Genomic, transcriptomic and proteomic analysis of *Pleurotus ostreatus* secreted proteins

Memoria presentada por

Manuel Alfaro Sánchez

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Tesis dirigida por:
Prof. Dr. Antonio G. Pisabarro
Catedrático de Microbiología
Grupo de Investigación de Genética y Microbiología
Universidad Pública de Navarra

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INFORMA:

Que la presente Tesis Doctoral, "Genomic, transcriptomic and proteomic analysis of *Pleurotus ostreatus* secreted proteins" elaborada por **D. Manuel Alfaro Sánchez**, ha sido realizada bajo su dirección, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.
Y para que así conste, firma la presente en Pamplona, a 31 de mayo de 2017.

Fdo. D. Antonio G. Pisabarro

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Summary

The objective of this thesis is to study the proteins secreted by the edible and worldwide cultivated white rot basidiomycete fungus *Pleurotus ostreatus* with three major goals: to determine the set of proteins secreted under different nutritional conditions, to determine the effect of the monokaryotic and dikaryotic mycelial conditions on the secretome, and to explore the relationship between the transcriptome of the secreted proteins and the actual secretome in different monokaryotic strains.

In the first chapter of this thesis, we will review several basidiomycete secretome analyses comparing the results obtained using different analytical techniques and discussing some representative examples. We will pay a special attention to the lignocellulolytic enzymes secreted and to the different fungal lifestyles. This chapter is an updated version of the paper entitled *Comparative analysis of secretomes in basidiomycete fungi* that we published in Journal of Proteomics in 2014 as a summary of the state of the art. The main conclusions of this chapter are that a combination of genomic, transcriptomic and proteomics techniques is still the best approach for analyzing fungal secretomes, allowing to the identification of secretion patterns associated to the different lifestyles.

In the third chapter, we screened two *P. ostreatus* monokaryotic genomes to identify bioinformatically the genes coding for proteins targeted for secretion. The study was made using the two monokaryotic protoclones (mkPC9 and mkPC15) whose genomes had been previously sequenced and annotated in a collaborative project carried out with the Joint Genome Institute. These two protoclones contain the two nuclei present in the commercial dikaryotic strains dkN001. The results obtained showed that, surprisingly, both strains differ in their lignocellulose degrading genomic capabilities. mkPC9 have less CAZy genes annotated, especially in the Glycosil hydrolases (GH) class. Nevertheless, mkPC9 grows better than mkPC15 on lignocellulosic substrates and has a higher enzyme secretion capacity when growing in the presence of wood. The transcription of the genes coding for secretable proteins was studied by RNAseq analysis and we could conclude that, whereas the genome profile of the secretome was similar in the two strains, the corresponding transcriptome

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profiles were different between them and in different culture conditions and we observed a concentrated transcriptional activity in few genes per function and an increased importance of the glycosil hydrolases and proteins without a functional classification. These results highlight the importance of adding additional data to the gene lists produced by genome sequence analysis for gaining a more accurate picture of the biological process under study. *P. ostreatus* secretes a huge variety of lignocellulose degrading enzymes when cultured in the presence of wood. More than 20% of them lack a known enzymatic function. Transcriptome analysis noted the importance of these proteins, further confirmed by proteomics. Using domain structure prediction, we were able to give an insight about the possible role of several proteins, including a xylanase and a AA10 LPMO. This chapter is a version of the manuscript entitled *Comparative and transcriptional analysis of the predicted secretome in the lignocellulose-degrading basidiomycete fungus Pleurotus ostreatus* published in Environmental Microbiology.

Finally, in the fourth chapter, mass spectrometry analyses were used to confirm the presence of these enzymes acting on the lignocellulosic substrates. We compared the proteins secreted by the two monokaryons studied in the bioinformatics analysis with that of dikaryon that contains the two nuclei present in them. Interestingly, monokaryons behave in a very different manner; mkPC15 showed a weakest production of lignocellulose degrading enzymes than mkPC9 and dkN001 when cultured using wood as a carbon source. Moreover, dkN001 was able to secrete more plant cell wall decomposing enzymes, correlating with their superior capacity to grow on lignocellulosic substrates. Furthermore, the three strains were cultured in three different media using with glucose, wood or both (glucose and wood) as a carbon source. As expected, we identify a higher number of lignocellulose degrading enzymes in wood-containing media, especially glycosyl-hydrolases, carbohydrate esterases and polysaccharide lyases.

Fungal lignocellulose degradation is the result of the synergistic action of several enzymes. These thesis improve our overall understanding of plant biomass degradation as a step to achieve the goal of using biomass as a sustainable source of energy to support future needs.

Resumen

El objetivo de esta tesis es estudiar las proteínas secretadas por el hongo basidiomiceto de la podredumbre blanca *Pleurotus ostreatus*, un excelente comestible ampliamente cultivado por todo el mundo, con tres propósitos principales: determinar el conjunto de proteínas secretadas en diferentes condiciones nutricionales, determinar el efecto de las cepas monocarióticas o dicarióticas en el secretoma, y explorar la relación entre el transcriptoma de las proteínas secretadas y su presencia en diferentes medios de cultivo.

En el primer capítulo de esta tesis, revisaremos varios análisis de secretomas de basidiomicetos comparando los resultados obtenidos utilizando diferentes técnicas analíticas y discutiendo algunos ejemplos representativos. Prestaremos especial atención a las enzimas lignocelulolíticas secretadas y a los diferentes estilos de vida fúngicos. Este capítulo es una versión actualizada del artículo titulado "*Comparative analysis of secretomes in basidiomycete fungi"* que publicamos en Journal of Proteomics en 2014 como una revisión. En este capítulo, concluimos que una combinación de técnicas genómicas, transcriptómicas y proteómicas sigue siendo el mejor enfoque para analizar secretomas fúngicos, y para permitir la identificación de patrones de secreción asociados a los diferentes estilos de vida.

En el tercer capítulo, hemos seleccionado dos genomas monocarióticos de *P. ostreatus* para identificar bioinformáticamente los genes que codifican las proteínas dirigidas a la secreción. El estudio se realizó utilizando los dos protoclones monocarióticos (mkPC9 y mkPC15) cuyos genomas habían sido previamente secuenciados y anotados en un proyecto colaborativo llevado a cabo con el Joint Genome Institute. Estos dos protoclones contienen los dos núcleos presentes en las cepas dicarióticas de la cepa comercial dkN001. Los resultados obtenidos mostraron que, sorprendentemente, ambas cepas difieren en sus capacidades genómicas para degradar lignocelulosa. mkPC9 tiene menos genes CAZy anotados, especialmente glycosil hidrolasas (GH). Sin embargo, crece mejor que mkPC15 sobre sustratos lignocelulósicos y tiene una mayor capacidad de secreción enzimática cuando crece en presencia de madera. La transcripción de los genes codificantes para proteínas secretables fue estudiada por RNAseq, y se observó que mientras el número de genes clasificados para cada función es similar en las dos cepas, los correspondientes perfiles transcriptómicos son diferentes entre ellos y también diferentes según las condiciones de

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cultivo. La actividad transcripcional se concentra en pocos genes por función, y se observó que las glicosil hidrolasas y las proteínas sin una clasificación funcional tienen una mayor importancia de lo que se infería analizando su número. Estos resultados ponen de relieve la importancia de agregar datos adicionales a las listas de genes producidos por el análisis genómico para obtener una imagen más precisa del proceso biológico en estudio. *P. ostreatus* secreta una gran variedad de enzimas degradadoras de lignocelulosa cuando se cultiva en presencia de madera. Más del 20% de las proteínas secretadas carece de una función enzimática conocida. El análisis de transcriptoma subraya la importancia de estas proteínas, confirmada además por la proteómica. Usando la predicción de la estructura de las proteínas, pudimos inferir el posible papel de varias proteínas, incluyendo una xilanasa y un AA10 LPMO. Este capítulo es una versión del manuscrito titulado "*Comparative and transcriptional analysis of the predicted secretome in the lignocellulose-degrading basidiomycete fungus Pleurotus ostreatus*" publicado en Environmental Microbiology en 2016.

Finalmente, en el cuarto capítulo, se utilizaron análisis de espectrometría de masas para confirmar la presencia de estas enzimas actuando sobre los sustratos lignocelulósicos. Las tres cepas se cultivaron en tres medios diferentes usando glucosa, madera o ambos (glucosa y madera) como fuente de carbono. Como era de esperar, identificamos un mayor número de enzimas de degradación de lignocelulosa en medios que contienen madera, especialmente glicosil hidrolasas, carbohidrato esterasas y polisacárido liasas.

Además, observamos que las tres cepas se comportan de manera diferente. mkPC15 mostró una producción más débil de enzimas de degradación de lignocelulosa que mkPC9 y dkN001 cuando se cultivó usando madera como fuente de carbono. La cepa dkN001 fue capaz de secretar más enzimas de descomposición de la pared celular de las plantas, como corresponde a su mayor capacidad para crecer en sustratos lignocelulósicos.

La degradación fúngica de la lignocelulosa es el resultado de la acción sinérgica de varias enzimas. Esta tesis mejora nuestra comprensión general de la degradación de la biomasa vegetal para lograr el objetivo de utilizar la biomasa como una fuente sostenible de energía para el futuro.

Chapter 1:

Comparative Analysis of Secretomes in Basidiomycete Fungi

Part of this chapter has been published as:

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1. – INTRODUCTION

The fungal lifestyle depends on the absorption of environmental nutrients that are made available via the action of sets of secreted hydrolyzing enzymes. Consequently, protein secretion is crucial for fungal life and growth. This high capacity of enzyme secretion has been exploited by industry, where compounds secreted by fungi have been used for decades (Conesa, Punt, van Luijk, & van den Hondel, 2001; Shoji, Arioka, & Kitamoto, 2008). The secretome is defined as the set of proteins secreted by a cell or an organism at a given time (Tjalsma, Bolhuis, Jongbloed, Bron, & van Dijl, 2000). There are two points that must be kept in mind in this context. First, in a broad sense, the secretome includes both the proteins that are released into the surrounding medium and those that remain bound to the membrane or cell wall or that are integral membrane proteins. Second, the secretome, like the transcriptome, is highly variable depending on environmental conditions, including the growth substrate, temperature and growth phase. Although the number of fungal transcriptomic studies is steadily increasing, it is not sufficiently large to draw global conclusions to explain how a secretome is modulated in response to environmental conditions in different species. Hence, in this chapter, we review the characteristics of the secretomes of some model organisms that are representative of different basidiomycete lifestyles, paying special attention to the enzymes involved in lignocellulose degradation.

Lignocellulose, the major reservoir of organic carbon on Earth, is recalcitrant to turnover and resistant to microbial and enzymatic attacks because of the combination of the protective action of lignin, the cementing action of hemicellulose and the crystalline structure of cellulose. From the perspective of a geological time frame, lignocellulose could have served as a major carbon sink until the appearance of saprophytic lignin-degrading fungi at the end of the Carboniferous period (Floudas et al., 2012). Filamentous fungi play an important role in carbon cycling because some of these fungi, grouped in the phylum Basidiomycota, secrete large amounts of lignocellulose-degrading enzymes. These fungi are the only organisms known to degrade lignocellulose at a global scale. Lignocellulolytic basidiomycetes use two alternative strategies for attacking lignocellulose. White rot basidiomycetes degrade the lignin moiety extensively before attacking cellulose, whereas brown rot basidiomycetes cause limited alterations of lignin while primarily degrading cellulose. These two processes

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are carried out by complex portfolios of secreted enzymes whose expression and export are modulated in response to environmental and substrate changes. White rot basidiomycetes attack the lignin moiety of lignocellulose using oxidative enzymes classified as manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC 1.11.1.16), lignin peroxidases (LiP, EC 1.11.1.14) and phenol oxidases (benzenediol:oxygen oxidoreductase Pox, laccase, EC 1.10.3.2). Not all white rot basidiomycetes contain all of these enzymes. For instance, *Phanerochaete chrysosporium* does not produce laccases, while *Pleurotus ostreatus* does not produce lignin peroxidases. In contrast, the production of manganese peroxidases and versatile peroxidases seems to be a common feature of this group. In addition to these main ligninolytic enzymes, there are a number of accessory enzymes that participate in the process, including hemeperoxidases such as cytochrome c peroxidases (EC 1.11.1.5), chloroperoxidases (EC 1.11.1.10) and dye decolorizing peroxidases (DyP, EC 1.11.1.19), in addition to glyoxal oxidases (GLOX) and aryl-alcohol oxidases (AAO, EC 1.1.3.7), pyranose dehydrogenases (EC 1.1.99.29), and methanol oxidases (EC 1.113.13).The cellulose moiety of the lignocellulose is degraded by carbohydrate-active enzymes (CAZy) classified as exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91), subclassified as types I and II depending on their attack of the reducing or to the non-reducing end of cellulose, respectively, as well as endocellulases (EC 3.2.1.4) and cellobiases (beta-glucosidases, EC 3.2.1.21).

Finally, the hemicellulose moiety of lignocellulose is degraded by endoxylanases (EC 3.2.1.8), α-glucuronidases (EC 3.2.1.131), acetyl-xylan esterases (EC 3.1.1.72), arabinofuranosidases (EC 3.2.1.55), ferulic acid esterases (feruloyl esterases, EC 3.1.1.73) and β-xylosidases (EC 3.2.1.37), among other enzymes(Sun, Tian, Diamond, & Glass, 2012).

In addition to the enzymatic complexity described above, there is another level of complexity that should be considered: each of these enzymes is encoded by multigenic families whose members are differentially regulated (R Castanera et al., 2012). For instance, the genome of the white rot basidiomycete *P. ostreatus* encodes 10 phenol oxidase genes, while the brown rot basidiomycete *Postia placenta* encodes 34 putative glycoside hydrolases (GH). This multilayered complexity precludes us from understanding the process of lignocellulose degradation a gene-to-gene level and from exploiting this process in more efficient industrial applications.

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The vast renewable carbon resource represented by lignocellulose not only serves as the raw material of the paper industry but is also the focus of attention as a potential source of second-generation biofuels. However, to fully utilize the potential of lignocellulose, various problems associated with its practical utilization must be solved. One of these key problems is the low susceptibility of lignocellulose to the hydrolysis processes used to produce fermentable sugars. This low susceptibility is due to the crystalline structure of cellulose fibrils surrounded by hemicellulose and to the presence of the lignin seal, which prevents penetration by degrading enzymes. To overcome these structural barriers, thermochemical lignocellulose pretreatments are necessary to break down the lignin protective shield and the cellulose crystal structure. However, these pretreatments are expensive in terms of energy costs and are environmentally unfriendly. Biological pretreatments based on the enzymatic activity of white rot basidiomycetes are considered safe and environmentally friendly methods for the removal of lignin from lignocellulose (Hatakka & Helsinki, 2013). However, our limited understanding of the ways in which environmental or the culture conditions affect the expression of the ligninolytic enzyme-encoding genes and the export of the corresponding enzymes results in a low efficiency of these processes.

There are currently more than 781 available fungal genomes sequenced, more than 272 come from basidiomycetes (JGI Mycocosm, March 2017(I V Grigoriev et al., 2012). These sequences offer us the opportunity to predict proteins that can be secreted (bioinformatics secretome) before performing more costly and complex secretome analyses. However, one question that arises concerns the correlation between the bioinfosecretome and the actual secretome. This question is pertinent not only because the actual secretome represents the response to particular environmental culture conditions or developmental stages, whereas current bioinfosecretomes include all secretion possibilities, as discussed above, but also because novel secretion mechanisms and pathways have been discovered.

In the following sections, we review the strategies for producing bioinformatics and experimental secretomes in basidiomycetes (Figure 1), comparing the results obtained through both approaches and discussing some representative examples of these secretomes, with special attention given to the lignocellulolytic enzymes and the different fungal lifestyles.

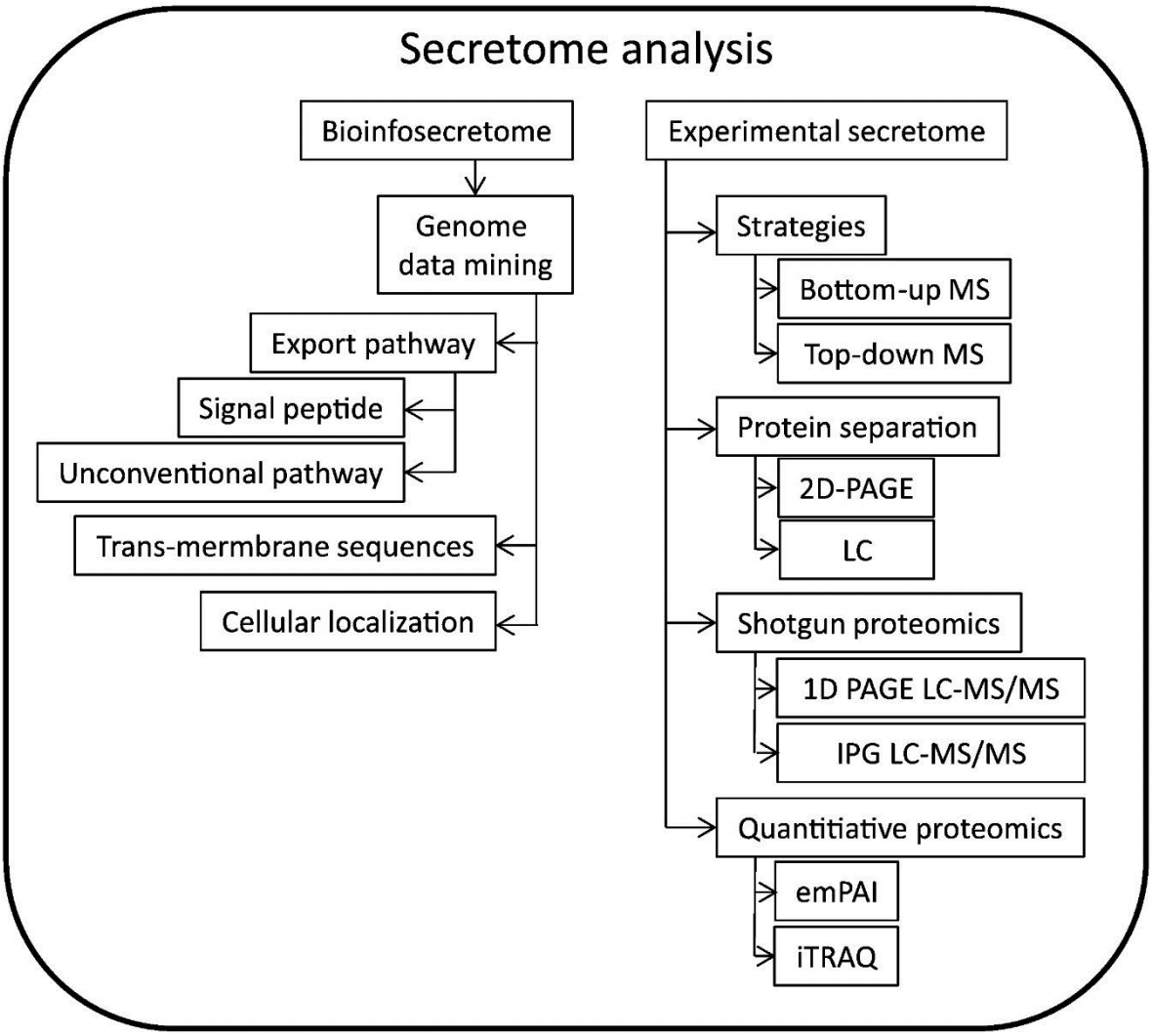

Fig. 1. Secretome analysis flowchart.

2. - BIOINFORMATIC SECRETOMES IN BASIDIOMYCETES

Bioinfosecretomes (bioinformatics secretomes) are in silico-predicted secretomes based on the identification of secretion signals in the putative proteins corresponding to gene models that are automatically annotated in a genome sequence. A preliminary consideration to bear in mind is that the quality of a bioinfosecretome will depend on the quality of the genome annotation, and consequently, these secretomes will evolve along with new versions of genome assemblies and annotations.

The conventional fungal protein secretion pathway requires secretion-oriented proteins to contain an amino-terminal peptide sequence (N terminal sequence, Signal Peptide, SP) that targets them to the endoplasmic reticulum (ER), where the proteins will be correctly folded, post-translationally modified (through glycosylation, disulfide bridge formation, phosphorylation, and subunit assembly), and pass through an ER quality control process, where misfolded proteins will be degraded via the unfolded protein response (UPR) and the ER-associated protein degradation (ERAD) mechanisms (Conesa et al., 2001). Subsequently, the secretion-targeted proteins enter vesicles of the *cis*-Golgi apparatus, where they dock and fuse, and additional modifications can take place. Finally, the proteins are ferried by the secretory vesicles to the plasma membrane, where vesicles fuse, and the proteins are secreted (Conesa et al., 2001; Fonzi, 2009; Shoji et al., 2008).

The requirement for an SP sequence in secreted proteins can be used to predict the subcellular location of a protein via in silico methods. For a detailed review of the bioinformatics tools available for secretome analysis see Caccia *et al.*(2013). A commonly used protocol to initiate the analysis of fungal bioinfosecretomes was described by Müller *et al.* (2008), who predicted the occurrence of secretory targeting signals using SignalP software (http://www.cbs.dtu.dk/services/SignalP/), which takes into account the N-terminal region (70 amino acids) of the protein sequence (J Bendtsen at al., 2004). An alternative to the SignalP program is PHOBIUS software (http://phobius.sbc.su.se/), which predicts transmembrane topology and signal peptides from the amino acid sequence of a protein [13]. The initial bioinfosecretome prediction can be refined using other programs or utilities designed to predict the subcellular localization of the proteins, such as ProtComp software (www.softberry.com), TARGETP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson,

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2000) or WoLF PSORT (http://wolfpsort.org/) (P Horton et al., 2007), to scan the protein for the presence of multiple transmembrane motifs (TMHMM; http://www.cbs.dtu.dk/services/TMHMM/), selecting those models with 0 or 1 transmembrane motifs located at the N-terminus, and to predict GPI-anchor proteins (big-PI; http://mendel.imp.ac.at/gpi/fungi_server.html) (Eisenhaber at al., 2004). Most genome servers at a minimum contain unrefined information about the presence of SPs in annotated proteins, providing a starting point for a more complete bioinfosecretome analysis.

The first report on the bioinformatics prediction of basidiomycete secretomes was for *P. chrysosporium* (Wymelenberg et al., 2005). The authors predicted 268 secreted proteins based on the first version of the genome of this white rot fungus (Martinez et al., 2004), although this number could be an underestimation of the actual number of secreted proteins due to inaccurate and incomplete gene model annotation. Subsequently, based on the second version of the genome assembly, these researchers carried out new secretome predictions, identifying 769 secreted proteins (Vanden Wymelenberg et al., 2006). The final bioinformatic secretome included 7.6% of the total annotated protein models. This number was higher than that obtained using a similar approach in the yeast *Candida albicans* (Lee et al., 2003), and 407 of the predicted secreted proteins could be classified as glycosyl hydrolases (GH), oxidoreductases, peptidases and esterases-lipases. This secretome profile fits the saprotrophic lifestyle of *P. chrysosporium*.

The secretome of the maize smut, *Ustilago maydis*, was also studied *via in silico* methods (Mueller et al., 2008). The initial analysis using SignalP and TargetP identified 776 candidate secreted proteins, and further refinement with the TMHMM utility (retaining the proteins with one predicted transmembrane domain and with one potential GPI-anchor) reduced the prediction to 168 secreted proteins and 386 proteins with other functions. The authors identified 39 GPI-anchored proteins involved in plant cell wall modification and the degradation of other plant components as well as proteins involved in modification of the fungal cell wall and a set of extracellular metabolic proteins with potential roles in pathogenicity. In addition to the secreted proteins, a large number of proteins without a predicted function were identified. These proteins included secreted proteins with internal repeats, repetitive protein precursors that are likely to be processed into small peptides in the

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Golgi apparatus, hydrophobins, and effector proteins containing signals for targeting to the host nucleus. In summary, the *U. maydis* bioinfosecretome fits the biotrophic (in contrast to saprotrophic or necrotrophic) relationship established by this fungus and its host plant.

Unconventional protein secretion pathways (UPS pathways) are ER/Golgi independent and do not require the presence of an SP sequence in the protein to be exported (Nickel & Seedorf, 2008). UPS pathways can be experimentally demonstrated as the export process is not affected by the inhibitor brefeldin A (Nickel, 2010). Although the molecular mechanisms and machinery components underlying these processes are still unknown, there are two general types of UPS: non vesicular (direct translocation across plasma membranes and ABC transporters) and vesicular (autophagy-based secretion and proteins that bypass the Golgi complex) (Nickel, 2010; Nickel, 2012). The occurrence of UPS pathways in fungi is well documented in the case of yeasts (Nombela, 2006). Jain *et al.* (Jain, 2008) conducted a bioinformatics analysis of non-classically secreted proteins in *Laccaria bicolor* using the SecretomeP method (http://www.cbs.dtu.dk/services/SecretomeP/) (Bendtsen et al., 2004). Among the proteins found to be unconventionally exported, the authors identified proteins involved in carbohydrate metabolism, lectins, and proteases.

The use of bioinformatics screening procedures similar to those described above has led to the construction of databases such as the Fungal Secretome Knowledgebase (Lum & Min, 2011) and the Fungal Secretome Database (Choi et al., 2010).

3. - PROTEOMICS METHODS USED TO INVESTIGATE BASIDIOMYCETE SECRETOMES

In contrast to bioinfosecretomes, which provide information about the complete set of predicted secreted proteins present in a genome, wet secretomes provide information about the set of proteins secreted under given culture conditions, by specific sets of cells, and/or at a given time. Nevertheless, analyses of wet secretomes, like analyses of bioinfosecretomes, rely on the availability and quality of complete annotated genome sequences for the identification of protein tags. Additionally, there are three further considerations that must be

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kept in mind when working with wet secretomes: they are still representations of the proteins exported during particular culture conditions or developmental stages; in the applied sampling processes, proteins secreted at a low level can fall below the detection sensibility limit; and there is a chance of contamination of the sample with intracellular proteins during the extraction process. On the other hand, the nature of the sampling process permits quantitative results to be produced that are beyond the possibilities provided by bioinfosecretomes.

