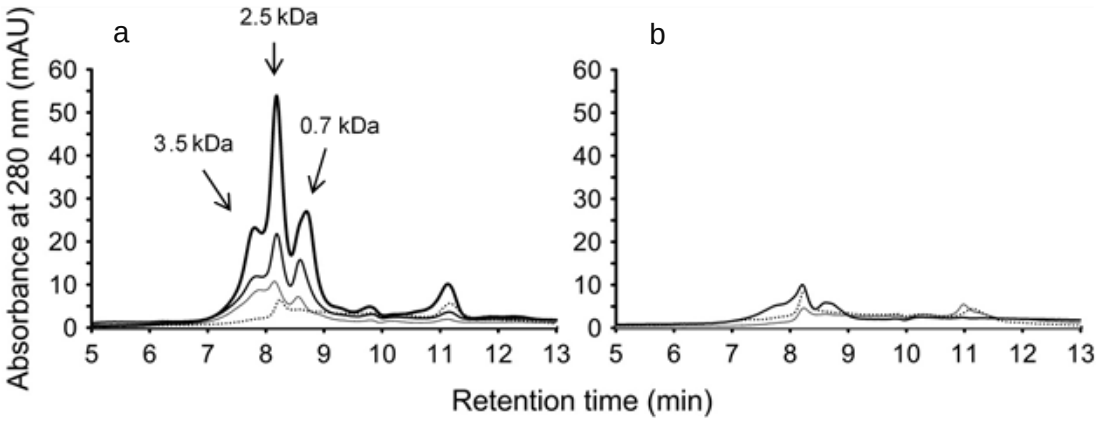

Fig. 5. Water-soluble aromatic fragments after DHP degradation with a versatile peroxidase of *B. adusta* (*Bad*VPII) (a) purified from ASC. Mn^{2+}-dependent lipid peroxidation reactions after 12 h (thin line), 24 h (medium line) and 48 h (thick line), dotted line = control (with heat-denatured *Bad*VPII). (b) Controls: -Mn^{2+}/+linoleic acid (dotted line), +Mn^{2+}/-linoleic acid (thick line), -Mn^{2+}/-linoleic acid (thin line).

Discussion

The secretome of *B. adusta* studied here, based on proteins taken 6 days after addition of an aqueous extract of agricultural waste material to enhance enzyme secretion, yielded an overall of 157 proteins. Of these, 98 were not represented in the KM secretome. The number protein identifications in fungal secretome analysis ranged from 70 until 796 (796, 574, 191 or 70 for e.g. wood-rotting fungi *Phanerochaete chrysosporium*, *Fusarium graminearum, Postia placenta*, *Irpex lacteus;* (Wymelenberg et al., 2006b; Wymelenberg 2009; Rampitsch et al., 2013; Salvachúa et al., 2013). Comparative secretome analyses of fungi focused usually on changes in the growth media (ligninolytic or cellulolytic conditions), or on comparisons among wood-rotters or phytopathogens (Salvachúa et al., 2013). Up to now, there are no comparative studies available that showed the differences of fungal secretomes caused by the addition of a natural phenolic elicitor such as ADOR; in addition, little is known which extracellular lignocellulolytic proteins are directly influenced by this polyphenols. In the *B. adusta* genome 15,473 genes are present of which 994 encode proteins with oxidoreductase activity (including 34 for class II peroxidases (e.g. 12 LiPs, 1 VPs, 6 MnP-short, 1 generic peroxidase (GP)), 10 for DyPs and 4 for heme-thiolate peroxidases (Ruiz-Dueñas et al., 2013). While 11 of these peroxidases were detected in the *B. adusta* secretome (Table S1), 7 of them were present only in ASC: 3 MnPs (175536, 172152, 306404), 1 LiP (41982), 2 DyP (172310, 169535) and 1 GP (173495). Oxidoreductases not enhanced in ASC were DyPs and AAOs. Although not much is known about the actual biological function of AAOs, they may support a redox cycle by supplying extracellular peroxides for peroxidases and perhaps can be related to an intracellular aryl alcohol dehydrogenase (Wymelenberg et al., 2009). The first function can be substituted by other oxidases, e.g. glyoxal or pyranose oxidases. DyP2 (72253) and DyP8 (287732) were more abundant in KM than in ASC. It is proposed that DyPs are secreted as a response to media with limited nutrients (Colpa et al., 2014; Salvachúa et al., 2013), which could explain its abundance in KM.

Notably, the addition of Mn^{2+}-ions or lignocelluloses (e.g. wheat straw or wood) to glucose-rich and nitrogen-limited media is a reliable method to induce MnP production in wood-degrading fungi (Kaal et al., 1993). Thus, in the secretome analysis of lignocellulose-containing solid and liquid cultures from *Pleurotus*, *Irpex* and *P. chrysosporium* peroxidases and oxidases (glyoxal oxidase) were detected but the versatile peroxidase VP2 was uniquely most abundant in *P. eryngii* and *P. ostreatus* (Salvachúa et al., 2013). Therefore, our findings confirmed the inductive effect on peroxidases from *B. adusta*, *A. auricula-judae* and *Coprinellus radians* elicited by solid

olive mill residues (DOR) supplemented to a barley-based solid medium (Reina et al., 2013). DOR and subsequently ADOR are glucose-rich and nitrogen-limited by-products, containing Mn^{2+}-ions (~16.5 ppm in solid DOR), numerous phenolic and other plant substances responsible for a certain enzyme induction effect (Salvachúa et al., 2013).

In addition, several proteins responsible for lignocellulose degradation and certain detoxification processes were generally identified in KM and ASC (e.g. glyoxal and pyranose oxidase - copper radical oxidases (EC 1.2.3.5 and 1.2.3.3)). They are required for the function of particular enzymes e.g. for the action of extracellular peroxidases via the production of H_2O_2. Indeed under the culture conditions used no enzymes potentially involved in the generation of highly-reactive hydroxyl radicals via iron reduction and subsequent Fenton-based degradation of lignocellulose (e.g. quinone reductase, cellobiose dehydrogenase, iron ferroxidase) were found in both *Bjerkandera* secretomes (Wymelenberg et al., 2005; Kersten and Cullen, 2007).

In the *B. adusta* genome, 1,524 genes encode for hydrolases, 92 of them were detected in this study and 26 corresponded to the GH family including α-amylase, α- and β-glucosidases, chitosanase and glucanases. The presence of one half of these GHs is inconsistent; few polysaccharide degrading one are more abundant in KM (e.g. GH15, GH16, GH128), whereas just one is more abundant in ASC (GH13). Indeed, it is implicated that hydrolytic enzymes involved in lignocellulose degradation were induced by cellulose-rich solid olive mill residues (Aranda et al., 2004), the aqueous part of ADOR does not significantly induce hydrolases. Rather it seems to repress certain GHs (e.g. GH15 and GH128) that were found in the *B. adusta* KM secretome and may be involved in plant cell-wall degradation and carbohydrate metabolism.

Two isoforms of the VPs produced by *B. adusta* in ASC were isolated from a phenol-rich culture fluid (*Bad*VPI & II). The specific activities and the internal peptide fragments proved their affiliation to the VP1 (43095). These enzymes are obviously part of the fungus ligninolytic and detoxification system, as proved by the conversion of a synthetic lignin model compound (DHP) by means of Mn^{3+}-mediated lipid peroxidation. Although it is assumed that Mn^{2+}-oxidizing peroxidases contribute to Mn^{3+}-ions that act as a diffusible oxidants of minor phenolic lignin moieties (Wariishi et al., 1992), the involvement of polyunsaturated fatty acids as redox mediators is postulated also for the enzymatic attack of non-phenolics (Kapich et al., 2010; Kapich et al., 2011).

Conclusion

The utilization of solid olive mill residues and their water-soluble extractives is a suitable method for inducing protein and enzyme secretion in wood-degrading fungi. This method can be applied to other eco-physiological groups like coprophilous, compost- or litter-dwelling fungi and may enable protein profiling to reveal fungal degradation and detoxification strategies as well as adaption processes for growth on this phenol-rich material.

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CHAPTER 4

Enzymatic mechanisms and detoxification of dry olive mill residue by Agrocybe aegerita, Marasmius alliaceus and Chondrosterum purpurem.

Abstract

The detoxification of Dry Olive Mill Residue (DOR), a lignocellulosic by-product, was investigated in three agaric fungi: *Agrocybe aegerita*, *Marasmius alliaceus* and *Chondrosterum purpureum*. The lignin modifying enzyme (LME) secretion patterns of the above mentioned fungi were also investigated while growing in cultures supplemented with this agro-waste. Due to its role as a potential LME enhancer, oxidases such as DyP-type peroxidase (DyP), laccase (Lac), unspecific peroxygenase (UPO) and manganese peroxidase (MnP) were determined in presence and absence of DOR. In *A. aegerita,* a Lac and UPO induction effect was found when DOR was added (2 and 6-fold of these activities). Thus, these enzymes seemed to be responsible for DOR detoxification and indirectly, its plant growth promoting effect. In the experiment performed with *M. alliaceus,* no differences in DyP secretion were found when the basal barley medium was supplemented with DOR. However, MnP and Lac activities in DOR-barley were maximal after 5 weeks of incubation with a concomitant decrease of DOR phytotoxicity. Since a soft basal secretion of the novel enzyme UPO was detected in *C. purpureum*, a deeper study was performed including soy as an organic support medium. Interestingly, the usage of soy (where UPO secretion was more notable) or barley as fungal growth support, had no noticeable effect on the outcome of the experiments: in both cases the plant toxicity of DOR was completely removed. In order to study the mechanisms involved in DOR phytotoxicity removal with *C. purpureum*, other enzymatic mechanisms were also investigated, such as hydrolytic enzymes and the intracellular system CytP450. Our results suggest the participation of a complex enzymatic system (intra and extracellular) in *C. purpureum* in the biotransformation of DOR which could shed light on the possible mechanism of this notwell-known fungus in ligninolysis.

Keywords: *Agrocybe aegerita*, *Marasmius alliaceus*, *Chondrostereum purpureum*, class II peroxidases, laccases, olive mill residues, unspecific peroxygenase, CytP450, phytotoxicity.

Introduction

Olive oil production is mostly centered in the Mediterranean basin countries (Mateo and Maicas, 2015), with Spain topping the list of producers (1.1 million metric tons in 2013) (FAOSTAT, 2013). The current predominant method for the extraction of olive oil is a two-phase system which consists of a two-phase centrifugation that separates the oil from a semisolid waste which is known as “alpeorujo” or two-phase olive mill waste (TPOMW) (Morillo et al., 2009). TPOMW goes through a process of drying and is subsequently extracted with the aid of organic solvents (e.g. hexane) in order to generate “orujo” oil and a new lignocellulosic by-product called dry olive residue (DOR) (Arjona et al., 1999). DOR can be considered a phytotoxic agro-industrial waste due to its high organic load, polyphenols content and low molecular weight organic acids (Vlyssides et al., 2004). This by-product could also be considered a source of phenolic compounds with technological and pharmaceutical properties, but a previous complex purification process is required (Forbes-Hernández et al., 2014). Despite their antioxidant properties, phenolic compounds have been pointed out as being responsible for the high phytoxicity of olive mill wastes (OMWs). Their negative effects are due to the generation of reactive oxygen species, some of which undergo an auto-oxidation reaction (García-Sánchez et al., 2012). Therefore, improper disposal of olive mill residues can cause environmental problems (Dermeche et al., 2013).

In order to create a more environmentally-friendly and sustainable industry, proposals have been made to use agricultural wastes as raw material for new products or applications. Several physicochemical approaches have been employed to treat OMWs such as ozonization, electrocoagulation and physical fractionation (Chedeville et al., 2009; Hanafi et al., 2010; Aranda et al., 2012). They are, however, difficult to apply in practise due to several problems derived from large-scale implementation such as cost-efficiency and environmental or technical concerns, since physicochemical treatments can produce toxic sludge or air pollutants (Kapellakis et al., 2008).

The phytotoxic and antimicrobial properties of OMWs impede their direct application to soil. However, its elevated content in organic matter and plant nutrients makes them potential candidates for bioconversion as fertilizers and/or amendments (Dermeche et al., 2013; Siles et al., 2013). The biological treatment of OMWs is an alternative approach to achieve their detoxification. Composting is one of the most popular techniques for phytotoxicity reduction, phenolic removal and fertilizer production from such wastes (Monetta et al., 2014). Nonetheless, the composting of the residue requires a longer incubation time to reach maturity than it requires in monoculture fermentations with specific microorganisms. The bioconversion of DOR by the

traditionally so-called white rot fungi (WRF) in monoculture fermentations represents one of the most efficient tools for its treatment (Reina et al., 2013).

WRF comprise numerous fungi with the ability to secrete class II peroxidases (PODs), including lignin peroxidases (LiPs, EC 1.11.1.14), manganese peroxidases (MnPs, EC 1.11.1.13), and versatile peroxidases (VPs, EC 1.11.1.16) (Hatakka, 1994). These fungi can transform lignocellulosic wastes into valuable nutritional ingredients for plants by degrading lignin and converting complex polysaccharides into simple sugars with the aid of a hydrolytic system (Sharma and Arora, 2015). Among the oxidative enzymes implicated are: PODs, heme-thiolate peroxidases, dye-decolorizing peroxidases (DyPs) and laccases (Lacs, EC 1.10.3.2). Heme-thiolate peroxidases are a different class of peroxidase enzymes, which catalyze the transfer of peroxide-oxygen to substrate molecules. The unspecific peroxygenases (UPOs, EC 1.11.2.1) belong to this group. DyPs are able to oxidize a wide range of organic substrates, some of which are difficult to transform by the typical peroxidases (e.g. dyes derived from anthraquinone). Lacs have a low oxidation potential (compared to peroxidases), but they are able to oxidize chains of non-phenolic aromatic rings with the aid of a small diffusible mediator which is previously activated by them (Areskogh et al., 2010). There is a broad agreement about the participation of PODs and Lacs in lignin degradation (Hammel and Cullen, 2008; Munk et al., 2015). However, the role of DyPs and UPO in ligninolysis requires further investigation, although UPOs seem to participate in the polymerization of aromatic water soluble compounds of lignocellulose residue extracts (Reina et al., 2013). These biocatalysts are also able to oxidize non- phenolic lignin model compounds and they seem to be implicated in the bioconversion of methoxylated compounds that can derived from lignin (Kinne et al., 2011). In addition to the secreted enzymes, intracellular enzymes included in the microsomal fractions can also take part in the ligninolytic process, since they contribute to deal with the myriad of potential toxic compounds derived from wood and organic matter transformation (Morel et al., 2013). In this intracellular enzymatic system, represented by multigenic families, cytochrome P450 monooxygenases are included (CytP450s, EC 1.14). CytP450s are also heme-thiolate proteins which are involved in different xenobiotic detoxification pathways, the most important of which is the hydroxylation of substrates. In the basidiomycetes, these groups of enzymes can break down various xenobiotics and lignin metabolites released by the action of extracellular peroxidases (Ide et al., 2012; Doddapaneni et al., 2013). In addition, non-enzymatic mechanisms participate in the conversion of lignocellulose, which can be achieved with the aid of reactive oxygen species generated via Fenton reactions (Hammel et al., 2002).

The agaric fungus *Agrocybe aegerita* has been extensively studied in the last decade for its ability to secrete a stable UPO (Ullrich et al., 2004), as have been *Marasmius* sp*, which* are well known for their DyP secretion (Ferreira Gregorio et al., 2006; Scheibner et al., 2008*). Chondrostereum purpureum* is a ligninolytic fungus due to its ability to colonize hardwoods and produce oxidative enzymes (Vartiamäki et al., 2008; Bellgard et al., 2014). Although extensive research has been carried out on the role of *C. purpureum* as a biocontrol agent in which it appears as a good candidate for lignin components degradation, no single study exists concerning its ligninocellulolytic enzyme secretion.

In this chapter, we have addressed the participation of the above mentioned fungi in the degradation of DOR, focusing on the implication of the non-classical PODs and studying possible detoxification effects and the mechanism by which this detoxification occurs.

Results

Agrocybe aegerita

Low activities, both for Lac and UPO, were detected in barley control cultures of *A. aegerita* (Fig. 1a). However, UPO activity was stimulated when cultures where supplemented with DOR, reaching its peak after 4 weeks of incubation (1.2 U g^{-1}). No DyP or MnP activity was detected under these conditions (data not shown). The maximal decrease in phenol content of the fermented DOR occurred between the 3rd and the 4th week of incubation which corresponds to the maximal increment in UPO activity. pH suffered a progressive increment in both supplemented and non-supplemented cultures.

The polymerization pattern showed an increment in the aromatic soluble fragment size during the incubation of DOR with *A. aegerita*. In extracts of non-inoculated control cultures, the peak corresponded to fragments of 14.55 kDa, and after five weeks of incubation with the fungus the peak was 183 kDa (Fig. 1b).

DOR toxicity disappeared after one week of incubation with *A. aegerita* since there were no significant differences in SDW and RDW between control and T1 treatments (Fig. 1c). After one week of fermentation, half of the soluble phenols were removed. At T5 (DOR incubated during 5 weeks) SDW and RDW were significantly bigger than controls.

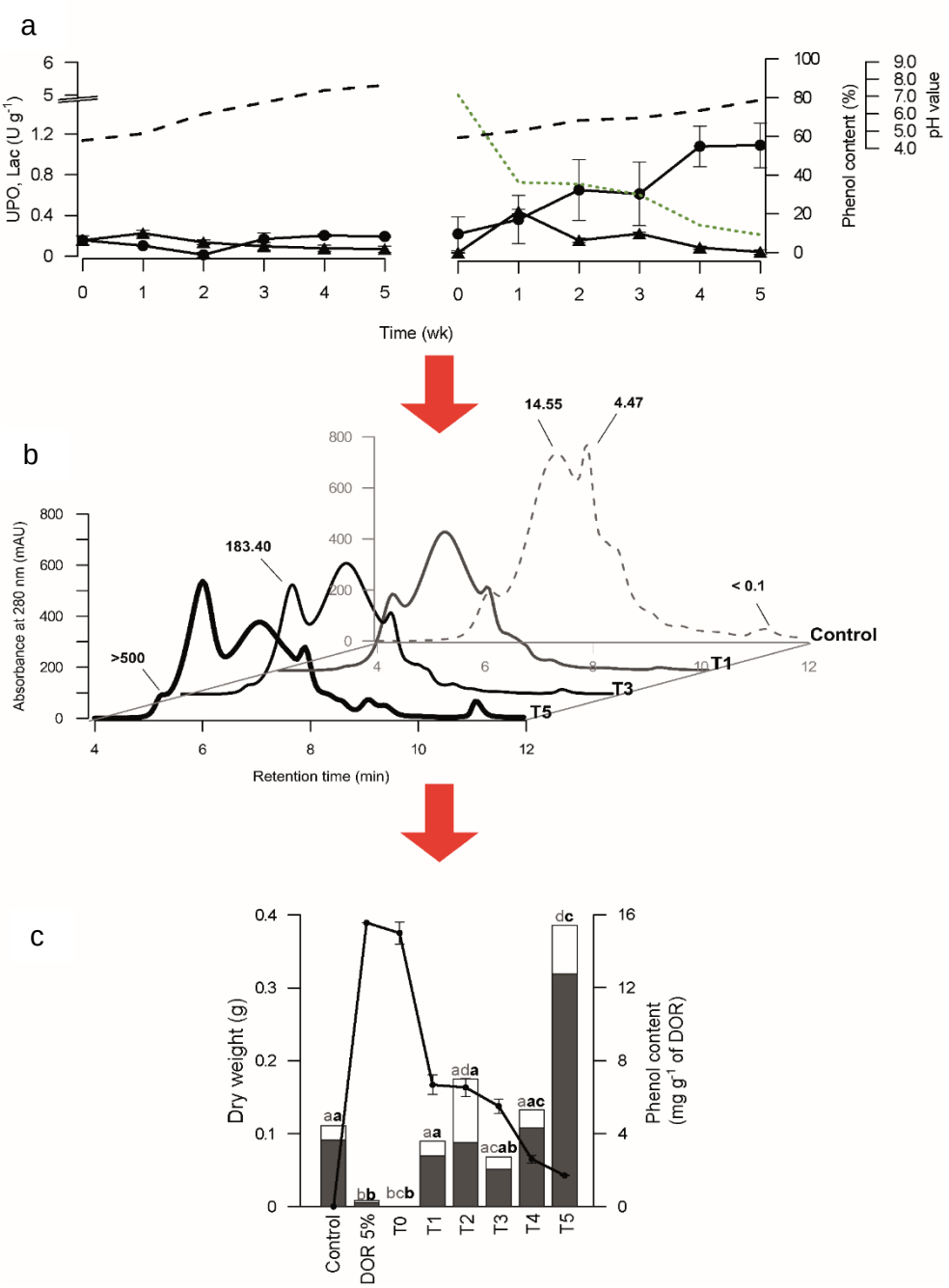

Fig. 1. (a) Time course of laccase (Lac, *triangles*) and peroxygenase (UPO, *circles*) activity of *Agrocybe aegerita* during SSF on barley (*left*) and DOR-supplemented barley cultures (*right*). Enzyme activities (*solid lines*), total percentage of phenol content (*green dotted line*) and pH value (*dashed line*). Mean values of triplicate measurements were calculated and error bars represent standard deviation. (b) Changes in the molecular mass distribution of water-soluble aromatic fragments formed by *A. aegerita* during growth on unfermented DOR (control, *dashed line*) and on DOR-barley media after, 1 (T1), 3 (T3) and 5 (T5) weeks of cultivation (*solid line*). (c) SDW (*upper bar*) and RDW (*lower bar*) of tomato plants (Control) cultivated in the presence of dry olive mill residue (DOR), non-fermented (DOR 5%) and fermented by *A. aegerita* during 0, 1, 2, 3 ,4 and 5 weeks (T0–T5). Lowercase letters distinguish between statistically significant different groups for each treatment, bold letters are used for SDW and normal letters for RDW (p <0.05 Dunnet test). The solid line represents the total phenol content (mg g^{-1} DOR).

Marasmius alliaceus

M. alliaceus MnP, Lac and DyP activities were moderate during solid state fermentation in barley media (Fig. 2a). No UPO activity was detected under these conditions (data not shown). Non-significant differences in DyP secretion were found when this medium was supplemented with DOR, but MnP and Lac activities in DOR-barley media were found to be 5 and 12 fold than control values at the 5th week of incubation, respectively. An 80% of total phenolic content was removed after 5 weeks of incubation (Fig. 2a). pH varied from 4.5 to 6 in barley cultures and from 4 to 5.5 in DOR-barley media.

A shift was detected in the polymerization profile of DOR fermented with *M. alliaceus*. In the 5th week of incubation, bigger aromatic soluble fragments of DOR treated with the fungus were found in the DOR-barley mixture than in the control medium (untreated DOR) (Fig. 2b). Peaks in SEC profiles varied from 4.63 in control samples to 190 kDa in DOR incubated during 5 weeks with barley as a support medium.

The experiments performed in *Solanum lycopersicum* plants (Fig. 2c) showed a toxicity depletion of the residue at the 5th week of incubation with a concomitant decrease of phenolic content since there were significant differences between SDW and RDW values of plants treated with the residue incubated with *M. alliaceus* during 5 weeks and control plants without the residue.