The increasing number of available fungal genomes (I V Grigoriev et al., 2012; Igor V Grigoriev et al., 2011) and the advances in complex protein mixture separation and analysis technologies have led to a great increase in research on fungal secretomes (Bouws, Wattenberg, & Zorn, 2008). In this section, we will review the fundamentals of the different workflows used to carry out these laboratory-based analyses. The analysis of a secretome can be divided into two steps: separation and identification of the secreted proteins. The separation procedure can be based on electrophoretic (2D-PAGE) or chromatographic techniques (LC), or a combination of the two. The identification process is based on mass spectrometry (MS). For a detailed review of secretome methodologies, see Mijkherjee & Mani, 2013(2013).

3.1. – PROTEIN AND PEPTIDE IDENTIFICATION

Protein and peptide identification in proteomic analyses is based on mass spectrometry (MS, see Graham *et al.* (2011) and Han *et al.* (2008) for comprehensive reviews). MS permits the accurate measurement of the molecular mass of a protein or peptide and its identification by searching its mass in the complete set of molecular masses of the proteins or peptides produced by an organism whose genome sequence is known.

Current mass spectrometers permit the analysis of intact proteins (top-down MS), but MS of proteolytic peptides (bottom-up MS) is more generally applied. Top-down MS provides information about the molecular mass, amino acid sequence, and positions and types of post-

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translational modifications of a protein (Collier, Hawkridge, Georgianna, Payne, & Muddiman, 2008; Waanders, Hanke, & Mann, 2007); however, its application to large-scale proteomic analyses is still a major challenge, and it requires the use of mass spectrometers with particularly high resolving power, which are therefore very expensive. Bottom-up proteomics can follow two different strategies: either the proteins are first separated and then digested (sort-then-break approach), or the digestion is carried out directly in the complex protein sample, and the proteolytic peptides are then separated (break-then-sort approach). Under the sort-then-break approach, a peptide mixture can either be directly analyzed to produce a peptide mass fingerprint (PMF, (Henzel et al., 1993)), or the peptides can be further sorted via LC, interfaced with a tandem mass spectrometer. In the break-then-sort approach, the peptides in the complex digestion mixture are separated and then analyzed through MS. This second approach is also referred to as shotgun proteomics and will be discussed below.

3.2. – SEPARATION TECHNIQUES

3.2.1. – 2D-PAGE

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been the principal technique employed to analyze secretomes for the last 10 years (Awdeh, Williamson, & Askonas, 1968; Dale & Latner, 1969; Macko & Stegemann, 1969; Margolis & Kenrick, 1969; O'Farrell, 1975; Shevchenko, Wilm, Vorm, & Mann, 1996). Using this technique, the proteins in a sample are separated in two dimensions, e.g., using different acrylamide gel concentrations, or they are first separated on the basis of their isoelectric point (isoelectric focusing, IEF) and then on the basis of their molecular mass (SDS-PAGE). With the introduction of the 2D-PAGE using IEF, as many as 300 protein spots can be separated, although the obtained spots are frequently smeared (Issaq & Veenstra, 2008).

The orthogonal combination of both separation procedures permits the identification of new proteins and the measurement of their relative abundance within a sample. In addition, 2D-PAGE permits the detection of post-translational modifications and isoforms of the studied

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proteins. However, 2D-PAGE has general limitations in resolving proteins that are too basic or too acidic (outside the pH 3 to 10 range) or too large or too small (outside the range of 10^4 to 10^6) and in detecting proteins that are present in low amounts (low sensitivity, dynamic range of 10^3 to 10^7 copies per cell) (Issaq & Veenstra, 2008; Minden, 2007). Once a high-quality 2D-PAGE gel has been obtained, the secretome analysis continues, involving the identification, isolation and protein sequencing of spots corresponding to single proteins. Protein spot detection requires high-sensitivity staining techniques (i.e., silver staining) compatible with ulterior MS analysis of the samples (Rabilloud, Carpentier, & Tarroux, 1988). The protein spots identified via 2D-PAGE analysis can be individually excised and digested within the gel according to the procedure initially described by Shevchenko *et al.* (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2007; Shevchenko et al., 1996) using trypsin. These tryptic peptides can be subsequently analyzed via MS (see below). The low gel-to-gel reproducibility of 2D-PAGE has made it difficult to use this technology to compare different samples. To overcome this problem, the difference gel electrophoresis (DIGE) method has been developed, in which the two protein samples are covalently labeled with different fluorescent dyes and co-electrophoresed on the same 2D gel (Minden, 2007). It is important to emphasize that 2D-PAGE provides experimental data in which the observed size is larger than the predicted size due to post-transcriptional modifications. In the case of higher lignocellulolytic basidiomycetes, all of the secreted enzymes that have been purified are glycoproteins (Henzel et al., 1993). Moreover, these fungi produce large amounts of extracellular polysaccharides and other low-molecular-weight compounds that interact with the secreted proteins, making difficult the production of 2D-PAGE of secretomes with sufficient quality and resolution for spot analysis. A number of methodological techniques have been developed to avoid these problems (Abbas, Koc, Liu, & Tien, 2005; Fragner, Zomorrodi, Kues, & Majcherczyk, 2009; Ravalason et al., 2008; Vanden Wymelenberg et al., 2006), but they have only been applied in submerged or semi-solid cultures, and high-quality secretomes from solid fermentation cultures have not been produced to date.

3.2.2. – Liquid chromatography

A highly efficient method for proteomic analysis is the separation of proteins or peptides

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using liquid chromatography (LC). A more generalized method is the use of C18 reverse-phase columns loaded with proteome digestion products, working under nano-flow conditions (≈ 100 nl/min). These columns are connected to mass spectrometers to allow on-line analysis of the resolved peptides.

For carrying out a powerful proteomics analysis, it is necessary to attain a high resolving power and high sensitivity to achieve a dynamic range of at least 10^5. These resolution and sensitivity levels can be reached using two-dimensional nano-liquid chromatographic (2D-nLC) techniques in which strong cation exchange (SCX) columns are employed for the first dimension and reversed-phase (RP) columns for the second (MudPIT approach, (Nagele, Vollmer, & Horth, 2003, 2004).

3.3. – SHOTGUN PROTEOMICS

Under a shotgun proteomics approach, mass spectra are collected for as many peptide fragments as possible, and these masses are then compared against the complete set of peptide masses deduced from the genome sequence of the organism(s) being analyzed with bioinformatics tools such as SEQUEST [52], MASCOT (Perkins, Pappin, Creasy, & Cottrell, 1999) and X!Tandem (Craig & Beavis, 2004). The results of shotgun proteomics are highly dependent on the quality of the peptide separation procedure and on the limits of the MS dynamic range , which causes peptides present at a high relative abundance to be preferentially sampled. Moreover, this approach is also affected by the presence of unexpected post-translational modifications that make the identification of the corresponding peptide difficult. Consequently, the results of this approach cannot be considered strictly quantitative.

The separation of the peptidic fragments under the shotgun proteomics approach is more complex than in the top-down approach discussed above. There are at least three main strategies applied for peptide separation: pre-fractionation *via* one-dimensional electrophoresis (1D-PAGE LC-MS/MS)(Simpson et al., 2000); immobilized pH gradient isoelectric focusing (IPG-LC-MS/MS, (Cargile, Sevinsky, Essader, Stephenson Jr., & Bundy,

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2005); and the gel-free LC-MS shotgun approach (Link et al., 1999; Washburn, Wolters, & Yates, 2001).

The 1D-PAGE LC-MS/MS technique involves the pre-fractionation of proteins *via* one-dimensional electrophoresis, followed by in-gel digestion and automated LC-MS/MS. Intact proteins are fractionated through 1D-SDS-PAGE, and approximately 25 contiguous slices are cut from the gel lane. Each gel slice is digested *in situ* with trypsin, and the resulting peptides are separated *via* reverse-phase liquid chromatography (RP-HPLC) and analyzed *via* MS. In 2D-PAGE, the pH range of the IPG strips is a limiting factor for the identification of proteins. Using a 1D-PAGE LC/MS approach, Vanden Wymelenberg *et al.* (2009) identified secreted proteins with an alkaline isoelectric point (10.6) in *P. chrysosporium*. Proteins in this alkaline isoelectric point range are very difficult to detect using 2D-PAGE.

Under the IPG LC-MS/MS approach, the protein sample is digested, and the resulting peptide mix is loaded and focused onto an immobilized IEF strip. The IEF strip is cut into sections, and peptides in each section are eluted and subjected to LC/MS analysis. This strategy allows better separation of peptides than classical 2D-PAGE (Bjellqvist et al., 1982), and it has been used by Vincent *et al.* to study the secretome of *L. bicolor* (Vincent et al., 2009).

Under the gel-free LC-MS shotgun approach (LC-LC-MS/MS), proteins are digested in solution to generate a complex mixture of peptides that are subsequently separated through multidimensional liquid chromatography. Typically, strong cation exchange (SCX) and reversed-phase (RP) liquid chromatography columns are used to separate peptides into fractions, prior to tandem mass (MS-MS) spectrometry analysis (Link et al., 1999; Washburn et al., 2001). This technique has allowed the detection of the largest number of secreted proteins in fungal secretomes. Vanden Wymelenberg *et al.* (2010) studied the secretome of *P. placenta* using two different techniques, 1D-PAGE-LC-MS/MS and the LC-LC-MS/MS shotgun approach, and detected 19 proteins *via* 1D-PAGE-LC-MS/MS and 63 proteins *via* LC-LC-MS/MS. Following the same strategy, these authors analyzed the *P. chrysosporium* secretome and identified 30 proteins through 1D-PAGE LC-MS/MS and 73 through LC-LC-MS/MS (Vanden Wymelenberg et al., 2010). Several proteins (four in *P. placenta* and seven in *P. chrysosporium*) were detected only by 1D-PAGE LC-MS/MS, demonstrating the effectiveness of using several techniques in the analysis of fungal secretomes.

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Vincent *et al.* (Vincent et al., 2012) carried out a comparative analysis of different separation techniques (IPG-IEF shotgun, 1D-PAGE LC-MS/MS and 2D-PAGE) when studying the secretome of *Laccaria bicolor* (see below). They found that the IPG-IEF LC-MS/MS approach detected the greatest number of proteins (142). In contrast, 1D-PAGE LC-MS/MS identified 116 proteins, and 2D-PAGE was the least efficient technique in terms of the number of proteins identified, detecting 77 proteins from 201 analyzed spots. This is a common characteristic of fungal secretome 2D-PAGE: many of the spots analyzed cannot be identified, and acidic proteins are more easily detected than alkaline proteins, demonstrated by the fact that among all of the spots excised, 134 were found in the gels at pH 3-11, 46 at pH 4-7, and 21 at pH 7-11. Another interesting difference between these techniques was that glycosylphosphatidyl inositol (GPI) small secreted proteins were identified only through SDS and IPG and not by 2D-PAGE, indicating the difficulty of separating small proteins using the last technique. Nevertheless, 16 proteins were only found in the 2D-PAGE experiments, again highlighting the need to combine various analytical approaches to identify as many proteins as possible in a given sample.

3.4. – QUANTITATIVE PROTEOMICS

A major drawback of conventional proteomics approaches is that they produce poor quantitative results. There are two basic issues addressed by quantitative proteomics: the estimation of protein abundance in a given sample and comparisons between proteins present in different samples.

For the estimation of protein abundance in a given sample, the Protein Abundance Index (PAI) (Rappsilber, Ryder, Lamond, & Mann, 2002) and the exponentially modified Protein Abundance Index (emPAI) (Ishihama et al., 2005) have been developed. The rationale for these indexes is that larger proteins can give rise to more peptides, and the PAI represents the number of peptides identified (Nobserved) divided by the number of theoretically observable (Nobservable) tryptic peptides (PAI = Nobserved/Nobservable), whereas the emPAI is an exponential modification of the PAI (emPAI = 10^{PAI-1}). The Nobserved peptides are determined experimentally, and Nobservable peptides are determined via *in silico* digestion

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of the proteins.

Several methods have been developed for using data acquired in MS to quantify the amount of proteins in a sample. The basic rationale underlying such quantitative methods involves the differential labeling of two or more samples being compared, combining the labeled samples and analyzing the samples using the proteomics approaches discussed above. One option for labeling samples is to provide stable isotope-labeled (i.e., using ^{13}C or ^{15}N) metabolic precursors to an organism that then incorporates them into proteins (see Beynon and Pratt for a review (Beynon & Pratt, 2005)). However, chemical labeling methods are required for samples to which labeled precursors cannot be added. There are three main strategies for chemical labeling for quantitative proteomics: the isotope-coded affinity tag (ICAT) (Gygi et al., 1999), isotope-coded protein label (ICPL) (Schmidt, Kellermann, & Lottspeich, 2005) and the isobaric tag for relative and absolute quantitation (iTRAQ) methods (Ross et al., 2004).

The ICAT approach is based on the labeling of the cysteine residues present in a protein, prior to its digestion with the appropriate protease. The labeled peptides are enriched through affinity chromatography and subsequently analyzed. ICAT has some limitations: its results are not sufficiently robust; the requirement for cysteine residues limits the number of possible labels per protein; and the technique cannot be used in cysteine-free proteins (Wiese, Reidegeld, Meyer, & Warscheid, 2007). The requirement for cysteine residues is avoided in the ICPL approach, in which the primary amino groups present in lysine residues are labeled. This procedure shows a higher sensitivity than ICAT and is compatible with gel-based separation techniques. On the other hand, ICPL results in changes in the migration of peptides during IEF (Schmidt et al., 2005).

The iTRAQ technique was developed to solve most of the drawbacks of the other quantitative techniques. In iTRAQ, peptides from different samples are labeled with different compounds that have been designed to have the same total mass but can be cleaved to produce different fragments. Consequently, the derivatized peptides are indistinguishable in MS but exhibit intense low-mass MS/MS signature ions that support quantitation (Ross et al., 2004). Up to four different isobaric tags can be simultaneously used in multiplex experiments. In the classical iTRAQ approach, the N-terminal and lysine residues present in the peptides of the

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two samples to be compared are labeled with two different isobaric labels. After mixing the two samples, due to the isobaric design of the labels, the differentially tagged peptides will appear as a single peak in the MS analysis. Then, a second tandem MS analysis of the spots fragments the isobaric label, producing residues that can be differentiated, and the relative proportion of each peptide can be deduced from the relative intensities of the corresponding reporters (Ross et al., 2004). The iTRAQ approach can be used either with the proteolytic peptides from a protein sample or with intact proteins (Wiese et al., 2007). A limitation of iTRAQ is that its requirement for MS/MS limits the obtained quantitative information to the most abundant peptides, and consequently, this technique shows a greater propensity to identify the most abundant proteins. The iTRAQ approach has been successfully used to compare the proteomes of *P. chrysosporium* produced using different carbon sources (A. Manavalan, Adav, & Sze, 2011) or natural biomass (Adav, Ravindran, & Sze, 2012) and to study the proteins secreted by lignocellulolytic fungi in single cultures and in consortia (Adav, Ravindran, Cheow, & Sze, 2012).

4. - BASIDIOMYCETE SECRETOMES

4.1. – WHITE ROT BASIDIOMYCETES

4.1.1. – *Phanerochaete chrysosporium*

P. chrysosporium is a model white rot basidiomycete that contains lignin (LiP) and manganese (MnP) peroxidases but not phenol oxidases (Pox). The genome of this fungus has been sequenced by Martinez *et al.*(Martinez et al., 2004), and its secretome has been studied under different conditions. Automatic annotation of the *P. chrysosporium* genome predicted 10,048 gene models (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). Vanden Wymelenberg *et al.* (Vanden Wymelenberg et al., 2006) predicted a *P. chrysosporium* bioinfosecretome of 769 proteins, accounting for nearly 7.7% of the predicted proteins. More than half of the secreted predicted proteins (407) were similar to other known proteins. Among these proteins, the most prominent groups were GHs (87), oxidoreductases (84), peptidases (52), esterases–lipases (21), hydrophobins (14), LiPs (10) and MnPs (5).

The use of lignocellulose-derived products as carbon source or as inducers in the analysis of white rot fungi secretomes often results in the co-extraction of brown water-soluble extractives and smearing in 2D-PAGE (Abbas et al., 2005; Zorn, Peters, Nimtz, & Berger, 2005). These extractives and smears impede the correct visualization and cut off of the obtained spots, which has prompted the use of different approaches to analyze the *P. chrysosporium* secretome. Some examples of carbon sources or inducers that will be discussed below include purified cellulose, xylan, starch or synthetic lignin, wood or other natural lignocellulose substrates (Abbas et al., 2005; Zorn et al., 2005).

Vanden Wymelenberg *et al.* (Vanden Wymelenberg et al., 2006) studied the *P. chrysosporium* secretome in shaken submerged cultures produced in synthetic media under carbon or nitrogen limitation using 1D-PAGE and LC-LC-MS/MS and identified 40 secreted proteins, including 13 peptidases, 6 esterases-lipases, 8 GHs, 5 LiPs and 3 MnPs. On the other hand, Manavalan *et al.* (A. Manavalan et al., 2011) analyzed the secretome in shaken submerged cultures supplemented with synthetic lignin, cellulose or a mixture of the two, using an iTRAQ-based approach. The largest group of secreted proteins identified in this experiment consisted of GHs (66), followed by lignin-degrading enzymes (16) and proteases (16). Surprisingly, LiPs and MnPs were absent, suggesting that synthetic lignin might not be an efficient inducer of these enzymes. The quantitative results of this experiment revealed that three different cellobiohydrolases were the most abundantly secreted proteins in the cellulose-containing cultures, whereas a pyranose 2-oxidase was the most oversecreted protein in the lignin-containing cultures. Hori *et al.* (C Hori, Igarashi, Katayama, & Samejima, 2011) studied the effect of starch or xylan addition in shaken submerged cultures containing cellulose as a carbon source. They identified 47 proteins in this secretome and found that xylan addition increased the synthesis of several GHs. These authors did not identify peptides corresponding to LiPs or MnPs.

Adav *et al.* (Adav, Ravindran, & Sze, 2012) employed different complex lignocellulosic substrates (corn stover, sugarcane bagasse, wheat bran, hay, wood chips and sawdust) to quantitatively study protein secretion induction in shaken submerged cultures. In the obtained secretomes, they identified GHs (52%), lignin-depolymerizing enzymes (10%) and proteases (20%). However, LiPs and MnPs were absent, as in the experiment conducted by Manavalan

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et al. The authors concluded that the expression and iTRAQ quantification of copper radical oxidase, cellobiose dehydrogenase, glucose oxidase, isoamyl alcohol oxidase, peroxiredoxins, pyranose 2-oxidase, quinone oxidoreductase, and iron-containing alcohol dehydrogenase indicated that *P. chrysosporium* degrades lignin through oxidases.

All of these previous studies were performed in submerged cultures. In contrast, Abbas *et al.* (Abbas et al., 2005) followed a different approach and carried out a preliminary study of the proteins secreted in solid cultures on red oak (*Quercus rubra*) hardwood. They identified 45 2D-PAGE spots, which corresponded mainly to GHs (15, including the two cellobiohydrolases indicated above). These authors also detected a signal corresponding to LiP. Subsequently, using 2D-PAGE and 1D-PAGE LC-MS/MS in liquid and solid media, Sato *et al.* (Sato, Liu, Koc, & Tien, 2007) identified GHs and proteases in solid cultures and GHs in submerged ones.

Ravalason *et al.* (Ravalason et al., 2008) compared the *P. chrysosporium* secretome in shaken submerged cultures performed using synthetic medium (developed to promote peroxidase production under carbon limitation, ligninolytic culture) and polyurethane foam cubes to immobilize the fungus with the secretome produced under biopulping conditions (static semisolid cultures containing basal medium and wood chips from *Pinus nigra*, with final moisture content of 75%). They identified 18 spots in the ligninolytic and 19 in the biopulping cultures. The secretome in the ligninolytic medium was mainly comprised of peroxidases (LiPs and MnPs), whereas several wood-degrading enzymes and enzymes involved in fungal metabolism (mainly GHs) were detected in the biopulping cultures. Based on the comparison of these results with the other findings discussed above, it becomes clear that ligninolytic culture conditions produce a secretome profile similar to that observed in submerged cultures carried out under basal conditions (i.e., high expression of MnPs, LiPs, proteases and lipases), whereas the presence of complex lignocellulosic substrates promotes the predominant secretion of enzymes belonging to the group of GHs with respect to the relative abundance of proteases and lipases.

The correlation of the changes in the transcriptome and secretome profiles under different culture conditions has been addressed by Vanden Wymelenberg *et al.* (Vanden Wymelenberg et al., 2006, 2009) using expression microarrays and LC-LC-MS/MS. These authors

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compared the genes upregulated under carbon limitation (CLB, 33 genes), nitrogen limitation (NLB, 63 genes) and avicel *versus* glucose as a carbon source (HBA, 70 genes) with the secreted proteins detected under the same conditions (CLB, 46 proteins; NLB, 23 proteins; and HBA, 37 proteins). The expression of genes involved in nitrogen and in carbohydrate metabolism was dramatically upregulated under the NLB and HBA culture conditions, respectively. In general, a good correlation between expression levels and the LC-LC-MS/MS identification of secreted proteins was observed, except for highly transcribed genes with putative secretion signals without LC-LC-MS/MS support and high-scoring peptides with low transcript levels. In some cases, these discrepancies can be associated with the presence of unconventional export signals. Using aspen or pine wood as a substrate, they carry out a comparative transcriptome and secretome analysis (Vanden Wymelenberg et al., 2011) and identified 118 proteins in both media. Carbohydrate-active enzyme (CAZy)-encoding genes represented 33% (20 of 61 genes) and 45% (40 of 88 genes) of the total *P. chrysosporium* protein genes identified in aspen and pine containing media, respectively. These differences show the influence of wood species in the secretome and may reflect the diversity of the strategies for degrading lignocellulose.

4.1.2. – *Phanerochaete carnosa*

P. carnosa is a *P. chrysosporium* relative that shows a preference for softwood, in contrast to the preference for hardwood shown by *P. chrysosporium*. Mahajan and Master (Mahajan & Master, 2010) conducted an LC-LC-MS/MS based analysis of the secretome of these species when cultivated statically with either microcrystalline cellulose (avicel) or with *Picea glauca* (white spruce) wood chips as a carbon source. This study was carried out before the *P. carnosa* complete genome sequence was available (H. Suzuki et al., 2012), and *P. chrysosporium* gene models were used for the identification of peptides. The major difference in ligninolytic genes between these species is that the *P. carnosa* genome encodes seven MnPs and four LiPs, whereas the *P. chrysosporium* genome encodes five MnPs and ten LiPs. Moreover, although *P. carnosa* does not appear to encode laccases (Pox) *sensu stricto*, there are nine gene models annotated as copper oxidases in this fungus. In contrast, Pox genes are absent in the *P. chrysosporium* genome. The main difference between the *P. carnosa*

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secretomes obtained in cellulose and spruce-based cultures was the increase in GHs in the cellulose-based cultures (representing 46% of the identified proteins *vs*. 29 % in the spruce-based cultures) and the increase in peptidases in the spruce-based cultures (40% *vs*. 25% in the cellulose-based cultures).

4.1.3. – *Ganoderma lucidum*

G. lucidum (Reishi or Ling Zhi, Polyporales) is an edible white rot basidiomycete that has been extensively studied because of its use in traditional Asian medicine. This fungus shows strong wood degradation ability, associated with potential in bioenergy production. The *G. lucidum* genome has been sequenced by Chen *et al.* (Chen et al., 2012). Its genome size is 43.3 Mbp, presenting 16,113 annotated gene models. It contains a large set of cytochrome P450-encoding genes and one of the largest repertoires of wood-degrading enzymes. The secretome of *G. lucidum* cultivated in solid cultures on sugarcane bagasse following fruiting body harvesting (day 40 of culture) was studied by Manavalan *et al.* (T. Manavalan, Manavalan, Thangavelu, & Heese, 2012) using 1D-PAGE-LC-MS/MS. The authors identified 71 proteins that could be grouped into the GHs (cellulases, 24%; hemicellulases 5%; and other 10%), lignin depolymerizing enzymes (24%, including five laccases, and a manganese peroxidase), proteases (2%), phosphatases (7%), transport proteins (10%) and hypothetical proteins (10%).

4.1.4. – *Pleurotus sapidus*

Using 2D-PAGE, Zorn *et al.* (Zorn et al., 2005) analyzed the secretome of the white rot basidiomycete *P. sapidus* in shaken submerged cultures in nitrogen-limited medium containing either *Arachis hypogaea* (peanut) shells or glass wool as a carrier material and glucose as a carbon source. The lignin-degrading strategy of *P. sapidus* differs from that of *P. chrysosporium* due to the presence of Poxs and the absence of LiPs in the genome. The role of LiPs in these organisms seems to be carried out by versatile peroxidases (VP). This work was carried out before a genome sequence for a *Pleurotus* species was available, and

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the number of enzymes identified was consequently limited. Comparison of the two *P. sapidus* secretomes showed a broader spectrum of peptidases in the cultures grown on peanut shells in comparison with those grown on glass wool, where the versatile peroxidases clearly dominated.

4.1.5. – *Ceriporiopsis subvermispora*

C. subvermispora and *P. chrysosporium* are both members of the order Polyporales, but they differ in their ability to selectively degrade lignin. *C. subvermispora* depolymerizes lignin but may do so while causing relatively little cellulose degradation (Fernandez-Fueyo et al., 2012). Fernandez-Fueyo *et al.* (Fernandez-Fueyo et al., 2012) conducted LTQ-Orbitrap (LC-LC-MS/MS) analysis of TCA-precipitated filtrates from 5-day-old shake flasks containing ball milled aspen, glucose or avicel to compare the protein expression profiles of *C. subvermispora* and *P. chrysosporium*. They unambiguously identified 60 and 121 proteins in filtrates from aspen wood medium cultures of *P. chrysosporium* and *C. subvermispora*, respectively, among which 18 and three corresponded to GHs. A total of three MnPs were identified in the *C. subvermispora* filtrates, but no peroxidases were found in the *P. chrysosporium* cultures.