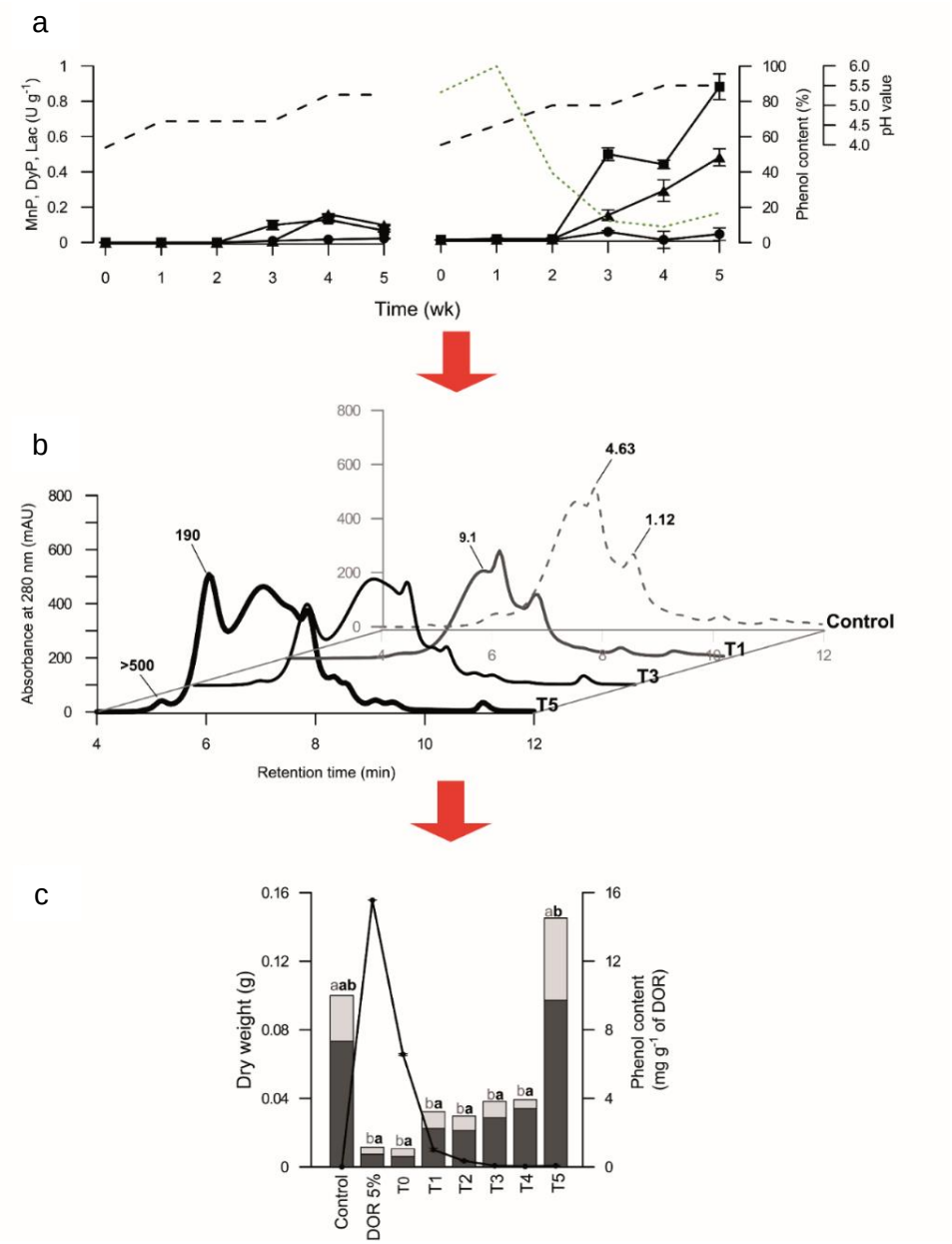

Fig. 2. (a) Time course of laccase (Lac, *triangles*), manganese peroxidase (MnP, *squares*) and DyP-type peroxidase (DyP, *circles*) activity of *Marasmius alliaceus* during SSF on barley (*left*) and DOR-supplemented barley cultures (*right*). Enzyme activities (*solid lines*), total percentage of phenol content (*green dotted line*) and pH value (*dashed line*). Mean values of triplicate measurements were calculated and error bars represent standard deviation. (b) Changes in the molecular mass distribution of water-soluble aromatic fragments formed by *M. alliaceus* during growth on unfermented DOR (control, *dashed line*) and on DOR-barley media after, 1 (T1), 3 (T3) and 5 (T5) weeks of cultivation (*solid line*) (c) SDW (*upper bar*) and RDW (*lower bar*) of tomato plants (Control) cultivated in the presence of dry olive mill residue (DOR), non-fermented (DOR 5%) and fermented by *M. alliaceus* during 0, 1, 2, 3 ,4 and 5 weeks (T0–T5). Lowercase letters distinguish between statistically significant different groups for each treatment, bold letters are used for SDR and normal letters for RDW (p <0.05 Dunnet test). The solid line represents the total phenol content (mg g^{-1} DOR).

Chondrostereum purpureum

Barley medium

Figure 3a illustrates the enzyme secretion of *C. purpureum* in *a* barley support medium with or without DOR. The most significant activity was UPO, but the highest peak level was only 0.15 U g^{-1} during the 2nd week of incubation. Lac and MnP activity secretion was very low and no DyP was detected under the conditions tested. No induction of any enzymes was detected with DOR supplementation. The total phenol content was reduced to 40%.

The SEC chromatogram showed no increment or decrease in soluble aromatic fragment sizes (data not shown).

With regards to DOR plant toxicity properties, no statistically significant differences were found between SDW and RDW values of tomato control plants without DOR and plants in which DOR, fermented after 1 week with *C. purpureum* in a barley-based medium, was added. This indicates a complete removal of phytotoxicity after a week of fermentation (Fig. 3b).

Soy medium

The secretion of UPO when *C. purpureum* were grown in a soy-based medium was similar to the values detected with barley. However, when DOR was added to the fungus culture, a stimulation in the UPO secretion pattern occurred after one week of incubation. The peak activity was determined at week 5 of incubation in DOR-supplemented soy media (0.32 U g^{-1}) that was paralleled with the phenol depletion at 40% (Fig. 4a). pH varied from 4 to 8 whereas it remained stable in DOR-barley culture extracts.

DOR incubated in a soy-based medium with *C. purpureum* were not phytotoxic for tomato after two weeks of incubation (Fig. 4b). SDW and RDW values of tomato plants treated with DOR and incubated in a soy support medium during 4 and 5 weeks were significantly higher than control plants without DOR.

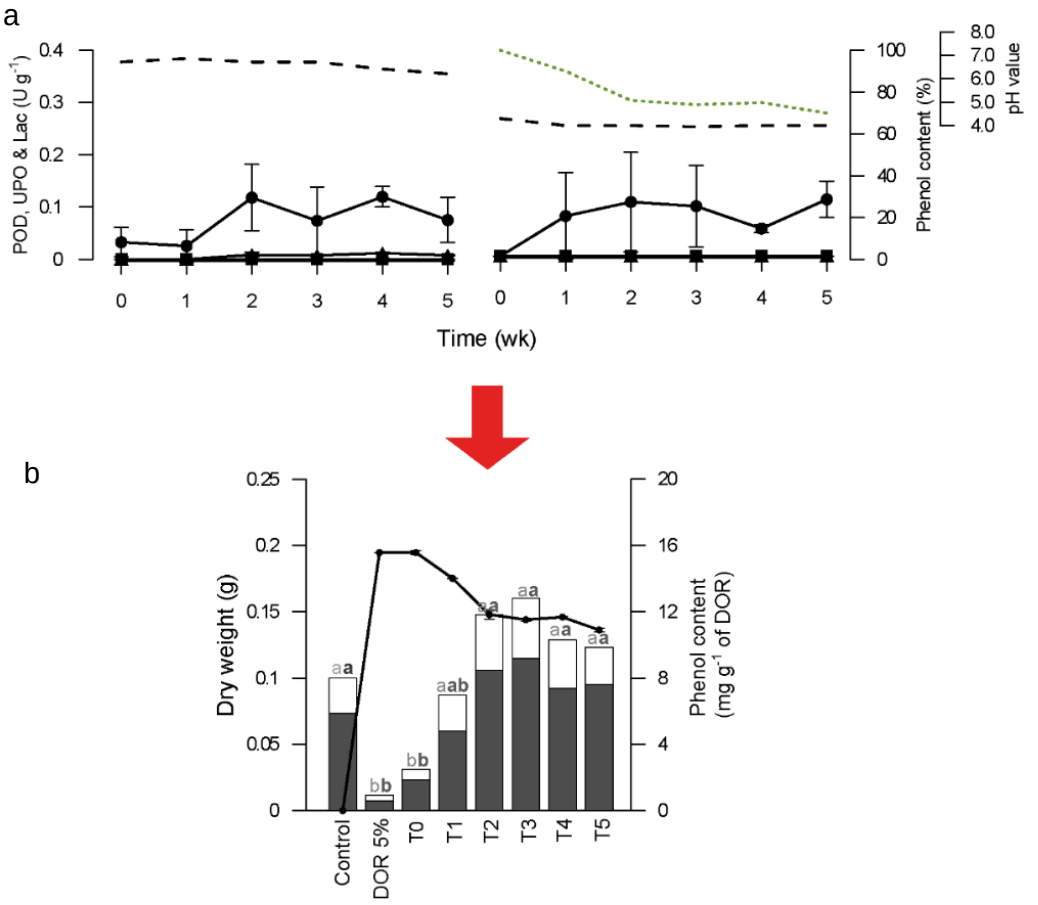

Fig. 3. (a) Time course of laccase (Lac, *triangles*), manganese peroxidase (MnP, *squares*) and peroxygenase (UPO, *circles*) activity of *Chondrostereum purpureum* during SSF on barley (*left*) and DOR-supplemented barley cultures (*right*). Enzyme activities (*solid lines*), total percentage of phenol content (*green dotted line*) and pH value (*dashed line*). Mean values of triplicate measurements were calculated and error bars represent standard deviation. (b) SDW (*upper bar*) and RDW (*lower bar*) of tomato plants (Control) cultivated in the presence of dry olive mill residue (DOR), non-fermented (DOR 5%) and fermented by *C. purpureum* during 0, 1, 2, 3 ,4 and 5 weeks (T0–T5). Lowercase letters distinguish between statistically significant different groups for each treatment, bold letters are used for SDR and normal letters for RDW ($p < 0.05$ Dunnet test). The solid line represents the total phenol content (mg g^{-1} DOR).

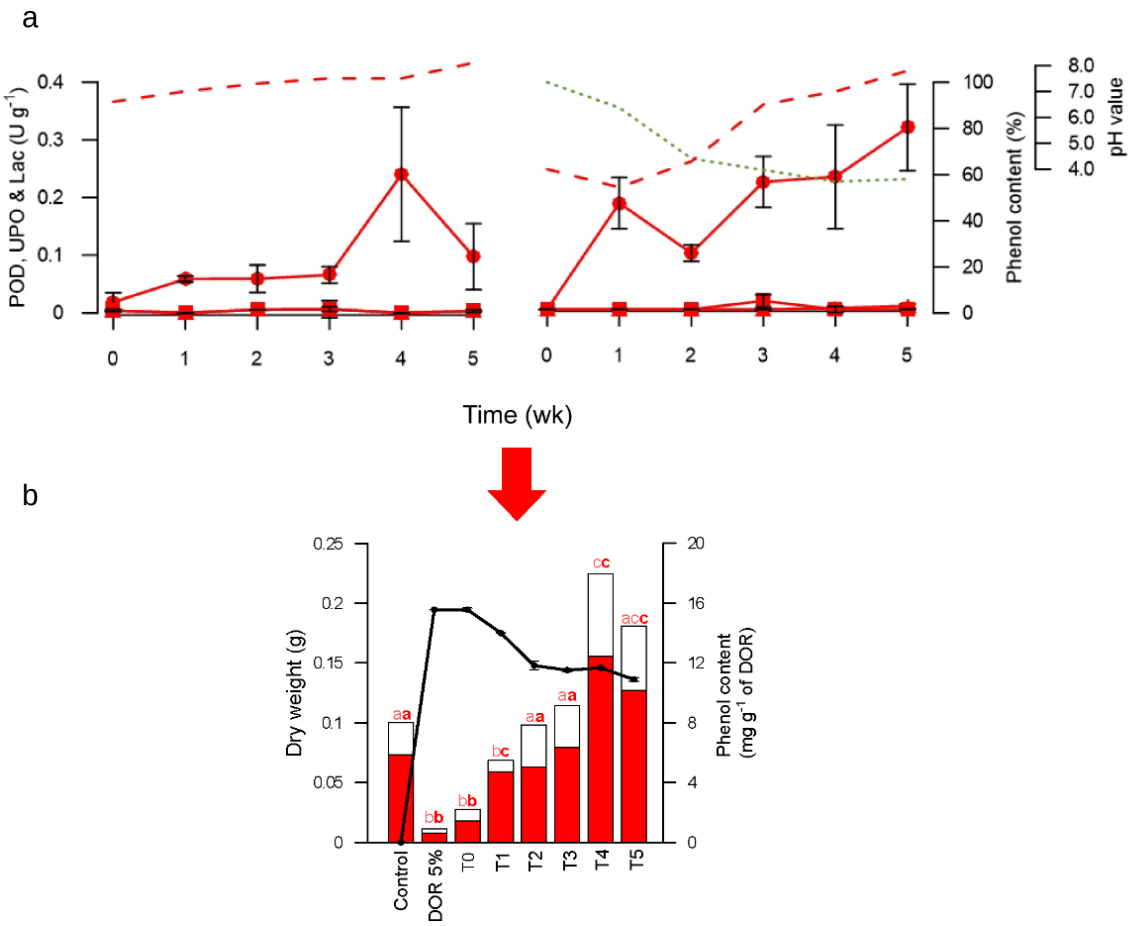

Fig. 4. (a) Time course of laccase (Lac, *triangles*), manganese peroxidase (MnP, *squares*) and peroxygenase (UPO, *circles*) activity of *Chondrostereum purpureum* during SSF on soy (*left*) and DOR-supplemented soy cultures (*right*). Enzyme activities (*solid lines*), total percentage of phenol content (*green dotted line*) and pH value (*dashed line*). Mean values of triplicate measurements were calculated and error bars represent standard deviation. (b) SDW (*upper bar*) and RDW (*lower bar*) of tomato plants (Control) cultivated in the presence of dry olive mill residue (DOR), non-fermented (DOR 5%) and fermented by *C. purpureum* during 0, 1, 2, 3 ,4 and 5 weeks (T0–T5). Lowercase letters distinguish between statistically significant different groups for each treatment, bold letters are used for SDR and normal letters for RDW ($p < 0.05$ Dunnet test). The solid line represents the total phenol content (mg g^{-1} DOR).

Hydrolytic enzymes

The hydrolases CMC-ase and Xyl-ase activities of *C. purpureum* during SSF in the different media with or without DOR were higher than the PO-ase activity (Table 1). CMC-ase activity was highest in extracts of barley, but Xyl-ase and PO-ase activity were higher in soy control cultures. Our results show significant differences in PO-ase activity between DOR-supplemented and control cultures after week 4 of incubation. On the

other hand, the CMC-ase was also higher in DOR-supplemented soy at week 3 of incubation with respect to its soy control medium showing a 1.6-fold increase.

Intracellular enzymatic activity

The CytP450 activity of *C. purpureum* in a soy medium supplemented or not with ADOR was determined in cytosolic and microsomal fractions. No activities were found in microsomal fractions (Table 2). However, in cytosolic fractions we found values of 29 and 50 U mL^{-1}. The highest CytP450 activity was found in ADOR-supplemented cultures after 5 days of ADOR addition (170 U mL^{-1}). No CytP450 activity was detected in the presence of ABT.

Table 1. Carboxymethylcellulase (CMC-ase), xyloglucanase (Xyl-ase) and endopolymethylgalacturonase (PO-ase) relative activity of *Chondrostereum purpureum* during SSF in soy, DOR-soy, barley and DOR-barley.

	Wk	Soy	DOR-soy	Barley	DOR-barley
CMC-ase	0	50 ± 5	1.6 ± 0.6	83 ± 7	1.4 ± 0.5
	1	67 ± 5	19 ± 1	69 ± 7	6 ± 1
	2	56 ± 9	31 ± 1	65 ± 7	21 ± 2
	3	45 ± 2	73 ± 8	66 ± 3	36.9 ± 0.8
	4	30 ± 3	27 ± 2	92 ± 2	42 ± 5
	5	51 ± 3	45 ± 4	37 ± 4	23 ± 2
Xyl-ase	0	36 ± 2	5 ± 2	48 ± 4	1.5 ± 0.5
	1	83 ± 5	35 ± 8	59.8 ± 0.0	4 ± 2
	2	97 ± 6	35 ± 7	27 ± 2	16 ± 3
	3	122 ± 3	104 ± 7	51 ± 1	19 ± 2
	4	97 ± 7	56.2 ± 0.0	50 ± 4	14 ± 1
	5	70 ± 3	55 ± 7	83 ± 8	27 ± 2
PO-ase	0	8 ± 3	0 ± 0	9 ± 1	0 ± 0
	1	27 ± 1	21 ± 4	11 ± 2	8 ± 1
	2	11.3 ± 0.2	4 ± 1	5.7 ± 0.6	4.8 ± 0.0
	3	10 ± 1	7.1 ± 0.7	15 ± 3	15.4 ± 0.9
	4	3 ± 1	4.5 ± 0.4	2.9 ± 0.0	5 ± 1
	5	4.6 ± 0.6	4 ± 1	7 ± 1	7 ± 2

Table 2. Cyt P450 activity (U mL^{-1}) in microsomal and cytosolic fractions of soy and soy + ADOR *C. purpureum* cultures respectively.

	Microsomal fraction		**Cytosolic fraction**	
Day of incubation	Soy	ADOR-soy	Soy	ADOR-soy
5	0	0	29 ± 6	170 ± 20
15	0	0	50 ± 10	12 ± 4

Discussion

The phytotoxicity of OMW has been mostly attributed to phenolic acids and the decrease of phytotoxicity to a polymerization process that avoids the entrance of phenolics through the plant cell wall (Barakat et al., 2009). The polymerization of phenolic compounds by PODs has been studied in detail; these enzymes can easily polymerize phenolic acids with electron-donating groups (Xu et al., 1995). Laccases as well are able to oxidize phenolic substrates, either by polymerizing them by oxidizing phenolic end groups to phenoxy radicals that can couple to form covalent bonds, or by degrading them (Areskogh et al., 2010). The agaric fungi *A. aegerita* and *M. alliaceus* were able to grow in the presence of the toxic DOR. The polymerization of soluble aromatic fragments in DOR by *A. aegerita* is correlated to a high UPO activity. There is previous evidence of a UPO polymerization effect on DOR aromatic soluble fragments (mainly phenolic acids) by the fungus *Coprinellus radians* (Reina et al., 2013). The finding that the fungus *M. alliaceus* was able to polymerize aromatic soluble ingredients in olive mill wastes was somehow expected since it was able to secrete MnP and Lac which have previously been reported as being responsible for phytotoxic decrease (Reina et al., 2013). The ability of DyP to form polymerization products, previously suggested by (Büttner et al., 2015), could not be confirmed in this study due to low secretion values in all the media tested. In supplemented media, DOR stimulated UPO secretion by *A. aegerita* and MnP and Lac secretion by *M. alliaceus*, which confirms the effectiveness of DOR in LME stimulation. Thus, these results suggest a different method for valorizing agroindustrial OMW regardless of the already proposed alternatives (Morillo et al., 2009). In both fungi, the secretion of lignin modifying enzymes (LME) together with their polymerization effect on DOR, leads to a decrease in the total phenolic content, a complete removal of phytotoxicity and a promotion of plant growth which represents an interesting finding in the field of agronomic use of by-products. The bioremediation of OMW by WRF has also been observed in fungi like *Funalia floccosa, Phanerochaete flavido-alba*, *Trametes versicolor, Bjerkandera adusta* and *Auricularia auricula-judae* among others (Linares, 2003; Ergül et al., 2009; Díaz et al., 2010; Reina et al., 2013).

LME secretion of *C. purpureum* in SSF in the media tested was not significant and no evidence of phenolic acids polymerization was observed. However, the phytotoxic effect of DOR was not only entirely removed in the tested media, but also, interestingly, this fungus promoted the growth of tomato plants to which biorremediated DOR was added. It was unclear whether *C. purpureum* was able to completely remove DOR phytotoxicity to produce a 40% of phenol depletion and to transform DOR into a

plant growth promoting substrate. In order to detect alternative possible enzymatic systems involved in DOR conversion to a fertilizer, we analysed the most important carbohydrate active enzymes from the extracellular medium as well as the intracellular enzymatic system. In the case of hydrolytic enzymes, an induction effect by DOR or a possible relation between them was not clearly found. CytP450 was analyzed in the presence and absence of ABT, which is highly expanded in wood degrading fungi (Kües, 2015). In the current study, CytP450 activity in the cytosolic fraction of *C. purpureum* grown under SF in an ADOR-soy medium after 5 days of ADOR addition was clearly enhanced. This suggests a detoxification pathway activation, probably promoted by the phenolics presents in the ADOR as well as by the metabolites and radicals formed during the LME attack. By-products from the primary extracellular attack could enter through the mycelia continuing the transformation in the cytoplasm, as occurs in transformations of aromatic compounds (Aranda, 2016). Thus, this data could indicate the implication of intracellular mechanisms (direct and indirect) in the detoxification of the residue within the cells, which is for the first time being studied in this work using these agrowastes. However, the factors related to intracellular regulatory networks of lignocellulolysis for this fungus are completely unknown. Finally, it is important to take into account that non-enzymatic degradation mechanisms can occur as well, such as Fenton reactions with the aid of peroxide producing enzymes (Fig. 5) which have already been shown to occur in OMW (Vlyssides et al., 2004), but they are not considered in this work.

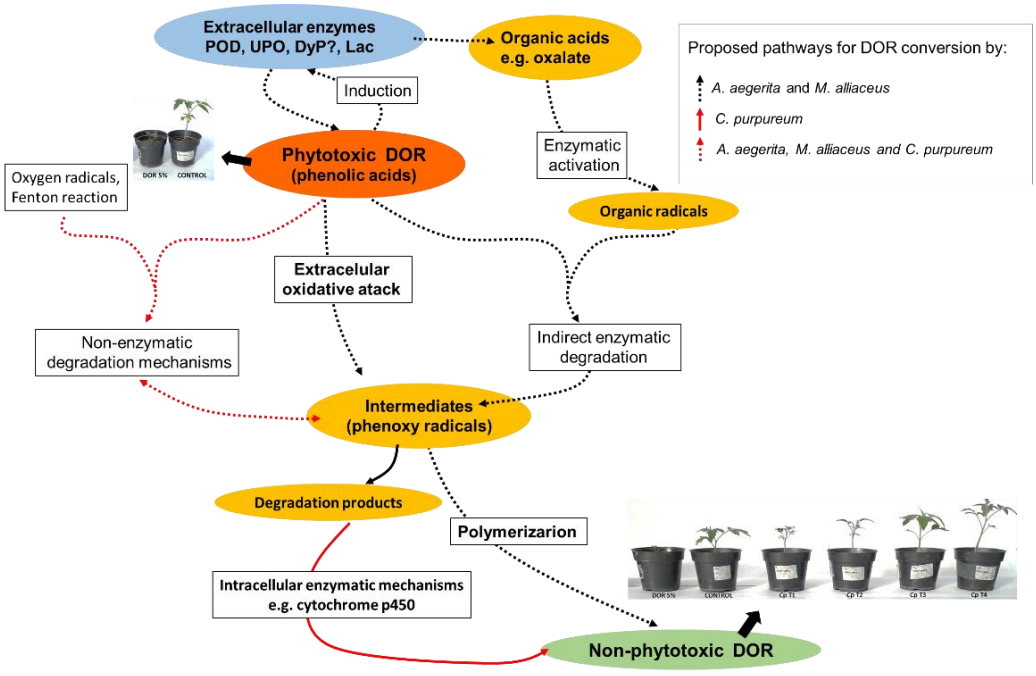

Fig. 5. Possible mechanisms for DOR bioremediation by the litter-decomposing fungi *Agrocybe aegerita, Marasmius alliaceus* and *Chondrostereum purpureum* found in this study. Based on Kellner et al., 2014; Kües, 2015.

Conclusions

According to the results of this study, the fungi *A. aegerita*, *M. alliaceus* and *C. purpureum* are suitable organisms for OMW plant toxicity depletion. In addition, a different approach for the broad valorization alternatives of the residue is proposed. It consists of the utilization of DOR as an enzyme inductor of MnP and Lac in *M. alliaceus* and the multifunctional UPO in *A. aegerita*. To further understand the oxidative pathways that *C. purpureum* uses for DOR bioremediation, genomic and proteomic analyses are needed.