4.1.6. – *Trametes sp.*

Trametes is a white rot basidiomycete distributed worldwide that is commonly found growing on tree stumps. The sequenced genome *of T. versicolor* is 44.8 Mbp in size, presenting 14,296 annotated gene models (http://genome.jgi.doe.gov/Trave1/Trave1.info.html) (Floudas et al., 2012). Analysis of the secretome of *T. versicolor* cultivated under the same conditions used for *P. chrysosporium* and *P. placenta* (see below) resulted in the identification of 218 proteins, including 44 uncharacterized proteins, 65 GHs, 27 peptidases, 8 oxidases related to lignin degradation, 5 lipases, 4 Poxs, and 3 MnPs and Vps. (Floudas et al., 2012).

Lebrun *et al.* (Lebrun et al., 2011) conducted a 1D-PAGE-LC-MS analysis of the effect of

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metal ions (Zn, Cu, Pb and Cd) on the secretion profile of *T. versicolor*, with a particular focus on the extracellular hydrolases and ligninolytic oxidases. Their results showed that exposure of *T. versicolor* to metal ions modified its secretion profile in different ways. While the activity of hydrolases was inhibited by single metals or metal cocktails, oxidase activities were specifically stimulated by Cu and Cd (individually or in cocktails). Moreover, the glycosylation pattern of 2 laccases was affected by the presence of the metal ions.

Using a more secretome-oriented approach, Ji *et al.* (Ji et al., 2012) studied the proteins secreted by *T. trogii via* 1D-PAGE-LC-MS. The identified proteins (64) were sorted into five categories: cell wall and lignin-degrading enzymes (29 %), carbohydrate metabolism proteins (20 %), fatty acid metabolism proteins (11 %), protein metabolism proteins (11 %) and other proteins (29 %).

4.1.7. – *Bjerkandera adusta, Ganoderma sp.* and *Phlebia brevispora*

Hori *et al.* (Chiaki Hori et al., 2013) conducted a comparative LC-LC-MS/MS secretome analysis of monokaryotic strains of the white rot fungi *Bjerkandera adusta, Ganoderma sp.* and *Phlebia brevispora* grown in wood cultures under identical conditions. In the *B. adusta* cultures, among the 187 proteins detected, 33% were GHs, and 6% were other CAZys. In *Ganoderma sp.*, a total of 105 proteins were identified, 37% of which were GHs, whereas 6% were other CAZys. In *P. brevispora*, 178 proteins were identified; 39% and 4% of these proteins were GHs and other CAZys, respectively. Aldose 1-epimerase (ALE) was previously detected together with cellobiose dehydrogenase (CDH) and cellulases in the culture filtrates of white rot fungi (Vanden Wymelenberg et al., 2006), and genes encoding ALE were also present in the genomes of these 3 white rot basidiomycetes, suggesting a physiological connection between ALE, CDH, cellulase and possibly LPMO (lytic polysaccharide monooxygenase).

4.1.8. - *Schizophyllum commune*

Using LC-MS/MS, the authors (Zhu et al., 2016) compared the *S. commune* secretome with those of *P. chrysosporium, C. subvermispora,* and *G. trabeum* (BR) during solid-state

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fermentation on Jerusalem artichoke stalks. A total of 229 proteins were identified in the *S. commune* secretome, 112 proteins in the *P. chrysosporium* secretome**,** 95 proteins in the *C. subvermispora* secretome, and 109 in the *G. trabeum* secretome. In this analysis, *S. commune* produced a much larger set of GH family enzymes than the other three species.

As expected, oxidoreductase secretion differed substantially between the four fungi. *P. chrysosporium* secretes three lignin peroxidases and one manganese peroxidase, while *C. subvermispora* secretes two manganese peroxidases and one laccase. In contrast, neither of the *S. commune* and *G. trabeum* secretomes contained ligninolytic enzymes, such as manganese peroxidases, lignin peroxidases, or laccases. *P. chrysosporium* cannot produce laccases because it does not have laccase genes in its genome, but *G. trabeum and S. commune* have laccases (two and four, respectively) (Riley et al., 2014a). Both *G. trabeum* and *S. commune* share some genomic characteristics of brown rot fungi, such as the lack of class II peroxidases (PODs). *S. commune* was indeed not classified as white or brown rot when looking at its enzymatic arsenal for lignocellulose degradation (Riley et al., 2014a), despite how it has always been seen as a white rot fungus. Protein identifications in this paper support the use of the Fenton mechanism for lignin attack by *S. commune*, which was also suggested by Ohm *et al.* (2010).

4.1.9. - *Pycnoporus cinnabarinus*

Levasseur *et al*. (2014) analyzed the *P. cinnabarinus* secretome (1D LC-MS/MS) and detected 184 proteins in maltose medium, 166 proteins in maltose and micronized birch-wood medium, 121 proteins in maltose, maize bran, and Avicel medium, and 139 proteins in SSF cultures (five different substrates: sugarcane bagasse, banana skins, wood shavings, hemp, and micronized birch wood.

The authors also identified LPMOs of the AA9 family only in the conditions that included complex substrates, and no AA9 proteins were found in the control condition with maltose. Furthermore, different AA9 proteins were produced in response to different growth conditions, a result that could indicate growth substrate- or time-dependent regulation of the LPMO genes. In contrast, three AA1_1 laccases were identified in all the studied conditions,

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indicating constitutive production by the fungus and a wide range of media where these enzymes can have roles.

4.1.10. - *Pycnoporus coccineus*

Couturier *et al*. (2015) analyzed *Pycnoporus coccineus* because of its ability to grow on both softwood and hardwood. This fungus displays similar sets of CAZymes when grown on these two types of wood. 1D LC–MS/MS was subsequently used to identify a total of 115, 135, and 135 CAZymes in maltose, pine, and aspen cultures, respectively; most of the detected proteins (84%) were common to the two wood cultivations. Among the CAZymes identified in pine and/or aspen that were not detected in maltose the authors identified proteins attributed to cellulose, hemicellulose, and pectin degradation, including GH families GH3, GH5_5, GH5_7, GH6, GH10, GH12, GH16, GH28, GH43, and GH45, as well as CE family CE1, two AA9 LPMOs, and AA2 and AA3 family enzymes with activities attributed to lignin degradation. Finally, only one protein bearing a CBM1 module was detected in the maltose secretome, whereas those of pine and aspen comprised 10 and 12 CBM1-containing CAZymes, respectively, which were largely attached to CAZy catalytic modules known to target cellulose or xylan and related to a white rot-type fungus (Riley et al., 2014a).

Two AA1_1 laccases were detected in the secretomes of the fungi grown on pine and aspen, but low transcript levels were observed on these substrates. On the other hand, two other laccase genes were most abundantly transcribed on maltose. In the analyses of *P. cinnabarinus* (Levasseur et al., 2014), laccases were found in all the conditions studied. These facts suggest that laccase enzymes could be involved in other functions than lignocellulose degradation.

Overall, the authors identified similar protein types in the two wood conditions, and the numbers of identified peptides were comparable, but the total amount of released sugars was higher in the pine preparations than in the aspen preparations.

In summary, while *P. coccineus* seems to prefer hardwood for growing in nature, this fungus also contains the enzymatic arsenal appropriate for an efficient conversion of softwood.

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Several parameters noted in previous studies, such as the expression of pectinolytic enzymes, P450 monooxygenases, and manganese peroxidases, might be critical for fungi to degrade coniferous wood.

4.1.11. - *Phlebiopsis gigantea*

Phlebiopsis gigantea acts as a pioneer colonizer of softwood because it can tolerate and utilize resinous extractives. Hori *et al.* (Chiaki Hori et al., 2014) studied this basidiomycete growing on freshly-harvested ground loblolly pine wood that had been 'spiked' with acetone and thoroughly dried (NELP) or on the same material after extended acetone extraction (to remove these extractives) and drying (ELP). Nano-LC-MS/MS analysis identified extracellular proteins in the culture filtrates harvested after 5, 7, and 9 days, corresponding to a total of 319 gene products. Most proteins were observed in both the NELP and ELP culture filtrates, which contained 294 and 268 proteins, respectively.

P. gigantea's gene expression patterns revealed an important role of intracellular lipid and oxalate metabolism, together with the TCA and glyoxylate cycles, in the oxidation of the triglyceride and terpenoid components of resinous sapwood. As in all the other fungi analyzed in this review, many *P. gigantea* genes and proteins lack a functional classification (4744 genes annotated as hypothetical in the genome). Among them, the authors found genes that were highly expressed, regulated, and/or secreted. To fully understand the strategies used by *P. gigantea* to colonize resinous sapwood faster than other fungi, the biochemical characterization of these proteins is unavoidable.

4.1.12. - *Phlebia radiata*

Kuuskeri *at al.* (Kuuskeri et al., 2016) analyzed the total proteome (both extracellular and intracellular proteins) of *Phlebia radiata* growing on spruce wood solid-state cultures after 7, 14, 21, 28, and 42 days of cultivation. In total, 1356 proteins were identified by peptide LC–MS/MS proteomics; among them, N-terminal signal peptide was predicted for 15% (210) of them. When looking at the functions of the identified proteins, they found that the

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majority (77%) were classified as proteins with other functions, including many intracellular proteins involved in translation and metabolic processes. The authors pointed out the importance of a perfect annotation of the 5' starting codon to correctly identify the secreted proteins by *in silico* methods, as mentioned previously (Manuel Alfaro et al., 2016). These annotation problems could be solved by the use of proteogenomics methodologies that include the mapping of identified peptide data to the translation of the three frames of the genome to achieve more accurate nucleotide gene and protein annotations.

The authors confirmed, in this *P .radiata* extensive analysis, the difficulty of directly comparing gene expression analyses, protein detection, and enzymatic activity measurements. As an example of very well-known proteins implicated in wood degradation, laccase-encoding genes were not upregulated in the wood cultures (had a constant expression level in all the media included) and only one secreted protein was identified, but laccase activity was found in all the cultures. Differences between them at specific time points of cultivation could be the result of time-dependent regulation, including transcriptional and post-transcriptional regulation; distinct isozyme proteins may be active only under specific environmental conditions. Furthermore, difficulties that are inherent to LC/MS-MS, such as those derived from proteins without trypsin cleavage sites, proteins attached to the wood matrix, or quickly degraded proteins, produced an underestimation or complete loss of detection of these proteins. Despite these limitations, transcriptome analysis supported the proteomic results for CAZy enzymes, and peroxidases show a clear upregulation in the early stages of wood degradation, confirming the white rot behavior of *P. radiata.*

4.1.13. - *Agaricus bisporus*

Patyshakuliyeva *et al*. analyzed the secreted proteins of *A. bisporus* mycelium growing on compost at six different time points related to the industrial production of this widely cultivated edible fungus. Interestingly, and in contrast to many other articles reviewed here, the correlation between the transcriptomics and proteomics data seems to be good in *A. bisporus* under these cultivation conditions; 641 proteins were identified, and among them, 168 (26%) proteins were classified as CAZymes, and the cellulose-degrading enzymes GH6

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and GH7 cellobiohydrolases and GH5_5 endoglucanases showed high correlations between the proteomics and RNA-seq data. Also, several hemicellulose hydrolyzing enzymes were identified and correlated with transcriptomic data: the GH10 and GH11 endoxylanases, GH27 α-galactosidase, GH31 α-xylosidase and α-glucosidase, GH35 β-galactosidase, and GH51 α-arabinofuranosidase were the most abundant enzymes.

Ligninolytic enzymes were found in these growing conditions; the AA1_1 laccases were the most abundant and most highly expressed genes in the transcriptome data. Surprisingly, *A. bisporus* is grown in a substrate that suffers changes in its cellulose and hemicellulose composition, but the lignin content remains very constant during fungus growth. As can be seen in other papers analyzed here (Marie Couturier et al., 2015; Kuuskeri et al., 2016; Levasseur et al., 2014), laccases do not seem to be particularly influenced by the presence of lignin compounds in the substrate, highlighting the possibility of other functions for these interesting enzymes.

Furthermore, a similar pattern of concordance between the secretome and transcriptome profiles was observed regarding differences between the various growth stages of *A. bisporus,* with the only episode of no correlation after the harvesting of the first flush of mushrooms, where the CAZy transcriptome profile showed a decrease but the corresponding CAZymes with cellulolytic activities were still present on the mushrooms grown in compost. The authors explained these results by assuming that proteins remained on the media from the previous sampling time, when the corresponding genes were highly expressed. This is certainly a possibility, but a bad correlation between transcriptomics and proteomics is also feasible.

4.1.14. - *Pleurotus ostreatus*

Fernández-Fueyo *et al*. (2016) studied the secretome of *P. ostreatus* after 21 days of growth on woody (poplar chips) and non-woody (wheat straw) lignocellulosic substrates (with distilled water as the only additive) by nLC-MS/MS, and compared the secreted proteins with

those found in a glucose medium. A total of 241, 391, and 206 extracellular proteins were identified in the poplar, wheat straw, and glucose fungal cultures, respectively.

In agreement with data from *A. bisporus* (Patyshakuliyeva et al., 2015) and in contrast to other fungi described here (Marie Couturier et al., 2015; Kuuskeri et al., 2016; Levasseur et al., 2014), *P. ostreatus* broadly uses laccases when growing on lignocellulosic substrates and significantly reduces their lignin content, as shown by 2D NMR analyses (Fernández-Fueyo et al., 2016). Oxidoreductases are overrepresented when this fungus grows on wood lignocellulosic substrates, compared to what is observed in glucose medium. One laccase occupied the first position in both wood secretomes, and three more were overproduced, together with one Versatile Peroxidase (VP) and one Manganese Peroxidase (MnP).

4.1.15. – Summary of white rot basidiomycetes

In summary, GHs are the principal group of proteins secreted by white rot basidiomycetes. Peptidases are the second most commonly detected group, especially when using complex substrates in these experiments. Among the lignin-degrading enzymes, the oxidoreductases are the most prominent group, and the detection of MnPs and LiPs is less frequent, with the exception of cultures performed in media designed to induce their production.

4.2. – BROWN ROT BASIDIOMYCETES

4.2.1 – *Postia placenta*

P. placenta is a brown rot basidiomycete that is closely related to the white rot model species *P. chrysosporium*. Brown rot fungi rapidly depolymerize the cellulose in wood (especially conifer wood, (Hibbett & Donoghue, 2001) without significant lignin removal). The *P. placenta* genome sequence is available (Martinez et al., 2009), and 17,173 gene models have been annotated. The genome contains two Pox genes, whereas no LiP, MnP or VP genes were detected. Surprisingly, genes encoding exocellobiohydrolases and cellulose-binding

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domains, typical of cellulolytic microbes, are absent in this efficient cellulose-degrading fungus. The lack of exocellobiohydrolases is especially relevant because these enzymes are predominant among the GHs secreted by *P. chrysosporium*. On the basis of these results, a role for Fenton chemistry has been proposed as reaction mechanism, in which Fe(II) and H_2O_2 react to form hydroxyl radicals, which are highly reactive oxidants capable of depolymerizing cellulose (Martinez et al., 2009).

Signal peptides have been annotated in 834 *P. placenta* gene models (4.9 % of the models) (data not shown). This number is lower than that reported for *P. chrysosporium*, but it may be underestimated due to the large number of gene models predicted in this draft genome.

Vanden Wymelenberg *et al.* (2010) conducted an LC-LC-MS/MS comparison of the *P. chrysosporium* and *P. placenta* secretomes in shaken submerged cultures using either ball milled aspen (*Populus*) or glucose as the sole carbon source. A total of 73 and 67 secreted proteins were identified in *P. chrysosporium* and *P. placenta*, respectively. *P. chrysosporium* secreted an array of extracellular GHs to simultaneously attack cellulose and hemicelluloses, while *P. placenta* secreted an array of hemicellulases (34), but few potential cellulases. In addition, experimental evidence of the secretion of enzymes involved in the extracellular peroxide generation was found (including a copper radical oxidase, a FAD-linked oxidoreductase, and a glucose oxidase). An exceptional characteristic of the *P. placenta* genome is its impressive set of 236 cytochrome P450 genes, some of which can participate in the biodegradation of lignin, and experimental evidence of the secretion of a P450 protein has been obtained.

In another transcriptome and secretome comparison between *P. Placenta and P. chrysosporium* (Vanden Wymelenberg et al., 2011) a total of 413 *P. placenta* proteins were identified, of which a total of 71 were identified in either aspen or pine containing media. Among differentially regulated *P. placenta* gene products, they identified eight extracellular proteins using LC-MS/MS with no apparent homologs in *P. chrysosporium* and no function assigned, opening an interesting field for future research.

On the other hand, Ryu *et al.* (Ryu et al., 2011) performed another LC-LC-MS/MS analysis of the proteins secreted by *P. placenta* while colonizing aspen chips in solid cultures. These

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authors were able to recover four secreted proteins (3 endo-1,4-β-D-glucanases and an endo-1,4-β-xylanase) that exhibited cellulase activity when expressed heterologously, and they concluded that non-highly processive cellulases can participate in the degradation of cellulose by *P. placenta*.

4.2.2. – *Serpula lacrymans*

S. lacrymans (Boletales) is a brown rot basidiomycete that is phylogenetically distant from *P. placenta* (Polyporales). Eastwood *et al.* (Eastwood et al., 2011) performed an LC-LC-MS/MS analysis of the *S. lacrymans* secretome when grown for 30 days in solid cultures using *Picea abies* wood as a substrate, with or without $CaSiO_3$ supplementation. Of the 39 identified proteins, only 29 contained a signal peptide, which were classified as follows: 16 GHs (including an endo-1,4-β-D-glucanase and an endo-1,4-β-xylanase, as found in *P. placenta*), four oxidoreductases (including a laccase), six proteases, and five esterases/lipases. Comparison of the *S. lacrymans* and *P. placenta* genomes revealed the presence of cellobiohydrolases and proteins containing a cellulose-binding motif (CBM) in *S. lacrymans*, while these proteins are absent in *P. placenta*. Finally, analysis of the *S. lacrymans* secretome and genome indicated non-enzymatic disruption of cellulose by this brown rot basidiomycete (Fenton mechanism).

4.2.3. - *Wolfiporia cocos*

Gaskell *et al.* (Gaskell et al., 2016) studied the secretome of *Wolfiporia cocos* using *Populus grandidentata* (aspen, Asp), *Pinus contorta* (lodgepole pine, LP) or microcrystalline cellulose (MCC) as the sole carbon source and 704 *W. cocos* proteins were identified. In order to avoid analyzing proteins likely to be involved in intracellular processes, the authors focused on proteins with a secretion signal (132 proteins), noting that inaccurate 5′ termini and non-classical signals may have reduced the number of proteins included in the analyses. A relevant example includes a laccase model. The authors underlined the fact that among 12,746 predicted *W. cocos* genes, only 48% had assigned Pfam domains. Consequently, the transcriptomic study found many upregulated genes without a functional classification.

Among them, 33 proteins with predicted secretion signals were upregulated on MCC, Asp, or LP relative to a glucose (Glc) control medium; 51, 84, 117, and 112 proteins were identified in Glc, MCC, Asp, and LP media, respectively. More proteins were found in media that contained wood, in accordance with the higher carbon source complexity. Asp and LP wood differ substantially in their structures and compositions; nevertheless, *W. cocos* gene expression patterns were remarkably similar on these substrates, despite some differences in specific genes.

Furthermore, the authors used the transcriptome and secretome of *W. cocos* to analyze the production of key reactants (H_2O_2 and Fe^2) for Fenton chemistry, thought to be used by brown rot fungi. Among others, genes encoding benzoquinone reductases (BQR), laccases (Lac), ferroxidase (FET3), ferric reductase transmembrane component (FRE), iron permease (FTE), and oxalate decarboxylase (ODC) were observed. The upregulation of FET3 and FTR1 in the *W. cocos* transcriptome supports enhanced iron acquisition, consistent with a Fenton-based system. Hydroquinone redox cycling has been proposed to generate hydroxyl radicals for Fenton chemistry, but in *W. cocos*, in contrast to *P. placenta,* BQR and MOX genes are not upregulated when the fungus grows on wood, suggesting that the Fenton mechanism is less important and that a unique repertoire of genes is involved in lignocellulose degradation.

4.2.4. - *Gloeophyllum trabeum*

In a very interesting approach to studying the secretome in this species (Presley & Schilling, 2017; Zhang et al., 2016a), wood wafers were colonized by *S. lacrymans* or *G. trabeum* in a directional way that allowed the researchers to observe a spatial separation of the decay stages linearly along the substrate. *G. trabeum* produced more proteins (209 in total) than *S. lacrymans* (93), especially at the section nearest the hyphal front, the earlier stages of wood colonization. In *S. lacrymans* cultures, 65, 81, and 72 proteins were found at distances of 0 to 5, 10 to 15, and 20 to 25 mm behind the hyphal front, respectively, and 200, 150, and 120 proteins in the equivalent wafer sections colonized by *G. trabeum*. After transcriptomic and proteomic studies of the spatially separated stages of wood colonization, the authors proposed a two-step decay mechanism where oxidoreductases were more important at the hyphal front

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and endoglucanase and hemicellulase activities increased in a second stage of wood colonization.

The existence of an actual differentiation between white and brown rot basidiomycetes was questioned by Riley *et al.* (2014). Some fungi showed characteristics from both white and brown rot fungi, making it very difficult to classify them as one specific type of rot.

4.2.5. – Comparison of the secretomes of white and brown rot basidiomycetes

In a comparative study of 31 diverse saprotroph fungi, Floudas *et al.* (Floudas et al., 2012) examined the secretomes of 11 basidiomycetes, comprising six white rot species (*Auricularia delicata, Dichomitus squalens, Fomitiporia mediterranea, Punctularia strigosozonata, Stereum hirsutum* and *Trametes versicolor*) and five brown rot species (*Coniophora puteana, Dacryopinax sp., Fomitopsis pinicola, Gloeophyllum trabeum* and *Wolfiporia coccos*). In the white rot species, 977 secreted proteins were identified, while 1012 were identified in the brown rot species. To compare the two types of basidiomycetes, the following principal functional groups of proteins were examined (white rot / brown rot): uncharacterized proteins, 230 / 205; GHs, 302 / 204; 132 / 92 peptidases; 29 / 15 oxidases related to lignin degradation; 34 / 25 esterases and lipases; 14 / 1 Poxs; and 11 / 0 MnPs and Vps.

More recently, Hori *et al.* (2013) conducted a comparative LC-LC-MS/MS secretome analysis of seven Polyporales (the white rot species *D. squalens, T. versicolor*, *Bjerkandera adusta, Ganoderma sp.* and *Phlebia brevispora* and the brown rot species *F. pinicola* and *W. cocos*) grown in wood cultures under identical conditions. They found that genes encoding cellulases belonging to families GH6, GH7 and GH9 and the carbohydrate-binding module family CBM1 were lacking in the brown rot polyporales. In addition, the presence of CDH and expansion of LPMO were observed only in the white rot genomes. Indeed, GH6, GH7, CDH and LPMO peptides were identified only in white rot polyporales. In contrast, peptides corresponding to quinone reductase (QRD) were identified only in brown rot fungi in this study. This finding is consistent with previous studies in the brown rot fungus *P. placenta* (Martinez et al., 2009; Vanden Wymelenberg et al., 2010) and may constitute a key

component of a redox cycle supporting Fenton chemistry (Paszczynski, Crawford, Funk, & Goodell, 1999; M. R. Suzuki, Hunt, Houtman, Dalebroux, & Hammel, 2006). Regarding hemicellulose degradation, genes and peptides corresponding to GH74 xyloglucanase, GH10 endo-xylanase, GH79β-glucuronidase, CE1 acetyl xylan esterase and CE15 glucuronoyl methylesterase were significantly increased in white rot compared to brown rot basidiomycetes. As proposed by Hori *et al.*, these results collectively suggest that white rot basidiomycetes employ both extracellular hydrolytic and oxidative reactions for cellulose degradation, a strategy that is distinct from the Fenton systems of brown rot basidiomycetes (Hori et al., 2013).

The existence of an actual differentiation between white and brown rot basidiomycetes has been questioned by Riley et al. (Riley et al., 2014). Some fungi showed characteristics from both white and brown rot fungi, making very difficult to classify them in a type of rot.

4.3. – SYMBIOTIC BASIDIOMYCETES

4.3.1. – *Laccaria bicolor*

L. bicolor has a life cycle that includes a soil saprotrophic phase and a long mutualistic ectomycorrhizal (ECM) interaction with its host tree species, which is preferentially a pine (*Pinus*) or birch (*Betula*). The *Laccaria* genome (60.71 Mbp) has been sequenced by Martin *et al.* (Martin et al., 2008). Bioinformatics analysis of this genome revealed 2,931 secreted proteins (14.2 % of the predicted protein models), although a function could not be ascribed to most of them (69%), and 89% of these proteins appeared to be specific of *L. bicolor* (Vincent et al., 2012). Among these proteins, a large number were cysteine-rich small secreted proteins (SSP) that play a role in the interaction between the host and the symbiont (Mycorrhizal-induced Small Secreted Proteins, MiSSP). An unexpected finding was a lack of carbohydrate-active enzymes (CAZymes) involved in the degradation of the plant cell wall. However, *L. bicolor* maintained the ability to degrade non-plant cell wall polysaccharides (Martin et al., 2008; Vincent et al., 2012).

The *L. bicolor* secretome was studied in a shaken submerged culture by Vincent *et al.*

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(Vincent et al., 2012) using three different fractionation approaches (IPG-IEF, 1D-PAGE and 2D-PAGE), followed by LC-ESI-MS/MS. A total of 224 proteins were identified, 103 of which were annotated as being secreted: 41 of these 103 proteins had an unknown function, and 15 of the 41 unknown proteins could be classified as MiSSPs. Many CAZymes that are likely involved in modifications of the fungal cell wall were also identified in the secretome. Additionally, these authors proposed that the dual lifestyle of this fungus requires two sets of proteins to be secreted in the two phases of the life cycle: enzymes involved in nutrient acquisition via the degradation of substrates (proteases, GHs) and proteins participating in morphogenetic and defense mechanisms prevail in the saprotrophic phase; whereas proteins involved in recognition, adherence and host-defense modulation are predominant in the symbiotic phase.