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CHAPTER 5

Genome and Secretome of *Chondrostereum purpureum*

Abstract

The *Agaricomycetes* are among the few organisms capable of causing a complete degradation of lignocellulosic material. The fungus *Chondrostereum purpureum* is a biological control agent of which little is known about its role as a wood degrader. No conclusive studies about its lignocellulolytic machinery and its phylogenetic position within the *Agaricomycetes* have been carried out. In this chapter, we report a 41.2 Mbp genome assembly of the fungus and present a detailed study of its pool of lignin modifying (LME) and (hemi) cellulolytic enzymes. We found a large number of protein-coding genes for the oxidative enzymes such as laccases, unspecific peroxygenases, lytic polysaccharide, monooygenase and some class II peroxidases coding sequences. We claim that *C. purpureum* is a white rot fungus which belongs to the order *Agaricales*. In addition, we performed a proteomic study including potential elicitor substrates such as the phenolic rich dry olive mill residue or wood material. We also confirm the role of the olive mill by-product as an enhancer for LME secretion. Our results shed light on the complex extracellular machinery of *C. purpureum* for lignocellulose degradation.

Keywords: *Chondrostereum purpureum*, Ion Torrent, genome, secretome, unspecific peroxygenase, laccase, lytic monosaccharide monooxygenase, dry olive mill residue, beech wood, birch wood.

Introduction

Wood decomposition is a major part of the global carbon cycle and basidiomycetes belong to the most important eukaryotes involved in wood decay (Kirk and Farrell, 1987). Wood-decomposing fungi are traditionally divided into brown rot fungi (BRF) and white rot fungi (WRF), depending on their decay patterns. BRF are able to degrade all the polysaccharides of plant cell walls and partially depolymerize the lignin polymer with the aid of enzymatic and non-enzymatic mechanisms. The latter include free radical systems which can act as oxidants driven by Fenton reactions (e.g. hydroxyl radicals) (Hori et al., 2013; Arnstadt et al., 2016). By contrast, WRF are known to degrade all polymers of the lignocellulosic complex involving a battery of lignin modifying enzymes, as well as a large set of (hemi) cellulases (Van Den Brink and De Vries, 2011). In a strict sense, the term WRF refers to fungi that secrete class II peroxidases (Riley et al., 2014). The enzymes implicated in the conversion of celluloses and hemicelluloses are mainly hydrolases and esterases, although the copper-dependent lytic polysaccharide monooxygenases (LPMO; EC 1) are able to cleave the glycosidic bonds in cellulose by an oxidative mechanism (Beeson et al., 2015). Lignin is mainly depolymerized by oxidative enzymes including high-redox potential class II peroxidases (PODs; EC 1.11.1) such as lignin peroxidase (LiP), manganese peroxidase (MnP), or versatile peroxidase (VP) and low-redox potential generic peroxidase (GP; EC 1.11.1.7) (Martínez, 2002; Hofrichter et al., 2010; Lundell et al., 2010). Nevertheless, laccase (Lac; EC 1.10.3.2), cellobiose dehydrogenase (CDH; EC 1.1.99.18) and peroxide-producing enzymes (e.g. aryl alcohol oxidase (AAO; EC 1.1.3.7)) are also involved in ligninolysis and aromatics conversion (Baldrian, 2006; Langston et al., 2011). Furthermore, a potential role for recently described oxidoreductases in ligninolysis has been proposed, as is the case for the unspecific peroxygenase (UPO; EC 1.11.2.1) and the dye-decolorizing peroxidase (DyP; EC 1.11.1.19) (Hofrichter et al., 2010), which occur abundantly in wood-rotting basidiomycetes.

The array of enzymes responsible for plant cell wall polysaccharide degradation are classified on the basis of their amino acid sequences into carbohydrate-active enzyme (CAZy) families such as glycoside hydrolase (GH; EC 3.2.1), glycosyltransferase (GT; EC 2.4), polysaccharide lyase (PL; EC 4.2.2), carbohydrate esterase (CE; EC 3), and carbohydrate-binding modules (CBMs) (Lombard et al., 2014). In addition, the CAZy database integrates auxiliary enzymes (auxiliary activities; AAs) comprising mainly oxidative representatives that are also important for the decomposition of lignocellulose (Levasseur et al., 2013).

Chondrostereum purpureum is a common pathogen on hardwoods, found on broad-leaved trees, deciduous trees, shrubs and fruit trees within the temperate zones world-wide (Wall, 1990). Due to its ability to colonize soft and hardwood, *C. purpureum* has been proposed as a biocontrol agent against sprouting and root suckering of tree species such as red alder (*Alnus Kda*), black cherry (*Prunus serotina*), white birch (*Betula papyfiera*) and aspen (*Populus* spp.) (Bellgard et al., 2014; Hamberg et al., 2014; Hamberg et al., 2015). Aside from its ability to cause wood decay, little is known about the selective mechanisms and the responsible lignocellulolytic enzymes by which the fungus achieves the wood decay. Current studies also show discrepancies about the taxonomic classification of *C. purpureum*, it being included either within *Polyporales* or *Agaricales* (Djeddour and Shaw, 2014; Hamberg et al., 2015). Therefore, there seems to exist a definite need to reveal the phylogenetic position of *C. purpureum,* as well as its lignocellulolytic potential and enzymatic system.

In this chapter, we report a draft genome of *C. purpureum* in combination with a mass spectrometry-based analysis of the secretome during the fermentation of different solid and liquid media supplemented with natural substrates such as hardwood and agricultural waste material (dry olive mill residue; DOR) (Reina et al., 2013; Reina et al., 2014).

Results

Assembly and quality assessment of *Chondrostereum purpureum* draft genome

The genome of *C. purpureum* was assembled from 3,971,460 reads obtained from the Ion Torrent PGM System. Previously, all the reads obtained from polyclonal and low quality ISPs, were excluded (Table 1). The average fragment size was 168 bp.

Table 1. *C. purpureum* Ion Torrent PGM Run information.

Addressable Wells	1303570
With ISPs	7754617 (68.6%)
Enriched ISPs	7721461 (99.6%)
Test Fragment	34817 (0.5%)
Library ISPs	7686644
Polyclonal ISPs	1657917 (21.6%)
Low Quality ISPs	2055596 (26.7%)
Primer Dimer	1671 (00.0%)
Final Library ISPs	**3971460 (51.7%)**

A final 41.2 Mbp draft genome organized in 3435 contigs was obtained after the assemblies. (Table 2). The empirical average "depth-of-coverage" (number of reads * read length / assembly size) was 15.2x. From the subset of 248 ultra-conserved CEGMA CEGs considered, we found 229 complete and 237 partial proteins (data not shown). This means a completeness of 92.32%. A number of 13730 protein-coding gene models were predicted

Table 2. Statistical assembly of *C. purpureum* genome.

Assembly statistics	
Genome assembly size (Mbp)	41.2
Mean genome coverage	15.2
Max contig length	207970
Min contig length	390
Number of contigs	3435
N50	23869
Annotation statistics	
Number of predicted CDS	13739
Maximal CDS length (bp)	15468
GC content (%)	47.5
Completeness (%)	92.34
Number of ultra-conserved CEGs proteins*	229
Total number of CEGs	304
Average number of orthologs per CEG	1.33
%Ortho **	25

* Number of 248 ultra-conserved CEGs present in genome.

** Total number of CEGs present including putative orthologs.

Taxonomy

In the multi-gene phylogenetic analysis, a hundred of well-known species were distributed across 20 orders such as the orders *Auriculariales*, *Dacrymycetales* and *Tremellales* from the former fungal taxonomic group of heterobasidiomycetes, now classified within the subphylum *Agaricomycotina* (*Agaricomycetes*, *Tremellomycetes*, *Dacrymycetes*; (Hibbett et al., 2007)). The resulting NJ tree demonstrated that *C. purpureum* belongs to the order of the *Agaricales* (SM, Fig. S1). The sequence analysis situated *C. purpureum* within the monophyletic *Marasmioid* clade. *C. purpureum* was closely related to *Mycena, Tricholomopsis* and *Hygrocybe* sp.

Gene Ontology classification of predicted gene models

The global functional annotation of the 13739 predicted gene models was based on the analysis of the Gene Ontology (GO). The identification of coding sequences by launching the BLAST tool against the NCBI non-redundant (nr) protein database was carried out prior to the annotation of GO terms with the aid of Blast2GO program. For 99% of the sequences, we obtained a blast hit, and 30% (4016 sequences) of the gene models contained a GO term associated.

Considering the biological process, 480 (12.6%) of the predicted models with a GO were related to *C. purpureum* metabolism, 267 (7%) were implicated in oxidation processes (e.g. decarboxylation, fatty acid beta-oxidation), 181 (4.8%) in transmembrane transport and 116 (3%) in carbohydrate metabolism (e.g. glycolytic process, glyoxylate cycle). Regarding molecular function, oxidoreductase and hydrolase activities occupy the second and fourth position in the number of predicted models: 280 (16%) and 237 (14.7%) respectively. 316 (26.2%) of the predicted proteins with a GO term were associated with the membrane and only 7% were extracellular (Fig. S2).

Hydrolases and transferases were the most abundant predicted proteins found with 31% (250 sequences) and 30% (247 sequences) of all the predicted gene models with a GO term and a defined activity associated respectively, followed by oxidases with 14.4% (155 sequences, Fig. S3).

CAZy genes classification of predicted gene models

The analysis of the predicted gene models of oxidases that participate in lignocellulolysis is shown in Table 3. In the *C. purpureum* genome, 6 DyPs and 10 UPOs (7 complete) coding sequences were found. Regarding the AA1 family, 50 Lacs and 2 ferroxidases (FOXs) sequences were detected. Within the AA2 family composed by class II peroxidases, we distinguished 2 short MnPs, a long MnP, and 4 GPs. In addition, we also identified 3 class I peroxidase protein-coding genes: 2 ascorbate peroxidases (APXs) and 1 cytochrome C peroxidases (CcPs). The number of peroxide-producing predicted proteins corresponding to auxiliary activities AA3 and AA5 was 46, but it fell to 37 when considering the complete protein sequence. In the AA3 category, glucose-methanol-choline oxidoreductases (GMC) such as CDH, glucose oxidase (GOx), AAO and alcohol oxidase (AO) and 12 more copper radical oxidase (CRO) coding sequences were found.

Table 3. Global composition of oxidases encoding genes found in *C. purpureum***,** including UPOs, DyPs, class I peroxidases (CcPs and APXs), AA1 (Lacs and FOXs), class II peroxidases (MnPs and GPs), AA3 (GMCs), AA4 (Vanillyl-alcohol oxidase (VAO)), AA5 (glyoxal oxidases (GLXs), and CROs), AA7 (Glucooligosaccharide oxidase (GOO)), AA8 and AA9 (LPMO)

Proteins	Total	Expressed*
UPOs**	10	5
DyPs**	**6**	**1**
class I peroxidases**	**3**	**2**
CcP	1	1
APX	2	1
AA1	52	26
Lac	50	24
FOXs	2	2
AA2 class II peroxidases (PODs)	7	5
MnPs	3	1
GP	4	4
AA3 GMC	34	16
AA4 VAO	3	0
AA5 GLX and CRO	12	9
AA7 GOO	14	3
AA8 iron reductase domain	8	0
AA9 LPMOs	30	17

*Number of genes expressed in the secretome of the different media tested

** Non CAZy protein families

The analysis of the CAZys occurring in the genome indicated that almost half of them were glycoside hydrolases (GH, 49.5%). The most abundant GHs were GH16 and GH5 with 43 and 25 sequences respectively. Carbohydrate esterases (CEs) represented 21.75% of the predicted CAZy gene models with hydrolytic activities, among them CAZyme CE10 being the most abundant with 59 predicted genes. Glycosyltransferases and polysaccharide lyases 90 and 41 sequences were found (Fig 1).

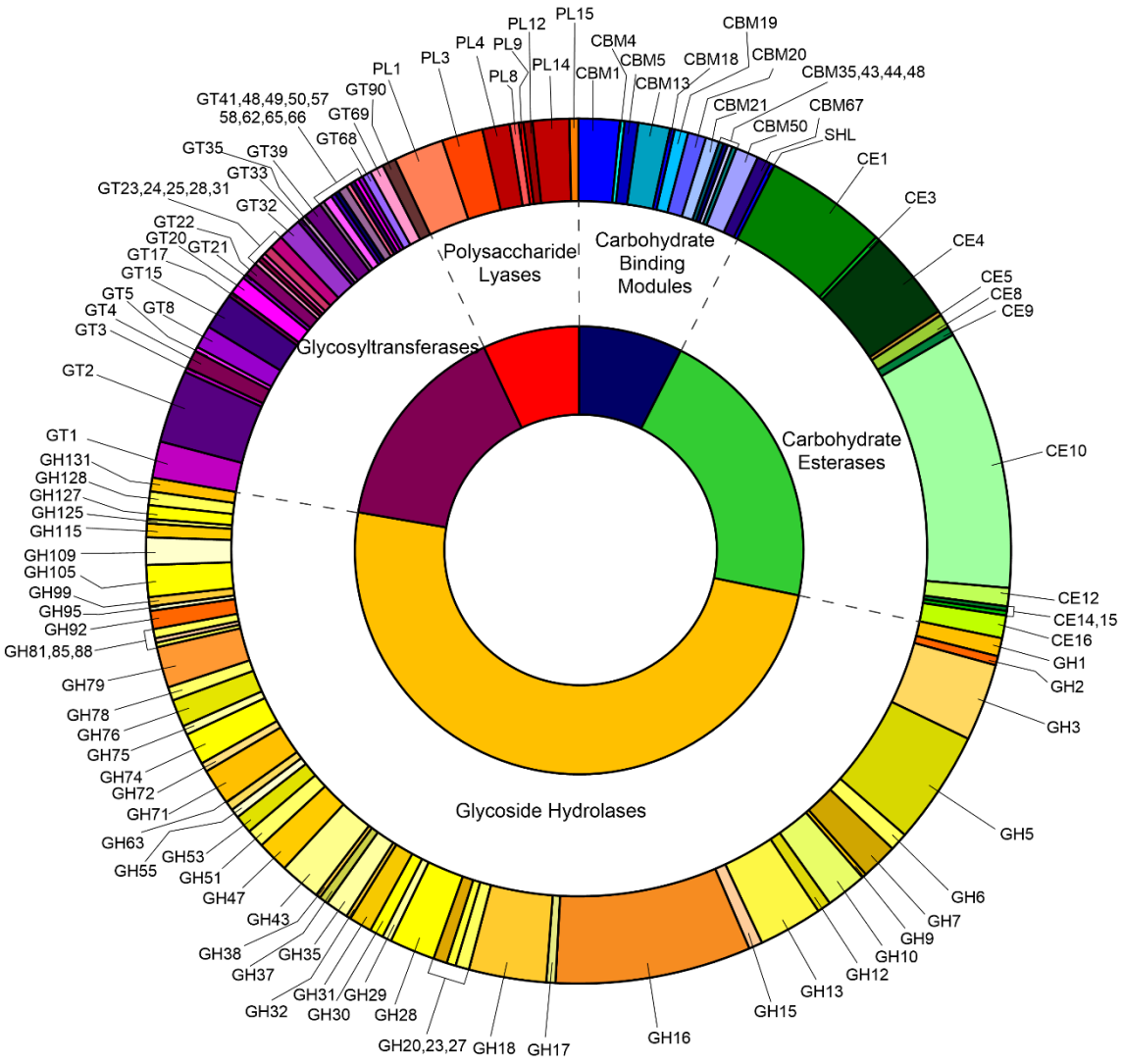

Fig. 1. *C. purpureum* CAZy genes classification (excluding auxiliary activities of oxidoreductases from AA1 to AA9).

We compared the number of oxidoreductases and CAZymes genes that are implicated in wood decay in *C. purpureum* with 12 WRF, 7 BRF and 3 undetermined fungal species that produce wood decay (Table 4). Concerning the enzymes implicated in lignin degradation, the number of PODs genes in *C. purpureum* was below the average for white rot whereas the number of Lacs was the highest of all the fungal species compared. CAZy coding sequences acting on crystalline cellulose are abundant in WRF genomes. The number of genes that belong to the GH6 family was superior to the rest of the white and brown rot species and the number of GH7 and LPMO genes was comparable to white rot fungi species and superior to brown rot species. No remarkable differences were found in the described genes implicated in the secondary metabolisms.

Table 4. Lignocellulose-degrading and secondary metabolism enzymes in wood-decaying fungi. Based on Ryley et al., (2014).

	White rot fungi												Undetermined				Brown rot fungi								
	Tvers	*Galma*	*Aurde*	*Fomme*	*Phchr*	*Cersu*	*Dicsq*	*Punst*	*Phaca*	*Pleos*	*Hetan*	*Stehi*	*Schco*	*Jaaar*	*Botbo*	*Chopur*	*Fompi*	*Pospl*	*Wolco*	*Dacsp*	*Glotr*	*Conpu*	*Serla*		
Crystalline cellulose	23	54	43	6	30	17	17	21	27	33	17	17	5	24	28	**9**	0	0	0	1	1	2	8	CBM1	Carbohydrate-binding module family 1
	1	3	2	2	1	1	1	1	1	3	1	1	1	3	3	**6**	0	0	0	0	0	2	1	GH6	Glycoside hydrolase family 6
	4	8	8	2	9	3	4	5	5	16	1	3	2	5	7	**8**	0	0	0	0	0	2	0	GH7	Glycoside hydrolase family 7
	18	19	20	13	15	9	15	14	11	29	10	16	22	15	32	**30**	4	2	2	0	4	10	5	AA9	Lytic polysaccharide monooxygenase
Lignin	25	10	5	16	15	15	12	10	8	9	8	5	0	0	0	**7**	0	0	0	0	0	0	0	POD	Class II peroxidase
	17	32	30	24	27	18	30	19	37	36	29	40	18	16	21	**21**	15	21	9	8	20	14	8	AA3(2)	GMC oxidoreductase
	9	15	8	4	7	3	9	9	6	16	5	8	2	4	5	**12**	4	3	4	3	2	6	3	AA5(1)	Copper radical oxidase
	7	8	0	10	0	7	11	12	0	11	14	15	2	1	0	**50**	5	2	3	0	4	6	4	AA1(1)	Laccase
	4	6	6	3	3	3	4	4	4	4	3	7	4	2	3	**12**	5	4	5	1	2	5	5	AA3(3)	Alcohol oxidase
	0	9	2	0	0	0	4	1	0	3	3	3	4	1	4	**10**	5	0	0	3	0	0	0	AA7	Glucooligosaccharide oxidase
	2	1	1	1	1	1	1	1	1	1	1	2	0	1	1	**2**	1	1	1	3	1	1	1	AA1(2)	Ferroxidase
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	**1**	0	0	0	0	1	2	2	AA3(1)	Cellobiose dehydrogenase
	1	0	3	0	1	0	0	1	0	0	0	0	0	1	2	**0**	0	0	0	0	1	0	0	AA3(4)	Pyranose oxidase
	1	0	7	1	4	1	1	0	8	0	2	3	4	1	4	**0**	1	1	1	2	0	1	1	AA1*	Multicopper oxidase
	1	3	4	3	4	0	1	2	3	2	2	1	4	3	1	**0**	1	1	1	1	3	2	2	AA6	Benzoquinone reductase
	2	1	2	1	2	2	2	1	2	1	2	2	3	2	2	**8**	0	0	0	0	0	4	4	AA8	Iron reductase domain
	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	**3**	0	0	0	2	3	0	0	AA4	Vanillyl alcohol oxidase
Secondary metabolism	1	2	1	1	1	1	1	1	1	1	1	1	2	1	1	**2**	1	1	1	1	1	1	1	FAS	Fatty acid synthase
	2	1	1	1	1	1	2	1	9	1	0	1	7	0	3	**1**	0	0	0	0	0	1	8	NRPS	Non-ribosomal peptide synthase
	1	1	0	1	0	1	1	1	1	1	1	2	1	1	5	**5**	1	1	1	0	1	1	1	NR-PKS	Non-reducing polyketide synthase
	1	4	1	4	0	2	2	4	0	0	2	5	0	2	1	**0**	6	5	6	1	10	4	9	R-PKS	Reducing polyketide synthase
	5	4	3	13	1	6	9	3	1	7	6	6	2	11	5	**1**	8	6	6	2	6	7	5	TS	Terpene synthase

Abbreviations: Aurde, *Auricularia delicata*; Botbo, *Botryobasidium botryosum*; Cersu, *Ceriporiopsis subvermispora*; Chopur, *Chondrostereum purpureum* , Conpu, *Coniophora puteana*; Dacsp, *Dacryopinax sp*.; Dicsq, *Dichomitus squalens*; Fomme, *Fomitiporia mediterranea*; Fompi, *Fomitopsis pinicola*; Galma, *Galerina marginata*; Glotr, *Gloeophyllum trabeum*; Hetan, *Heterobasidion annosum*; Jaaar, *Jaapia argillacea*; Phaca, *Phanerochaete carnosa*; Phchr, *Phanerochaete chrysosporium*; Pleos, *Pleurotus ostreatus*; Pospl, *Postia placenta*; Punst, *Punctularia strigosozonata*; Schco, *Schizophyllum commune*; Serla, *Serpula lacrymans*; Stehi, *Stereum hirsutum*; Tvers, *Trametes versicolor* and Wolco, *Wolfiporia cocos.*

Protein secretion of *Chondrostereum purpureum*

Ligninolytic activities in SSF and SF

In SSF, MnP and Lac activities were lower than UPO activities during the 7 weeks of incubation either in beech wood (BW) and beech wood–DOR (BWD) cultures. Maximal UPO activity was reached at week 6 of incubation in BWD cultures (0.51 U g^{-1}) whereas it was 5-fold lower in non-supplemented cultures (0.11 U g^{-1}, BW) (Fig. 2).

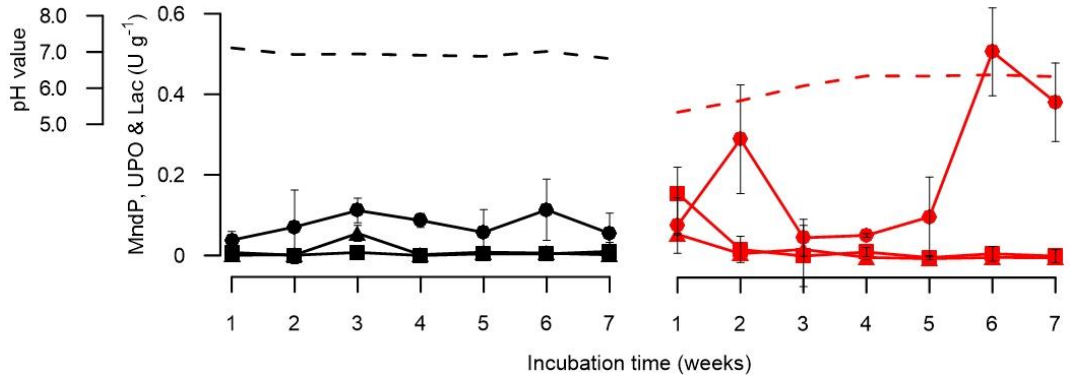

Fig. 2. Time course of extracellular oxidoreductase production by *C. purpureum* during growth on SSF containing beech wood (BW, left) and beech wood supplemented with the olive mill residue-DOR (BWD, right). MnP, (*squares*), UPO (*circles*) and Lac (*triangles*) and pH value (dashed line). The error bars denote standard deviation of three replicates.

MnP, Lac and UPO activities of *C. purpureum* were detectable during liquid cultivation in the six different media tested, even though in Kirk medium (KM), the oxidative activities were moderate (e.g. 2.7 U L^{-1} of UPO at day 7 of incubation). The highest titer of MnP (31.2 U L^{-1}) was detected in soy medium cultures (SM) at day 7 of incubation and it was nearly 10-fold in comparison to KM (3.6 U L^{-1}). The UPO activities proceeded in both media just at a moderate level of approx. 12 U L^{-1}. A decrease in MnP activity was observed when SM was enriched with ADOR or birch wood. However, UPO activity remained unaffected after the addition of the two natural additives to the soy-based media (peaks varied from 14 to 15 U L^{-1} in the three soy-based media tested) and only a marginal increase in UPO activity was observed in KM media supplemented with ADOR and wood (from 1 to 12 U L^{-1} at day 12 of incubation for ADOR-supplemented KM (ASKM) and 0.2 to 12 U L^{-1} at day 10 of incubation for Birch-supplemented Kirk media (BSKM) (Fig. 3).

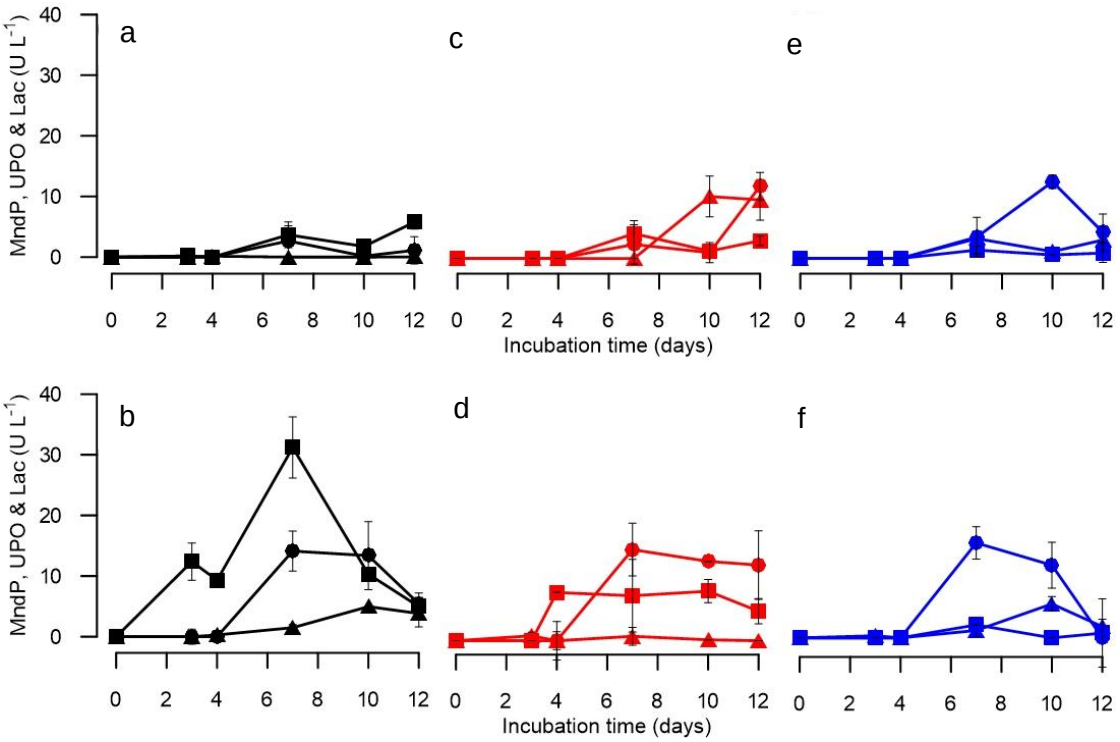

Fig. 3. Time course of extracellular oxidoreductase production by *C. purpureum* in SF cultures after ADOR and birch wood addition. (a) Kirk medium (KM) and (b) soybean meal suspension (SM), (c) KM-ADOR (ASKM), (d) SM-ADOR (ASSM), (e) KM-birch wood (BSKM) and (f) SM-birch wood (BSSM). Manganese dependent peroxidase (MnP, squares), unspecific peroxygenase (UPO, circles) and laccase (Lac, triangles). The error bars denote standard deviation of three replicates.

Secretome analysis

Enzymes implicated in the lignocellulose degradation and therefore classified within the CAZy database, represent a 46 and a 53 of NSAF (%) in BW and BWD respectively (Fig. 4). Within the carbohydrate-active enzyme classification, GH was the major enzyme family in the secretome and "CAZyome". This fact was demonstrated by an abundance of 34 and 32 of NSAF (%) and by a diversity of 112 and 124 different GHs sequences detected in BW and BWD cultures respectively (SM, Table S1). The addition of DOR on *C. purpureum* during SSF seems to stimulate the secretion of PLs, which is 3-fold higher NSAF (%) in BWD than in BW cultures. This stimulating effect of DOR was also observed for CAZy oxidases e.g. for LPMO (2-fold increase of the relative abundance in BWD than in BW), and non-CAZy hydrolases such as peptidases and for UPOs. Furthermore, the latter enzyme is only secreted when DOR is added (0.12 of NSAF (%) in BWD).

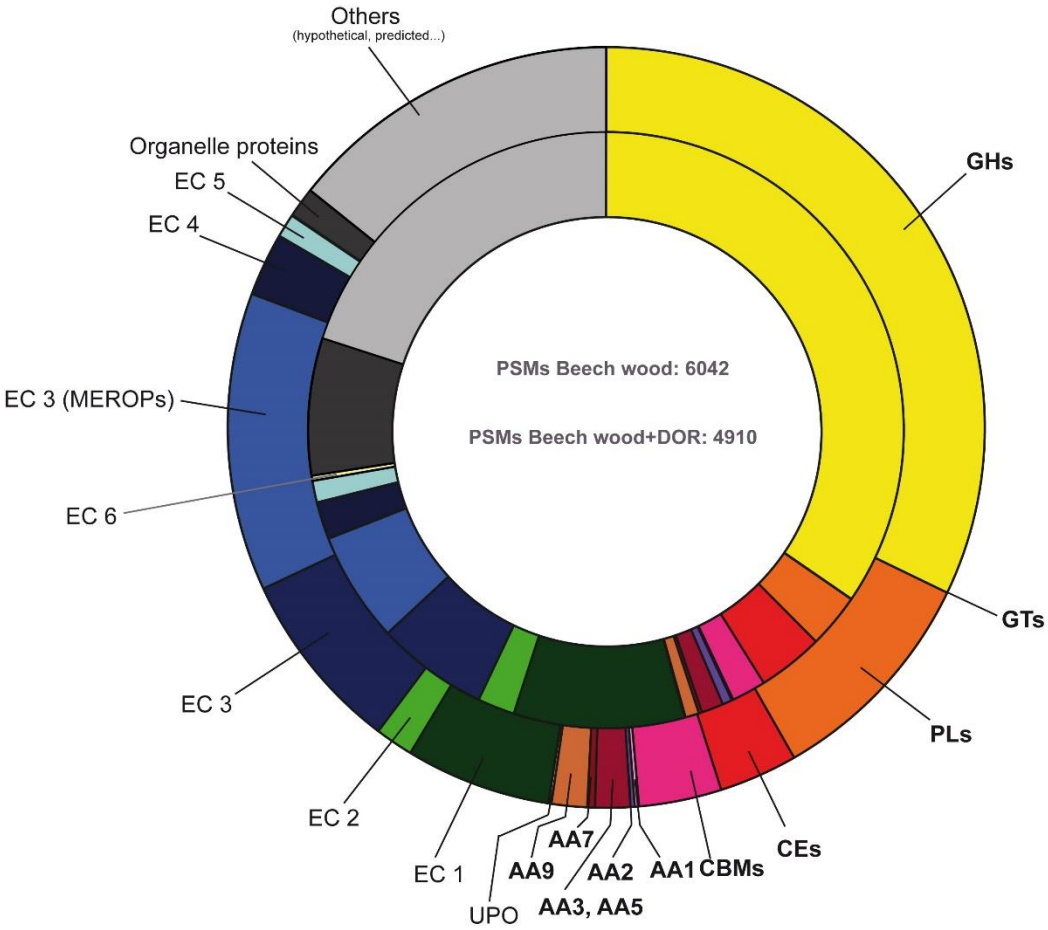

Fig. 4. Relative protein abundance (normalized spectral abundance factor, NSAF (%)) of secreted proteins from *C. purpureum* during growth on SSF: BW (inner ring) and BWD (outer ring). CAZy activities are represented in bold letters. Organelle proteins include ribosomal, peroxisomal and vacuolar proteins without defined catalytic properties.

No distinct differences were found in the protein secretion profile of the three Kirk-media based cultures with and without additives (Fig. 5), with the exception of UPO and Lac secretion, which were 2 and 3-fold higher in ASKM than in KM, (0.3 and 0.08 of NSAF (%) for UPOs in ASKM and KM and 2.8 and 1% of Lacs in ASKM and KM respectively). The protein secretion profile of *C. purpureum* was in a similar pattern and CAZy classified enzymes were represented by approx. ~35% NSAF in all three KM media (KM, ASKM and BSKM) (SM, Table S2). GHs again were the predominant proteins representing a 29, 26 and 23% of the NASF in KM, ASKM and BSKM respectively.

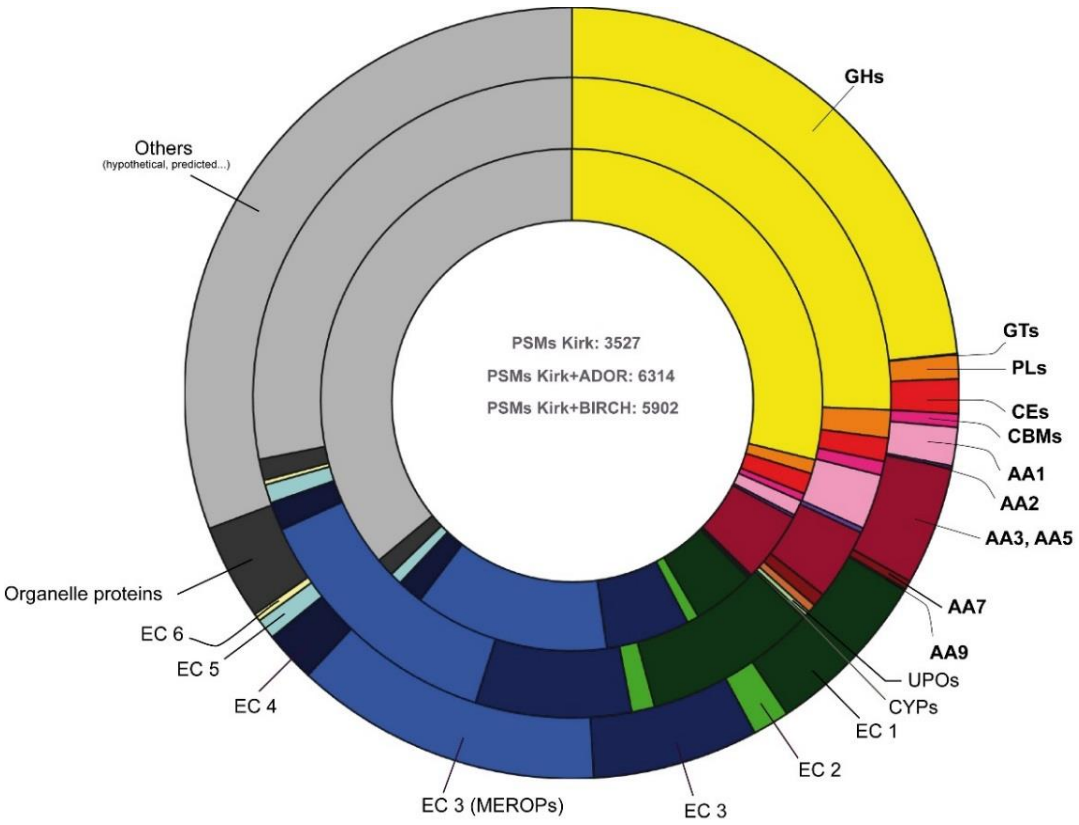

Fig. 5. Relative abundance (NSAF (%)) of identified proteins according to enzymatic activities secreted by *C. purpureum* under SF: KM (inner ring) ASKM, (middle ring) and BSKM (outer ring). CAZy activities are represented in bold letters.

However, these numbers are different from those obtained in SSF in lignocellulose-based solid media (Fig. 4) or in SF in soy-based media (Fig. 6).

The most abundant protein family secreted by *C. purpureum* in soy-based media was GH, as was already observed for Kirk-media based cultures (23-29%). Nevertheless, in SM and BSSM, these CAZy groups reached a remarkable value of 44% of the overall set of secreted proteins (Fig. 6). A GH6 (g13545) was the most abundant protein representing 12, 8 and 13 NSAF(%) (SM, Table S2) which is also indicated in the PCA bi-plot (Fig. 8). Probably, the protein is closely related to soybean-based components that are contained in the media. The data showed that also in soy media, the ADOR addition marginally enhances the secretion of UPOs. The NSAF was 0.15% in ASSM but only 0.04 and 0.03% in SM and BSSM, respectively (SM, Table S2). Furthermore, there was a remarkable increase in peptidase secretion and relative abundance when cultures were supplemented with ADOR (8 and 15 of NSAF (%) in SM and ASSM respectively). Two aminopeptidases M28 (g1529 and g13177) and two peptidyl-lys metalloendopeptidases M35 (g1048 and g5146) were the most abundant proteins in ASKM with a 3 and 4.6 of NSAF (%).

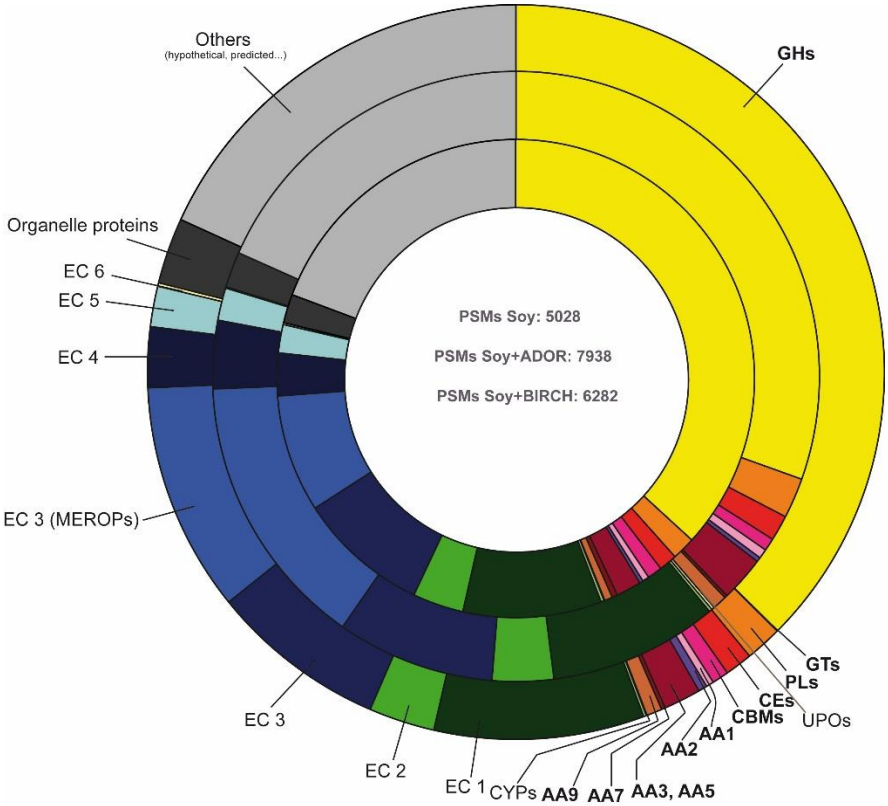

Fig. 6. Relative abundance (NSAF (%)) of identified proteins according to enzymatic activities secreted by *C. purpureum* under SF: SM (inner ring), ASSM (middle ring) and BSSM (outer ring). CAZy activities are represented in bold letters.

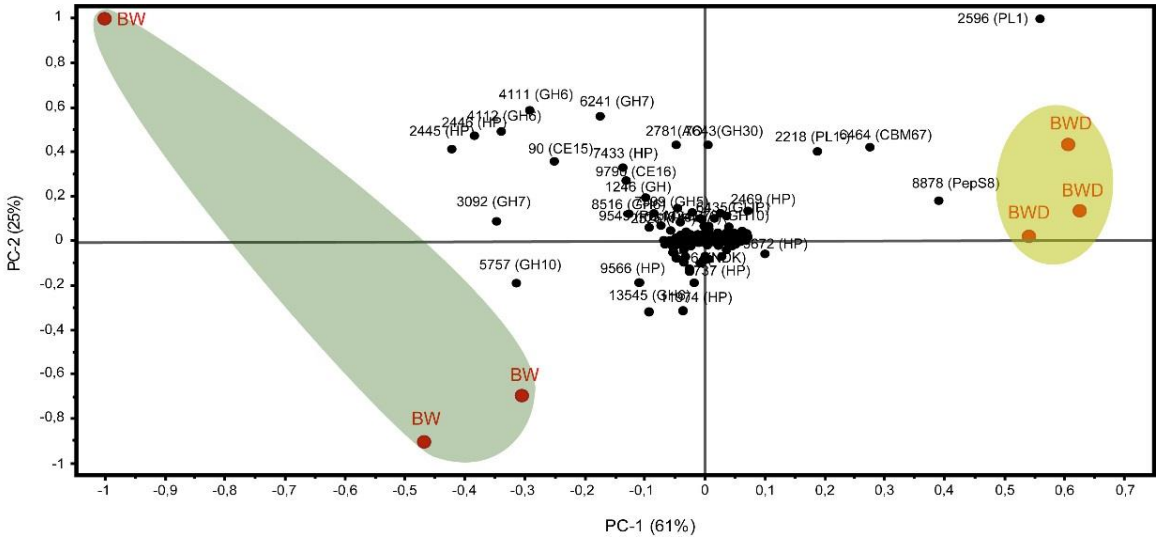

Fig. 7. Principal component analysis (PCA) bi-plot of *C. purpureum* secretome on SSF using NIPALS algorithms; BW and BWD loading replicates are represented in red.

In SSF, the PCA bi-plot shows that the two main components (PC1 and PC2) are responsible for 86% (Fig. 7) and in SF for 90% of the variance (35 and 55%, respectively, Fig. 8). Fig. 8 shows that the samples are clustered in 6 groups: KM, ASKM, BSKM, SM, ASSM and BSSM. Each one is composed by the three replicates of each treatment. The three categories containing soybean-based media replicates are clearly distinguishable from those of Kirk-media based cultures. Peptidase g8878 is closely related to ASKM media and the hypothetical protein without an associate function (HP) g12046 to KM. Generally speaking, GHs are closer to clusters composed by soy media (e.g. GH6 g8516 and GH7 g3092).

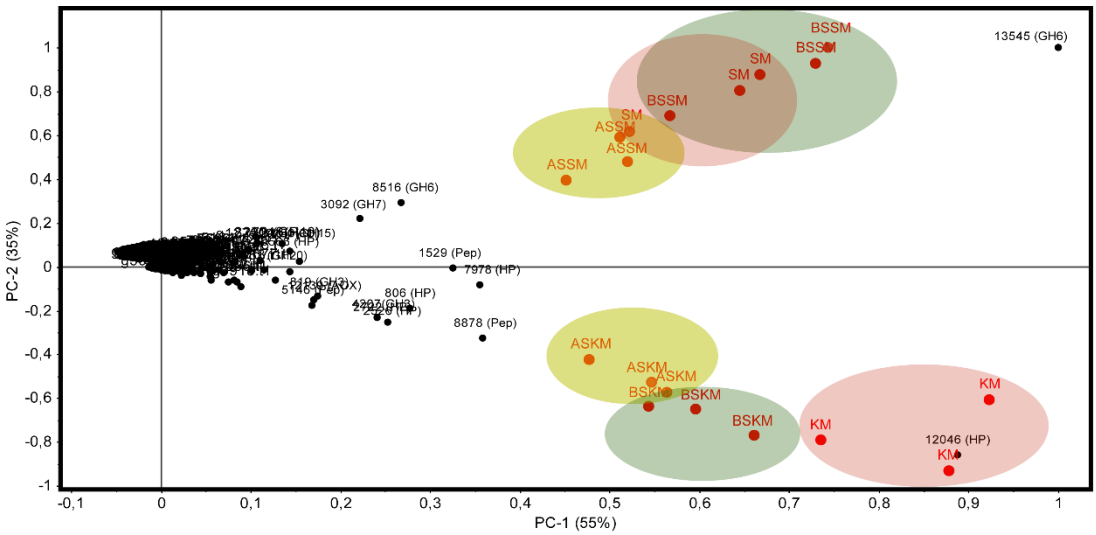

Fig. 8. Principal component analysis (PCA) bi-plot of *C. purpureum* secretome under SF using NIPALS algorithms; SM, ASSM, BSSM, KM, ASKM and BSKM loading replicates are represented in red.

The heat map represents 67 GHs and 53 further CAZymes as well as AAs. Only proteins which showed significant differences between culture media are included. While 30 of the 67 GHs were significantly increased in the soybean-containing media, 37 were stronger expressed in the Kirk-media based cultures. For example, GH5 proteins were more abundant in soybean-based media than in the three KMs, with the exception of the GH5 gene variants g6838 and g10169 (Fig. 9). Lacs were significantly more abundant in the supplemented and control KM than in the soy media, especially in ASKM, where Lacs were strongly overexpressed. A total of 15 LPMO proteins were found in the secretome, none was present in KM, whereas all of them were expressed in ASSM. 6, 1, 12 and 13 were found in ASKM, BSKM, SM and BSSM, respectively (SM, Table S2). 7 of these LPMO showed statistically significant differences between treatments, being up-regulated in soybean-containing media (Fig. 9).

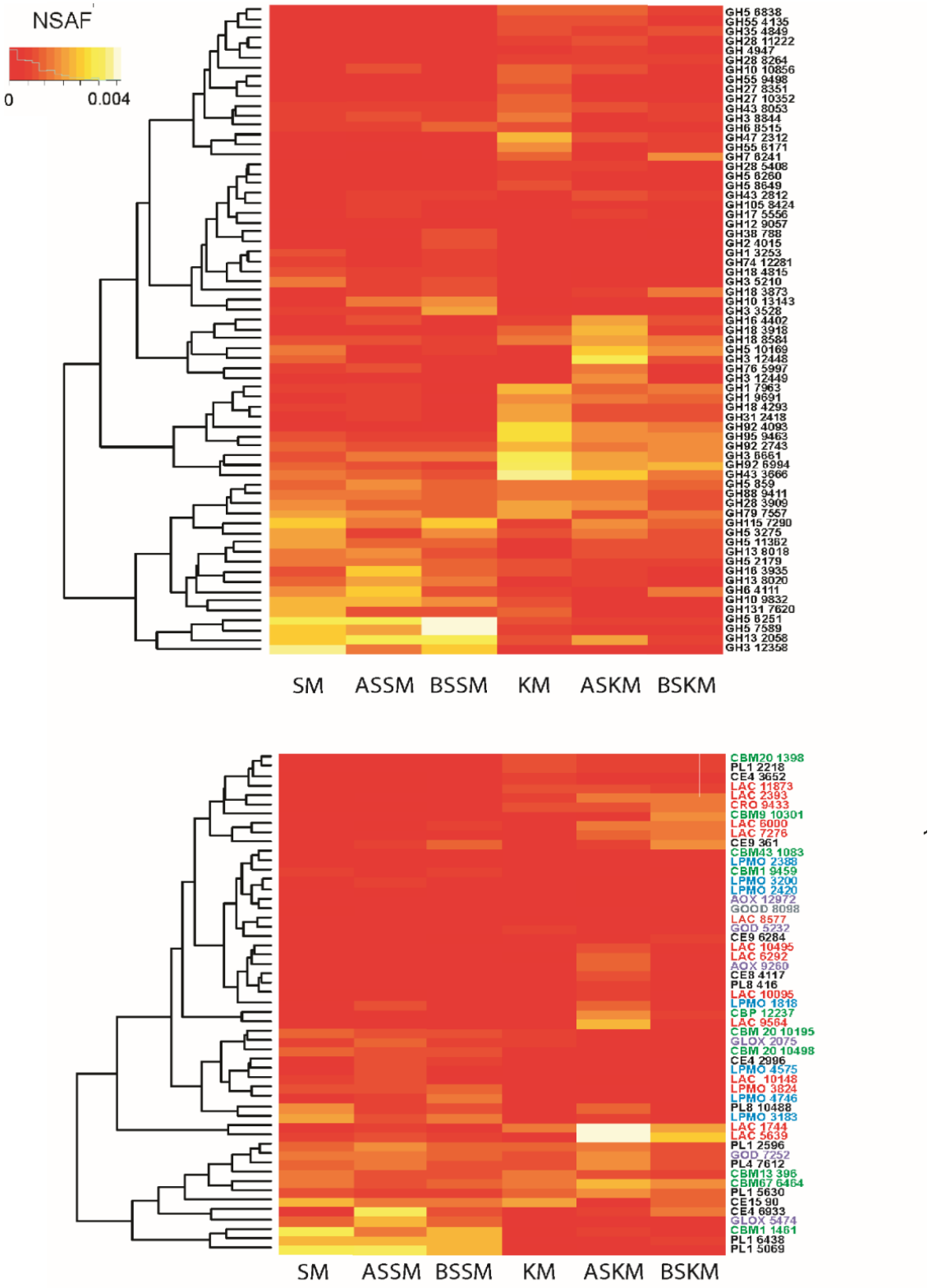

Fig. 9. Heat map of the secreted CAZymes of *C. purpureum* which showed significant differences during SF in KM, SM, ASKM, ASSM, BSKM and BSSM. Differences between treatments were corroborated with Hotelling's T2 test. Abundance was represented by the Normalized Spectral Abundance Factor (NSAF). GHs are represented in the upper figure, while the rest of CAZymes, including AAs, are represented in the lower figure

Discussion

Genome assembly and size

The average genome size of fungi is 37.7 Mbp overall and 49.9 Mbp for basidiomycetes (Kullman et al., 2005). The genome size of *C. purpureum* lies between these averages and is similar to the genome sizes of some of the most well-known WRF, such as the polyporal *Pycnoporus cinnabarinus*, *Phanerochaete chrysosporium,* and the agarical *Agaricus bisporus,* with genome sizes of 33.67, 30 and 30.5 Mbp, respectively (Martínez et al., 2004; Morin et al., 2012; Levasseur et al., 2014).