4.3.2. - *Tricholoma vaccinum*

Tricholoma vaccinum, a fungal symbiont of Norway spruce (Picea abies), was cultivated *in vitro* to identify secreted proteins (Wagner et al., 2015). After the addition of spruce root exudates, the authors looked for differentially regulated proteins that acted as communication signals in early ectomycorrhizal interaction, before physical contact happens. Using 2D-PAGE to separate proteins prior to MS identification, the authors identified 22 different proteins from the 2D gels, including five small secreted proteins. Twelve proteins showed a typical secretion signal at their N-termini. Five proteins were predicted to be excreted via a non-classical pathway, and five more were not predicted to be intracellular proteins. Seven proteins were regulated after the addition of root exudates. The root exudates changed the secretome of the fungus, but in an unspecific manner. The small number of proteins identified (usually when using 2D PAGE secretomes) prevented the authors from determining the regulation of more ectomycorrhizal-related proteins.

4.3.3. - *Hebeloma cylindrosporum*

Doré et al. (2015) analyzed the exoproteome of *H. cylindrosporum* in Melin-Norkrans (MMN) medium and identified proteins of unknown function; SSPs and CAZymes were among the 10% most abundant proteins. An extensive analysis of *H. cylindrosporum*'s capacity to use several carbon sources showed that this fungus cannot grow when using any polysaccharide constituents of the plant cell wall (PCW) as carbon sources; however, it can grow on starch and β-1,3-glucan, two other polysaccharides that are normally absent from PCWs. This is a common feature of ectomycorrhizal fungi (Martin et al., 2008; Vincent et al., 2012). Interestingly, *H. cylindrosporum* possesses the genetic arsenal necessary to degrade cellulose, although the corresponding proteins either could not be detected by mass spectrometry in culture filtrates or were detected at low concentrations. The only exception was a GH28-encoded polygalacturonase that was highly concentrated in the filtrates. This protein was also present in the *L. bicolor* genome and was suggested to be involved in cell wall remodeling during fungal tissue differentiation because it was upregulated in fruiting bodies and ectomycorrhizae (Martin et al., 2008). Most of the CAZymes found in high concentrations in the filtrate could be involved in fungal cell wall metabolism or related to the degradation of cellobiose, starch, or β-1,3 glucans. Besides CAZymes, *H. cylindrosporum* abundantly secreted proteases, one laccase, and four glucose-methanol-choline (GMC) oxidoreductases.

Based on these findings, the authors proposed an adaptation of *H. cylindrosporum* to use soil organic nitrogen, although avoiding host damage. Furthermore, SSP regulation suggests a role for these proteins in symbiosis and the degradation of organic matter.

4.4. – PLANT PATHOGENIC BASIDIOMYCETES

4.4.1. – *Ustilago maydis*

U. maydis is a biotroph pathogen that causes smut disease in maize. The *U. maydis* genome (Kämper et al., 2006) consists of 20.5 Mbp and contains 6,902 predicted proteins. At the level of gene cataloging, *U. maydis* displays few of the pathogenicity signatures found in the genomes of aggressive pathogenic fungi (for instance, cell wall-degrading enzymes such as cellobiohydrolases and endoglucanases; polyketide synthases; and enzymes involved in antibiotic or mycotoxin production). The presence of GH enzymes is also reduced in *U.*

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maydis in comparison with the white rot species *P. chrysosporium* and *Coprinopsis cinerea* (30 GH genes in *U. maydis* vs. 50 in *P. chrysosporium* and 42 *in C. cinerea*).

However, the *U. maydis* genome contains several clusters of small secreted proteins (SSPs) that are co-regulated and induced in the infected tissue in most cases. Of the 554 proteins predicted to be secreted, 70% could not be ascribed to any function, and 65 % were only found in *U. maydis*. Some of these proteins are essential for the infection to proceed. SSPs were also prevalent in the symbiotic *L. bicolor* and were not found in the saprotrophic basidiomycetes discussed above.

Müller *et al.* (Muller et al., 2008) employed a combination of molecular (signal sequence trap) and bioinformatics tools to determine the secretome of *U. maydis.* They identified 29 proteins, including hydrophobins and proteins containing repetitive structures, some of which are essential for the infection process ((Kämper et al., 2006).

Using a solely bioinformatic approach, Müller *et al.* (Mueller et al., 2008) identified 554 predicted secreted proteins. Only 168 of them(39%) could be functionally annotated, which included enzymes involved in the degradation of cellulose and hemicellulose and modification of the fungal cell wall as well as peptidases esterases-lipases, oxidases and laccases. Surprisingly, shaken submerged cultures of *U. maydis* performed using 15 g/l maize bran as a carbon source were found to efficiently release sugars from micronized wheat straw (M Couturier et al., 2012). In this work, the *U. maydis* secretome was analyzed via 1D-SDS-PAGE LC-MS/MS, and the 86 proteins identified were functionally annotated as follows: 29 unknown proteins, 22 GHs, 11 esterases and lipases, 10 proteases, and 7 oxidases.

4.4.2. – *Armillaria mellea*

Armillaria spp. are among the most serious plant root pathogens and have been studied because of their virulence, bioluminescent properties and ability to produce natural products (Collins et al., 2013). Collins *et al.* identified a total of 293 proteins in culture supernatants using several different carbon sources (lignin, xylan, cellulose, rutin or yeast extract). Through 1D-LC–MS/MS analysis, 157 unique proteins were identified, while 37 proteins

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were uniquely identified via a shotgun strategy without SDS-PAGE fractionation. SignalP analysis predicted the presence of a signal peptide in 50.2% (147) of the secreted proteins. Furthermore, SecretomeP indicated that 99 of the secreted proteins (34%) contained a non-classical secretion signal, meaning that less than 20% (55) of these proteins do not contain a secretion signal. GO analysis showed peptidase activity as the largest functional category (15).

Additionally, the supernatant from co-cultures of *A. mellea* and *Candida albicans* was analyzed using a LC-LC-MS/MS approach, and 30 *A. mellea* proteins were identified that have not been observed under any other culture conditions. The presence of these proteins and the existence of a significant killing effect on *C. albicans* induced by *A. mellea* appears to demonstrate the existence of a defensive response of *A. mellea* against *C. albicans* (Collins et al., 2013).

4.5. – OTHER BASIDIOMYCETES

4.5.1. – *Coprinopsis cinerea*

C. cinerea is a classic experimental model of multicellular development in fungi, as it grows on defined media, completes its life cycle in 2 weeks, produces a total of 10^8 synchronized meiocytes and can be manipulated in all stages in development via mutation and transformation (Stajich et al., 2010). Based on analysis of the annotated genome of *C. cinerea* (formerly *C. cinereus*), 1,769 proteins were predicted to be potentially secreted (data not shown), and 76 extracellular proteins have been experimentally confirmed thus far by means of 2-D PAGE and MS. The secretome of *C. cinerea* in a liquid complex medium containing glucose, yeast extract, peptone, and mineral salts includes glyoxal oxidases, β-glucosidases, β-1,3-glucanases, glucoamylases, metallopeptidases, and serine peptidases. However, a significant number of spots could not be identified on basis of sequence homology with known proteins (Bouws et al., 2008; Hoegger et al., 2007; Stajich et al., 2010).

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5. – CONCLUDING REMARKS

The use of several complementary proteomic techniques to analyze secretome samples appears to be the best method for obtaining broad insight into the complex and highly dynamic mixture of proteins that basidiomycetes use to degrade lignocellulose. Among the proteins identified in these secretomes, there is a large number of proteins that are not predicted to be secreted. Avoiding intracellular contamination of secretome samples can be highly challenging due to cellular lysis occurring before or during secretome sample extraction; however, the extracellular functions of some non-secretome predicted proteins detected in these analyses demonstrate the importance of non-classical secretion among fungi. Eastwood *et al.* (2011) identified 39 GHs in extracellular media, but only 29 were predicted to be secreted. These data also indicate the importance of in vitro experiments to demonstrate the accuracy of in silico data.

Quantitative proteomics techniques are beginning to be widely applied in fungal studies. However, at the time of this review, Dr. Sze's (Adav et al., 2012; Adavat al., 2012b; Manavalan et al., 2011) studies were the only ones that applied iTRAQ techniques to basidiomycete secretomes, allowing them to compare enzyme regulation when different carbon sources are used. Other quantitative approaches range from 2D-PAGE spots quantitation to the use of label free proteomics (like emPAI (Ishihama et al., 2005)), based on the peptide peak intensity or number of peptides identified from a given protein, which is increasing its use in fungal secretomes. One of the main aims of the basidiomycete secretome analyses reviewed here is to search for valuable enzymes that can be utilized to obtain energy from lignocellulosic materials, and quantitative proteomics can contribute valuable data on the importance of each enzyme involved in every step of fungal decay.

Furthermore, the increasing number of available sequenced fungal genomes, 278 of which come from basidiomycetes (April 2017), and the increasing quality of the annotated genomes will increase the number of proteins detected using proteomic techniques and improve our overall understanding of plant biomass degradation, with the goal of using such biomass as a sustainable source of energy to support future needs. This can yield a reciprocal benefit, because the use of proteogenomics methodologies (Nesvizhskii, 2014) can provide protein-level evidence of gene expression to help refine gene models annotation.

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The number of experimentally determined secretomes in basidiomycetes is increasing, but there is a lack of information about the function of many proteins that seem to be involved in how fungal interact with its environment. This is now the main reason why only coarse pictures of the secretome differences in association with fungal lifestyles can be drawn

We can generalize, however, as a preliminary model that saprotrophs secretomes are characterized by the abundance of glycosyl hydrolases and peptidases, whereas these two groups are more reduced in symbionts and pathogens. Among the saprotrophs, the two lignin-degrading styles (white and brown rot) can be clearly correlated with the presence of cellulases and lignin degrading enzymes (WR) and the limited presence of these enzymes and the occurrence of enzymes involved in the Fenton-based cellulose degradation reactions (BR).

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Chapter 2:

Introducing the Oyster Mushroom *Pleurotus ostreatus*

P. ostreatus (Jacq.) P. Kumm. is one of the most widely cultivated edible fungi in the world under the common name of the Oyster mushroom. It is broadly accepted as an excellent comestible because of its organoleptic characteristics. This industrial background provides a deep knowhow about its cultivation needs and behavior and makes *P. ostreatus* a good scientific model for basidiomycete laboratory work. Furthermore, this already stablished fungal cultivation industry is a nice starting point for exploring new applications of fungi, such as biofuel production.

In nature, *P. ostreatus* is a saprophyte that lives on decaying wood and stands out among other wood rotters as a specific lignin degrader (van Kuijk, Sonnenberg, Baars, Hendriks, & Cone, 2015). *P. ostreatus* takes the nutrients necessary to survive from lignocellulosic materials by means of proteins secreted to the extracellular media. These proteins are a complete catalog of tools targeted to each one of the compounds that form lignocellulose, which are known for being recalcitrant against degradation by the majority of living organisms; therefore, this decomposition is a major step in the carbon cycle, considering that cellulose and lignin are the most abundant carbon polymers in nature (J. Pérez, Muñoz-Dorado, De La Rubia, & Martínez, 2002).

This extracellular digestion carried out by fungi requires enzyme secretion. Fungi need to access the lignocellulose sugars and produce enzymes specifically suited to digest lignocellulose in different environments, including those that are hostile for the enzymes.

Different strategies were followed by basidiomycete fungi to degrade wood. In a rough classification based on the aspect of the rotting material, they were classified as white or brown rot fungi. White rot fungi are generally noted to degrade all the lignocellulosic compounds, but degrade lignin in a higher percentage than brown rotters, which employ a different mechanism and don't need extensive lignin degradation. These different strategies were shown to have a blurred separation, as some fungi shared characteristics from both rot types (Riley et al., 2014).

The *P. ostreatus* dikaryotic strain N001 is a hybrid strain that has been used as a source for commercial cultures for many years. The two nuclei present in this dikaryotic strain were separated, and two monokaryotic strains (PC9 and PC15) were produced and maintained

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(Luis M. Larraya et al., 1999). PC15 was sequenced with the Sanger whole-genome shotgun approach (Riley et al., 2014a) and PC9 was sequenced using Sanger whole genome shotgun and 454 paired end sequencing reads. PC15 genome assembly version 2.0 (34.3 Mb) was subjected to targeted genome improvement, which led to a complete assembly of 12 scaffolds with a very low gap content (1 gap of 91 base pairs in the whole assembly) that matched the corresponding *P. ostreatus* chromosomes (eleven nuclear plus one mitochondrial chromosome) (Luis M. Larraya et al., 1999). In contrast, PC9 assembly v1.0 (35.6 Mb) contains 572 scaffolds and a total of 476 gaps that cover 9.72% of the whole assembly (Raúl Castanera et al., 2016) (Figure1).

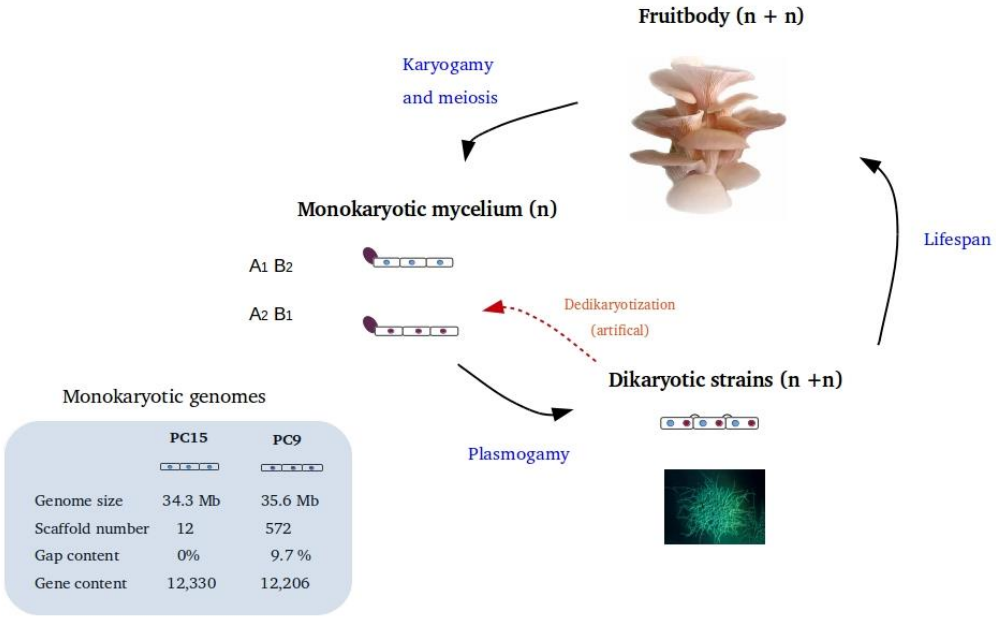

Figure 1. *P. ostreatus* lifecycle and genomes. Data from DOE Joint Genome Institute (I V Grigoriev et al., 2012).

In this thesis, we use the term 'strain' to refer to these two monokaryons that are genetically compatible and reconstitute the dikaryotic strain N001 when mated. Sequencing shows that

the two monokaryotic strains are very similar in terms of genome size or number of genes, despite the poorest assembly of the PC9 sequence.

1. – *P. ostreatus* LIGNINOLYTIC CAPABILITIES

P. ostreatus possesses an impressive arsenal of enzymes involved in degrade lignocellulose decomposition. Comparing to other well–known basidiomycetes, such as the brown rot *Laccaria bicolor (Martin et al., 2008)*, the ectomycorrhizal symbiont *Postia placenta (Martinez et al., 2009)*, the white rot model *Phanerochaete chrysosporium (Martinez et al., 2004),* and the edible agarical *Agaricus bisporus* (Morin et al., 2012)*, P. ostreatus* monokaryons PC15 and PC9 exhibit its huge genomic potential: they have more enzymes involved in peroxide generation and lignin degradation (AA), more glycosil hydrolases (GHs) and carbohydrate esterases (CEs) to break down constituents of the PCW, and more polysaccharide lyases (PLs) that cleave uronic acid-containing polysaccharide chains (Lombard et al., 2010) and thus are involved mainly in pectin degradation. Glycosil transferases (GTs) are more involved in polysaccharide biosynthetic pathways than in polymer degradation, acting in the formation of the fungal cell wall.

Total	Lacbi2	Pospl1	Agabi_H97	Phchr1	PleosPC15	PleosPC9
AA	55	29	81	84	114	82
CBM	29	32	42	56	74	66
CE	18	15	33	15	28	20
GH	170	129	174	175	235	162
GT	96	24	54	65	65	47
PL	7	1	9	4	23	14
EXPN	12	12	9	9	8	7
Total	**387**	**242**	**402**	**408**	**547**	**398**

Table 1. Brown rot *Laccaria bicolor* (Lacbi2), Symbiont *Postia placenta* (Pospl1), White rot *Phanerochaete chrysosporium* (Phchr1), Edible *Agaricus bisporus var. bisporus H97* (AgabiH97) and *Pleurotus ostreatus* Monokaryons PC15 (PleosPC15) and PC9 (PleosPC9). Data from JGI Mycocosm (Igor V Grigoriev et al., 2014).

The Carbohydrate-Active enZYmes database (CAZy) (Lombard et al., 2010) is today the most broadly accepted classification of enzymes involved in lignocellulose degradation. This classification is based on sequence similarity, giving us the opportunity to classify enzymes without any biochemical evidence about their catalytic properties, as is the case for the

thousands of uncharacterized protein sequences generated by genome sequencing. In contrast to other enzyme classification databases that are based on the catalytic properties of the enzymes, enzymes belonging to one CAZy family can have different substrates. Most of the GH families comprise enzymes with different functions, and up to 28 different enzyme activities have been described for proteins belonging to a single GH family (Busk & Lange, 2013). It is therefore not possible to predict the activity of a GH simply by assigning it to a GH family. Likewise, the prediction of function is complicated by the fact that proteins with the same enzymatic function can belong to different GH families (Busk & Lange, 2013). Having said that, proteins with very similar sequences and subsequently belonging to the same CAZy family have structurally-related catalytic activities, like transglycosylases that are mechanistically related to retaining GHs. Some enzymes can even share both enzymatic activities (Bissaro, Monsan, Fauré, & O'Donohue, 2015).

1.1. – CELLULOSE-DEGRADING ENZYMES

Lignocellulose is the carbon source of *P. ostreatus* in nature. Consequently, this fungus can degrade all the constituents of the PCW, which is mainly composed of cellulose, hemicellulose, pectin, and lignin. Cellulose is, because of its composition based on only one sugar monomer (D-glucose) linked by β(1→4) glycosidic bonds, a highly ordered molecule. This spatial conformation allow hydroxyl groups on the glucose from one chain to form hydrogen bonds with oxygen atoms on the same or on a neighbor chain, forming microfibrils with high tensile strength that are arranged in layers (Alberts et al., 2002).

Cellulose-degrading enzymes	
Endoglucanase	
GH5,CBM1	endoglucanase
GH5_5	endo-β-1,4-glucanase
GH9	Endoglucanase
GH12	β-glycanase
GH12	endo-1,4-β-glucanase
GH45	endo-β-1,4-glucanase

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Exoglucanase	
GH6	exocellobiohydrolase CBH II
GH7	reducing end-acting exocellobiohydrolase CBH I
β-glucosidase	
GH1	β-glucosidase
GH3	β-glucosidase
GH30	β-glucosidase

Table 2. Cellulose-degrading enzymes

P. ostreatus possesses all the enzymes necessary to degrade cellulose (Table 2). Three types of enzymes are involved in this process: endoglucanases, exoglucanases, and beta-glucosidases. Endoglucanases can hydrolyze internal bonds (preferably in cellulose amorphous regions), releasing new terminal ends. Exoglucanases act on the existing or endoglucanase-generated chain ends. Both enzymes can degrade amorphous cellulose but, with some exceptions, exoglucanases are the only enzymes that efficiently degrade crystalline cellulose. Exo- and endoglucanases release cellobiose molecules. Beta-glucosidases break down cellobiose, releasing two glucose molecules (Kirk & Cullen, 1998; J. Pérez et al., 2002).

1.2. – HEMICELLULOSE-DEGRADING ENZYMES

Hemicellulose links cellulose fibers, creating a complex network of bonds that provide structural strength to the PCW (Rubin, 2008). Hemicelluloses include xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan. Contrary to cellulose, these polysaccharides include several monosaccharides apart from glucose: xylose, mannose, galactose, rhamnose, and arabinose, among others. In accordance with this huge variety of compounds, fungi can use a broad portfolio of enzymes to break down this barrier. Again, *P. ostreatus* possesses more enzymes dedicated to this task than other basidiomycete fungi (Table x). Carbohydrate esterases are also involved in the degradation of hemicelluloses, especial acetyl xylan esterases belonging to CE families 1, 4, 5, 8, 9, 12, 15, and 16.

1.3. – OTHER PROTEINS RELATED TO CELLULOSE AND HEMICELLULOSE DEGRADATION

Carbohydrate binding modules (CBM) are usually found in the protein domains involved in lignocellulose degradation. They are usually within a carbohydrate-active enzyme and target the enzyme to the appropriate substrate, binding polysaccharides, including cellulose, xylan, pectin, mannans, starch, and chitin(Table 3). These domains present the so-called "CBM promiscuity" in ligand binding; they can discriminate strongly against some polysaccharides while remaining relatively promiscuous toward other compounds. This domain can target fungal enzymes to different components to efficiently degrade complex substrates like hemicellulose (Charnock et al., 2002).

List of Carbohydrates and Interacting CBM Families (From CAZypedia)	
Cellulose	CBM1, CBM2, CBM3, CBM4, CBM6, CBM8, CBM9, CBM10, CBM16, CBM17, CBM28, CBM30, CBM37, CBM44, CBM46, CBM49, CBM59, CBM63, CBM64
Xylan	CBM2, CBM4, CBM6, CBM9, CBM13, CBM15, CBM22, CBM31, CBM35, CBM36, CBM37, CBM44, CBM54, CBM59, CBM60
Plant Cell Wall - Other (eg: beta-glucans, porphyrans, pectins, mannans, gluco- and galacturonans)	CBM4, CBM6, CBM11, CBM13, CBM16, CBM22, CBM23, CBM27, CBM28, CBM29, CBM32, CBM35, CBM39, CBM42, CBM43, CBM52, CBM56, CBM59, CBM61, CBM62, CBM65, CBM67
Chitin	CBM1, CBM2, CBM3, CBM5, CBM12, CBM14, CBM18, CBM19, CBM37, CBM50, CBM54, CBM55
Alpha-glucans (starch/glycogen, mutan)	CBM20, CBM21, CBM24, CBM25, CBM26, CBM34, CBM41, CBM45, CBM48, CBM53, CBM58

Table 3. Several CBM families can target different ligands (from CAZypedia).

In primary cell walls, the matrix in which the cellulose network is embedded is composed of pectin, a highly hydrated network of polysaccharides rich in galacturonic acid (Alberts et al., 2002). PLs are the main enzymes that degrade these components. The *P. ostreatus* genome presents PL1, 3, 4, 8, and 14 family enzymes, which are mainly pectate and

rhamnnogalacturonan lyases. GH16, 28, 43,53,78,79, and 93 and CE8 and 12 are also involved in pectin degradation.

Secondary cell walls contain additional components, such as lignin, which is recalcitrant to degradation and occupies the interstices between the other components, making the walls rigid and permanent.

Recently, the lytic polysaccharide monooxygenases (LPMOs) (Vaaje-Kolstad et al., 2010) have broken into the category of enzymes involved in the degradation of cellulose, chitin, and hemicelluloses (Agger et al., 2014). These enzymes, previously classified as GH61, cleave the β-(1→4) glycosidic bonds by inserting oxygen into C-H bonds that are adjacent to the glycosidic linkage of the polysaccharide backbone (Phillips et al., 2011). The majority of lignocellulose-degrading fungi contain genes that encode LPMOs, whose function is proposed to be the creation of access points for classical hydrolytic enzymes, such as cellulases (Kracher et al., 2016).

1.4. – LIGNIN DEGRADATION

The AA1 enzymes are multicopper oxidases, including laccases (EC 1.10.3.2), ferroxidases (EC 1.10.3.-), and laccase-like multicopper oxidases (EC 1.10.3.-). Family AA2 contains class II lignin-modifying peroxidases, including manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16), lignin peroxidase (EC 1.11.1.14), and peroxidase (EC 1.11.1.). Both protein families are among the best studied families in basidiomycete fungi because of their ability to degrade lignin, a highly recalcitrant polymer that protects hemicelluloses and cellulose from enzymatic attack. The main function of lignin is to provide strength and rigidity to plant tissues by infiltrating the intricate web formed by cellulose, hemicellulose, and pectin.

Family AA4 contains vanillyl-alcohol oxidases (VAO) that catalyze the conversion of a wide range of phenolic compounds produced during lignin degradation (Levasseur, Drula, Lombard, Coutinho, & Henrissat, 2013).

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AA6 (1,4-benzoquinone reductases) enzymes are involved in the fungal degradation of aromatic compounds in a quinone redox cycle that generates extracellular reagents (Levasseur et al., 2013).

Along with AA8 iron reductases, both CAZy classes are involved in generating H_2O_2 and reduced iron and carry out a non-enzymatic Fenton reaction ($H_2O_2 + Fe^{2+} + H+ \rightarrow H_2O + Fe^{3+} + OH$) in which highly reactive hydroxyl radicals depolymerize lignocellulosic compounds (Cullen, 2013; Xu & Goodell, 2001). It is widely accepted that brown rot lignocellulose degradation involves oxidation by hydroxyl radicals as a first step for hydrolysis (Cullen, 2013).

AA3 enzymes belong to the glucose-methanol-choline (GMC) oxidoreductase family and can be divided into 4 subfamilies: AA3_1 (mostly cellobiose dehydrogenases), AA3_2 (including both aryl alcohol oxidase and glucose 1-oxidase), AA3_3 (alcohol oxidase), and AA3_4 (pyranose 2-oxidase) (Levasseur et al., 2013).

Family AA5 is comprised of copper radical oxidases and the family includes two subfamilies: AA5_1 contains characterized glyoxal oxidase and AA5_2 contains galactose oxidase and alcohol oxidase enzymes.

The AA3, AA5, and AA7 (Glucooligosaccharide oxidases) families are involved in peroxide generation. New substrates for these enzymes are still being discovered, broadening the spatial localization of peroxide generation by these enzymes (Yin et al., 2015).

The enzyme cellobiose dehydrogenase (CDH, AA3_1) can deliver electrons to LPMOs, initiating the cellulose attack. Other enzymes belonging to the AA3 class can also reduce LPMOs through plant/fungal phenols that act as mediators (Kracher et al., 2016). There is evidence of AA3 and LPMO co-expression during lignocellulose degradation (Marie Couturier et al., 2015). Taken together, these findings show new connections between the decomposition of polysaccharide and lignin in fungal lignocellulose degradation (Martínez, 2016). Products from lignin degradation act as electron donors for cellulose-degrading LPMOs, linking the whole fungal lignocellulose degradation process.

2. – CONCLUDING REMARKS

This enzymatic diversity gives basidiomycete fungi (and specifically *P. ostreatus)* a broad portfolio of enzymatic possibilities to act on a wide variety of compounds and in different environments. As an example, *P. ostreatus* possesses several gene models whose enzymatic function is presumably the same. Among them, ligninolytic peroxidases are broadly studied because of their lignin-degrading capabilities. These enzymes show different levels of transcription and activity at different pH levels (Fernández-Fueyo et al., 2014). In a *P. ostreatus* previous study, the weight of the transcriptional activity was mainly supported by only one gene model per enzymatic function (Alfaro et al., 2016). Nevertheless, the advantages of genetic redundancy are clear (Kafri et al., 2009) and have been demonstrated to be useful in maintaining *P. ostreatus'* activity level by inactivating the most expressed MnP genes (Salame et al., 2013). Genome editing technologies like CRISPR-CASP9 applied to basidiomycete fungi (Schuster et al., 2016) will shed light on the role of genetic redundancy in lignocellulose fungal degradation.

Surprisingly, the two *P. ostreatus* monokaryotic strains seem to be different in their lignocellulose degrading genomic capabilities. PC9 have less CAZy genes annotated (403 versus 549 in PC15), especially in the Glycosil hydrolases(GH) class (86 versus 114 in PC15). Nevertheless, PC9 grows better than PC15 on lignocellulosic substrates and has a higher enzyme secretion capacity when growing in the presence of wood, as we will see in the next chapters of these dissertation.

	Lacbi2	Pospl1	Agabi_H97	Phchr1	Pleos PC15	Pleos PC9
AA Auxiliary Activities family	**55**	**29**	**81**	**84**	**114**	**86**
AA1 Auxiliary Activity Family 1	**15**	**6**	**13**	**5**	**12**	**11**
AA1 Auxiliary Activity Family 1	0	1	0	4	0	0
AA1_1 Auxiliary Activity Family 1 / Subf 1	10	4	12	0	11	10
AA1_2 Ferroxidase	3	1	1	1	1	1
AA1_dist Multicopper oxidase	2	0	0	0	0	0
AA12 Auxiliary Activity Family 12	**0**	**0**	**1**	**1**	**1**	**0**
AA2 Auxiliary Activity Family 2	**1**	**1**	**2**	**15**	**9**	**9**
AA3 Auxiliary Activity Family 3	**12**	**18**	**37**	**36**	**41**	**29**
AA3 Auxiliary Activity Family 3	0	2	0	0	0	0
AA3_1 Cellobiose dehydrogenase	0	0	1	1	1	1
AA3_2 GMC oxidoreductase	10	15	31	31	36	26
AA3_3 Alcohol oxidase	2	1	5	3	4	2
AA3_4 Auxiliary Activity Family 3 / Subf 4	0	0	0	1	0	0
AA5 Auxiliary Activity Family 5	**11**	**2**	**9**	**7**	**16**	**11**

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AA5_1 Auxiliary Activity Family 5 / Subf 1	11	2	9	7	16	11
AA6 Auxiliary Activity Family 6	**2**	**0**	**4**	**4**	**2**	**2**
AA7 Auxiliary Activity Family 7	**1**	**0**	**3**	**0**	**3**	**2**
AA7 Auxiliary Activity Family 7	1	0	1	0	3	2
AA7_dist Glucooligosaccharide oxidase	0	0	2	0	0	0
AA8 Auxiliary Activity Family 8	**0**	**0**	**1**	**2**	**1**	**1**
AA9 Auxiliary Activity Family 9	**13**	**2**	**11**	**14**	**29**	**21**
AA9 Auxiliary Activity Family 9	8	2	11	14	29	21
AA9_dist Lytic polysaccharide monoox. (GH61)	5	0	0	0	0	0
CBM Carbohydrate-Binding Module family	**29**	**32**	**42**	**56**	**74**	**66**
CBM1 Carbohydrate-Binding Module Family 1	1	0	17	30	32	31
CBM12 Carbohydrate-Binding Module Family 12	2	0	1	0	1	1
CBM13 Carbohydrate-Binding Module Family 13	9	18	8	8	22	19
CBM18 Carbohydrate-Binding Module Family 18	1	2	1	1	1	1
CBM20 Carbohydrate-Binding Module Family 20	1	2	1	2	3	3
CBM21 Carbohydrate-Binding Module Family 21	2	3	2	2	2	2
CBM35 Carbohydrate-Binding Module Family 35	0	0	1	1	1	1
CBM43 Carbohydrate-Binding Module Family 43	5	1	1	1	1	1
CBM48 Carbohydrate-Binding Module Family 48	3	1	2	3	2	2
CBM5 Carbohydrate-Binding Module Family 5	1	5	3	2	1	0
CBM50 Carbohydrate-Binding Module Family 50	3	0	5	6	1	1
CBM52 Carbohydrate-Binding Module Family 52	1	0	0	0	0	0
CBM67 Carbohydrate-Binding Module Family 67	0	0	0	0	7	4
CE Carbohydrate Esterase family	**18**	**15**	**33**	**15**	**28**	**20**
CE1 Carbohydrate Esterase Family 1	**0**	**0**	**1**	**4**	**2**	**2**
CE12 Carbohydrate Esterase Family 12	**0**	**0**	**2**	**0**	**2**	**1**
CE15 Carbohydrate Esterase Family 15	**0**	**2**	**1**	**2**	**1**	**0**
CE16 Carbohydrate Esterase Family 16	**2**	**4**	**11**	**2**	**9**	**6**
CE4 Carbohydrate Esterase Family 4	**11**	**2**	**9**	**4**	**11**	**9**
CE5 Carbohydrate Esterase Family 5	**1**	**0**	**6**	**0**	**0**	**0**
CE8 Carbohydrate Esterase Family 8	**4**	**4**	**2**	**2**	**2**	**2**
CE9 Carbohydrate Esterase Family 9	**0**	**3**	**1**	**1**	**1**	**0**
EXPN Distantly related to plant expansins	**12**	**12**	**9**	**9**	**8**	**7**
GH Glycoside Hydrolase family	**170**	**129**	**174**	**175**	**235**	**162**
GH1 Glycoside Hydrolase Family 1	**0**	**2**	**1**	**2**	**3**	**3**
GH10 Glycoside Hydrolase Family 10	**0**	**1**	**2**	**6**	**3**	**2**
GH105 Glycoside Hydrolase Family 105	**0**	**0**	**2**	**0**	**2**	**2**
GH11 Glycoside Hydrolase Family 11	**0**	**0**	**2**	**1**	**2**	**1**
GH115 Glycoside Hydrolase Family 115	**0**	**0**	**2**	**1**	**1**	**1**
GH12 Glycoside Hydrolase Family 12	**3**	**2**	**2**	**2**	**2**	**1**
GH125 Glycoside Hydrolase Family 125	**0**	**0**	**1**	**1**	**1**	**1**
GH128 Glycoside Hydrolase Family 128	**5**	**2**	**2**	**5**	**4**	**3**
GH13 Glycoside Hydrolase Family 13	**10**	**3**	**7**	**9**	**10**	**3**
GH13_1 Glycoside Hydrolase Family 13 / Subf 1	3	2	1	2	2	0
GH13_22 Glycoside Hydrolase Family 13	1	0	1	1	1	0
GH13_25 Glycoside Hydrolase Family 13	1	0	1	1	1	1
GH13_32 Glycoside Hydrolase Family 13 / Subf 32	0	0	1	1	3	1
GH13_40 Glycoside Hydrolase Family 13	3	0	1	2	1	0
GH13_5 Glycoside Hydrolase Family 13	1	0	1	1	1	1
GH13_8 Glycoside Hydrolase Family 13	1	1	1	1	1	0
GH131 Glycoside Hydrolase Family 131	**1**	**0**	**0**	**3**	**2**	**1**
GH133 Glycoside Hydrolase Family 133	**1**	**0**	**1**	**1**	**1**	**1**
GH135 Glycoside Hydrolase Family 135	**0**	**0**	**0**	**1**	**0**	**0**
GH15 Glycoside Hydrolase Family 15	**2**	**2**	**2**	**2**	**3**	**3**

GH16 Glycoside Hydrolase Family 16	**31**	**23**	**24**	**23**	**30**	**18**
GH17 Glycoside Hydrolase Family 17	**4**	**2**	**6**	**2**	**3**	**2**
GH18 Glycoside Hydrolase Family 18	**11**	**15**	**13**	**11**	**12**	**13**
GH2 Glycoside Hydrolase Family 2	**2**	**5**	**2**	**2**	**3**	**2**
GH20 Glycoside Hydrolase Family 20	**2**	**2**	**4**	**3**	**2**	**1**
GH23 Glycoside Hydrolase Family 23	**1**	**2**	**1**	**0**	**1**	**1**
GH24 Glycoside Hydrolase Family 24	**3**	**0**	**1**	**0**	**3**	**2**
GH25 Glycoside Hydrolase Family 25	**0**	**0**	**4**	**1**	**2**	**2**
GH27 Glycoside Hydrolase Family 27	**1**	**2**	**4**	**3**	**7**	**2**
GH28 Glycoside Hydrolase Family 28	**7**	**7**	**6**	**4**	**6**	**2**
GH29 Glycoside Hydrolase Family 29	**0**	**0**	**1**	**0**	**0**	**0**
GH3 Glycoside Hydrolase Family 3	**2**	**8**	**8**	**10**	**13**	**8**
GH30 Glycoside Hydrolase Family 30	**9**	**0**	**3**	**2**	**4**	**1**
GH30 Glycoside Hydrolase Family 30	1	0	1	1	2	0
GH30_3 Glycoside Hydrolase Family 30 / Subf 3	7	0	2	1	2	1
GH30_dist Glycoside Hydrolase Family 30	1	0	0	0	0	0
GH31 Glycoside Hydrolase Family 31	**5**	**6**	**6**	**6**	**8**	**4**
GH32 Glycoside Hydrolase Family 32	**0**	**0**	**0**	**0**	**1**	**1**
GH35 Glycoside Hydrolase Family 35	**1**	**2**	**1**	**3**	**4**	**2**
GH37 Glycoside Hydrolase Family 37	**2**	**7**	**2**	**2**	**2**	**2**
GH38 Glycoside Hydrolase Family 38	**1**	**1**	**1**	**1**	**1**	**0**
GH43 Glycoside Hydrolase Family 43	**0**	**0**	**4**	**4**	**8**	**8**
GH44 Glycoside Hydrolase Family 44	**0**	**0**	**1**	**0**	**1**	**1**
GH45 Glycoside Hydrolase Family 45	**0**	**1**	**2**	**0**	**3**	**3**
GH47 Glycoside Hydrolase Family 47	**11**	**7**	**7**	**6**	**9**	**5**
GH5 Glycoside Hydrolase Family 5	**23**	**12**	**19**	**19**	**21**	**16**
GH5_12 Glycoside Hydrolase Family 5	4	3	2	2	2	2
GH5_15 Glycoside Hydrolase Family 5 / Subf 15	3	1	1	1	1	0
GH5_22 Glycoside Hydrolase Family 5	0	1	2	2	2	2
GH5_30 Glycoside Hydrolase Family 5 / Subf 30	2	0	1	0	1	1
GH5_31 Glycoside Hydrolase Family 5 / Subf 31	0	0	0	1	0	0
GH5_5 Glycoside Hydrolase Family 5	1	3	3	2	4	3
GH5_50 Glycoside Hydrolase Family 5 / Subf 50	2	0	2	2	1	0
GH5_7 Glycoside Hydrolase Family 5	0	1	1	3	4	4
GH5_9 Glycoside Hydrolase Family 5	11	3	7	6	6	4
GH51 Glycoside Hydrolase Family 51	**0**	**2**	**1**	**2**	**3**	**2**
GH53 Glycoside Hydrolase Family 53	**0**	**0**	**1**	**1**	**1**	**1**
GH55 Glycoside Hydrolase Family 55	**2**	**1**	**1**	**2**	**2**	**2**
GH6 Glycoside Hydrolase Family 6	**0**	**0**	**1**	**1**	**3**	**3**
GH62 Glycoside Hydrolase Family 62	**0**	**0**	**0**	**0**	**1**	**1**
GH63 Glycoside Hydrolase Family 63	**1**	**2**	**1**	**1**	**1**	**1**
GH7 Glycoside Hydrolase Family 7	**0**	**0**	**1**	**8**	**16**	**11**
GH71 Glycoside Hydrolase Family 71	**4**	**4**	**3**	**3**	**5**	**3**
GH72 Glycoside Hydrolase Family 72	**7**	**1**	**1**	**1**	**1**	**1**
GH74 Glycoside Hydrolase Family 74	**0**	**0**	**1**	**4**	**3**	**2**
GH76 Glycoside Hydrolase Family 76	**0**	**0**	**0**	**0**	**2**	**2**
GH78 Glycoside Hydrolase Family 78	**0**	**1**	**4**	**1**	**2**	**2**
GH79 Glycoside Hydrolase Family 79	**11**	**0**	**6**	**5**	**7**	**6**
GH85 Glycoside Hydrolase Family 85	**1**	**2**	**1**	**1**	**1**	**1**
GH88 Glycoside Hydrolase Family 88	**2**	**1**	**1**	**1**	**1**	**2**
GH89 Glycoside Hydrolase Family 89	**0**	**0**	**0**	**2**	**0**	**0**
GH9 Glycoside Hydrolase Family 9	**1**	**0**	**1**	**1**	**1**	**1**
GH92 Glycoside Hydrolase Family 92	**2**	**0**	**5**	**4**	**6**	**2**
GH95 Glycoside Hydrolase Family 95	**1**	**1**	**1**	**1**	**1**	**1**
GT Glycosyl Transferase family	**96**	**24**	**54**	**65**	**65**	**47**
GT1 GlycosylTransferase Family 1	**6**	**1**	**3**	**6**	**3**	**2**

GT15 GlycosylTransferase Family 15	**7**	**1**	**3**	**2**	**4**	**4**
GT17 GlycosylTransferase Family 17	**1**	**0**	**1**	**1**	**1**	**0**
GT18 GlycosylTransferase Family 18	**2**	**0**	**0**	**0**	**1**	**0**
GT2 GlycosylTransferase Family 2	**21**	**7**	**11**	**13**	**12**	**10**
GT20 GlycosylTransferase Family 20	**4**	**1**	**3**	**3**	**3**	**3**
GT21 GlycosylTransferase Family 21	**1**	**0**	**1**	**1**	**1**	**1**
GT22 GlycosylTransferase Family 22	**4**	**1**	**3**	**2**	**3**	**2**
GT24 GlycosylTransferase Family 24	**1**	**0**	**1**	**1**	**1**	**0**
GT3 GlycosylTransferase Family 3	**1**	**0**	**1**	**1**	**1**	**1**
GT31 GlycosylTransferase Family 31	**1**	**0**	**1**	**1**	**1**	**0**
GT32 GlycosylTransferase Family 32	**1**	**0**	**1**	**1**	**0**	**0**
GT33 GlycosylTransferase Family 33	**1**	**0**	**1**	**1**	**1**	**1**
GT35 GlycosylTransferase Family 35	**1**	**0**	**1**	**1**	**1**	**1**
GT39 GlycosylTransferase Family 39	**8**	**0**	**3**	**3**	**3**	**3**
GT4 GlycosylTransferase Family 4	**6**	**2**	**4**	**4**	**4**	**4**
GT41 GlycosylTransferase Family 41	**0**	**0**	**0**	**1**	**0**	**0**
GT48 GlycosylTransferase Family 48	**2**	**0**	**2**	**2**	**2**	**2**
GT49 GlycosylTransferase Family 49	**0**	**0**	**1**	**1**	**1**	**1**
GT5 GlycosylTransferase Family 5	**3**	**0**	**1**	**1**	**1**	**0**
GT50 GlycosylTransferase Family 50	**1**	**0**	**1**	**1**	**1**	**0**
GT57 GlycosylTransferase Family 57	**2**	**0**	**2**	**2**	**2**	**0**
GT58 GlycosylTransferase Family 58	**1**	**0**	**1**	**1**	**1**	**0**
GT59 GlycosylTransferase Family 59	**1**	**0**	**1**	**1**	**1**	**0**
GT66 GlycosylTransferase Family 66	**1**	**1**	**1**	**1**	**1**	**1**
GT69 GlycosylTransferase Family 69	**3**	**2**	**1**	**2**	**1**	**0**
GT76 GlycosylTransferase Family 76	**1**	**0**	**0**	**1**	**1**	**1**
GT8 GlycosylTransferase Family 8	**7**	**8**	**2**	**9**	**7**	**6**
GT90 GlycosylTransferase Family 90	**8**	**0**	**3**	**1**	**6**	**4**
Myosin_motor Glycosyltransferase Family 2	**4**	**1**	**1**	**1**	**2**	**1**
PL Polysaccharide Lyase family	**7**	**1**	**9**	**4**	**23**	**14**
PL1 Polysaccharide Lyase Family 1	**0**	**0**	**2**	**0**	**10**	**6**
PL1 Polysaccharide Lyase Family 1	0	0	0	0	5	3
PL1_2 Polysaccharide Lyase Family 1 / Subf 2	0	0	1	0	1	0
PL1_7 Polysaccharide Lyase Family 1	0	0	1	0	4	3
PL14 Polysaccharide Lyase Family 14	**6**	**1**	**4**	**3**	**4**	**2**
PL14 Polysaccharide Lyase Family 14	2	1	0	1	1	1
PL14_4 Polysaccharide Lyase Family 14 / Subf 4	3	0	3	2	2	1
PL14_5 Polysaccharide Lyase Family 14 / Subf 5	1	0	1	0	1	0
PL3 Polysaccharide Lyase Family 3	**0**	**0**	**1**	**0**	**3**	**3**
PL3_2 Polysaccharide Lyase Family 3	0	0	1	0	3	3
PL4 Polysaccharide Lyase Family 4	**0**	**0**	**1**	**0**	**2**	**2**
PL4_1 Polysaccharide Lyase Family 4 / Subf 1	0	0	1	0	2	2
PL8 Polysaccharide Lyase Family 8	**1**	**0**	**1**	**1**	**4**	**1**
PL8_4 Polysaccharide Lyase Family 8	1	0	1	1	4	1
Total	**391**	**243**	**403**	**409**	**549**	**403**

Supplementary Table 1. Comparison between *P. ostreatus* monokaryons(PleosPC15 and PleosPC9) and 4 basidiomycetes: *Laccaria bicolor*(Lacbi2), *Postia placenta* (Pospl1) *Agaricus bisporus var. bisporus H97*(Agabi_H97) and *Phanerochaete chrysosporium*(Phchr1) Data from JGI Mycocosm.

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Chapter 3:

Comparative and transcriptional analysis of the predicted secretome in the lignocellulose-degrading basidiomycete fungus *Pleurotus ostreatus*

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Manuel Alfaro, Raúl Castanera, Jose Luis Lavin Trueba, Igor V Grigoriev, Jose Antonio Oguiza, Lucia Ramirez, Antonio Gerardo Pisabarro
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1. – INTRODUCTION

Fungi in the *phylum* Basidiomycota occupy a large variety of ecological niches: ectomycorrhizal (ECM) fungi form symbiotic relationships with the roots of most tree species (Hess et al., 2014; Plett & Martin, 2011; Vincent et al., 2012); saprotrophic fungi (SAP), such as white rot (WR) (Martinez et al., 2004) and brown rot (BR) (Eastwood et al., 2011), use decaying matter as carbon sources (Stajich et al., 2010); plant pathogens (PPT) invade living tissues of plants to obtain nutrients (Kämper et al., 2006); and animal pathogens (APT) (Loftus et al., 2005) infect animals, including humans. Furthermore, some fungi can switch between different lifestyles over their life cycles (e.g., *Moniliophthora perniciosa*) (Mondego et al., 2008). These different lifestyles are associated with the ability of the fungi to produce high amounts of secreted enzymes that are crucial tools to obtain nutrients, a characteristic that has been widely exploited by industry (Conesa et al., 2001; Shoji et al., 2008). The secretome is defined as the set of proteins secreted by a cell or an organism at a given time (Manuel Alfaro, Oguiza, Ramírez, & Pisabarro, 2014; Tjalsma et al., 2000). This definition includes proteins released into the surrounding medium and also proteins that persist anchored to the membrane or cell wall, including integral membrane proteins. The secretome is a very dynamic fungal tool that varies over time depending on the growth substrate, temperature or growth phase. One of the main objectives of fungal secretome studies is to understand secretome modulation due to environmental changes.

Lignocellulose is the largest reservoir of organic carbon on Earth and is a renewable resource that serves not only as the raw material of the pulp and paper industry but also as a promising potential source of second-generation biofuels. Basidiomycetes are the only organisms known to degrade lignocellulose on a global scale by producing a large amount of lignocellulolytic secreted enzymes and playing a key role in the global carbon cycle. From a geological time frame perspective, lignocellulose could have served as a major carbon sink until the appearance of SAP lignin-degrading fungi at the end of the Carboniferous period (Floudas et al., 2012). Lignin degradation can be achieved using two strategies: WR fungi secrete enzymes that remove the lignin polymer before they start to enzymatically degrade the cellulose, whereas BR fungi barely degrade the lignin and attack the cellulose layer via a Fenton reaction-based mechanism. However, these two lignocellulose-degrading strategies do not seem to be mutually exclusive because the *Jaapia argillacea* and *Botryobasidium*

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botryosum genome sequences simultaneously show similarities to the WR (presence of gene families coding for carbohydrate and lignin active enzymes) and BR (absence of genes coding for the class II peroxidases) fungi (Riley et al., 2014b).

The degradation of the lignin moiety of lignocellulose is achieved by a set of principal enzymes that include manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC 1.11.1.16), lignin peroxidases (LiP, EC 1.11.1.14) and phenol oxidases (benzenediol:oxygen oxidoreductase Pox, laccases, EC 1.10.3.2) that are accompanied by other accessory enzymes, such as cytochrome c peroxidases (EC 1.11.1.5), chloroperoxidases (EC 1.11.1.10), dye decolorizing peroxidases (DyP, EC 1.11.1.19), glyoxal oxidases (GLOX), aryl-alcohol oxidases (AAO, EC 1.1.3.7), pyranose dehydrogenases (EC 1.1.99.29), and methanol oxidases (EC 1.113.13). The cellulose moiety is attacked by secreted carbohydrate-active enzymes (CAZy) (Cantarel et al., 2009) such as exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91) that are subclassified as types I and II depending on their attack of the reducing or non-reducing cellulose ends, endocellulases (EC 3.2.1.4), and cellobiases (beta-glucosidases, EC 3.2.1.21). Finally, the cementing role of the hemicellulose is disrupted by the action of endoxylanases (EC 3.2.1.8), α-glucuronidases (EC 3.2.1.131), acetyl-xylan esterases (EC 3.1.1.72), arabinofuranosidases (EC 3.2.1.55), ferulic acid esterases (feruloyl esterases, EC 3.1.1.73) and β-xylosidases (EC 3.2.1.37), among other enzymes (Sun et al., 2012). All of these enzymes are frequently encoded by multigenic families whose members are differentially regulated [see (R Castanera et al., 2012) as an example in *P. ostreatus*].

The identification of the set of secretable proteins encoded in the genome of a fungal species should permit the development of hypotheses about its lifestyle. Bioinfosecretomes are *in silico*-predicted secretomes based on the identification of secretion signals in the predicted proteins using the automatically annotated gene models of a genome sequence (Manuel Alfaro et al., 2014). Therefore, the quality of a bioinfosecretome depends on the quality of the genome sequence and annotation. Fungi secrete proteins using either the conventional (CSP) or the unconventional (USP) secretion pathways. CSPs require that secretion-oriented proteins contain an amino-terminal peptide sequence (N-terminal sequence, signal peptide, SP) that targets them to the endoplasmic reticulum (ER), where they will be correctly folded and post-translationally modified (Conesa et al., 2001). Subsequently, proteins pass through

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the Golgi apparatus and are transported in secretory vesicles that fuse to the plasma membrane to perform the secretion process (Conesa et al., 2001; Fonzi, 2009; Shoji et al., 2008). Alternatively, proteins secreted by the UPS pathways do not require the presence of an SP sequence to guide the protein through the ER/Golgi pathway (Nickel & Seedorf, 2008). The occurrence of UPS pathways in fungi has been well documented in the case of yeasts (Nombela et al., 2006). The computational prediction of the bioinfosecretome is based on the initial screening of the required SP sequences in the secreted proteins (Caccia et al., 2013) using programs such as SignalP (Bendtsen *et al.*, 2004) and TargetP (Olof Emanuelsson, Brunak, von Heijne, & Nielsen, 2007), followed by a subsequent scan to determine the presence of transmembrane motifs. This approach was used to identify 168 secreted proteins, including 39 glycosylphosphatidyl inositol (GPI)-anchored proteins, encoded in the genome of the basidiomycete maize smut *Ustilago maydis* (Mueller et al., 2008). Additionally, the first bioinfosecretome of a WR basidiomycete was constructed for *Phanerochaete chrysosporium* (Vanden Wymelenberg *et al.*, 2005) using the first genome version. It consisted of 268 secretion-predicted proteins (Martinez et al., 2004), although this number could be an underestimation due to inaccurate and incomplete gene model annotation. Subsequently, 769 secreted proteins were identified based on the second assembled version of this genome (Vanden Wymelenberg et al., 2006). Many predicted secreted proteins were classified as glycosyl hydrolases (GH), oxidoreductases, peptidases and esterases-lipases, as expected for the SAP lifestyle of *P. chrysosporium*. Finally, Jain et al. (Jain et al., 2008) focused on non-classically secreted proteins using the SecretomeP method (Bendtsen *et al.*, 2004) to analyze genome sequences of *Laccaria bicolor, Botrytis cinerea, P. chrysosporium*, and five other fungal species and found proteins involved in carbohydrate metabolism, lectins and proteases. There are currently more than 135 basidiomycete genome sequences available in the MycoCosm database (Igor V Grigoriev et al., 2014) from the Joint Genome Institute (JGI, October 2014) (I V Grigoriev et al., 2012). These sequences offer the opportunity to predict the bioinfosecretomes of these basidiomycetes and correlate them with their corresponding lifestyles in order to identify characteristics that permit the deduction of the set of secreted proteins.