Downstream analyses in NGS approaches are not always immune to mistakes such as misalignments of short reads or difficulties in counting homopolymer stretches, which can result in insertion or deletion errors (Yang et al., 2013; Robasky et al., 2014). It is therefore indispensable to perform a quality assessment of the draft genome. However, this evaluation is complicated when comparing genomes with different sizes and assemblies that are performed with different NGS technologies producing different read lengths and read counts (Alkan et al., 2011). Coverage and N50 are relevant within the key metrics, especially in genomes that have been sequenced to high depths by short read technologies (e.g. by Illumina- Solexa, 454 pyrosequencing or Ion Torrent PGM). The coverage of the draft genome of *C. purpureum* (15x) is higher than that described for *P. chrysosporium* (10.5x) (Martinez et al., 2004) and half of that reported for *P. cinnabarinus* (31x) (Levasseur et al., 2014). Higher coverages do not necessarily imply better assemblies when comparing different species and sequencing methods. In fact, the draft genome of *P. chrysosporium*, which was sequenced by a Sanger method, is considered a high quality genome (Martinez et al., 2004). Longer sequencing reads are derived from larger insert libraries and can be assembled using improved mature assembly algorithms (Sims et al., 2014). In addition, one of the major weaknesses of the majority of *de novo* genome assemblers is that they consider the tandem repeat DNA sequences as a single non-repetitive region (Pop and Salzberg, 2008). Therefore, the coverage of these regions is artificially higher than the rest of the single copy genes. The N50 length of the *C. purpureum* genome assembly indicates that a lot of information is contained in medium and small contigs. CEGMA analysis (Parra et al., 2007) revealed a high completeness since 229 complete core eukaryotic genes were found out of the 248 considered by CEGMA. This completeness rate rises to 96% when partial matches are considered. Quantifying the quality of a genome draft can be somewhat subjective since comparisons to other genomes are not always possible and since it is necessary to establish artificial cut-offs for some parameters based on the objective of the research.

Phylogenetic position

The vast majority of fungi belong to the subkingdom *Dikarya*, which consists of the two monophyletic phyla *Ascomycota* and *Basidiomycota* (James et al., 2006). Whereas there is a general agreement on the position of the three *Basidiomycota* major classes (*Agaricomycotina*, *Pucciniomycotina* and *Ustilaginomycotina*), there is still some ambiguity concerning the position of *Wallemiomycetes* and *Entorrhizomycetes* (Hibbett et al., 2007). Besides, it is difficult to resolve into which lower clade (e.g. order, family or genus) some species should be included based on rDNA studies alone (18S, 25S, and 5.8S) (Matheny et al., 2007). This is the case of *C. purpureum*, which has been included in the order *Polyporales* by some studies (Hamberg et al., 2014) and by the well-established nomenclatural database Index Fungorum (http://www.IndexFungorum.org). Furthermore, the genus *Stereum*, which seems to be related to the genus *Chondrostereum*, belongs to the order *Russulales* (Martin et al., 2015). The phylogenetic tree in Figure S1, which is based on both rDNA sequences and the protein coding data *rpb2* and *tef1*, shows that the sequenced *C. purpureum* belongs to the order *Agaricales* and, more specifically, to the *Marasmioid* clade, a fact corroborated by previous phylogenetic studies (Matheny et al., 2006). The *Marasmioid* clade is a taxonomically diverse group in which seven monophyletic families and one clade are included: the *Omphalotaceae*, *Marasmiaceae*, the hydropoid clade, *Cyphellaceae*, *Physalacriaceae*, *Lachnellaceae* and *Schizophyllaceae* (Matheny et al., 2006). The majority of fungal species contained in this clade are leaf and litter decomposers. The alignment performed with the sequences of *C. purpureum* obtained from the sequenced genome suggests that it is closely related to species such as *Nia vibrissa* (*Lachnellaceae*) or the formerly known *Mycena scabripes* (*Mycopan scabripes*, *Cyphellaceae*). The current phylogenetic analysis alone cannot classify the sequenced fungus into a family; a specific analysis of the species within the *Agaricales* clade would be necessary to establish the family to which it belongs. However, some studies suggest that it belongs to the *Cyphellaceae* family (Matheny et al., 2007; Ranadive et al., 2013).

Degradation of lignocellulose

C. purpureum is a well-known plant parasite (De Jong, 2000) with the ability to efficiently colonize hardwoods (Becker et al., 2005; Mirić et al., 2012). Because of this behaviour, it was expected to be an efficient lignin modifying enzyme producer. However, the secretion of PODs and Lacs by this fungus during SSF and SF in different media was moderate in comparison to other well-known hyper-secretory species such as *P. chrysosporium* or *Trametes versicolor* (Kapich et al., 2004; Carabajal et al., 2013). It is therefore interesting to determine which kind of wood degrader it is. We addressed this question by using a genomic approach which included the analysis of genes, encoding

for lignocellulolytic enzymes and the protein profile in different lignocellulosic and synthetic media.

Lacs (AA1 family) may participate in the transformation of lignin moieties (Baldrian, 2006). An overwhelming number of Lac genes in the genome of *C. purpureum* (50) have not been described in a WRF so far. In addition, a large number of them were also expressed in SSF and SF. This is in contrast to the small number of Lac sequences found in BRF genomes. For instance, *Gloeophyllum trabeum* and *Wolfiporia cocos* contain just 3 Lac genes each (Floudas et al., 2012). Olive mill residues (e.g. water-soluble parts of the solid DOR) clearly stimulate Lac secretion in *Funalia floccosa* (formerly *Coriolopsis rigida*) in ADOR-supplemented cultures, as has previously been reported (Díaz et al., 2010). The synthesis and secretion of Lacs are influenced by nutrient levels, culture conditions, developmental stage and the addition of a wide range of inducers to the media (such as metal ions, like Cu, and aromatic compounds related to lignin) (Piscitelli et al., 2011). The phenolic compounds present in olive mill residues (e.g. tannic acids or phenolic acids such as caffeic acid, (Sampedro et al., 2004)) might be among the aromatic compounds responsible for Lac induction observed in our studies (Baldrian, 2006).

According to the traditional classification of WRF and BRF, the former are able to degrade all the plant cell wall components, whereas BRF mainly attack celluloses and hemicelluloses and produce a partial depolymerization of lignin (Pandey and Pitman, 2003). Nevertheless, certain fungi, such as *Jaapia argillacea* or *Botryobasidium botryosum,* lack PODs but are able to degrade all plant cell wall components. Therefore, this functional classification does not include all the wood-rotting species and consequently, species with PODs genes should, strictly speaking, be considered as white rot species but the ability to degrade lignin should not be attributed exclusively to them (Riley et al., 2014). Thus, we can consider *C. purpureum* as a real WRF, since it possesses 7 POD genes, 5 of which were expressed in the different media tested, although this number of POD genes is lower than in other typical WRF like *T. versicolor* or *Fomitoporia mediterranea* (39 and 33 predicted PODs proteins respectively) (Floudas et al., 2012). A single short MnP (5041) was secreted, but only in lignocellulosic media (ASKM or soybean-based media). Short-type hybrid MnPs show some homology with VP, maintaining the Mn^{+2}-binding sites (2 Glu, 1 Asp) but without the tryptophan (Trp-164) (Hildén et al., 2005; Hofrichter et al., 2010). PODs are considered AA2 according to the CAZy classification (Levasseur et al., 2013).

The effects of lignocellulosic substrates on UPO secretion have previously not been studied in great detail. A closer look at the data indicates that DOR and its aqueous extract (ADOR) were able to stimulate UPO secretion whereas either beech or birch

wood supplemented media were not. These UPOs could act on the phenolic compounds that emerge as the result of aromatic peroxygenation and subsequently, due to the peroxidative activity, transform them into phenoxyl radicals, which may couple and polymerize (Hofrichter and Ullrich, 2014).

Peroxide-producing enzymes are classified into different CAZy families (Levasseur et al., 2013). The AA3 family is composed of GMC oxidoreductases (e.g. CDH, AAO, GOX), whereas the AA5 family is composed of GLXs and galactose oxidases (GAO), which efficiently catalyse the oxidation of diverse aliphatic alcohols (Yin et al., 2015). GLX genes appear to have emerged early in the diversification of *Agaricomycetes*, coinciding with the expansion of POD genes. The majority of BRF do not possess GLX genes and their presence in WRF suggests arelation with lignin degradation. However, their expression in WRF does not always concomitantly occurs with PODs secretion (Kohler et al., 2015). 34 of them, in the draft genome of *C. purpureum,* were classified in AA group 3 (16 expressed as proteins) and 12 in AA family 5 (9 expressed proteins). The number of AA3 gene copies is slightly higher than in other white rot species (e.g. 37 in *Auricularia delicate* (Floudas et al., 2012)) while the number of AA5 genes is in the average range for agaric species (e.g. 14 in *Galerina marginata* (Kohler et al., 2015)). No differences among supplemented and non-supplemented cultures were found, although these proteins were relatively more abundant in Kirk- than in soybean-based media. They therefore obviously follow a different regulation mechanism than PODs. The peroxide produced by proteins within the AA3 and AA5 families can be thought of to act as a co-substrate for ligninolytic peroxidases (e.g. in the case of WRF; (Martínez et al., 2005, Ferreira et al., 2009, Hernández-Ortega et al., 2012)) or to be be reduced by ferrous iron to hydroxyl radicals during the so-called Fenton-type reaction. This highly reactive radical subsequently acts as a strong diffusible oxidizer initiating cellulose depolymerization (for BRF) (Baldrian and Valášková, 2008; Gomez-Toribio et al., 2009).

The (hemi)-cellulolytic system of *C. purpureum* identified by the genome data includes 6 GH6 and 8 GH7 coding genes (all of them expressed). Proteins belonging to the GH6 family were likely up-regulated in soy-based media, probably due to a higher cellulose content, while GH7 was equally expressed among all the media tested. GH6 and GH7 are related to the degradation of crystalline cellulose (Lynd et al., 2002; Baldrian and Valášková, 2008). These families include cellobiohydrolases and endoglucanases, which act on non-reducing (e.g. cellobiohydrolases, endoglucanase within GH6) and reducing ends (reducing end-acting cellobiohydrolase, endo-β-1,3-1,4-glucanase in the GH7) of the polysaccharide chain (Floudas et al., 2012; Riley et al., 2014). *Coprinopsis cinerea*, which appears to be closely related to *C. purpureum,*

possesses 5 and 6 GH6 and GH7 gene copies respectively, which is an average number of genes for white rot species, whereas the majority of brown rot species genomes do not contain any member of these CAZy families (Kolher et al, 2015).

Proteins belonging to the GH families 10, 30, 51, 74 and 43 were found among the GHs responsible for hemicellulose degradation. These genes encode for classical hemicellulolytic enzymes of WRF, whereas in BRF, the number of gene copies is lower and some representatives like e.g. GH74 do not exist, obviously due to a loss of enzymes during the evolutionary differentiation of both wood-rotting fungal groups (Floudas et al, 2012; Nagy et al., 2015).

Further hydrolytic enzymes found in the *Chondrostereum* genome belong to the GH families 28, 53, 78, 88, 105, the PL groups 1, 3 4 and to the CE class 8. It is assumed that these enzymes are related to pectin degradation (Riley et al., 2014), a process probably part of the invasive step of fungal plant pathogens (e.g. for *Botrytis cinerea* (Ten Have et al., 1998) and *Fusarium oxysporum* (Di Pietro and Roncero, 1998). The endopolygalacturonase (EndoPGI) belonging to GH 28 was the only enzyme identified so far as the probable responsible for the silver-leaf disease caused by *C. purpureum* (Simpson et al., 2001, Ogawa et al., 2009, described as "*Stereum*" *purpureum*; Hamada et al., 2015).

Among the families with more genes present in the genome of *C. purpureum* are GH5 and CE10. GH5 is a large protein family which contains a wide range of enzymes acting on β-linked oligo- and polysaccharides (Aspeborg et al., 2012). 17 of the 25 genes found in the genome were expressed predominantly in soy-based media. A number of 57 genes belonging to the CE10 family were detected, and nearly half of them expressed. However, according to recent updates in the CAZy database, the members of this family are esterases acting on a broad range of non-carbohydrate substrates (e.g. sterols, cocain, vitamin A, esters, Lombard et al., 2014).

Thus far, LPMOs (formerly GH 61) are thought to be important enzymes for the decomposition of recalcitrant biological macromolecules such as plant cell walls and chitin polymers by oxidizing the glycosidic bonds in the cellulose (Busk and Lange, 2015). They are included within class AA 9, 10 and 11 in the current CAZy classification. On the other hand, some studies suggest that due to the large sequence diversity, these proteins may also oxidize substrates other than cellulose (e.g. phenolic-related compounds, Li et al., 2012). In the genome of *C. purpureum*, 30 LPMOs-encoding genes were identified, which seems to be a comparably high number. 17 of them were expressed and induced during SF and SSF by olive mill wastes. No LPMOs were expressed in KM, while 6 were expressed in ASKM. In soy-based media, 12 were expressed in SM and 15 in ASSM. Although the number of genes in *C. purpureum* is

unusually high in comparison to the majority of WRF (e.g. 18 in *T. versicolor*, 15 in *P. chrysosporium*), it is similar to *C. cinerea* (35). The high number of LPMOs secreted in ADOR and DOR enriched cultures reflect their importance and probably role in the degradation of cellulose, chitin and related poly- and oligosaccharides (Busk and Lange, 2015) and perhaps further compounds such as lignin derivatives, present under natural conditions in the environment of the fungal organism.

Conclusions

C. purpureum is a saprophyte with a high tendency to a certain pathogenicity towards many hardwood species. To date, its potential lignocellulolytic activity has not been proven and controversies existed regarding its phylogenetic position. As a result of a genomic and a proteomic approach, we have shown that *C. purpureum* is a member of the order *Agaricales* (of the *Marasmioid* clade). In addition, we claim that this fungus is able to secrete a broad number of ligninolytic and cellulolytic enzymes. Our results show that *C. purpureum* has a moderate UPO and low Lac as well as MnP secretion during solid-state and liquid cultivation in plant-based and synthetically defined media, which was stimulated with the aid of solid olive mill residues and their water-soluble extractives.

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GENERAL

DISCUSSION

Dry olive mill residues: properties, comparison with other agro-wastes and potential role as a LME elicitors

Lignocelluloses are the most abundant aromatic biopolymers and the major components of plant cell walls. Their degradation represents a major aspect of the global carbon cycle because lignin, as well as celluloses and hemicelluloses, which are protected by lignin from the enzymatic attack, contains a large proportion of renewable carbons. Since some *Agaricomycetes* are capable of completely degrading these polymers (Hammel, 1997), many potential applications for these lignocellulose-degrading organisms and their extracellular enzymes have been proposed, such as the treatment of xenobiotic and toxic compounds, their utilization in the pulp and paper industry or their application in biorefinery processes to produce platform chemicals and biofuel, for example (Kuhad and Singh, 1993; Howard et al., 2004; Jørgensen et al., 2007). Due to its great potential for biotechnological applications, the enzyme-based bio-conversion of lignocellulosic materials has been extensively studied in the past, the main focus being the enhancement of lignocellulolytic biocatalysts (Keyser et al., 1978; Palmieri et al., 2000; Trupkin et al., 2003).

In the present thesis, we suggest an alternative approach to the valorization of Dry Olive Residue (DOR), a high-value agroindustrial by-product, rich in phenolic acids, that results from a two-phase olive oil extraction system (Alburquerque et al., 2004). Many alternatives for DOR valorization have been proposed, such as its use as an organic amendment thanks to its high content in organic matter and different important minerals from an agronomical point of view (e.g. K, P and Ca (López-Piñeiro et al., 2011)), or the utilization of some of its ingredients in cosmetics and pharmaceutical products (Vlyssides et al., 2004).

We started this study addressing the question of whether DOR could act as an appropriate enhancer (“elicitor”) of lignocellulolytic enzymes of fungal origin and, if so, which of these biotechnologically valuable enzymes are induced by it. The search for enzyme inducing components is not only a basic research question; it is also interesting from a biotechnological point of view, especially for industrially relevant oxidative biocatalysts, which are difficult to produce, even by molecular methods.

Not much is currently known about the inducing factors of fungal ligninolytic oxidoreductases such as laccases (Lac) and heme peroxidases (Scheel et al., 2000; Arora and Sharma 2010; Pecyna, 2016 (Diss.)). It has been found that a nitrogen-limited environment is required to trigger the lignin modifying enzyme (LME) secretion (mainly for classic ligninolytic fungal peroxidases) under natural conditions (e.g. when vegetative

growth comes to an end and secondary metabolism starts (Hatakka, 1994; Ten Have and Teunissen, 2001)), and that processes can be imitated under lab-scale conditions by using the well-known N-deficient Kirk media supplemented by Mn^{2+}-ions (Kirk and Farrell, 1987; Hofrichter et al., 1999). Notably, the addition of Mn^{2+}-ions or lignocelluloses (e.g. wheat straw or wood) to nitrogen-limited media is a reliable method to induce MnP production in wood-degrading fungi (Kaal et al., 1993; Kaal et al., 1995). These culture conditions can be used for MnP production even in a large-scale setting (Mester and Field, 1997). Other studies imply an important inducing effect achieved by the addition of nitrogen-rich ingredients like soybean meal (Ullrich et al., 2004; Anh et al., 2007), peptones, alfalfa (Anh et al., 2007; Gröbe et al., 2011) or by adding the storage proteins glycinin and β-conglycinin directly which was demonstrated for the biotechnologically relevant fungal peroxygenases (Pecyna 2016 (Diss.) and unpublished results). Further studies concerning induction effects on fungal oxidoreductases focused on other complex plant-based substrates like eco-tomato juice media (Ullrich et al., 2005; Michniewicz et al., 2006) or on the addition of natural compounds and lignocellulosic materials like various straw, leaf or hard wood sorts (Schlosser et al., 1997; Kapich et al., 2004).

Several reports suggest that phenol-rich DOR could also be a suitable component for stimulating fungal growth and the secretion of lignin modifying enzymes like MnP and Lac (Díaz et al., 2010; Sampedro et al., 2012). DOR possesses a high rate of C/N and a low concentration of soluble sugars, conditions which can be required for LME synthesis (Keyser et al., 1978, Lechner and Papinutti, 2006). A valorization of DOR was performed with the intensively studied polyporal fungus *Trametes versicolor* during solid state fermentation (SSF) of soy and barley grain cultures supplemented with DOR and grape pomace and stalks (GPS) (cf. Chapter 1, p. 85). Moreover, the aqueous extract of DOR (ADOR) and GPS (AGPS) was investigated during liquid submerged fermentation (SF) in soybean meal, barley meal and eco-tomato juice media (cf. Chapter 1, p. 81). Among the broad set of lignocellulolytic enzymes secreted, Lac was clearly stimulated when DOR or ADOR were added either in SSF or SF, respectively. This fact could be also observed for *Funalia floccosa* (formerly *Coriolopsis rigida)* in ADOR-supplemented cultures in which Lac activity was stimulated approx. 10-fold from ~100 U L^{-1} to ~1000 U L^{-1} (Díaz et al., 2010). Previously published studies have also demonstrated an enhancement of Lac secretion in complex liquid media such as eco-tomato juice in *Xylaria polymorpha*, *Agrocybe blazei* and *Cerrena unicolor* (Ullrich et al., 2005; Michniewicz et al., 2006; Liers et al., 2007) or class II peroxidases (PODs) in peptone media in *Pleurotus eryngii* and *P. ostreatus* (Ruiz-Dueñas et al., 1999; Kamitsuji

et al., 2004). The stimulation of ligninolytic activity could be attributed to the phenolic compounds, e.g. flavonoids or tannic acids, which are present in plant extracts-based media (Carbajo et al., 2002).

These results could indicate that the expression and secretion of fungal Lacs were influenced by nutrient levels (especially N and C) apart from culture conditions, developmental stage and the addition of several probable inducers to the culture media such as metal ions (e.g. copper, (Ullrich et al., 2005; Michniewicz et al., 2006)) and aromatic compounds related to lignin (e.g. veratryl alcohol, guaiacol, xylidine, (Barbosa et al., 1996; D'Souza et al., 2006; Liers et al., 2007)) with variations among different fungal species (Piscitelli et al., 2011).

Despite being moderate, the enhancement of peroxidase activities of *Trametes* induced by DOR were also observed for manganese peroxidase (MnP) and lignin peroxidase (LiP). By contrast, the intensively studied agro-industrial by-product GPS did not show to this clear peroxidase stimulation. DOR and GPS contain insignificant amounts of Mn ions (16 and 12 ppm respectively (cf. Chapter 1, S2, p. 224)) when compared to other LME-stimulating media (Kaal et al., 1995). Winery by-products possess a high content of various sugars (e.g. glucose, xylose) which are renewable (Moldes et al., 2007). The enzymatic treatment could therefore be an inexpensive alternative for the production of biodiesel (Fernández et al., 2010; Mateo and Maicas, 2015). It is, however, a poor LME enhancer, which might due to the low penolic acid content of 6 mg g^{-1} (cf. Chapter 1, S2, pp. 224). Most studies concerning fungal fermentation of agroindustrial lignocellulosic wastes (e.g. wheat straw, peanut shells) focus on residue bioconversion rather than on enzymatic induction and secretion enhancement (Moyson and Verachtert, 1991; Philippoussis et al., 2001).