However, the bioinfosecretome is a static list of secretable proteins that does not produce a complete picture of how the toolbox of secreted proteins is actually used. To approximate to

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its actual use, we propose here to color the bioinfosecretome with data derived from whole transcriptome studies of the same individual cultured under different conditions as a proxy for the actual secretome. Moreover, we hypothesize that because the secretome reflects the fungal lifestyle, the analysis of the bioinfosecretome should permit the prediction of the fungal lifestyle. To test this hypothesis, we studied the secreted proteins released into the surrounding medium (membrane-bound proteins were excluded) and compared the bioinfosecretomes of different fungi and their expression under different culture conditions. We show that although the secretome is conserved in different strains of the same species, its expression varies among strains and culture conditions. Moreover, the secretome's transcriptional effort is more similar within strains, even when they are cultured under different conditions, than between different strains cultured under the same conditions. We also show that the expansion of gene families does not imply the expression of more genes in the expanded family. We identify new families of secreted proteins with unknown functions and suggest a function for some of these groups. Finally, we show that fungal secretomes cluster according to the fungal lifestyles rather than according to their taxonomic or phylogenetic classifications.

2. RESULTS AND DISCUSSION

The *P. ostreatus* dikaryotic strain N001 is a hybrid strain that has been used as a source for commercial cultures for many years. The two nuclei present in this dikaryotic strain were separated, and two monokaryotic strains (PC9 and PC15) were produced and maintained. The genomes of these two strains have been sequenced (Riley et al, 2014). In this paper, we use the term "strain" to refer to these two monokaryons that are genetically compatible and that reconstitute the dikaryotic strain N001 when mated.

P. ostreatus PC9 and PC15 bioinfosecretomes

The protein models of the *P. ostreatus* PC9 (12,206 proteins) and PC15 (12,330 proteins) monokaryons were analyzed with SECRETOOL to identify putatively secreted proteins. A total of 659 gene models were identified as encoding secretable proteins. These included 538 (PC9) and 554 (PC15) models that represented 4.41 % and 4.49 % of the total number of protein models, respectively (Fig. 1).

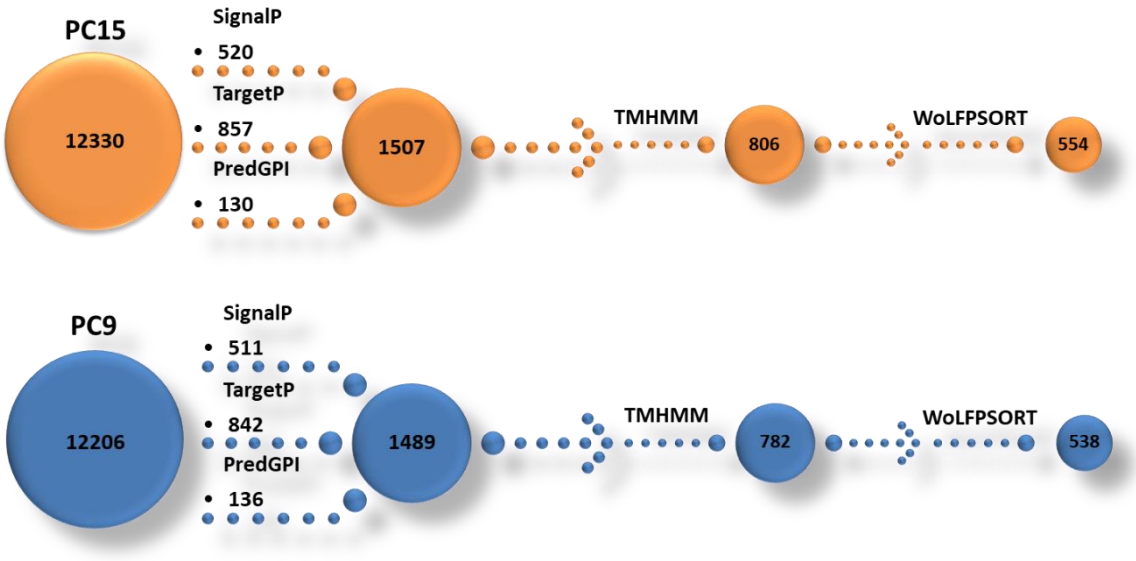
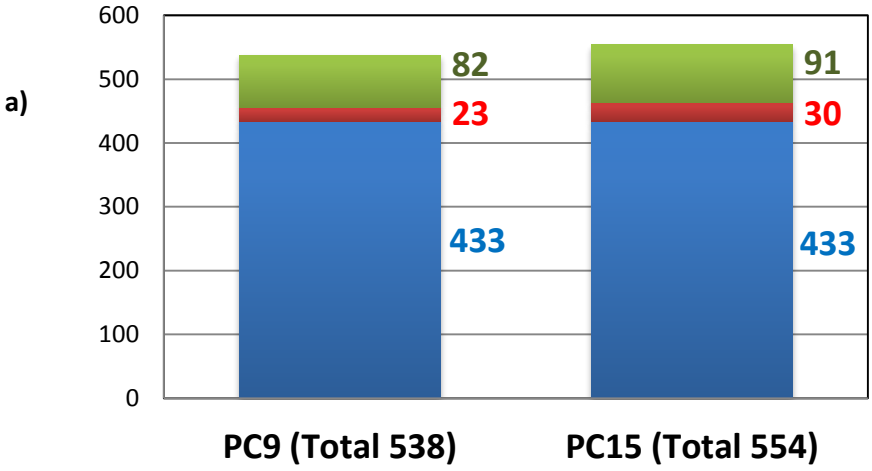
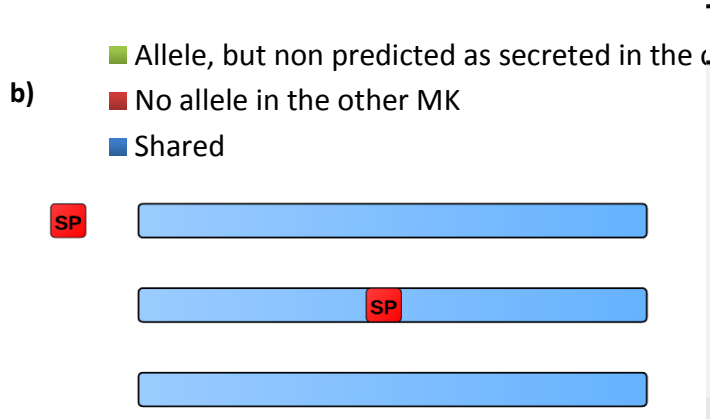


Figure 1. SECRETOOL pipeline for the prediction of secreted proteins in the *P. ostreatus* PC15 and PC9 monokaryons.

A total of 433 of these proteins were identified as secretable in both PC9 and PC15 (Fig. 2A), whereas 105 and 121 were predicted to be secreted exclusively by PC9 or PC15, respectively. There was no corresponding allele predicted in PC15 for 23 of the 105 PC9-exclusive proteins (i.e., they were encoded for genes solely identified in the PC9 genome); an allele for the remaining 82 proteins was found in PC15, although it encoded a protein that did not match the criteria for secretion used in the SECRETOOL pipeline. Conversely, an allele in PC9 was not found for 30 of the 121 PC15-exclusive proteins; for the remaining 91 proteins, an allele was found in PC9 that did not encode a protein meeting the secretion criteria established in the SECRETOOL pipeline.





PC9	PC15
38	24
36	40
14	18
3	0
91	**82**

Figure 2. a. Number of predicted proteins in the *P. ostreatus* monokaryons PC9 and PC15. . **b**. Signal peptide position in allelic protein models not predicted to be secreted.

Next, we took a closer look at the genes for which a secretable allele was found in one of the two monokaryons whereas the corresponding allele was annotated as non-secretable in the

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other monokaryon (91 PC9 alleles and 82 PC15 alleles =173 gene models, see above). To accomplish this, we used the first 210 bp of each model (starting at the initial ATG) as a query in a BLASTN search (Camacho et al., 2009) performed in the genome of the monokaryon in which the corresponding protein was not predicted to be secreted. The rationale was to search for the corresponding signal peptide in the non-secreted alleles. For 172 out of the 173 searches, a sequence similar to the signal peptide query was found in the model not predicted to be secretable (Fig. 2B). There was an exception that corresponded to a gene model identified in PC15 as carrying a signal peptide whose sequence was not found in the entire PC9 genome; thus, this gene model could correspond to a protein secreted by one of the monokaryons but not the other (PC9/PC15 gene model 91216/1109867).

The 172 (82+90) gene models could be grouped into three categories (Fig. 2B): (i) gene models in which a sequence similar to the corresponding signal peptide was found in the non-secreted allele at the expected position for a signal peptide (31 PC9 and 22 PC15 gene models); (ii) gene models in which the sequence similar to the corresponding signal peptide was found at different positions upstream of the expected position (36 in PC9 and 39 in PC15); and (iii) gene models in which the sequence similar to the corresponding signal peptide was found at different positions downstream of the start of the corresponding non-secreted model (23 in PC9 and 21 in PC15). In the case of genes in which the putative signal peptide was found upstream of the predicted gene model, the distance between the signal and the model varied from a few base pairs to 11 Kbp. The signal appeared in the inverted orientation in two of the PC9 models in which the putative signal peptide was far upstream from the gene model. Similarly, there was one PC15 gene model in which the sequence corresponding to the signal peptide was found downstream of the gene model and in an inverted orientation. Finally, in one of the models in which the signal peptide was far upstream from the protein start (PC15 gene model 1103939), the intervening sequence was identified as an LTR-*Gypsy* transposable element.

In summary, 53 gene models (31 PC9 and 22 PC15 gene models under the first category of the preceding paragraph) were discarded as secretable by the SECRETOOL pipeline although they had a *bona fide* signal peptide at the expected position. This discard is due to the stringent conditions of the SECRETOOL algorithm. For the remaining 120 gene models, the sequence differences between the alleles could reflect the dynamic nature of the genomes

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due to identifiable insertion of a transposon-related sequence and inversion events. This result emphasizes the need for refined automatic annotation systems that prevent artifactual biases in the annotation of genomes.

Because the 173 gene models described above had different types of alterations that prevented them from qualifying as encoding secretable proteins according to the standard established by SECRETOOL, we discarded them for the remainder of the studies described in this paper. Moreover, there could be protein models for which neither of the two alleles fulfill the conditions imposed by the SECRETOOL algorithm and have been undetected in this analysis. Consequently, the list of secreted proteins described in this work should not be taken as exhaustive, since stringency has prevailed over sensitivity in the process.

Functional categories of the proteins identified in the predicted *P. ostreatus* bioinfosecretome

Functional characteristics were associated with the *P. ostreatus* putative secreted proteins after the individual manually curated annotation. Figure 3 shows that the functional profiles of the PC9 and PC15 predicted secretomes were highly similar.

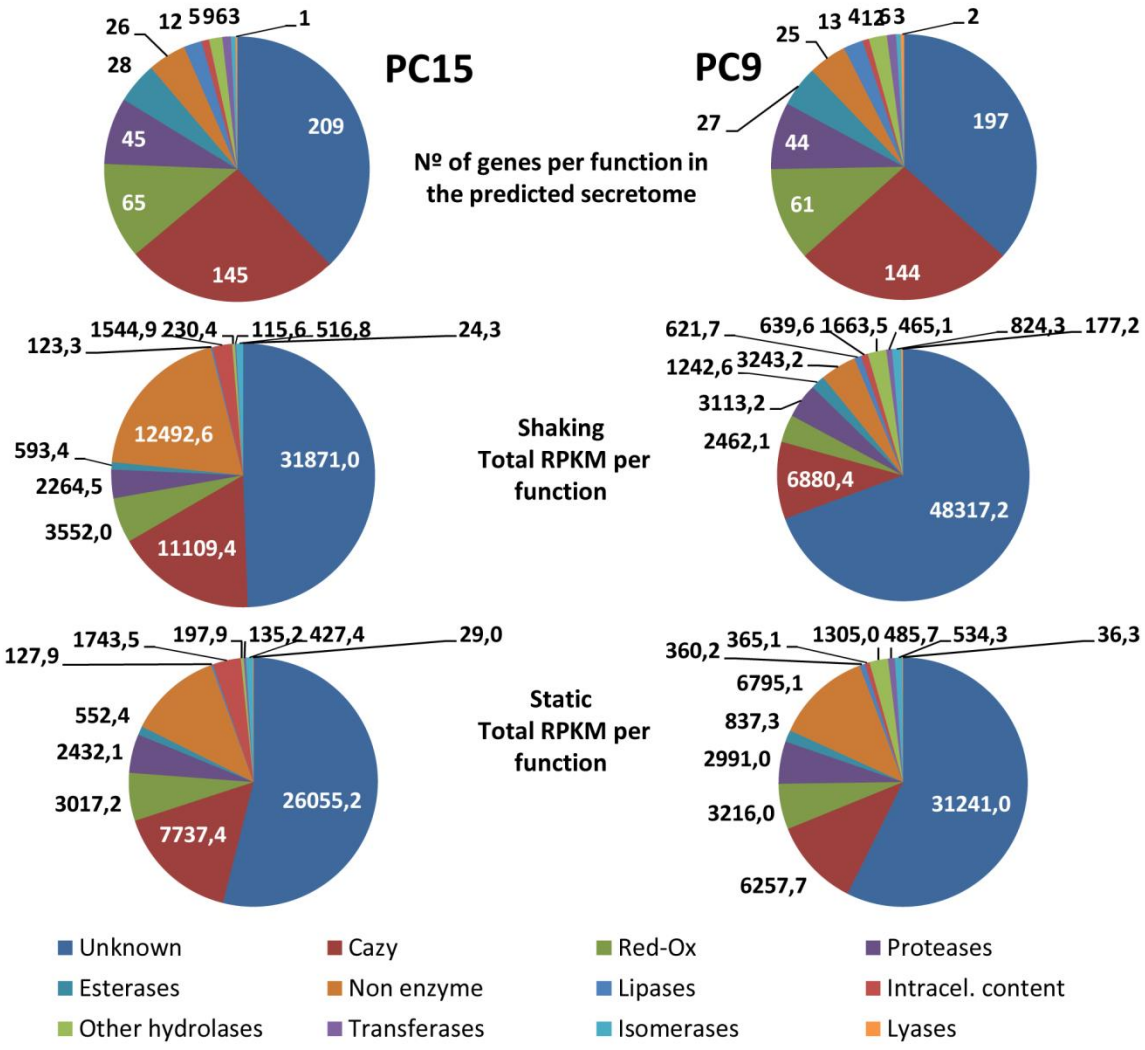

Figure 3. Function of proteins predicted to be secreted by the *P. ostreatus* monokaryons PC9 and PC15 and a comparison between the number of proteins predicted to be secreted and the number of RPKM by protein function

A protein function, being enzymatic or structural (i.e. hydrophobins), could not be predicted for 197 putative secreted proteins in PC9 (36.6% of the SECRETOOL positive models) and 209 in PC15 (37.7%). Most of these proteins with unknown function (145 gene models corresponding to 26% of the PC15 putative secreted protein set) appeared to be conserved in other basidiomycetes. For the rest of the models, the functional groups of carbohydrate active enzymes (CAZy), redox and proteases were the more abundant, accounting for more than 75% of the models with an assigned function.

A total of 166 gene models that could be grouped into 53 CAZy groups were identified as secretable. The most abundant groups corresponded to AA9 (copper-dependent lytic polysaccharide monooxygenases or LPMOs, formerly classified as group GH61, 20 gene models), GH16 (endoglucanase, 13 gene models) and GH7 (cellobiohydrolase I, 10 gene models). The most abundant Carbohydrate Binding Module (CBM domain) was the ricin-like CBM13 (8 gene models). A total of 70 gene models related to redox reactions were identified. These models could be further classified into 17 functional groups of which those corresponding to the GMC oxidoreductases (17 gene models), proteins containing a cupredoxin motif (11 gene models) and laccases (11 gene models) were the most abundant. A total of 53 gene models encoding different types of peptidases could be classified into 18 classes, among which peptidase type A1 (aspartic endopeptidases) and S8 (subtilases) were the most abundant (6 gene models each). A total of 12 different groups of secretable esterases were identified within 33 gene models that included the ribonucleases (7 gene models) and carboxylesterases (6 gene models, the most abundant). Finally, a total of 16 lipase genes were identified, of which 14 were classified as lipase 3. Four PC15 and three PC9 lipase 3 gene models were identified in only one of the two strains without an allele in the other strain.

Given the relevance of the group of gene models encoding proteins with unknown functions, we used a complementary approach to generate hypotheses about their functions. We used the Phyre2 web server (Kelley & Sternberg, 2009), which uses the more sensitive PSI-BLAST algorithm, to predict the structures and functions of these proteins, and then we used MCL (Enright, Van Dongen, & Ouzounis, 2002) to cluster them into families. This approach allowed us to identify two protein clusters with a common predicted function among the family members (Supplementary File 1, Table 4). The first cluster was formed by 16 proteins (10 of which could be folded with a level of confidence higher than 90%) as putative cell invasion proteins with a MAC/perforin domain (MACPF) (Lukoyanova et al., 2015). A protein with this domain that was involved in transmembrane pore formation (Pleurotolysin) was previously described as a cytolysin present in the basidiocarp of *P. ostreatus* (Bernheimer & Avigad, 1979; Tomita et al., 2004). Similarly, pore-forming proteins have been described in other eukaryotes and bacteria, and many play key roles in immunity and pathogenesis (Rosado et al., 2007). The second cluster was formed by four proteins whose predicted structures and functions matched a bacterial xylan esterase (confidence > 95%) of

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the soil bacterium *Cellvibrio japonicum*. This saprophytic bacterium has been experimentally shown to degrade all of the major plant cell wall polysaccharides, including xylan (DeBoy et al., 2008). Both groups have similar proteins that are present in many other basidiomycete genomes, where they have also been annotated as proteins without a predicted function.

These results emphasize the need to use structure–based algorithms for the prediction of functions in proteins with unknown functions because they greatly diverge in sequence but maintain a more conserved global structure.

Pfam enrichment in the bioinfosecretome of *P. ostreatus*

To elucidate whether there was an enrichment of functional categories in the secretome *versus* the whole genome, both the bioinfosecretome protein models and all the filtered models of the *P. ostreatus* PC15 genome were subjected to a Pfam domain search using the InterPro standalone program (Apweiler et al., 2000; Bateman et al., 2004). The Pfam categories with an enrichment higher than six-fold are shown in Figure 4.

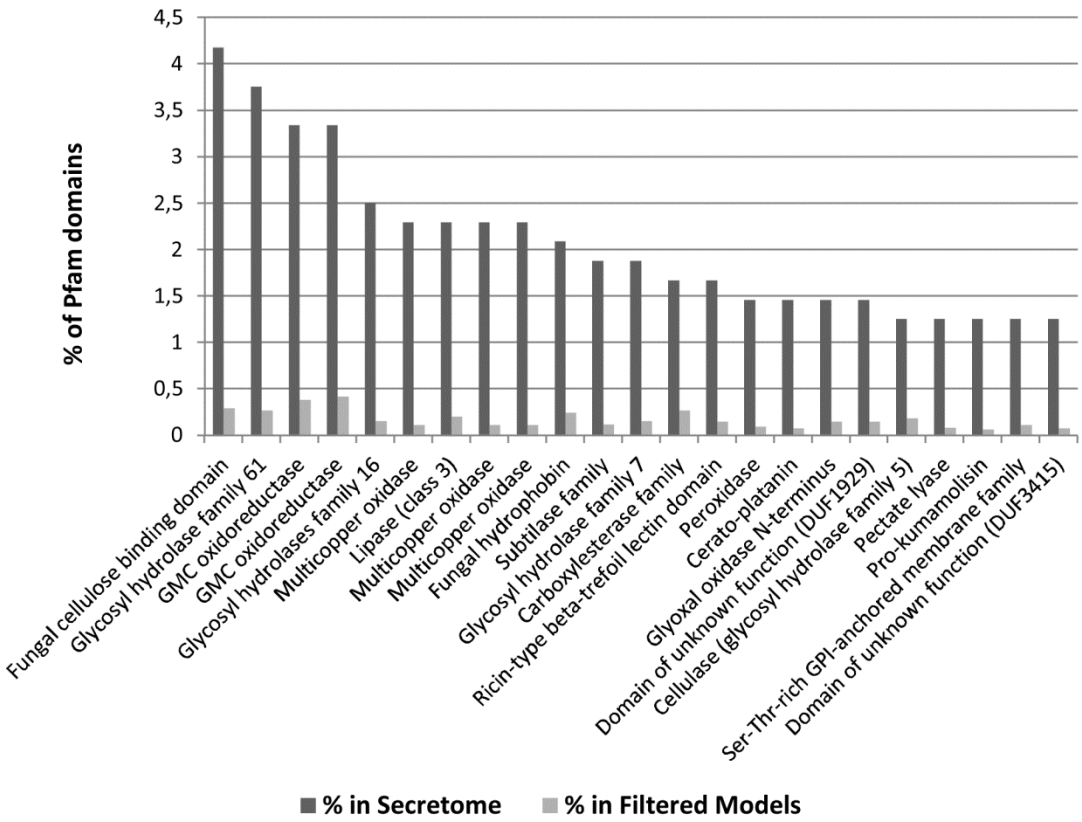

Figure 4. Percentage of domains from a Pfam category in the bioinfosecretome and in all filtered models of the genome sequence of *P. ostreatus* PC15. Only Pfam categories with at least 6 domains found in the secretome are shown.

Pfam domains involved in lignocellulose degradation were overrepresented in the *P. ostreatus* PC15 bioinfosecretome. The most overrepresented Pfam domains were the multicopper oxidase domain (which can be found in laccases and other proteins), the Pfam domains related to small secreted proteins [hydrophobins and ceratoplatanins, that are about 100 ± 25 amino acids long, cysteine-rich, hydrophobic fungal surface proteins able to self-assemble *in vitro* (Peñas, Rust, Larraya, Ramírez, & Pisabarro, 2002; Sbrana et al., 2007), and ricin type beta trefoil lectin domains] which are always among the most highly expressed genes in the *P. ostreatus* cultures as shown below, and domains involved in cellulose or hemicellulose degradation (families of GH5 and GH16 endoglucanases, GH7 cellobiohydrolase I, AA9/GH61, pectate-lyases and fungal cellulose-binding domains). Overall, the prevalence of Pfam domains related to lignocellulose degradation in proteins predicted to be secreted compared to the complete genome proteins demonstrated the influence of the ecological niche on the fungal secretome composition.

Transcriptional evaluation of the PC9 and PC15 secretomes

The bare enumeration of the genes encoding putative secreted proteins does not provide information about the relevance of each model or functional group in the performance of the organism. To investigate this relevance, we used the data obtained in four transcriptome analyses of the PC9 and PC15 monokaryons grown in rich medium (SMY) under static and shaking culture conditions. For this purpose, we scored the expression data values (RPKM) for each of the models identified with SECRETOOL. Expression was detected for 93.9% (505/538) and 91.5% (507/554) of the genes encoding secretable proteins in PC9 and PC15, respectively. Specifically, 99.3% (430/433) of the secretable proteins with alleles in the two protoclones were expressed by at least one of them. If we focused on the secretable proteins identified in only one of the two protoclones (105 and 121 for PC9 and PC15, respectively, Fig. 2), 100 (95.2%) and 109 (90.1%) showed expression signals in PC9 and PC15,

respectively. In summary, among the 659 *P. ostreatus* proteins predicted to be secreted by at least one monokaryon, transcripts of 639 (96.97%) were expressed in liquid cultures.

The transcriptome pie chart of the expression values of the different gene models encoding secretable proteins differs significantly from the chart containing only the gene models (Fig. 3). From the perspective of the large groups of secreted proteins (i.e., those described in the pie-chart), in all cases the weight of the genes without an assigned function increased to more than 50%.

If we focus our study to the gene or gene family level, it is possible to study the conservation of gene expression within strains cultured under different conditions and between different strains cultured under the same conditions (Figure 5).

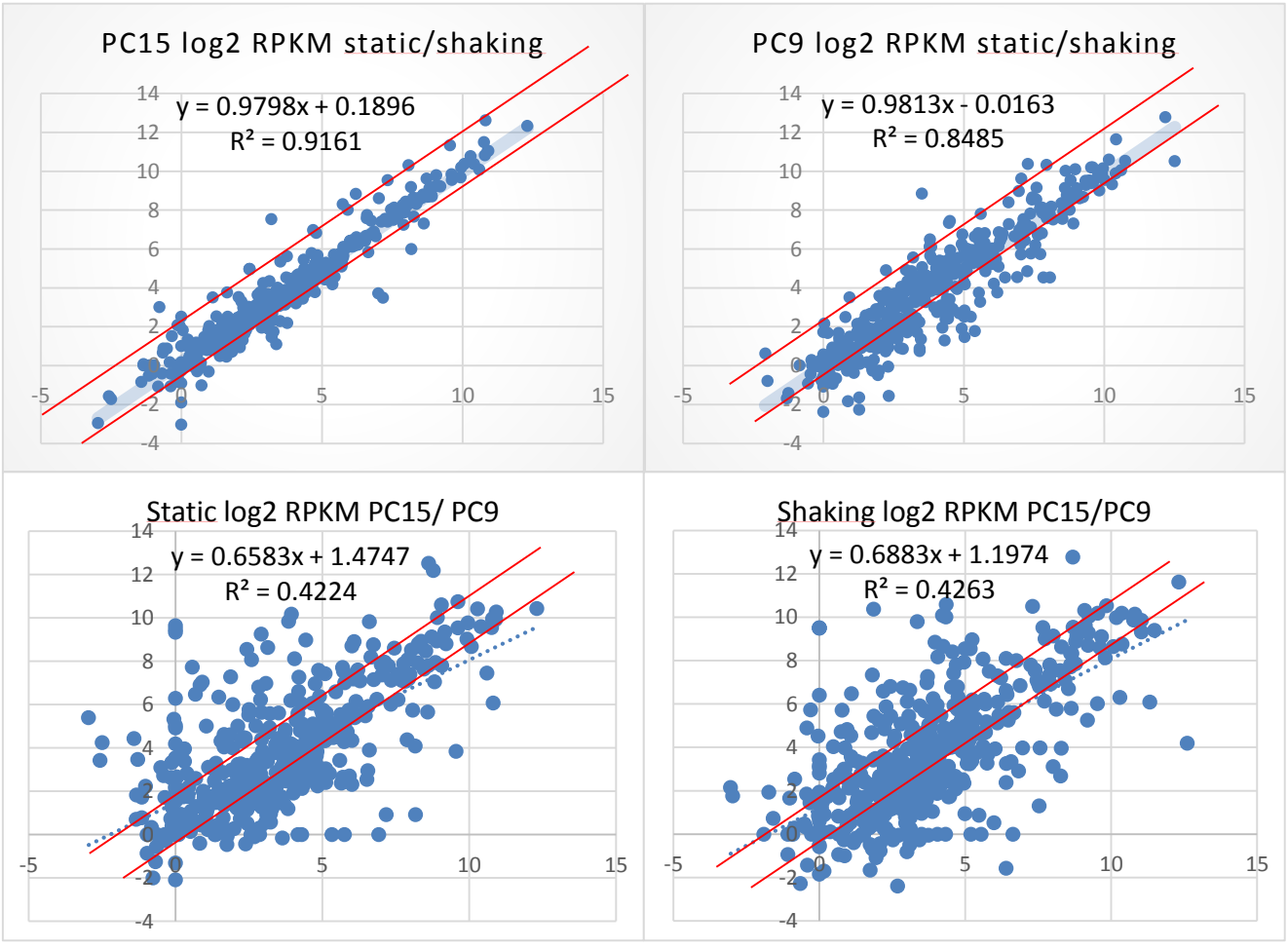

Figure 5. Gene expression (log2 RPKM) comparison within strains cultured under different conditions (static or shaking) and between different strains (PC15 and PC9) cultured under the same conditions.

This analysis revealed a high correlation of gene expression between samples of the same strain cultured under different conditions (static or shaking, determination coefficients higher than 85%), whereas there was a low correlation of gene expression between samples of different strains cultured under the same conditions (determination coefficient lower than 50%). This result suggests that the gene by gene expression profiles of the secretome differ more among strains than between the two conditions tested within a given strain and emphasizes the convenience of studying the transcriptome of different independent genetically related individuals to identify genes whose expression is environmentally regulated.

The study of the accumulated gene expression values indicated that between 11 and 16 genes were responsible for 50% of the total expression under each condition. In PC15, 61 (static) and 65 (shaken) genes scored RPKM values higher than 100, whereas these values were exhibited by 79 genes in the two culture conditions in PC9. The genes with transcription signals higher than 100 represented nearly 90% of the total expression signals of the bioinfosecretome. In summary, most genes encoding secretable proteins were expressed, although this expression was concentrated in less than 25% of them.

Four genes were among the most highly expressed genes in the four analyzed transcriptomes. Highly conserved counterparts for these four genes were found in other fungal genomes available. The first one (PC9/PC15 models 115427/1089609) encoded a small secreted protein (SSP, 128 amino acids) with unknown function that contains an 80 amino acid region well conserved in proteins found in other fungal genomes. Within this conserved region, there are some residues compatible with a putative Pfam10342 domain (glycosyl-phosphatidyl-inositol-anchored, GPI-anchored) suggesting that this protein can remain trapped in the cell wall when exported. The second gene (PC9/PC15 models 117301/1089988) was automatically annotated and KOG-described as β-1,6-acetylglucosaminyltransferase. The third (PC9/PC15 models 114382/172522) encoded a barwin-like protein that was distantly related to plant expansins containing a double-φβ-

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barrel fold; incidentally, this fold was also present in cerato-platanins, whose genes were also among the top expressed genes in PC15 under both culture conditions. The fourth (PC9/PC15 models 116525/1067734) encoded a protein conserved across the fungal kingdom for which no functional clues are available. In summary, the highest transcriptional effort in all strains and culture conditions was focused on the synthesis of proteins associated with cell wall constituents with different putative activities.

If we study the top expressed genes encoding secreted proteins in PC15 under both conditions, we can see that these genes are also among the most expressed in PC9 (both conditions). However, the four more expressed genes in PC9 (static growth) were not among the most expressed genes in PC15 (both conditions). These four PC9 expression-enhanced genes encoded hydrophobin Vmh3 (Peñas et al., 2002) (PC9/PC15 models 80078/1114379) which was the most expressed gene encoding for secretable proteins in PC9sta, and three SSPs (192, 256 and 145 aminoacids) with unknown functions.

CAZy-encoding genes

The expression of the genes encoding secreted CAZy was biased towards a limited number of families. The five most highly expressed families accounted for more than 66% of the total CAZy expression level, and the top ten represented nearly 90% of the total expression of this gene group. In all cases, the gene-rich families of the lytic polysaccharide monooxigenase (LPMO, AA9/GH61), the glucanase of the GH16 family and CBM13-containing proteins were among the top five most highly expressed CAZy genes. Surprisingly, two CAZy families contained few members with expression among the top three in all cases: the barwin-like endoglucanases (GH45) and the PL14 (glucuronan lyase) families contained only two genes each. The correlation coefficient of the global transcriptomes of the four secretomes was always higher compared to the corresponding coefficients for the top ten most highly expressed gene families. Finally, we observed that one gene model was preferentially expressed in each gene family, that the same gene model was the most highly expressed in all conditions and, that the expression of this predominant gene represented more than 75% of the gene family expression in most cases. The most obvious exception to this pattern was the gene family GH16, for which two gene models were preferentially expressed at similar

levels under each condition; their combined expression accounted for between 58 and 74% of the expression of this gene family.

In summary, the genes encoding secreted CAZy included 53 gene families and 166 gene models that represented roughly a quarter of the *P. ostreatus* bioinfosecretome. However, if we consider these genes weighted by their expression values, most of the CAZy expression was concentrated in a much smaller number of gene families and furthermore in only one or two genes per family. Notwithstanding, expression was detected for most of the CAZy-coding genes, albeit at a rather low level.

Redox enzymes

Among the 17 gene families classified as redox, genes encoding proteins containing the cupredoxin motif (cupredoxin family, 11 gene models in addition to the 11 laccase models that also contain the cupredoxin fold, see below) were the most highly expressed in all of the strains and conditions tested and accounted for 44-45% and 58-63% of the family expression in PC9 and PC15, respectively. The second and third most highly expressed gene families in all tested conditions were the glyoxal oxidase and the GMC oxidoreductase families, respectively. Within the cupredoxin family (11 members), more than half of the family's gene expression corresponded to a single gene model in PC15, whereas two genes (one that was the most expressed allele in PC15) were needed to accumulate this expression level in PC9. In the GMC oxidoreductases (17 genes), between two (PC9 shaken) and four genes (PC15 both conditions) were needed to accumulate 50% of the family expression. Gene expression within the glyoxal oxidase family was strongly biased towards the expression of a single gene that accounted for more than 90% of the family's gene expression under all conditions. Finally, the phenol oxidase family (laccases, 11 genes) was especially relevant in the WR fungus *P. ostreatus*. In this family, 50% of the accumulated expression was due to the gene model encoding laccase 6 either alone (PC9 static) or accompanied by a second gene model: laccase 3 in PC15 and laccase 12 in PC9 shaken. These results were similar to those observed by Castanera *et al.* in glucose-based liquid media using RT-qPCR (R Castanera et al., 2012; Raúl Castanera et al., 2013). The manganese-peroxidase and versatile-peroxidase encoding genes displayed a low expression level in all strains and conditions tested.

In summary, similar to the observations in the CAZy gene family, most of gene expression in the redox group of families depended on a limited number of gene families and a limited number of predominantly expressed genes within each family. Notably, the most highly expressed families in the CAZy and redox categories corresponded to proteins using copper as a cofactor.

Peptidases

The accumulated expression of the peptidase-encoding genes was similar under the four strains/conditions tested; however, the detailed expression profiles were different. In PC15, only four out of 18 gene families displayed an expression level higher than 100 RPKM, whereas six families were over this threshold in PC9. Three of the gene families ranked high for expression under all four conditions (S8 serine endopeptidases, M36 metalloendopeptidases using Zn as a cofactor, and a protein annotated as similar to the disintegrin). Moreover, one family was selectively expressed in PC15 (serine-carboxypeptidase S10) and two in PC9 (peptidase A1 and S53)

Esterases

Of the 12 gene families classified as encoding esterases, two accounted for more than 50% of the expression in PC9 and PC15, although they differed between the two strains. The phospholipase C-like gene family was the most highly expressed in all strains and conditions except in PC9-Shk, where it ranked second. However, the next most highly expressed esterase family was different in the two strains: arylesterase in PC15 cultured under static and shaken conditions and carboxylesterases and genes classified as putative esterases/lipases in PC9 cultured under static and shaken conditions. The two richer esterase gene families (carboxylesterases, six gene models, and ribonucleases, seven gene models) exhibited higher expression in PC9 than in PC15 (217.9 *vs.* 84.96 for the carboxylesterase family and 137.24 *vs.* 20.73 for the ribonuclease family for PC9 and PC15, respectively). In all case, this increase in the relative transcription was due to only one of the family members.

Lipases

For the 16 genes identified as lipases, the largest gene family was lipase 3 (14 genes). This family was the most highly expressed in all strains and conditions, accounting for between 56.86 and 95.25% of the family expression. Within this family, the most expressed genes

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differed in the four strains and conditions tested, suggesting that each strain adapted to the different culture conditions in different ways.

Non-enzymes

The group of secreted proteins functionally classified as non-enzymes was overrepresented in terms of its transcription level compared with its representation in the number of proteins (Figure 3). Hydrophobins are structural proteins widely studied in fungal systems. The nine hydrophobin genes identified by SECRETOOL have been found expressed in the *P. ostreatus* cultures. However, most of their expression corresponded to the gene coding for the Vmh3 protein, a glycosylated hydrophobin found detected during vegetative growth and in fruit bodies. This protein is supposed to play a role in development similar to that proposed for SC3 in *Schizophyllum commune*. (Peñas et al., 2002). Vmh3 expression represented more than 94% and 69% of the expression of the identified hydrophobins in PC9 and PC15, respectively. The expression of these genes was increased approximately eight-fold in PC9 compared to PC15; this difference was due to the high expression of the *vmh3* gene in PC9. In both strains, the total hydrophobin expression level was higher in the static compared to the shaken cultures. This difference in gene expression in the static cultures could be related to the aerial growth observed in the mycelium cakes produced in the static cultures compared with the pellet structure produced in the submerged shaken cultures.

Cerato-platanins are a group of small secreted proteins that were first identified in the fungal pathogen *Ceratocystis platani* as plant defense elicitors. There are seven genes encoding cerato-platanins in *P. ostreatus*. The expression of the cerato-platanin genes was concentrated in two genes that accounted for more than 50% of the family expression (one gene in PC9-Shk). Cerato-platanin genes were highly expressed in PC15 (especially in shaken cultures), where the two most highly expressed cerato-platanin genes ranked as the second and fifth most expressed genes encoding secretable proteins. The biochemical functions of cerato-platanins remain elusive. De Oliveira *et al.* (de Oliveira et al., 2011) showed that they had a structure similar to expansins and barwin-like endoglucanases, suggesting an activity involved in carbohydrate metabolism (Baccelli, Luti, Bernardi, Scala, & Pazzagli, 2014)(Gaderer, Bonazza, & Seidl-Seiboth, 2014). However, they have no activity against a variety of carbohydrates. If we consider the *P. ostreatus* cerato-platanin-

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like proteins as CAZY enzymes and analyze them in conjunction with the genes encoding proteins annotated as Barwin-like endoglucanases (which are the five most highly expressed CAZy genes), the sum of these two groups represents the majority of all secreted genes.

Genome mapping of the *P. ostreatus* secretome genes

We studied the mapping positions of the genes encoding secretable proteins using the PC15 strain because it had a better genome assembly. The gene models encoding the 554 secreted proteins appeared to be distributed across the 11 scaffolds (chromosomes) of the *P. ostreatus* PC15 genome (Figure 6).

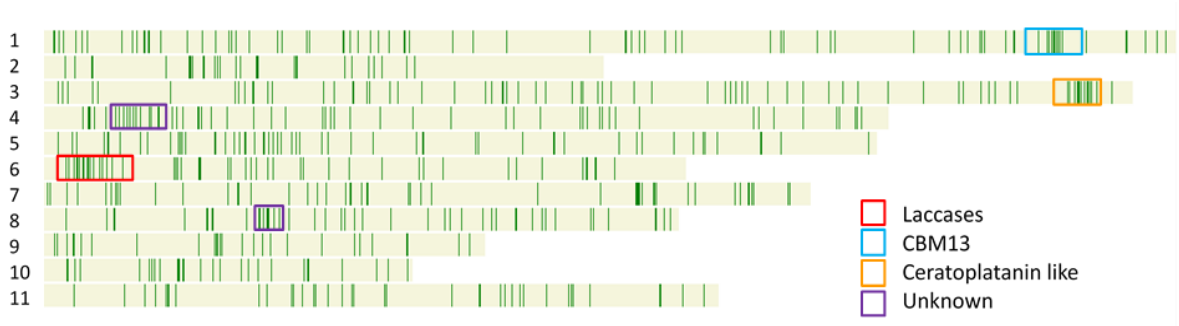

Figure 6. Location of secreted proteins in the chromosomes, three selected clusters of *P. ostreatus* PC15 and clusters of secreted proteins (REEF program, FDR corrected p-value < 0.05).

The number of genes encoding secretable proteins mapping to each chromosome increased linearly with the chromosome size (R^2=0.92) . Using the Reef program (Coppe, Danieli, & Bortoluzzi, 2006), we identified five clusters of secreted proteins: two clusters mapping to the subterminal region of chromosome 4 and to an internal region of chromosome 8 were mainly formed by proteins without a predicted function, a cluster formed by six laccase genes (out of 12 in PC15) appeared to be located at the subterminal region of chromosome 6, six CBM13 genes (out of eight in PC15) appeared to be clustered at a subterminal region of chromosome 1, and a cluster that included seven genes encoding cerato-platanin-related proteins was found at the subterminal region of chromosome 3. The occurrence of clusters of genes involved in environmental adaptation (species-specific) at subterminal chromosomal locations has been reported in several organisms and could be the result of the

exploitation of the high variability associated with these chromosomal regions (G. Pérez, Pangilinan, Pisabarro, & Ramírez, 2009; Ramírez, Pérez, Castanera, Santoyo, & Pisabarro, 2011).

Comparative analysis of the *P. ostreatus* bioinfosecretome with the proteomes of 54 basidiomycetes

The 554 proteins of the *P. ostreatus* PC15 bioinfosecretome were used as queries to search for similar proteins in other basidiomycete proteomes. The presence or absence of proteins similar to those of the *P. ostreatus* PC15 bioinfosecretome was used as a characteristic to classify the corresponding fungal species in a dendrogram. For a comparison, we built up a phylogenetic tree based on the predicted proteomes of all the fungal species used in this work (using *Aspergilus nidulans* as outgroup). The comparison of both trees revealed that the studied basidiomycetes appeared to be grouped according to their lifestyle (Figure 7).

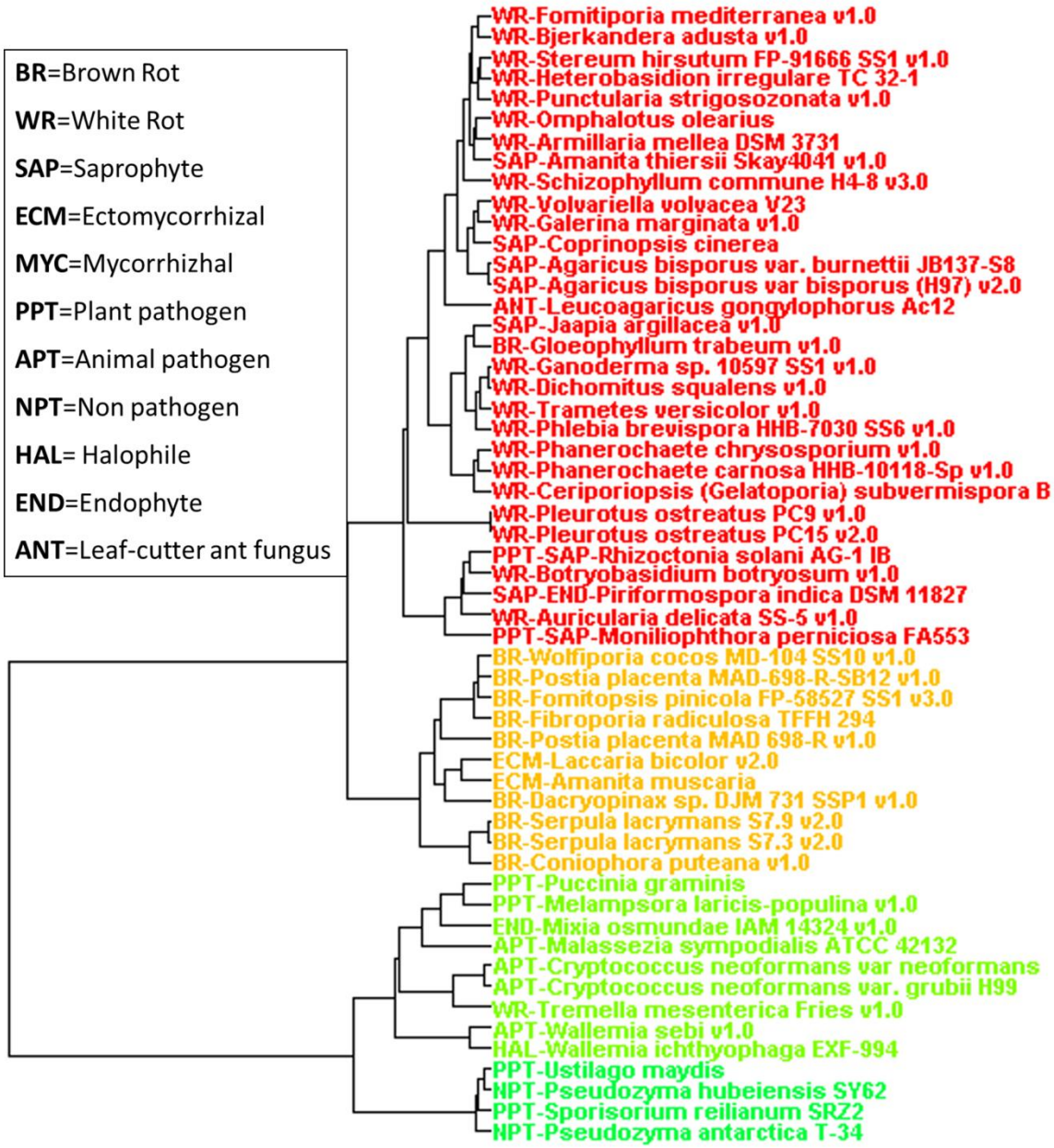

Figure 7a. Data from Table 1 arranged in a dendrogram (number of homologous proteins (e-20) to PC15 secreted proteins in basidiomycetes).

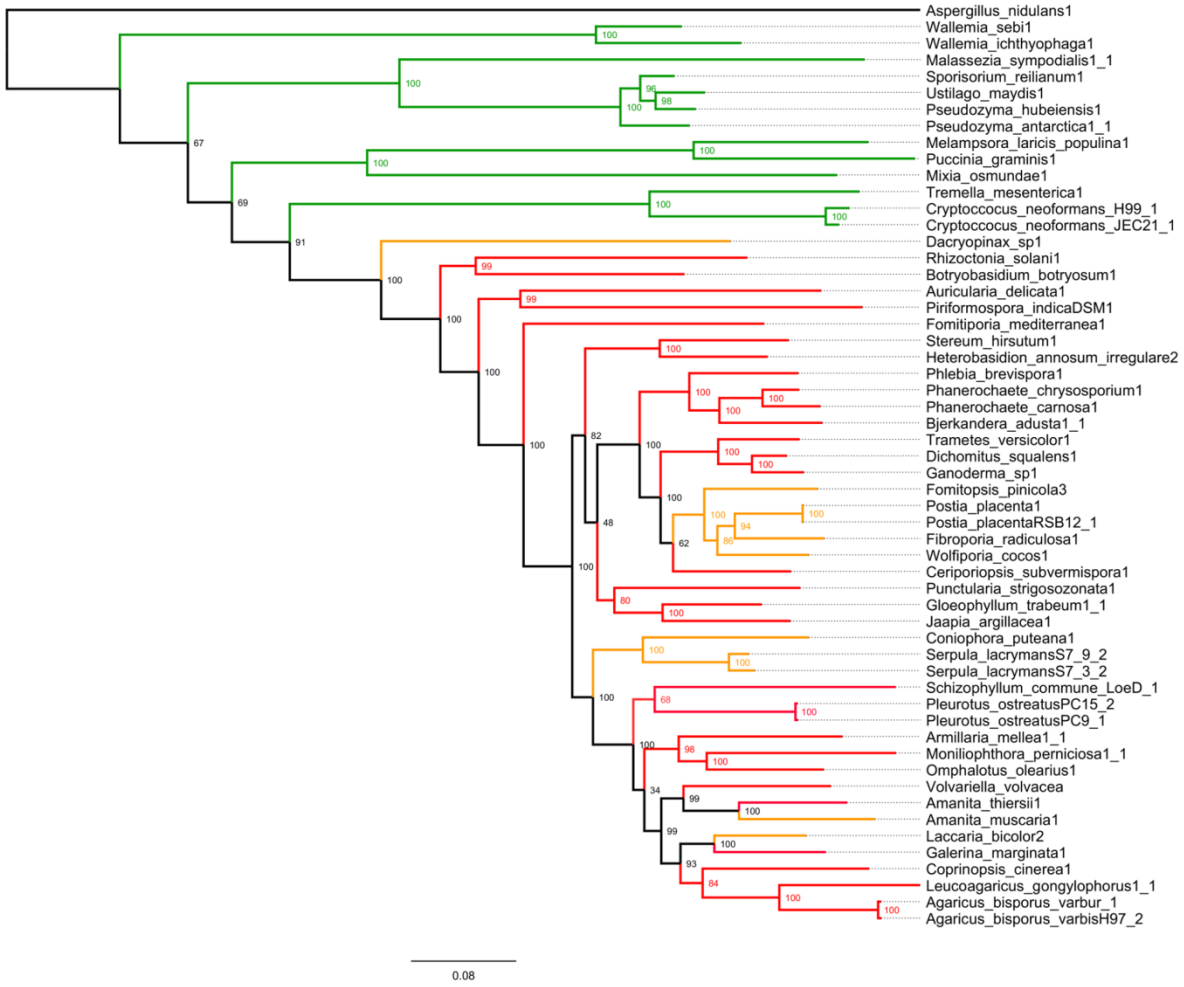

Figure 7b. Phylogeny of the fungal species analyzed in this study. Branches are colored following the pattern of colors in figure 7a.

Two major branches were observed in the dendrogram: the first branch could be subdivided into a group that included WR and SAP species and a second group of BR and ECM species, and the second branch included PPT, APT and endophytes (END) (Figure 7). The cluster of BR and ECM supports previous studies showing similarities in their organic matter degradation mechanisms (Rineau et al., 2012) and a parallel contraction of the lignin-degrading peroxidase gene family (Floudas et al., 2012; Kohler et al., 2015). The groups formed in this analysis reflected the fungal lifestyle rather than their evolutionary relationships, supporting the hypothesis that the fungal secretome could be used to predict the lifestyle because it is targeted to the ecological niche of the organism (Alfaro et al., 2014; Krijger et al., 2014; Lowe & Howlett, 2012; Martinez et al., 2009). Two species of the genus *Amanita* (*A. thiersii* (Hess et al., 2014) *and A. muscaria* (Kohler et al., 2015)) with different

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lifestyles were included in this analysis; they appeared to be grouped according to their lifestyle rather than their phylogenetic classification. Moreover, this analysis revealed that the secretomes of *J. argillacea* (classified as an uncertain WR) and *Gloeophyllum trabeum* (classified as a BR fungus) were highly similar, supporting the finding that both fungi belong to sister groups (Riley et al., 2014). Finally, the analysis placed *B. botryosum*, which is a species with enzymes typical of both WR and BR (Riley et al., 2014b), into a small sister group of the WR fungi. Interestingly, this group included two PPT (*Rhizoctonia solani* and *M. perniciosa*) and a root endophyte and growth promoting fungus (*Piriformospora indica*) that exhibited a dual lifestyle, with a switch to a SAP lifestyle during their infection cycles (Mondego et al., 2008; Wibberg et al., 2013; Zuccaro et al., 2011). Moreover, a mycorrhizal association of some species of the genus *Botryobasidium* with the orchid genus *Apostasia* has been suggested (Yukawa et al., 2009). The data presented here support a secretome profile for this fungus that is more closely related to other plant-interacting fungi than to pure WR or BR.

It is worth emphasizing that 209 predicted secreted proteins of *P. ostreatus* with unknown enzymatic function were included in this comparative analysis with other species of basidiomycetes. These proteins have been ignored in previous studies of lifestyle-associated proteins. Many of these proteins were present in nearly all the basidiomycetes compared in this study, and other proteins were clearly less represented in some lifestyles compared to others. Sorting fungi according to their lifestyles is not an easy issue, especially because many fungal species can switch between several lifestyles over the course of their life cycle. Our results suggest that the study of the secretome composition would provide a certain insight and add valuable data that could enable us to infer the roles of proteins with unknown functions.

Conclusions

The quality of a fungal bioinfosecretome will depend on the quality of the genome annotation. Thus, these in silico-predicted secretomes will evolve along with new versions of genome assemblies and annotations. The accuracy of bioinfosecretomes can be improved, but *in vivo* analyses will always be necessary to determine the pool of enzymes that fungi

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secrete to adapt to their ecological niches. Because mass spectrometry and transcriptomic analyses cannot identify every single enzyme involved in this process, a combination of *in silico* and *in vivo* techniques is still the best approach to analyze fungal secretomes. In all cases, the transcriptomic analyses of the data associated with bioinfosecretomes provides important information about the actual use of the available gene repertoire. This transcriptome analysis revealed that the secretome's transcriptome was more dependent on the strain genotype than on the environmental conditions. This finding indicates that the transcriptomes of a strain cultured under different conditions are more similar than the transcriptomes of other strains cultured under similar conditions.