Further studies on DOR bioremediation and its role in the enhancement of LME activities during SSF were performed with a set of fungi like the auricularial *Auricularia auricula-judae*, the polyporal *Bjerkandera adusta* and the agaric fungi *Coprinellus radians*, *Agrocybe aegerita, Marasmius alliaceus* and *Chondrostereum purpureum*. The objective of these experiments was not only to study the secretion of classical PODs like MnP, LiP and versatile peroxidase (VP) and Lacs, but also to learn more about the expression behaviour of the novel heme peroxidases like DyP-type peroxidases (DyP) and the unspecific peroxygenases (UPO). The time course of the DyP activity of *A. auricula-judae* and *M. alliaceus* was not comparable due to a marked shift in their secretion profiles after DOR addition. The experimental results were rather dissimilar, and no general conclusion could be drawn about whether the phenolic elicitor used could stimulate the DyPs secretion or not. The natural inducing components of DyPs as well

as their natural function (e.g. their probable degradative capabilities) are not yet fully understood. Larger amounts of these enzymes are reportedly produced in liquid cultures, e.g. in the presence of tomato juice (Liers et al., 2010; Liers et al., 2013) which is also a phenol rich medium. Nevertheless, tentative evidence indicates a potential role of DyPs in natural carbon turnover. Kellner et al. (2014) reported the detection of transcripts in soils, suggesting a role of DyPs in the transformation of soil organic matter. The presence of DyPs in cultures added with lignocellulosic and agro-waste ingredients has also been shown by using wheat straw and eucalypt pulp (Salvachúa et al., 2013; Yu et al., 2014). Thus, a possible involvement of DyPs in the degradation of plant biomass can be assumed.

UPOs are other oxidoreductases whose expression is obviously influenced by the addition of ADOR. These heme-thiolate proteins can act either as peroxidases or monooxygenases. Studies on the effects of inducing substrates were mainly done for nitrogen-rich compounds like soybean meal, but the effects of lignocellulosic substrates on UPO secretion have not been investigated in great detail. According to our results, DOR, which is indeed rather nitrogen-rich but also highly phenolic, was definitely able to stimulate the UPO secretion (by the factor of 7 and 6) of *C. radians* and *A. aegerita* (*Cra*UPO and *Aae*UPO), two enzymes which have already been characterized by the working group of Hofrichter (Ullrich et al., 2004; Anh et al., 2007). Kellner et al. (2014) compared the number of UPO transcripts in different types of forest litter and determined the maximum number of transcript copies in beech wood (up to 25 transcripts). Liers et al. (2011) found maximal UPO activity values in *A. aegerita* and *C. radians* of 125 and 20 mU g^{-1}. According to our results, the maximal UPO activity of *C. purpureum* was 17 mU g^{-1} during growth on beech wood, whereas in the beech wood-DOR medium, the peak activity was 51 mU g^{1} (cf. Chapter 5 p. 166).

Bjerkandera adusta ligninolytic secreted protein profile

The addition of agricultural by-products to fungal cultures may reflect complex growth conditions close to nature and can stimulate the secretion of various enzymes required for lignin degradation (Girard et al., 2013). This stimulation was corroborated by following the time course of PODs during growth of *B. adusta* in the presence of DOR. Methodological limits arose during the usage of the ADOR-containing dark coloured, culture aliquots, e.g. when we attempted the application of spectrophotometrical enzyme assays at wavelengths in the UV region for the measurement of enzymatic Mn^{2+}-oxidation at 270 nm (cf. Materials and Methods, p. 56). Furthermore, it is difficult to identify specifically induced PODs by ADOR addition with spectrophotometric methods. Therefore, a more sensitive method for the distinction of PODs with interfering catalytic

activities (e.g. Mn^{2+}-oxidation MnP but also VP, (cf. Introduction, p. 11) was necessary. To address these issues, a comparative analysis of the complete set of secreted proteins ("secretome") by *Bjerkandera* during growth in either synthetic (KM) or ADOR supplemented Kirk media (ASC) was performed by a high-throughput MS-based proteomics using a label-free proteomic approach. Our results confirm that ADOR can act as phenolic elicitor for POD induction. In its presence, the stimulation of 4 MnPs, 1 VP, 1 LiP, and 1 generic peroxidase (GP) was observed. ADOR showed an opposite effect on the expression of DyPs and AAOs. Two DyPs were up- and two others down-regulated in ASC, probably suggesting the existence of different regulatory secretion mechanisms. Indeed, the *Bjerkandera* AAOs, which belong to the AA3 family, were remarkably down-regulated in the presence of the phenol-rich extract. These enzymes may support a redox cycle by producing extracellular peroxides as co-substrates for peroxidases and could therefore participate indirectly in the degradation of lignocelluloses. The occurrence of this probable AAO substrate inhibition mediated by ADOR remains unclear, but the function of providing peroxide as a co-substrate can be performed by other oxidases (e.g. glyoxal, pyranose or glucose oxidases) which are also present in ASC (0.43, 0.12 and 0.02 (%) NSAF respectively, cf. Chapter 3, SM, Table S1 (Daniel et al., 1994)).

Two isoforms of the *Bjerkandera* VPs were isolated from the dark coloured phenolic culture liquid during growth in ASC (*Bad*VPI and II; cf. Chapter 3, p. 127). These heme proteins were assumed to be part of the fungal ligninolytic and detoxifying enzyme system (Camarero et al., 1999; Ruiz-Dueñas et al., 2009). The degradative capability was demonstrated by the conversion of a synthetic dehydrogenated lignin polymer (DHP) by means of Mn^{3+}-mediated lipid peroxidation in the presence of linoleic acid (cf. Chapter 3, p. 127). The *in vitro* polymerization of DHP has been previously reported by specific LMEs such as LiP, Lac and MnP in *Phlebia tremellosa*, *Phanerochaete chrysosporium* and *Pycnoporus cinnabarinus* (Wariishi et al., 1991; Vares et al., 1994; Eggert et al., 1996). In fact, studies of DHP transformation by *B. adusta* PODs and comparisons with other peroxidases such as horseradish peroxidase have been performed, demonstrating a more efficient polymerization by the *B. adusta* MnP (Iwahara et al., 2000).

92 hydrolases were detected in the *B. adusta* secretome, 26 of which corresponded to the GH family, including α-amylase, α- and β-glucosidases and glucanases. Previous studies suggest that hydrolytic enzymes involved in lignocellulose degradation can be induced in media supplemented with cellulose-rich residues such as wheat bran, mandarin peels or bleached spruce pulp from a paper mill (Doppelbauer et

al., 1987; Elisashvili et al., 2009). Nevertheless, the aqueous extract of DOR did not significantly induce hydrolases. It rather seems to repress certain GHs such as GH15 and GH128. Both were 10 and 12-fold in KM (3 and 1.5 (%) NSAF) as in ASC in the *B. adusta* secretome (cf. Chapter 3, p. 124). *B. adusta* has 2 GH15 and 5 GH128 (Grigoriev et al., 2012). Other well-known polyporal fungi like *T. versicolor* and *P. chrysosporium* have 4 and 2 copy genes for GH15 and 4 and 5 for GH128, respectively. None of these gene families show a preference for (hemi) celluloses and pectin susbtrates (Riley et al., 2014).

DOR (phyto)toxicity depletion and phenol polymerization

In this thesis, we have adopted a dual approach since we have not only addressed the question of LME induction, but have also studied the direct effects of fungal oxidoreductases on the *in-vivo*-treatment of (A)DOR (cf. Chapter 1, p. 81, Chapter 2, p. 106 and Chapter 4, pp. 142; 144; 146; 147). Our results show a reduction in the content of certain phenolic acids (e.g. caffeic and ferulic acid (cf. Chapter 2, p. 109)) combined with a polymerization effect of higher water-soluble aromatic fragments. The polymerization of the soluble phenolic acids could be attributed to the main LME secreted such as Lac, MnP and DyP in *A. auricula-judae* and *M. alliaceus*, MnP and Lac in *T. versicolor*, MnP in *B. adusta* and UPO in *A. aegerita* and *C. radians*. Polymerization effects of extracellular fungal Lacs and PODs on water-soluble phenols were described for both *in-vitro* (Bollag et al. 1988; Thurston 1994; Cozzolino et al. 2002; Kinne et al. 2011) and *in-vivo* systems e.g. for the synthesis of natural humic substances by these enzymes (Liers et al., 2006; Liers et al., 2011).

The polymerization, provoked by fungal Lacs and PODs, made these compounds inaccessible to plants and their cell walls, thus leading to a reduction in phytotoxicity. DOR phytoxicity is a dose dependent property since it is positively correlated to phenol concentration (Sassi et al., 2006). It has already been described how olive mill wastes have a negative effect on plant growth (Aranda et al., 2004; Sampedro et al., 2004; Morillo et al., 2009) and further studies demonstrate a reduction in seed germination on different plant species (Casa et al., 2003; Garrido et al., 2012). The LME stimulation that provoked phenol polymerization and the concomitant reduction of DOR phytotoxicity, was studied for all the fungi tested. The depletion of phenolic acids in olive mill residues by its treatment with ligninolytic fungi, not only appears to remove the phytotoxicity, but also reduces the microtoxicity (Martirani et al., 1996).

Lignocellulolytic enzyme system, genome and secretome analysis of *C. purpureum*

The fungus *C. purpureum* is a hardwood colonizer, responsible for the so-called “silver leaf disease”, which is commonly applied as a biological vegetation control agent (Becker et al., 2005; Hamberg et al., 2014). A screening performed by Casieri et al. (2010) provide some hints on the probable ligninolytic activity of *C. purpureum* since this fungus was able to decolorize different industrial dyes (e.g. Poly R 478 and Poly S119 (Casieri et al., 2010)). However, no previous studies have been able to identify a specific enzyme with such a degradative activity. Our results show that *C. purpureum* had a moderate UPO and a poor Lac and MnP secretion rate under submerged cultivation in complex plant-based liquid media supplemented with ADOR. No further significant induction effects were observed for any other enzyme and no changes in the polymerization profile of DOR during SSF were detected after several weeks of incubation by spectrophotometric methods. However, the fungal *in-vivo*-treatment leads to an unexpected conversion of DOR combined with a removal of the plant toxicity, transforming it into a plant growth promoting substrate.

In order to detect alternative enzymatic systems involved in DOR conversion, we analyzed the main hydrolytic activities from the fungal culture liquid since it was previously known that they could be involved in the detoxification of olive mill wastes (Aranda et al., 2004). No differences in the activity pattern of DOR supplemented and non-supplemented cultures were found. We also analyzed CytP450. This enzyme is involved in the metabolism of exogenous toxic compounds and in counteracting the effect of toxic compounds generated during ligninolysis by basidiomycetes (Morel et al., 2013; Syed et al., 2014). Our results show that levels of CytP450 activity increase when the olive mill residue extract is present in the medium, indicating a possible participation in the detoxification process and improving degradation properties of *C. purpureum* for valorization purposes.

Conclusive explanations concerning probably biological agents responsible for the observed DOR phytotoxicity removal by *C. purpureum* are difficult to make. Despite the moderate UPO level detected during this polymerization process, relevant publications fail to resolve whether *C. purpureum* is a real white rot fungus capable of secreting ligninolytic PODs since the activities obtained during SSF were not significant.

In order to find an explanation for the biodegradation of the lignocellulose mediated by the *C. purpureum* extracellular system, as well as to determine the

phylogenetic affiliation and position of a less investigated wood rotting fungus to date, a genomic and proteomic study was performed.

The *C. purpureum* genome was sequenced with the aid of an Ion Torrent PGM. A 200 bp library was prepared and after the complete annotation procedure, 13739 predicted genes were obtained. The genome size was 41.2 Mbp, which is similar to the genome sizes of the so far sequenced well-known white rot fungi, such as the *P. cinnabarinus, T. versicolor* and *P. chrysosporium* as well as the *Agaricus bisporus* (33.67, 42.88, 30 and 30.5 Mbp respectively; Martinez et al., 2004; Floudas et al., 2012; Morin et al., 2012; Levasseur et al., 2014). The quality assessment of the draft genome revealed a mean coverage of 15x, which is higher than the one reported for *Phanerochaete* (10.5X) (Martinez et al., 2004) and half of the obtained values for *P. cinnabarinus* (31x) (Levasseur et al., 2014). The N50 of *C. purpureum* draft genome is moderate, suggesting that much information is contained in medium and small contigs. The Core Eukaryotic Genes Mapping Approach (CEGMA) analysis (Parra et al., 2007) revealed a high completeness of 92% (229 of the 248 most-conserved CEGS were contained in *C. purpureum* draft genome (cf. Chapter 5, p. 162)). Nevertheless, the quantification of the quality of a genome draft is always subjective to a certain degree since a comparison with other genomes is not always possible, especially when different NGS technologies are used. Therefore, for some parameters, it is necessary to make subjective decisions on which level of genome assembly accuracy can be considered sufficient, based on the specific research goals (Alkan et al., 2011). *Chondrostereum* has been considered to affiliate within the order *Polyporales* by several reports (Hamberg et al., 2014; Hamberg et al., 2015) and by the nomenclatural database Index Fungorum (http://www.IndexFungorum.org). Formerly, it was known as *Stereum purpureum*, a genus that belongs to the order *Russulales* (Martin et al., 2015). In this work, we present a multi-gene analysis of RNA, *rpb2* and *tef1* genes of the *Basidiomycota*. The resulting phylogeny showed that *C. purpureum* belongs to the order *Agaricales* (cf. Chapter 5, Fig. S1, p. 229).

The hydrolytic machinery of *Chondrostereum* identified by the genome data includes four GH6 and eight GH7 coding genes. These CAZy-classified group preferably participates in the hydrolysis of the crystalline cellulose acting on non-reducing (e.g. cellobiohydrolases, endoglucanase within GH6) and reducing ends (reducing end-acting cellobiohydrolase, endo-β-1,3-1,4-glucanase in the GH7) of the polysaccharide chain (Floudas et al., 2012; Riley et al., 2014). Among the GHs responsible for hemicellulose degradation, protein-coding sequences of several representatives of the families 10, 30, 51, 74 and 43 were found. These enzymes and related proteins belong to a typical

enzymatic toolbox of white rot fungi, whereas in brown rot fungi, the number of gene copies is lower and some representatives like e.g. GH74 do not exist, obviously due to a loss of enzymes during the evolutionary differentiation of both wood-rotting fungal groups (Floudas et al, 2012; Nagy et al., 2015). Further hydrolytic enzymes found in the *Chondrostereum* genome belong to the GH families 28, 53, 78, 88, 105, the PL groups 1, 3 4 and to the CE class 8. It is assumed that these enzymes are related to pectin degradation (Riley et al., 2014), a process probably part of the invasive step of fungal plant pathogens (e.g. for *Botrytis cinerea* (Ten Have et al., 1998) and *Fusarium oxysporum* (Di Pietro and Roncero, 1998). The endopolygalacturonase (EndoPGI) belonging to GH 28 was the only enzyme identified so far as the probable responsible for the silver-leaf disease caused by *C. purpureum* (Simpson et al., 2001, Ogawa et al., 2009, described as "*Stereum*" *purpureum*; Hamada et al. 2015).

Beside the presence of typical enzymes responsible for certain phytophathogenic properties, the overall impression of *C. purpureum* annotated genes for oxidoreductases (Lacs, PODs, UPOs and DyPs) confirms the assumption that this fungus may participate in saprotrophic lignin degradation. The majority of ligninolytic white rot fungi characterized so far produce more than one Lac and also *Chondrostereum* possesses 25 complete Lac genes, 24 of which were expressed and secreted in the studied secretome (cf. Chapter 5, p. 163). However, Lac secretion is not that characteristic for white rot fungi, since there are numerous well-known white rotters which lack Lac genes, such as *Bjerkandera* or *Phanerochaete* (Heinfling et al., 1998; Martinez et al., 2004). LiP, MnP and VP families are distributed among the white rot species since they are highly reactive with strong redox potentials and are known as the key enzymes involved in lignin degradation (Hammel, 1997). The numbers of PODs genes found in the *C. purpureum* genome that correspond to AA2 families were four GPs, 2 short MnPs and one long MnP. The genome of the sequenced *C. purpureum* resembles that of a white rot fungus, although the total number of PODs (7) is smaller than that of other fungi such as *T. versicolor*, *Stereum hirsutum* or *P. chrysosporium* (25 and 11 and 15 PODs gene copies, respectively) (Floudas et al., 2012; Riley et al, 2014). However, the agaric *Coprinopsis cinerea*, which is closely related to *C. purpureum*, possesses just one POD gene (Kohler et al., 2015).

The extracellular oxidases which reduce molecular oxygen to hydrogen peroxide were also present in the *C. purpureum* genome; 34 of them were classified in the AA group 3 (16 expressed as proteins) and 12 in AA family 5 (9 expressed proteins). AA3 family is composed by glucose-methanol-choline oxidoreductases, whereas AA5 family is composed by glyoxal oxidases and galactose oxidases. Both families are competent

alcohol oxidases with a great catalytic diversity (Cavener, 1992; Yin et al., 2015). Representatives of these AA families are obviously important in lignocellulolysis since they provide peroxide. The later, can be coupled to ligninolytic peroxidases (e.g. in the case of white rot fungi; (Martínez et al., 2005, Ferreira et al., 2009, Hernández-Ortega et al., 2012)) or being reduced by ferrous iron to hydroxyl radicals during the so-called Fenton-type reaction, which subsequently acts as a strong diffusible oxidizer initiating cellulose depolymerization (for brown rot fungi) (Baldrian and Valášková, 2008; Gomez-Toribio et al., 2009).

An unusually high number (30) of lytic polysaccharide monooxygenases (LPMOs)-encoding genes were found in the sequenced *Chondrostereum* genome. LPMOs (formerly classified in the GH61) are currently grouped into AA families 9, 10 and 11 of the CAZy classification. Some studies suggest that due to the large sequence diversity and the high number of gene copies, these proteins may also oxidize other substrates than cellulose (e.g. phenol derivatives (Li et al., 2012)). Busk et al. (2014) found general LPMOs in numerous fungal organisms like saprotrophic soft-, brown- and white rot fungi (*Daldinia eschscholzii*, *Chaetomium globosum* and *Serpula lacrymans*, *Postia placenta* or *T. versicolor*, *P. cinnabarinus*), by database mining. The number of LPMO genes in *C. purpureum* is higher than in traditional white rot species such as *T. versicolor* and *P. chrysosporium* (18 and 15 respectively). Nevertheless, the litter decomposer *C. cinereus* also contains a remarkably high number of 35 LPMO genes (Kohler et al., 2015).

Heme peroxidases found in the *C. purpureum* genome which have not yet been classified into the CAZy database and whose role in lignocellulolysis is still not completely clarified (Hofrichter et al., 2010), are the UPOs and DyPs from which 10 and 6 coding-genes were assigned. These protein families are largely distributed among different basidio- and ascomycetes (Floudas et al., 2012). The number of DyP and UPO coding sequences is in the average range for white rot species. The polyporal *B. adusta*, which has a proficient ability to secrete DyPs (Sugano et al., 2009, cf. Chapter 3, p. 124), contains up to 10 DyPs copy genes (Kersten and Cullen, 2014) and *T. versicolor* has 2 DyPs genes which have also found to be expressed (Carabajal et al., 2013). *A. aegerita* is a UPO hypersecretor which possess 16 copy genes, however only one have been characterized so far (Pecyna et al., 2009; Pecyna et al., 2016 (Diss.) and unpublished results). *Sphaerobolus stellatus* genome contains 151 heme-thiolate peroxidases but no evidences of UPO activity have been reported so far (Kohler et al., 2015).

We performed a high-throughput MS-based proteomic analysis of the extracellular proteins expressed by *Chondrostereum* during growth in the presence of

dry olive mill residues (DOR and ADOR). We used both culture types, since in SF we obtained a faster fungal growth and higher titers of LME secretions and SSF was used for imitating and reflecting culture conditions closer to nature. The time course of induction of certain enzymes was similar in SSF and SF when DOR or ADOR was added, indicating the presence of water-soluble elicitors.

According to proteomic studies, four of those 6 POD genes were expressed in ADOR-supplemented media. As previously mentioned, *C. purpureum* should be considered as a white rot fungus since the secretion of ligninolytic PODs is a characteristic trait of this group (Riley et al., 2014). The proteome of *C. purpureum* also showed that Lacs were clearly upregulated in ADOR-supplemented media, as was also demonstrated by spectrophotometric enzyme assays for other fungi (*e.g. T. versicolor*)). In addition, we found an increase in the abundance of UPO and class II peroxidases when ADOR was added. Whereas the peroxide-producing proteins were downregulated in the *Bjerkander*a secretome in the presence of ADOR, no comparable differences were observed for these enzymes in the *Chondrostereum* secretome among the media tested. The abundance of CAZymes (GHs, GTs, PLs, CEs) and carbohydrate-binding modules (CBMs) was not affected by the supplementation of olive mill substrates or birch wood (in SF). CBM1 family is implicated in cellulose degradation whereas the preferable substrate for PL1, 3 and PL4 and CE8 and 12 is pectin (Riley et al., 2014). A number of 17 LPMO were expressed and induced during SF and SSF by olive mill wastes. There were no LPMO expressed in Kirk medium (KM), whereas 6 were expressed in ADOR-supplemented Kirk media ASKM. In soy based media 12 were expressed in control cultures (SM) and 15 in ADOR-supplemented soy cultures (ASSM) (cf. Chapter 5, Table S2). This feature reflects the important role of LPMOs in the degradation of natural substrates like cellulose, chitin and related poly- and oligosaccharides (Busk and Lange, 2015) and perhaps other compounds (e.g. phenolic-related compounds).

In this thesis, we have shown the potential use of olive mill by-products as enzyme elicitors, specifically for the production of Lacs, PODs, UPOs and LPMOs in different species of *Agaricomycetes*. The sequenced genome of the agaric *C. purpureum* and comparative proteomic analyses have shed light on the role of *C. purpureum* in lignocellulose transformation. This dissertation opens up a myriad of applications of different enzymes as a tool-box for lignocellulose degradation.

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CONCLUSIONS

1. *Trametes versicolor* is able to grow on solid-state and in liquid submerged cultures in the presence of agro-industrial by-products like the phenol-rich dry olive mill residue as well as grape pomace and stalks. The stimulation and induction of the secretion of ligninolytic enzymes (mainly laccases and manganese peroxidases) from *Trametes versicolor* is only achieved after the addition of (aqueous) dry olive mill residue, which is an elicitor suitable for enzyme studies.

2. Dry olive mill residue is a potential enzyme elicitor that leads to an enhancement of the secretion of class II heme peroxidases in *Auricularia auricula-judae*, *Bjerkandera adusta* and *Trametes versicolor*, laccases in *Marasmius alliaceus* and *Trametes versicolor* and unspecific peroxygenases in *Agrocybe aegerita*, *Chondrostereum purpureum* and *Coprinellus radians*.

3. The phytotoxic effects of dry olive mill residue at agronomic doses on *Solanum lycopersicum* plants are completely removed when the material is *in-vivo* treated with all the above-mentioned *Agaricomycetes*. This effect, probably due to the oxidation and polymerization of phenols, is in all cases mediated by a strong secretion of ligninolytic enzymes, with the exception of *Chondrostereum purpureum,* a less investigated moderate white rot fungus.

4. Certain heme peroxidases such as DyP-type peroxidases, auxiliary peroxide-producing enzymes such as aryl-alcohol oxidases and some glycoside hydrolases such as GH15 and GH128, are down-regulated in *Bjerkandera adusta* liquid cultures after the addition of the aqueous extract of dry olive mill residue.

5. Based on the enzymatic activities, the extracellular enzyme-encoding genes and the identified secretome profile of ligninolytic class II peroxidase proteins, we postulate the inclusion of *Chondrostereum purpureum* as a white rot fungus which belongs to the order *Agaricales* and, more specifically, to the taxonomically diverse *Marasmioid* clade.

6. *Chondrostereum purpureum* possesses different encoding sequences for laccases, class II peroxidases such as manganese peroxidases, DyP-type peroxidases and unspecific peroxygenases. The majority of them are secreted in both liquid and solid media and the enzyme secretion obviously varies according to the culture conditions and the presence of (aqueous) dry olive mill residue.

7. A different secretion pattern of oxidases is achieved when agro-industrial by-products (DOR but also wood) are added to the *Chondrostereum purpureum* cultures, either under liquid or solid-state conditions; class II peroxidases, laccases, unspecific peroxygenases and lytic polysaccharide monooxygenases are strongly up-regulated when (aqueous) dry

olive mill residue is supplemented to the cultures and only a moderate stimulation is observed by the addition of wood.

CONCLUSIONES

1. *Trametes versicolor* es capaz de crecer en presencia de subproductos agroindustriales tales como el alpeorujo (rico en fenoles) y el hollejo y los tallos de uva tanto en cultivos líquidos como sólidos. La estimulación e inducción de enzimas ligninolíticas por *Trametes versicolor* (principalmente lacasas y manganeso peroxidasas) tiene lugar solo en presencia de alpeorujo y su extracto acuoso, por lo que se considera que este residuo ces un inductor enzimático importante.

2. Se pone de manifiesto la capacidad inductora de diversos enzimas por el alpeorujo ya que estimula la producción de las hemo peroxidasas de clase II en *Auricularia auricula-judae*, *Bjerkandera adusta* y *Trametes versicolor*, las lacasas en *Marasmius alliaceus* y *Trametes versicolor* y las peroxigenasas inespecíficas en *Agrocybe aegerita*, *Chondrostereum purpureum* y *Coprinellus radians*.

3. El tratamiento *in vivo* del alpeorujo con los hongos *Agaricomicetes* mencionados anteriormente, elimina su fitotoxicidad sobre plantas de tomate cuando se aplica a dosis agronómica. Este efecto probablemente se debe a la oxidación y polimerización de sus fenoles que, en todos los casos, está mediado por una potente secreción de enzimas ligninolíticas, salvo en el caso de *Chondrostereum purpureum*.

4. El alpeorujo reprime la producción por *Bjerkandera adusta* de varias hemo peroxidasas, como las peroxidasas decolorantes de tintes, las enzimas auxiliares productoras de peróxido, las aril alcohol oxidasas y algunas glicósido hidrolasas, como las GH15 y la GH128.

5. El estudio del secretoma de *Chondrostereum purpureum* nos permite afirmar que es un hongo de la podredumbre blanca que pertenece al orden *Agaricales*, y más específicamente, a un clado taxonómicamente diverso como el *Marasmoide* en base a sus actividades enzimáticas, los genes de las enzimas extracelulares y las proteínas de las peroxidasas de clase II.

6. *Chondrostereum purpureum* tiene distintos genes que codifican lacasas y peroxidasas de clase II, como manganeso peroxidasa, peroxidasas decolorantes de tintes y peroxigenasas inespecíficas. La mayoría de estas enzimas se secretan tanto en

medios líquidos como sólidos aunque su secreción varia según las condiciones de cultivo y la presencia de alpeorujo.

7. El patrón de secreción de oxidasas por *Chondrostereum purpureum* es distinto en presencia de diferentes residuos agroindustriales. Así, mientras el alpeorujo induce gran estimulación de las peroxidasas de clase II, lacasas, peroxigenasas inespecíficas y monooxigenasas líticas de polisacáridos, la madera solo provoca una inducción moderada de las mismas.

SUPPLEMENTARY

MATERIALS

Supplementary Materials for Introduction

APPENDIX I, Oxidases Phylogenies

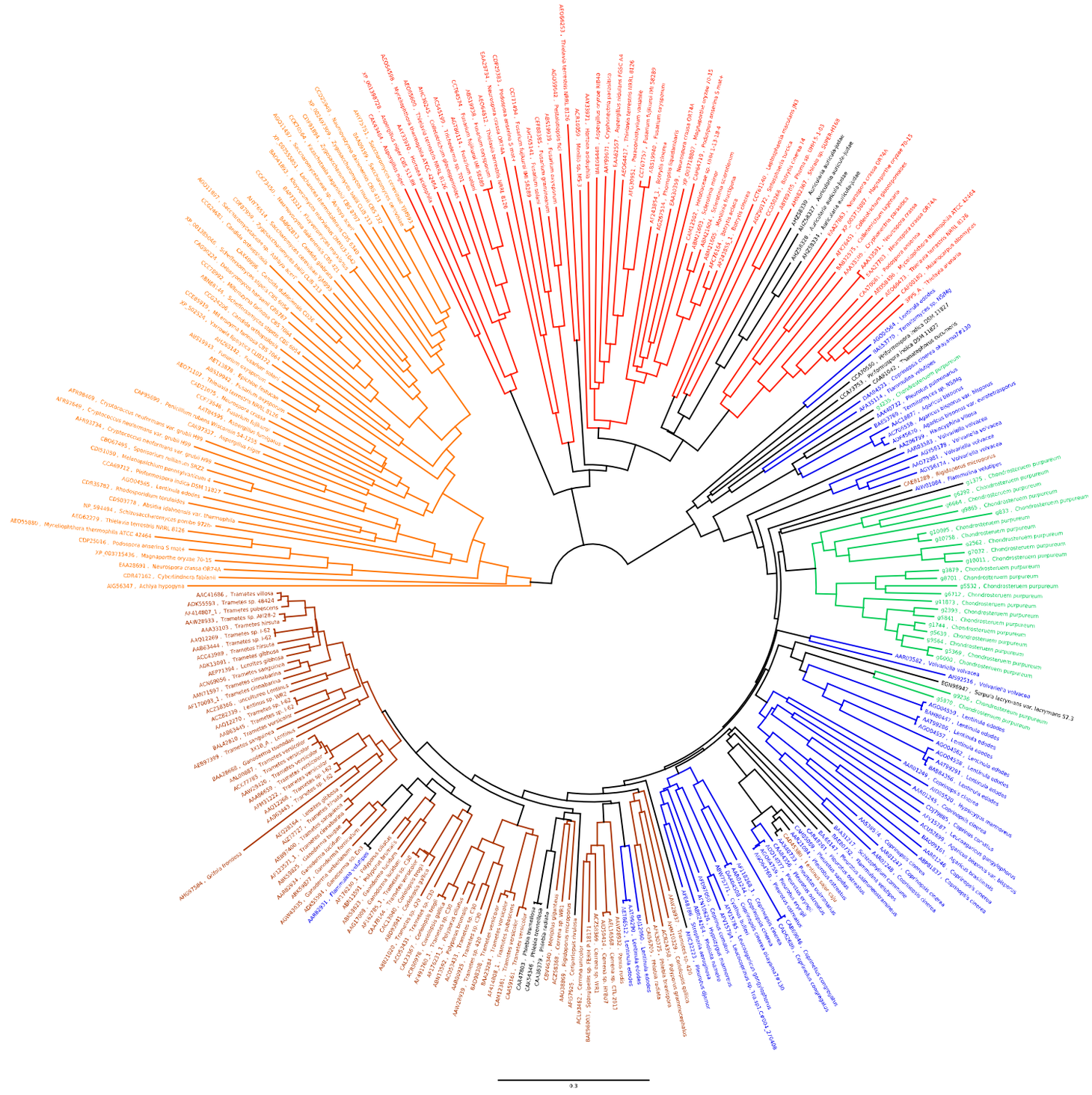


Fig. A1. Fungal Laccases Phylogenetic Tree. Clustal W aligment, NJ tree, JC distance model. Laccases of ascomycetes (red), ferroxidases (yellow), laccases of *Agaricales* (blue), laccases of *Polyporales* (brown) and *Chondrostereum purpureum* laccases (green).

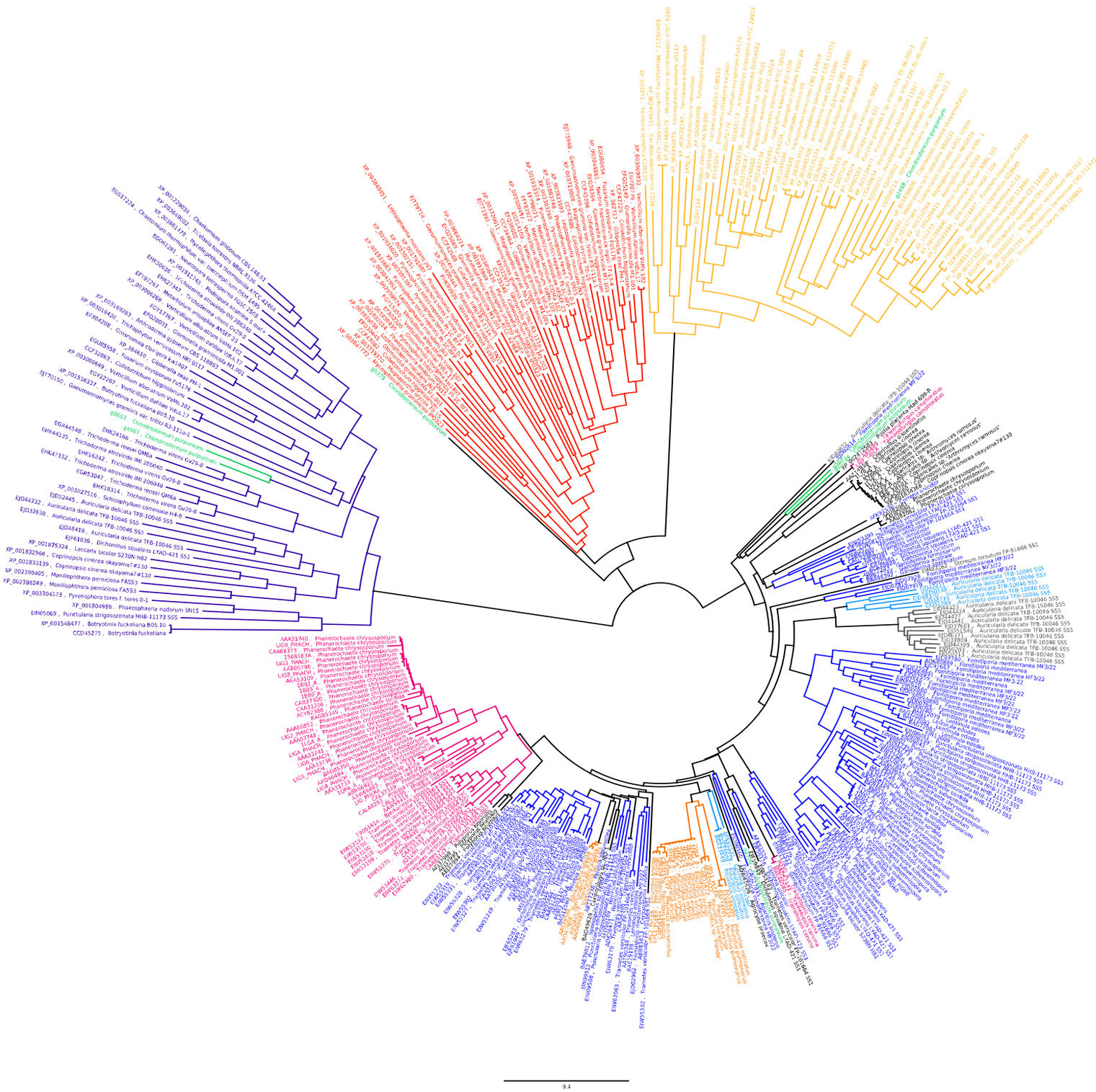


Fig. A2. Fungal class II peroxidases Phylogenetic Tree. Clustal W aligment, NJ tree, JC distance model. Heme peroxidases of ascomycetes(red), ascorbate peroxidases (purple), cytochrome C peroxidase (yellow), generic peroxidase (black), lignin peroxidase (pink), long-type manganese peroxidase (dark blue), versatile peroxidase (orange), short-type manganese peroxidase (pale blue) and *Chondrostereum purpureum* class II peroxidases (green).

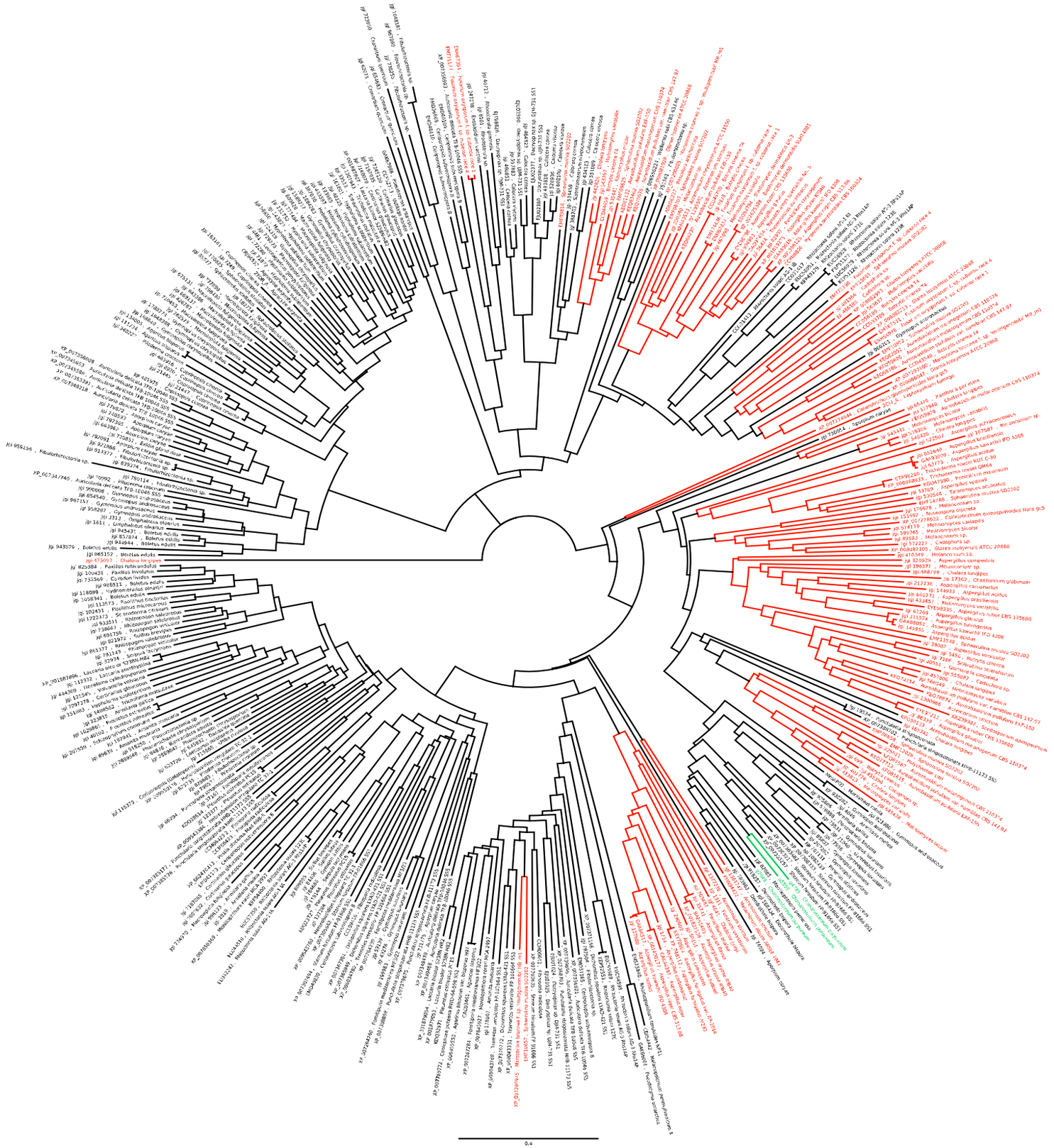


Fig. A3. Fungal Unspecific Peroxygenase Phylogeny Tree. Clustal W aligment, NJ tree, JC distance model. Unspecific peroxygenases of ascomycetes (red), basidiomycetes (black) and *Chondrostereum purpureum* (green).

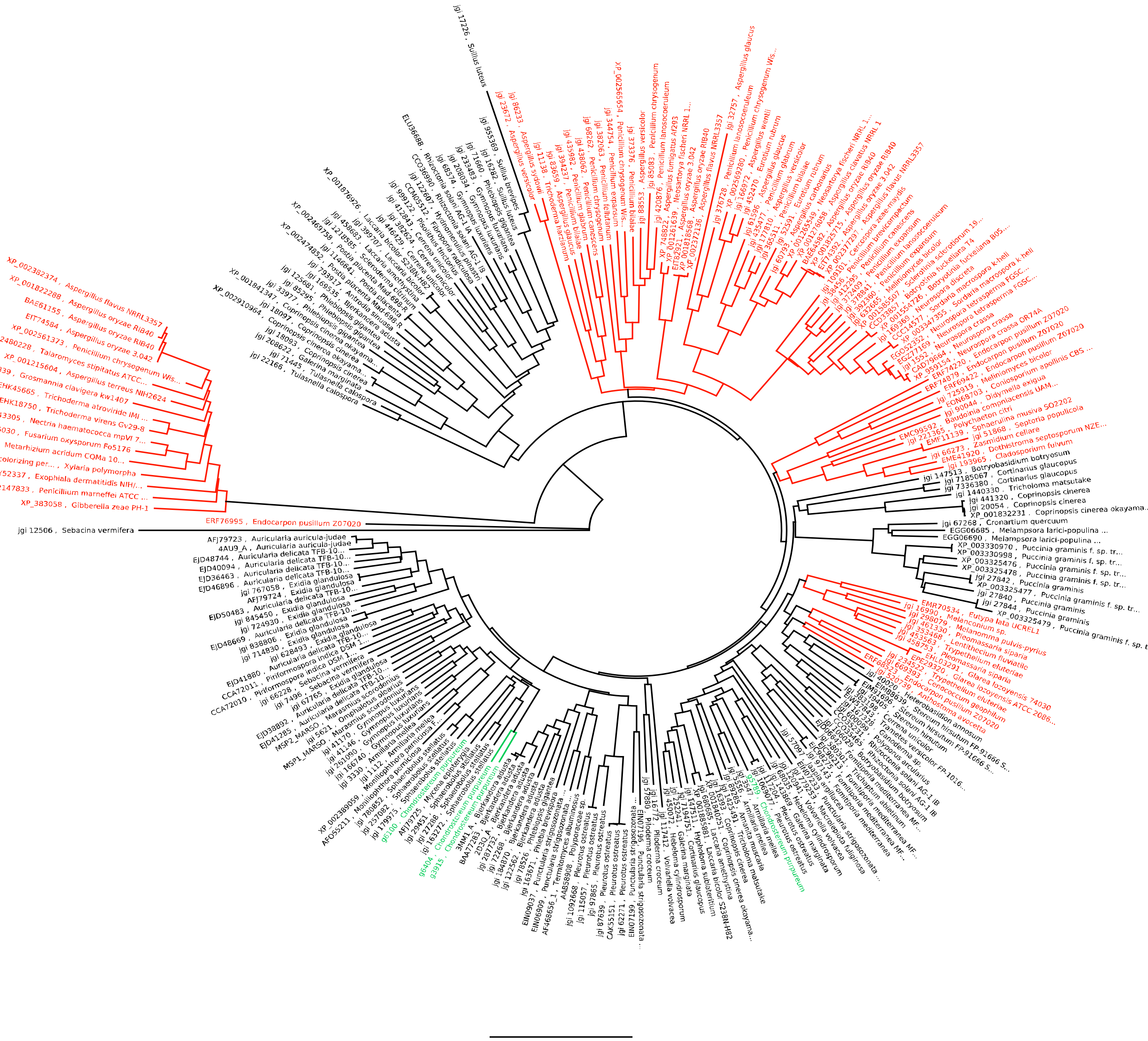


Fig. A4. Fungal DyP-type Peroxidase Phylogeny Tree. Clustal W aligment, NJ tree, JC distance model. DyP-type peroxidases of ascomycetes (red), basidiomycetes (black) and *Chondrostereum purpureum* (green).

APPENDIX II, NGS Platforms

Illumina/Solexa sequencing

Illumina performs next-generation sequencing (NGS) through a sequence by synthesis reaction (SBS), in which a fluorescently labelled reversible terminator is imaged as each dNTP is added and then cleaved to allow incorporation of the next base. Illumina achieves parallel sequencing by using a proprietary method that detects single bases as they are incorporated into growing DNA strands (Metzker, 2010). Read lengths vary, depending on users, between 36nt and 325nt per end.

Illumina offers the highest throughput of all platforms and the lowest per-base cost. Read lengths can reach up to 300 bp and are compatible with almost all types of applications. On the other hand, sample loading is technically challenging, with GGC motifs and inverted repeats being difficult to sequence (Nakamura et al., 2011).

454: pyrosequencing

This technique consists of the incorporation of each nucleotide by a DNA polymerase resulting in the release of pyrophosphate, which initiates a series of downstream reactions, leading to the production of light by luciferase. The amount of light produced is proportional to the number of nucleotides assimilated (Mardis, 2008). Pyrosequencing, specifically by the 454 GS Junior sequencer, delivered the longest read length but the lowest throughput (8 Mb/h during a 9-h run) and was adversely affected by errors in homopolymeric tracts, even when assembled at high coverage (Loman et al., 2012).

SOLiD: sequencing by ligation

This technology uses a special PCR called emulsion polymerase chain reaction (em-PCR) to amplify copies of DNA molecule templates. For sequencing, SOLiD uses a unique ligation-based strategy in which each data point represents two adjacent bases, and each base is sequenced twice.

The sequencing begins with the addition of a universal primer to all fragments attached to the magnetic beads. Then, a mixture of fluorescent probes is added, the complementary pair is ligated to the primer, the fluorescent signal generated is measured and the remaining unbound magnetic beads are washed out. The entire sequencing step consists of five rounds, with each round spanning approximately 5–7 cycles. This platform can produce approximately 20 Gb of short-read sequence data

(25–50 bases) per run, which makes it more suitable for resequencing than de novo assembly (Mitra et al., 2014).

Ion Torrent: semiconductor sequencing

The Ion Torrent PGM sequencer uses semiconductor technology to detect whether the protons released as nucleotides are incorporated during synthesis (Rothberg et al., 2011). Although Ion Torrent and pyrosequencing 454 technologies are similar in the basis, the Ion Torrent detected the proton released instead of pyrophosphate (Fig. SA).

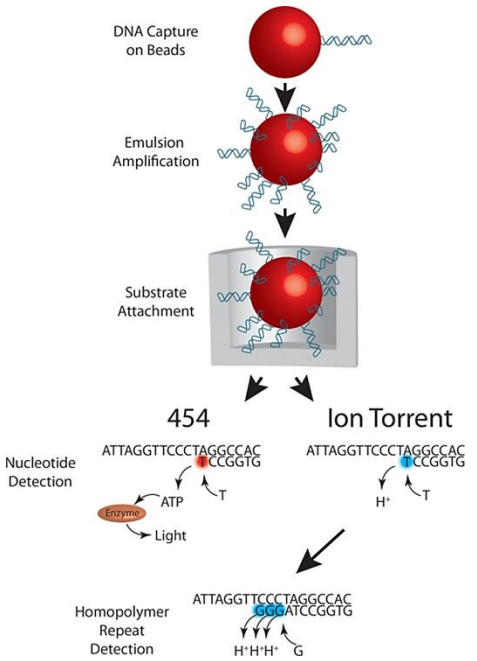


Fig. SA. Ion Torrent and 454 sequencing (Churko et al., 2013).

DNA fragments with specific adapter sequences are linked to and then clonally amplified by em-PCR on the surface of beads (Ion Sphere Particles). The templated beads are loaded into proton sensing wells that are fabricated on a silicon wafer, and sequencing is primed from a specific location in the adapter sequence (Quail et al., 2012). Since Ion Torrent performs sequencing-by-synthesis using electrochemical detection, imaging technology is not required. This technology attempts to simplify the detection process with the aid of a transistor-based sensor without using labelled nucleotides. The chemical by-products of synthesis are the release of a hydrogen ion (H^+) from the 3´ OH incorporation site on the growing strand, and the H^+ released is

then detected by a sensor. The transistor-based detection of the H^+ is a well-established technology. This transistor device is known as a pHFET (pH-sensitive field effect transistor). Each base is introduced sequentially. If more than one base of a single type is incorporated, protons are released and a signal, proportional to the number of bases incorporated, is detected. The advantages of this semiconductor technology include the independence of optical scanning and fluorescent nucleotides and very fast run times, with a typical run taking only a few hours. On the other hand, this technology suffers from the same problem as 454 pyrosequencing, with high error rates in homopolymers (Merriman et al., 2012).

PacBio

The PacBio system enables single molecule real time (SMRT) sequencing (Eid et al., 2009). A single DNA polymerase bound to a DNA template is attached to the bottom of a zero-mode waveguide (ZMW) well (Fig. B4). Each polymerase is allowed to carry out second strand DNA synthesis in the presence of γ-phosphate fluorescently labelled nucleotides. The ZMW is a small structure that creates an illuminated observation where it is only possible to observe a single nucleotide of DNA being incorporated by DNA polymerase. Every time a single nucleotide is incorporated, a fluorescent signal is detected (Quail et al., 2012).

Supplementary Materials for Chapter 1

Data S1. DNA FASTA sequences were obtained after DNA extraction, PCR and sequencing of the PCR products using the primers ITS4 (5´TCCTCCGCTTATTGATATGC3´) and ITS5 (5´GGAAGTAAAAGTCGTAACAAGG, 3´) White et al., 1999. a) Forward, b) Reverse.

a)

>Tra_ver.ITS4.scf

NNNNGCNGNCTACCTGATTTGAGGTCAGATGTTAAAAATGTTGTCCTAATGGACGGTTAGAAGCTCGCCAAAACA
CTTCACGGTCACAGCGTAGACAATTATCACACTGAGAGCCGATCCGTACGGAATCGAGCTAATGCATTCAAGAGG
AGCCGACCAACGAGGGCCAGCAAGCCTCCAAGTCCAAGCTTATAGATCACAAGGATTTATAAGTTGAGAATTCCA
TGACACTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAA
TTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTAAAAG
TTGTATTATAGATGCGTTAGACGCGTTTACATTCTGATACTTTAAAGTGTTTGTAGTATACATAGGCCGGCAGAATG
CTCCCGTTAAGGAGCCCACGCCAACCTACAGTAAGTGCACAGGGGTAGAGTGGATGAGCAGAGCGTGCACATGC
CTCGGAAGGCCAGCTACAACTCGTTTCAAAACTCGTTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTACA
TTTTNTTTTCCCCC

b)

>Tra_ver.ITS5.scf

ANGGGACTGCGGAGATCATTAACGAGTTTTGAAACGAGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCTCTGCT
CATCCACTCTACCCCTGTGCACTTACTGTAGGTTGGCGTGGGCTCCTTAACGGGAGCATTCTGCCGGCCTATGTATA
CTACAAACACTTTAAAGTATCAGAATGTAAACGCGTCTAACGCATCTATAATACAACTTTTAGCAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTTATAAAT
CCTTGTGATCTATAAGCTTGGACTTGGAGGCTTGCTGGCCCTCGTTGGTCGGCTCCTCTTGAATGCATTAGCTCGAT
TCCGTACGGATCGGCTCTCAGTGTGATAATTGTCTACGCTGTGACCGTGAAGTGTTTTGGCGAGCTTCTAACCGTC
CATTAGGACAACTTTTTAACATCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAGACCGGG
AAGGAAA

Table S2. Mineral composition of DOR and GPS was determined by ICP-OES, and N, C and organic carbon were determined by the Dumas method using a Leco Tru Spec CN analyser. Total phenol content and total sugar content were determined as described by Ribéreau-Gayon (1968) and Witham (1971), respectively.

Component	DOR	GPS
Al (ppm)	787.90	54.20
B (ppm)	30.12	undetermined
Ca (%)	0.62	0.36
Cd (ppm)	<0,5	<0,025
Cr (ppm)	4.31	2.90
Cu (ppm)	14.41	5.95
Fe (ppm)	566.37	93.73
K (%)	2.12	0.68
Mg (%)	0.16	800.85
Mn (ppm)	16.45	12.85
Na (%)	0.03	62.38
Ni (ppm)	2.38	1.23
Pb (ppm)	0.53	0.60
P (%)	0.20	0.21
S (%)	0.13	undetermined
Zn (ppm)	31.50	10.28
Hg (ppm)	<0.025	<0,025
Li (ppm)	0.62	0.047
Mo (ppm)	<0.025	<0,025
As (ppm)	0.90	1.27
Co (ppm)	<0.025	<0,025
Se (ppm)	<0,025	<0,025
Ti (ppm)	17.77	3.38
V (ppm)	0.69	0.08
N (%)	1.57	0.93
C (%)	51.47	43.69
Organic C (%)	49.20	41.31
Total phenols (mg g^{-1})	16.42	6.00
Total sugar content (mg g^{-1})	174.50	260.04

Table S3. Abundance of simple phenols in DOR and GPS analysed by LC-MS.

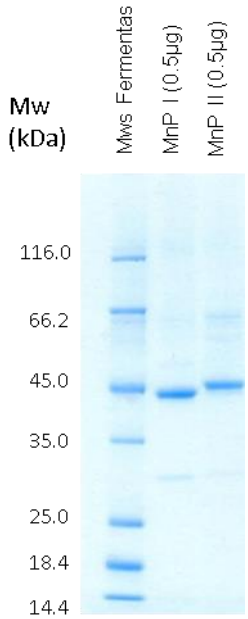
Phenol content (µg g^{-1} of DOR or GPS)	DOR	GPS
Gallic acid	Nd	133 ± 10
Protocatechuic acid	140 ± 10	18 ± 3
Hydroxybenzoic acid	100 ± 9	nd
P-tyrosol	105 ± 10	nd
Caffeic acid	47 ± 8	nd
Syringic acid	81 ± 8	nd
Ferulic acid	31 ± 3	nd
Methoxycinnamic acid	42 ± 3	nd
3,4-Dimethoxycinnamic acid	90 ± 5	nd
Hydroxytyrosol	128 ± 10	17 ± 1

nd: not detected

Table S4. Purification steps of MnP from *T. versicolor* grown in SF on ADOR-soy medium.

Purification step	Volume (ml)	Total activity (U)	Spec. activity (U mg $prot^{-1}$)	Gain (%)
Crude	2200	928.40	7.36	100
Ultrafiltration	380	752.02	8.78	81
Qseph 1	126	428.40	68.76	46.14
Qseph 2	9.00	95.40	100.95	10.28
MonoQ MnP II	0.38	42.17	72.56	4.54
MonoQ MnP II	0.24	44.54	211.86	4.80
HPSEC MnP I	0.07	24.90	427.99	2.68
HPSEC MnP II	0.06	18.21	471.38	1.96

Fig. S5. SDS-PAGE 10% of the purified MnP fractions



Supplementary Materials for Chapter 3

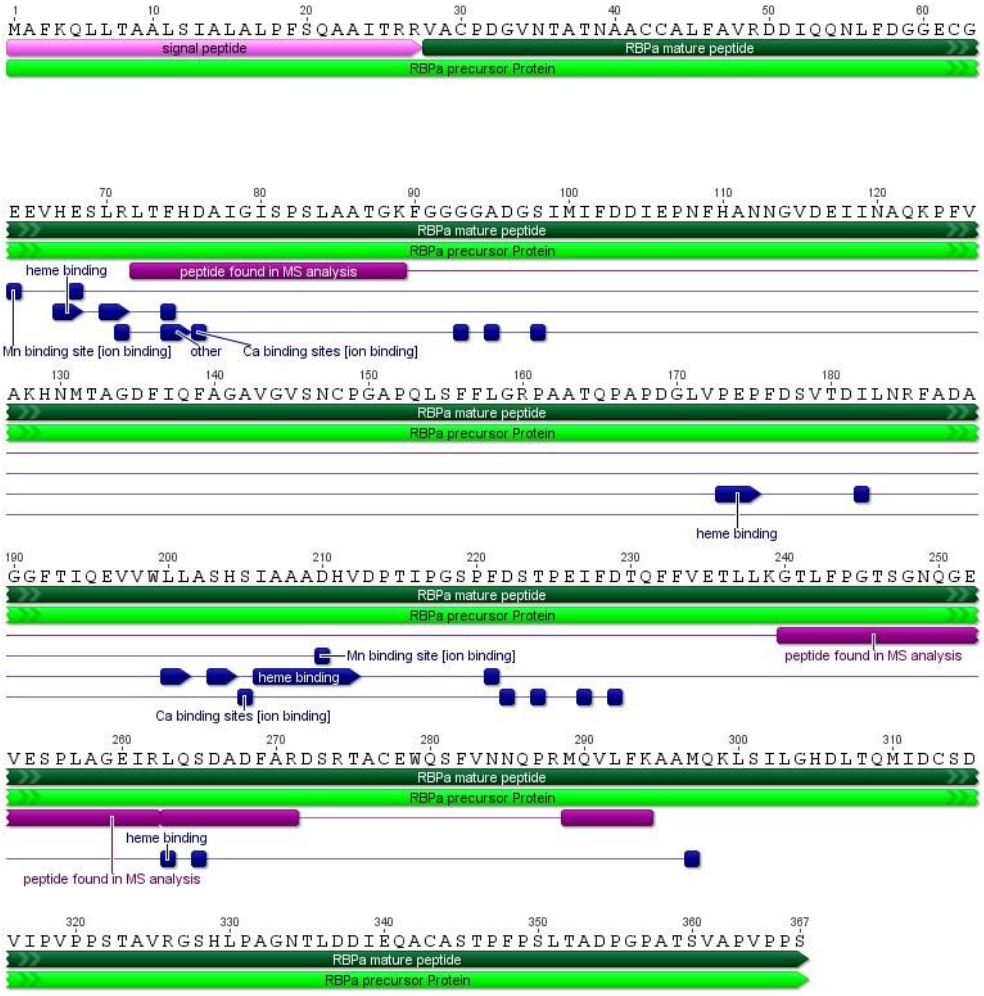


Fig. S1. *B. adusta* versatile peroxidase protein sequence (VP1) inclusive of annotations obtained from GenBank, i.e. binding sites of heme, calcium and manganese, and a predicted signal peptide. Four peptides identified in purified *Bad*VPI and *Bad*VPII obtained by mass-spectrometry analysis are shown (purple).

Supplementary Table S1. (xls file): Identified extracellular *B. adusta* proteins, selected according to a score higher than 2 and with more than 2 peptides from all proteins expressed in concentrated Kirk medium with (ASC) and without (KM) ADOR supplementation. The proteins are ordered according to EC number and the sum of PSM (Σ# PSM). PSM: peptide spectral match, NSAF: normalized spectral abundance factor, AA: amino acids, MW: molecular weight, pI: isoelectric point.

Supplementary Materials for Chapter 5

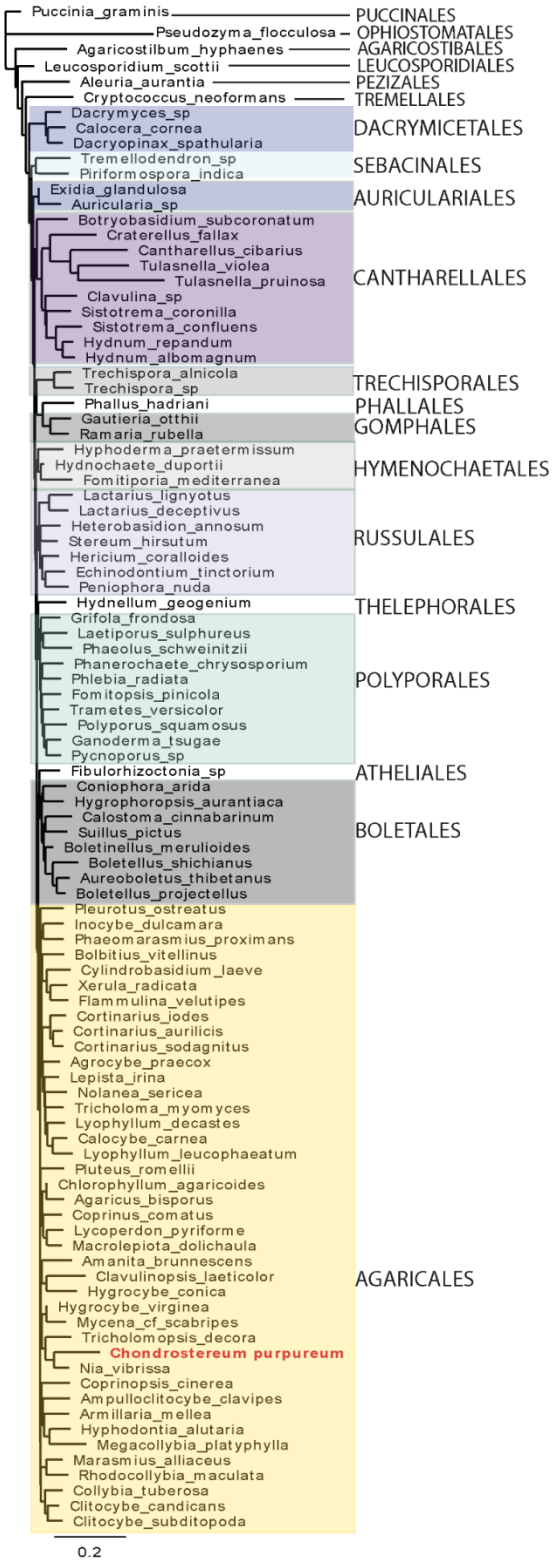


Fig. S1. NJ phylogeny of the *Basidiomycota* based on nucleotide sequences of 18s, 5.8s, 28s, *rpb2* and *tef1*. Clustal W aligment using a blocks substitution matrix. Sequences obtained from Matheny et al., 2007.

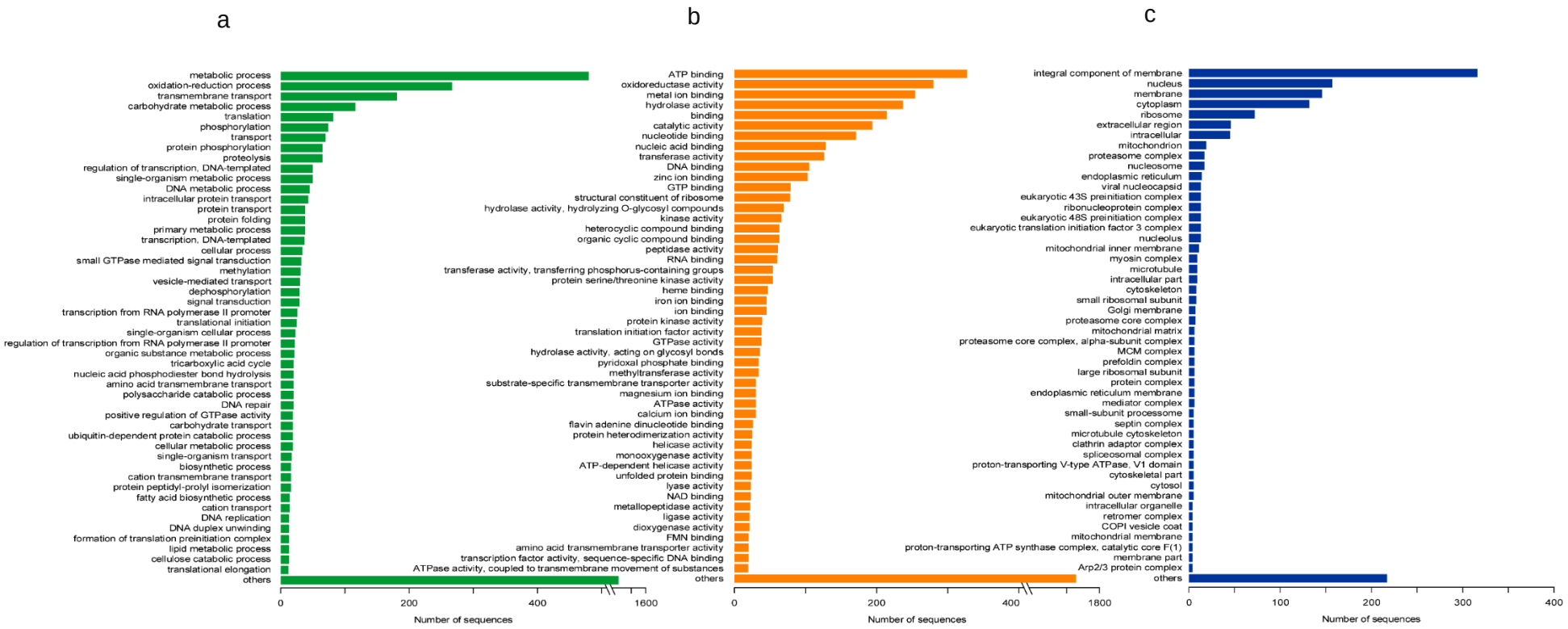


Fig. S2. *C. purpureum* gene sequences classified according gene ontology domains: (a) biological process, (b) molecular function and (c) cellular component.

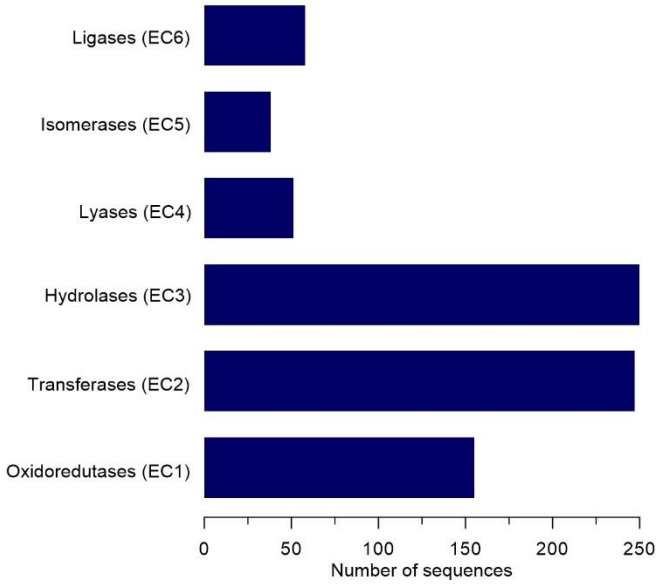


Fig. S3. *C. purpureum* gene sequences classified according enzymatic activities.

Supplementary Table S1. (xls file): Identified extracellular *C. purpureum* proteins in SSF cultures, selected according to a score higher than 5 and with more than 2 peptides from all proteins expressed in concentrated beech wood media (BW) and DOR-supplemented beech wood media (BWD). The proteins are ordered according to EC number and CAZy classification and the sum of PSM (Σ# PSM). PSM: peptide spectral match, NSAF: normalized spectral abundance factor, AA: amino acids, MW: molecular weight, pI: isoelectric point.

Supplementary Table S2. (xls file): Identified extracellular C. purpureum proteins in SF cultures, selected according to a score higher than 5 and with more than 2 peptides from all proteins expressed in concentrated Kirk media (KM) supplemented with ADOR (ASKM) and with birch wood media (BSKM) and soy media (SM) supplemented with ADOR (ASSM) and with birch wood (BSSM). The proteins are ordered according to EC number and CAZy classification and the sum of PSM (Σ# PSM). PSM: peptide spectral match, NSAF: normalized spectral abundance factor, AA: amino acids, MW: molecular weight, pI: isoelectric point.

LIST OF

ABBREVIATIONS

AA: Auxiliary activity

*Aae*UPO: *Agrocybe aegerita* unspecific peroxygenase

AAO: Aryl alcohol oxidase

ABT: Aminobenzotriazole

ABTS: 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

ADOR: Aqueous extract of DOR

AGPS: Aqueous extract of Grape pomace and stalks

AO: Alcohol oxidases

APX: Ascorbate peroxidases

AR: Relative activity

ASC: ADOR supplemented cultures

ASKM: ADOR supplemented kirk medium

ASSM: ADOR supplemented soy medium

BLAST: Basic local alignment search tool

bp: Base pair

BRF: Brown rot fungi

BSKM: Birch supplemented kirk medium

BSSM: Birch wood supplemented soy medium

BW: Beech wood

BWD: Beech wood DOR

CAZy: Carbohydrate active enzymes

CBM: Carbohydrate-binding module

CcPs: Cytochrome C peroxidases

CDH: Cellobiose dehydrogenase

CDS: Coding DNA sequence

CE: Carbohydrate esterase

CEG: Core eukaryotic genes

CEGMA: Core eukaryotic genes mapping approach

CiP: *Coprinopsis cinerea* peroxidase

CMC-ase: Carboxymethylcellulase

CPO: Chloroperoxidases

*Cra*UPO: *Coprinellus radians* unspecific peroxygenase

CRO: Copper radical oxidases

Cyt P450: Cytochromes P450

DHP: Dehydropolymer

DMP: 2,6-Dimethoxyphenol

DOR: Dry olive mill residue

DTT: Dithiothreitol

DyP: Dye-decolorizing peroxidases

EC: Enzyme Commission number

em-PCR: emulsion polymerase chain reaction

EndoPGI: Endopolygalacturonase

ESI: Electrospray ionization

ETJ: Eco tomatoe juice

FAD: Flavin adenine dinucleotide

FAE: Feruloyl esterases

FDR: False discovery rate

FOXs: Ferroxidases

FTIRC: Fourier transform infrared spectroscopy

FPLC: Fast protein liquid chromatography

GAO: Galactose oxidases

GC-MS: Gas chromatography–mass spectrometry

GH: Glycoside hydrolase

GLX: Glyoxal oxidase

GMC: Glucose-methanol-choline oxidoreductases

GOX: Glucose oxidase

GP: Generic peroxidase

GPS: Grape pomace and stalks

GT: Glycosyl transferase

GO: Gene ontology

GOO: Glucooligosaccharide oxidase

HCD: Higher energy collisional dissociation

HDM: Human drug metabolites

HPSEC: High performance size-exclusion chromatography

HRP: Horseradish peroxidase

IEA: International Energy Agency

ISP: Ion sphere particles

IT: Ion trap

ITS: Internal transcribed spacer

JGI: Joint Genome Institute

KM: Kirk medium

KOG: Eukaryotic Orthologous Groups

Lac: Laccase

LC: Liquid chromatography

LCAO: Short chain alcohol oxidase

LiP: Lignin peroxidase

LIT: Linear ion trap

LME: Lignin modifying enzyme

LPMO: Lytic polysaccharide monooxygenase

LRET: Long-Range Electron Transfer

LTQ: linear trap quadrupole

MALDI: Matrix-Assisted Laser Desorption/Ionization

MnP: Manganese peroxidase

MnoP: Manganese-oxidizing peroxidase

*Mro*UPO: *Marasmius rotula* unspecific peroxygenase

MS: Mass spectrometry

MW: Molecular weight

NAD(P): Nicotinamide adenine dinucleotide phosphate

NGS: Next-generation sequencing

NIPALS: Non-linear iterative partial least squares algorithms

NJ: neighbour joining

nr: Non-redundant database

NSAF: Normalized spectral abundance factor

OG: Orthologous group

OMWs: Olive mill wastes

OXO: Oxalate oxidase

PAH: Polycyclic aromatic hydrocarbons

PC: Principal component

PCA: Principal component analysis

PGM: Personal genome machine

pHFET: pH-sensitive field effect transistor

pI: Isoelectric point

PL: Polysaccharide lyase

PMO: Polysacharide monoxogenases

PMSF: Phenylmethylsulfonyl fluoride

*p*NP: of p-nitrophenol

*p*NPH: p-nitrophenyl acetate

P2Ox: pyranose 2-oxidase

PO-ase: Endopolymethylgalacturonase

PODs: Class II peroxidases

PSM: Peptide spectrum matches

QIT: Quadrupole ion trap

QUAST: Quality Assessment tool for genome assemblies

rpb2: RNA polymerase II

RDW: Root dry weight

ROS: Reactive oxygen species

SAO: Secondary alcohol oxidase

SBS: Sequence by synthesis

SCAO: Short chain alcohol oxidase

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDW: Shoot dry weight

SEC: Size exclusion chromatography

SF: Submerged fermentation

SM: Soy medium

SpC: Spectral counts

SSF: Solid state fermentation

tef1: Translation elongation factor 1-α

TOF: Time of flight

TPOMW: two-phase olive-mill waste

UPLC: Ultra performance liquid chromatography

UPO: Unspecific peroxygenase

VAO: Vanillyl-alcohol oxidase

VP: Versatile peroxidase

WRF: White rot fungi

Xyl-ase: Xyloglucanase