Classical genome analyses emphasize the expansion of gene families. However, gene family expansion does not correlate with an increase in the number of actively expressed genes. We suggest that gene family expansions are associated with an increase in potential rather than with an increase in activities in a given environmental condition.

Among the *P. ostreatus* PC15 predicted secreted proteins that were conserved in other basidiomycetes, more than 20% have an unknown enzymatic function. Transcriptome analysis noted the importance of these proteins, underlining the need for further studies to decipher their role in fungal biology. For example, we used domain structure prediction to predict the function of two new protein families: a putative xylanase and a putative MAC perforin domain protein.

The functional categories found in proteins predicted to be secreted showed that domains associated with lignocellulose degradation were overrepresented compared to the whole proteome. This finding reflects the matching between the secretome and the lifestyle. Indeed, a blind grouping of organisms using their secretomes as distinctive characteristics produces a dendrogram in which the fungi are grouped by their lifestyle rather than by their phylogeny. Taken together, these data emphasize the dynamic, environmentally dependent, and ecologically influenced characteristics of fungal secretomes (Alfaro et al., 2014; Krijger et al., 2014; Lowe & Howlett, 2012; Martinez et al., 2009).

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3. –EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

Two *P. ostreatus* monokaryotic strains were used in this work. PC9 and PC15 were produced by dedikaryotization of the original dikaryotic strain (N001). Details of their genetics and molecular biology have been published elsewhere (L. Larraya et al., 1999; L M Larraya, Pérez, Ritter, Pisabarro, & Ramírez, 2000; Luis M. Larraya et al., 1999). Their two genomes were sequenced by the Joint Genome Institute and are accessible at http://genome.jgi.do.gov/PleosPC9_1 and http://genome.jgi.do.gov/PleosPC15_2. Both strains were maintained in Petri dishes containing solid SMY medium (10 g/L sucrose, 10 g/L malt extract, 4 g/L yeast extract, and 15 g/L agar) at 24ºC in the dark and subcultured every 8 days. The liquid cultures were performed using SMY (same composition as above but without agar) in the dark at 24ºC under either static (Sta) or shaking (Shk, 200 rpm) conditions. The samples for the transcriptome experiments were harvested when the cultures were in the exponential growth phase (Alejandra Parenti et al., 2013), although the two growth conditions differed due to the inherent effects of the static (growth as a floating cake) or shaking (growth as pellet) conditions.

RNA-seq data analysis

Total RNA was purified from the corresponding cultures using a fungal E.Z.N.A. RNA kit (Omega Bio-Tek, Norcross, GA, U.S.) following the manufacturer's indications. Four samples were prepared corresponding to each of the two strains in the two culture conditions (static and shaking): PC9-Sta, PC9-Shk, PC15-Sta and PC15-Shk. The sequencing experiments were performed using the SOLiD platform at Sistemas Genomicos (Valencia, Spain). The RNA-seq reads were mapped to the *P. ostreatus* PC15 (assembled in 12 scaffolds) and PC9 (assembled in 572 scaffolds) genome sequences using TopHat (Trapnell, Pachter, & Salzberg, 2009). HTseq-count (Anders, Pyl, & Huber, 2014) was used to determine the number of reads mapped to every predicted gene model. Samtools (Li et al., 2009) and custom Python scripts were used to handle the data and calculate the RPKM(Reads Per Kilobase of exon per Million reads mapped) values, which were used to evaluate the transcriptional levels of the secreted proteins.

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Prediction of secreted proteins in *P. ostreatus* PC9 and PC15

The complete sets of proteins (best filtered models) predicted in PC9 and PC15 were obtained from MycoCosm (http://jgi.doe.gov/fungi) (I V Grigoriev et al., 2012; Igor V Grigoriev et al., 2014), and the prediction of the secreted proteins were performed using the web analysis tool SECRETOOL (Cortázar, Aransay, Alfaro, Oguiza, & Lavín, 2014). This tool starts processing the data with TargetP (Olof Emanuelsson et al., 2007), SignalP (Bendtsen *et al.*, 2004) and PredGPI (Pierleoni, Martelli, & Casadio, 2008). Then, the proteins predicted by these three methods are merged into a common list that is evaluated for transmembrane domains (TMD) using the TMHMM tool (Krogh, Larsson, von Heijne, & Sonnhammer, 2001). The candidate proteins with 0 or 1 TMDs are kept as input for WoLF PSORT (Paul Horton et al., 2007) (thereby eliminating them from our secretome analysis as transmembrane proteins) to retain the sequences labeled as extracellular. When proteins were identified as allelic pairs in PC9 and PC15 but only one of the alleles was predicted to be secreted, we used Python scripts to extract the sequence 210 bp upstream from the 5' region of the gene models used as queries to search for similar regions in the genome of the monokaryon with alleles not predicted to be secreted using the BLASTN standalone program (Camacho et al., 2009). Alleles were defined as Blast Reciprocal Best Hits (RBH) between the PC15 and PC9 gene models (Santoyo et al., unpublished data).

Hypothetical functions for the predicted secreted proteins were assigned using the utilities available at the JGI webpage (Igor V Grigoriev et al., 2014), Pfam database (Finn et al., 2006), Gene Ontology and Interpro Domain database (Apweiler et al., 2000).

If we could not assign a hypothetical function using this procedure (proteins classified as unknown), we used the MCL program to cluster proteins (I=2) (Enright et al., 2002) and the Phyre2 server (Kelley & Sternberg, 2009) to predict the structure and function of the proteins. BLASTP (cut-off $E<10^{-10}$) in JGI MycoCosm (Igor V Grigoriev et al., 2014) was used to identify similar proteins in other basidiomycetes.

The Pfam domain search was performed using the InterProScan standalone program (Apweiler et al., 2000; Bateman et al., 2004; Jones et al., 2014) and database version. Fisher's

exact test was used to reveal enriched Pfam domains ($p<0.05$) in the bioinfosecretome compared to the genome data of *P. ostreatus*.

The OmnimapFree program was used to visualize the position of individual and cluster genes on the *P. ostreatus* PC15 chromosomes (Antoniw et al., 2011). We used the REEF ("Regionally Enriched Features") program (Coppe et al., 2006) to identify genomic regions enriched in specific features using a statistical test based on the hypergeometric distribution with a sliding window approach and adopting the false discovery rate to control multiplicity. A window width of 100 kb, a shift of 20 kb and an FDR-corrected p-value of <0.05 were used. The minimum number of genes to form a cluster was 3.

Comparative analysis of the *P. ostreatus* PC15 bioinfosecretome with the basidiomycetes' proteomes

For the comparison of the *P. ostreatus* PC15 bioinfosecretome with 54 basidiomycetes proteomes, the protein filtered models were obtained from the JGI webpage (Igor V Grigoriev et al., 2014). BLASTP standalone (cut-off $E < 10^{-20}$) (Camacho et al., 2009) was used to identify proteins similar to the PC15 secreted proteins. Python scripts were developed to arrange the results of the BLASTP search into a matrix of the presence or absence of similar proteins in the basidiomycete proteomes. The R program (R Core Team, 2013) with the function hclust was used to plot the dendrogram.

Phylogenetic analyses

The predicted proteomes of all species were downloaded from Mycocosm database (http://genome.jgi.doe.gov/programs/fungi/index.jsf) and proteins were clustered with MCL (Enright et al., 2002) using an inflation value of 2. Clusters containing single copy genes of each genome were retrieved (allowing 2 missing taxa) and proteins were aligned with MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002). The alignments were concatenated after discarding poorly aligned positions with Gblocks (Castresana, 2000). Phylogeny was constructed using RaxML (Stamatakis, 2014) under PROTGAMMAWAGF substitution model and 100 rapid bootstraps.

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MA and JLL identified the genes encoding secretable proteins in PC9 and PC15, MA, RC and AGP performed the analysis of the transcriptome data associated with the secretome, MA and RC performed the comparative analyses of fungal secretomes, the Pfam comparison between secreted models and whole genome models and the clustering of proteins of unknown functions, IVG led the genome assemblies, JAO supervised the protein predictions, and AGP supervised the transcriptomics. MA and AGP wrote the manuscript, which was revised by IVG, JAO and LR. LR conceived the analyses and led the Genetics and Microbiology Research Group as PI of the project.

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Chapter 4:

The Pleurotus ostreatus secretome:

Lignocellulose degrading machinery

Introduction

Lignin synthesis has been a key step in the adaptation of plants from an aquatic to terrestrial environment. Traqueophytes are defined as those land plants that have lignified tissues (the xylem) for conducting water and minerals throughout the plant, enabling an efficient transport and higher sizes of plants. Lignin waterproofs the otherwise permeable (because of hydrophilic polysaccharides) xylem, and provides a structural rigidity crucial to reach these high sizes that provides vascular plants a competitive advantage to reach the sunlight (Boerjan et al., 2003). Furthermore, lignin is a recalcitrant polymer, due to its molecular architecture, where different non-phenolic phenylpropanoid units form a complex three-dimensional network linked by a variety of ether and carbon–carbon bonds (Ruiz-Dueñas and Martínez, 2009), constituting a protective layer against pathogens and predators.

Lignin is the second major sink for carbon in plants after cellulose it represents as much as 30% of the organic carbon produced in the biosphere (Boerjan et al., 2003; Weng and Chapple, 2010), and is the major precursor of coal (Robinson, 1990).

Besides some bacteria, the main lignin degraders are white rot fungi belonging to Agaricomycetes, that play a key role in recycling carbon from lignocellulose since the onset of lignified compounds (Floudas et al., 2012), even if late adaption of fungi to lignin degradation may not be the main reason of coal accumulation on the Carboniferous period (Nelsen et al., 2016).

These fungi have the ability to secrete large amounts of enzymes, are capable of adapt to near all the terrestrial ecosystems and show diverse strategies for attacking lignocellulose, ranging from white rot basidiomycetes that degrade lignin extensively before attacking cellulose, to brown rot basidiomycetes that cause limited alterations of lignin while primarily degrading cellulose, through fungi sharing characteristics of both rot types (Riley et al., 2014a).

Furthermore, each fungus possesses a huge library of diverse enzymes and is able to modulate their expression and export in response to environmental and substrate changes, even in a sequentially regulated way (Zhang et al., 2016a).

This set of proteins secreted by a cell or an organism at a given time is defined as the secretome (Tjalsma et al., 2000). The secretome includes the proteins that are released into

the surrounding medium, those that remain bound to the membrane or cell wall and the integral membrane proteins. Fungi use these enzymes as a tool to obtain nutrients from their environment, and consequently, protein secretion is crucial for fungal growth and the secretome is different depending on the ecological niche of the fungus (Alfaro et al., 2014; Lowe and Howlett, 2012). Furthermore, the secretome is highly variable depending on environmental conditions, including the growth substrate, temperature and growth phase.

Lignocellulose is composed by three main compounds: lignin, cellulose and hemicellulose. White rot basidiomycetes attack lignin using oxidative enzymes classified as manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC 1.11.1.16), lignin peroxidases (LiP, EC 1.11.1.14) and phenol oxidases (benzenediol:oxygen oxidoreductase Pox, laccases, EC 1.10.3.2). In addition to these main ligninolytic enzymes, there is a number of accessory enzymes that participate in the process, including hemeperoxidases such as cytochrome c peroxidases (EC 1.11.1.5), chloroperoxidases (EC 1.11.1.10), and dye decolorizing peroxidases (DyP, EC 1.11.1.19), in addition to glyoxal oxidases (GLOX) and aryl-alcohol oxidases (AAO, EC 1.1.3.7), pyranose dehydrogenases (EC 1.1.99.29), and methanol oxidases (EC 1.113.13).

Cellulose is degraded by enzymes classified as exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91), subclassified as types I and II depending on their attack of the reducing or to the non-reducing end of cellulose, respectively, as well as endocellulases (EC 3.2.1.4) and cellobiases (β-glucosidases, EC 3.2.1.21).

Finally, hemicellulose lignocellulose is degraded by endoxylanases (EC 3.2.1.8), α-glucuronidases (EC 3.2.1.131), acetyl-xylan esterases (EC 3.1.1.72), arabinofuranosidases (EC 3.2.1.55), ferulic acid esterases (feruloyl esterases, EC 3.1.1.73), and β-xylosidases (EC 3.2.1.37), among other enzymes (Sun et al., 2012) (Alfaro et al., 2014).

Many of these enzymes are classified in the CAZy database (Cantarel et al., 2009; Lombard et al., 2014), that has become the reference database for the carbohydrate active enzymes in bacteria and fungi.

The enzymes described above are not a fixed list and new enzymes involved in this process are still being discovered, like AA9, AA10 and AA11, previously classified as GH61, CBM33. All of them are copper dependent mono-oxygenases; but only AA9 was classified

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as Lytic polysaccharide Monooxigenase (LPMO) because its activity has been proved on polysaccharides by a novel oxidative mechanism (Quinlan et al., 2011; Vaaje-Kolstad et al., 2010). Furthermore, many of the genes transcriptionally upregulated during lignocellulose degradation do not belong to any of these classes. In some cases, more than 50% of the total transcripts belong to proteins without a known function (Alfaro et al., 2016).

The strategy of basidiomycetes to degrade lignocellulose has another level of complexity: many of these enzymes are encoded by multigenic families whose members are differentially regulated (Castanera et al., 2012). For instance, the genome of the white rot basidiomycete *Pleurotus ostreatus* encodes 21 glycosyl hydrolase family 5 (GH5, cellulase) genes, while the brown rot basidiomycete *Postia placenta* encodes 12 and the white rot *Phanerochaete chrysosporium* 19. Furthermore, fungi are able to regulate the expression and secretion of these enzymes in a temporal sequence, in accordance with the phase of degradation of lignocellulose. This staggered mechanism, proved in the brown rot fungus *P. placenta*, consist in two steps: first, reactive oxygen species are created to attack lignified compounds and, second, enzymes are secreted to hydrolyze polysaccharides from cellulosic compounds (Zhang et al., 2016b). Gene expression is, however, a multistep process that involves the transcription, translation and turnover of messenger RNAs and proteins, and transcript levels are not always related to protein levels and enzyme activities (Schwanhäusser et al., 2011), making the proteome analysis a crucial step to verify the presence of these enzymes acting on substrates.

Solar energy is collected by plants and stored as lignocellulose. It is an abundant renewable resource that can be used for the production of alternative transportation fuels (Rubin, 2008). Nevertheless, plant biomass has evolved complex structural and chemical mechanisms for resisting attacks from microbial and animal predators.

At the molecular level, the crystalline cellulose core of cell-wall microfibrils is highly resistant to chemical and biological hydrolysis because of its highly ordered structure. The hydrophobic face of cellulose sheets makes crystalline cellulose resistant to acid hydrolysis and the strong inter-chain hydrogen-bonding network makes crystalline cellulose resistant to enzymatic hydrolysis. (Himmel et al., 2007; Nishiyama et al., 2002). Hemicelluloses and lignin create a complex matrix, and lignin is highly recalcitrant as we discussed above.

White rot basidiomycetes are seen as an excellent environmental-friendly alternative to thermochemical treatments to overcome the recalcitrance of plant biomass, despite the cost of the alternative enzymatic treatment is still too high (Hatakka and Helsinki, 2013). A deeper understanding of the mechanism of lignocellulose degradation by fungi is necessary to reduce costs and make lignocellulosics ethanol a real alternative for liquid fuels.

There are currently 781 available fungal genomes sequenced, 272 come from basidiomycetes (JGI Mycocosm, March 2017) (Grigoriev et al., 2014, 2012, 2011). Among all these fungi, *P. ostreatus*, the oyster mushroom, stands out as an edible mushroom, the third most cultivated mushroom in the world, and a preferential lignin degrader (Martínez et al., 1994). These features make this fungus interesting for biofuel production and are good reasons to analyze which enzymes *P. ostreatus* secretes while using lignocellulose as a carbon source.

Two strains were sequenced at the JGI: PC15 and PC9, that are monokaryons (mk) derived by dedikaryotization from the dikaryon (dk) N001(Borgognone et al., 2017; Castanera et al., 2016; Larraya et al., 1999), a commercial strain used by the mushroom industry able to produce a high number of mushrooms with excellent organoleptic properties.

The genome sequence offers us the opportunity to predict proteins that can be secreted (bioinformatics secretome, (Alfaro et al., 2016) before performing more costly and complex secretome analyses. However, as said before, the correlation between the bioinfosecretome (which provide information about the complete set of predicted secreted proteins present in a genome and can be seen as the arsenal that fungi possess to attack lignocellulose), the *in vitro* secretome (the actual group of enzymes used in a precise step of lignocellulose degradation, under given culture conditions, by specific sets of cells, and/or at a given time) is going to be deciphered by using proteomics secretome analyses.

Nevertheless, analyses of *in vitro* secretomes, like analyses of bioinfosecretomes, rely on the availability and quality of complete annotated genome sequences for the identification of protein tags.

In this article analyze *P. ostreatus* secretome using wood, glucose or both as a carbon source to identify the proteins that *P. ostreatus* uses to adapt to this media using LC MS-MS. This is the first in vitro secretome analysis comparing *P. ostreatus* sequenced strains PC15, PC9 and the dikaryon from they are derived, N001 (Riley et al., 2014).

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2. – RESULTS AND DISCUSSION

General qualitative analysis

The secretomes of *P. ostreatus* grown in submerged shaking cultures using poplar (*Populus alba*) chips (wood, W), wood supplemented with glucose (WG) and glucose (G) as carbon sources were studied by LC-MS/MS. The strains used in the analysis were the dikaryon dkN001 and the monokaryotic protoclones mkPC9 and mkPC15 (which contain each one of the two nuclei present in the N001 dikaryon)(Luis M. Larraya et al., 1999). The purposes of the experiment were (i) to shed light on the regulation of protein secretion by wood and glucose (end product of cellulose biodegradation), and (ii) to determine the effect of the monokaryotic and dikaryotic condition of the fungus on the secretome profile. The rationale of the experiment was as follows: we considered the G cultures as a basal condition, the proteins detected in the W cultures were assumed to be induced by wood, and the proteins recovered in the WG cultures were considered induced by wood and insensitive to glucose repression.

A total of 552 proteins were identified in the nine secretome analyses with, at least, two positively matched peptides for each protein. The number of proteins identified in each experiment is shown in **Table 1**.

Strain	Glucose	Wood	Wood + glucose	Total
dkN001	52	107	63	222
mkPC9	60	69	38	167
mkPC15	54	62	47	163
Total	166	238	148	552

Table 1. Number of proteins identified in each strain and culture conditions

The number of proteins identified reflects the complexity of the corresponding secretome. The results shown in **Table 1** indicate that secretome complexity found was higher in the cultures made using wood as sole carbon source than in those that contained glucose (which were considered a basal state), and suggest that wood could be an inducer of the secretome

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complexity. Furthermore, the results obtained in the cultures containing glucose plus wood suggest that the presence of the sugar decreased the secretome complexity in the three strains, and suggest that glucose could act as a repressor of the secretome complexity.

A closer look at the number of proteins secreted by each of the strains in each culture shows that there the increase in the secretome complexity is more relevant in the dikaryon than in the monokaryons (**Figure 1**) suggesting that the dikaryon is more adapted to strive in a wood environment than the monokaryons. Furthermore, the global behavior of the two monokaryons is quite similar.

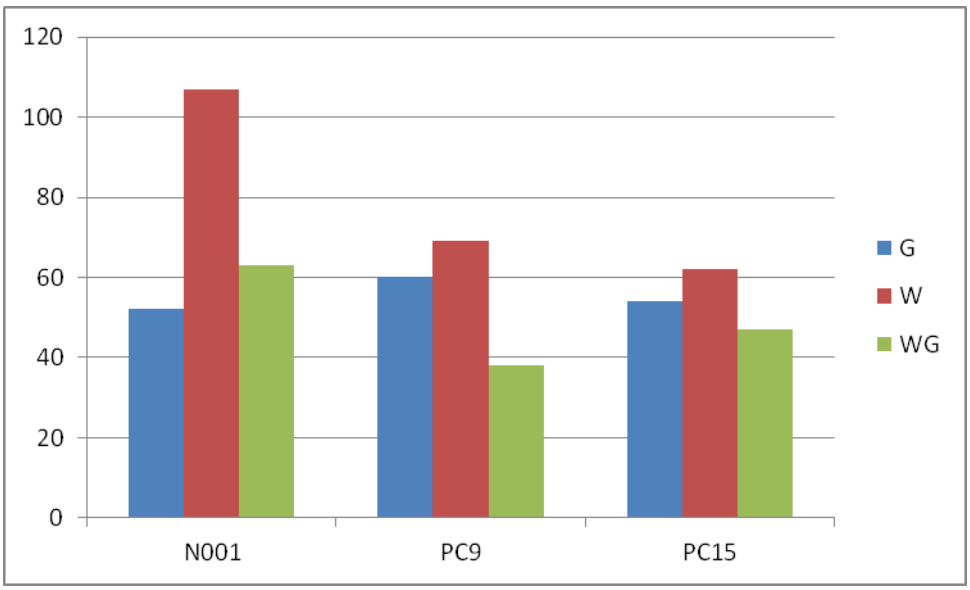

Figure 1. Number different proteins identified in the secretomes of dikaryon dkN001 and monokaryons mkPC9 and mkPC15 cultivated in liquid shaken media containing glucose (G), wood (W) or glucose plus wood (WG) as carbon sources.

If we compare the number of proteins identified in the three strains, it was found that dkN001 produced a secretome more complex (222 proteins) than those observed in mk PC9 and mkPC15 (167 and 163 proteins, respectively) (**Table 1, Figure 2)**. In a previous study of our group (Alfaro et al., 2016) we have shown that the number of proteins predicted to be secreted was similar in mkPC9 and mkPC15 (538 and 554, respectively). In this study we confirm that the complexity of the two monokaryotic secretomes was similar and we show that it was

higher in the dikaryon, suggesting an advantage of dikaryons for the adaptation to the environment.

275 different proteins were identified in the nine secretomes. This number is lower than the total number of proteins identified since many proteins were recovered from more than one experimental condition. **Figure 2** shows the Venn diagrams of the proteins identified in each culture/condition. As it was seen and discussed above, dkN001 was the strain in which a higher number of different proteins were identified (154), whereas the numbers in monokaryons mkPC9 and mkPC15 were lower (122 and 101, respectively).

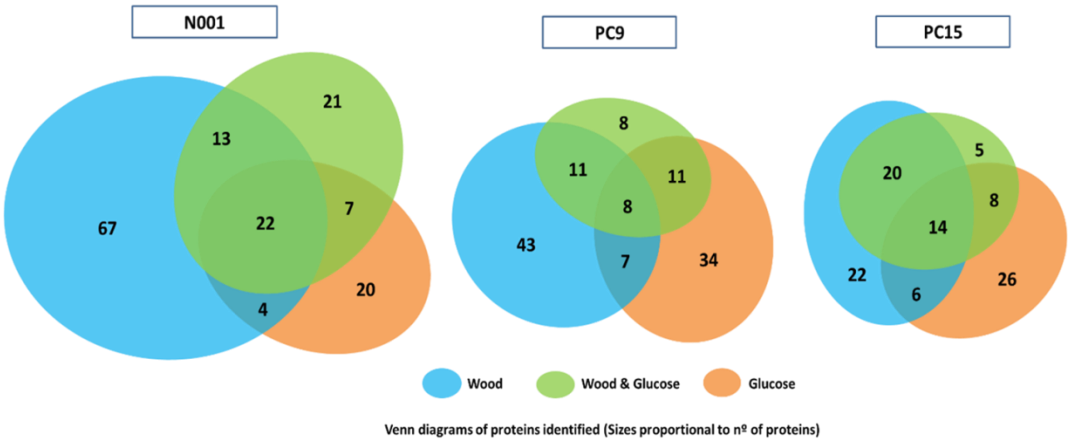

Figure 2. Venn diagram showing the number of proteins identified in the three strains (dkN001, mkPC9 and mkPC15) cultivated using wood (W), sugar (G) or wood plus sugar (WG) as carbon source. The numbers inside the diagram indicate the number of different proteins identified in each strain and condition.

The Venn diagrams reveal that most of the differences in the secretome complexity between strains were due to the proteins secreted when only wood was used as carbon source. These proteins, according to our working hypothesis, are proteins whose secretion is induced by wood but repressed by glucose. Moreover, previous results from our group and others ((A. Parenti et al., 2013)) indicate that shaking *P. ostreatus* cultures use preferentially glucose when it is available and that they exhaust this carbon source in six to seven days of cultivation. The long-term differences between the cultures started using only wood (W) or

wood plus glucose (WG) could suggest that this initial condition would have a long lasting effect on the lignocellulose degradation strategy displayed by the fungus.

Protein families found in the secretomes

The proteins identified in the LC-MS/MS experiments were individually annotated using different databases (JGI, Interpro, Pfam and Gene Ontology) and were manually classified into 11 groups: esterases/lipases, glycosyl hydrolases, intracellular proteins, isomerases, lyases, non-enzyme proteins, other hydrolases, phosphatases, proteases, RedOx enzymes and unknown proteins.

Figure 3 shows the functional classification of the proteins identified in the three secretomes. The functional classification of the whole set of secreted proteins identified revealed that glycosyl hydrolases (GH), proteins with unknown function, RedOx enzymes, proteases and esterases/lipases were the more represented groups and accounted for more than the 75% of the secreted proteins identified. These five groups correspond to the five larger groups of secretable proteins identified in the bioinfosecretome ((Manuel Alfaro et al., 2016)). The major difference between the predicted and the experimental secretomes appeared when comparing the proteins with unknown function, which includes a large number of Small Secreted Proteins (SSPs), whose relative importance seemed to be smaller in the recovered secretome than in the predicted one. In the analysis described in this paper, the low number of unknown proteins can reflect either an unintentional bias against the SSPs in protein sampling or analysis in the LC-MS/MS, a poor correlation between expression secretion and/or significant differences in protein lives.

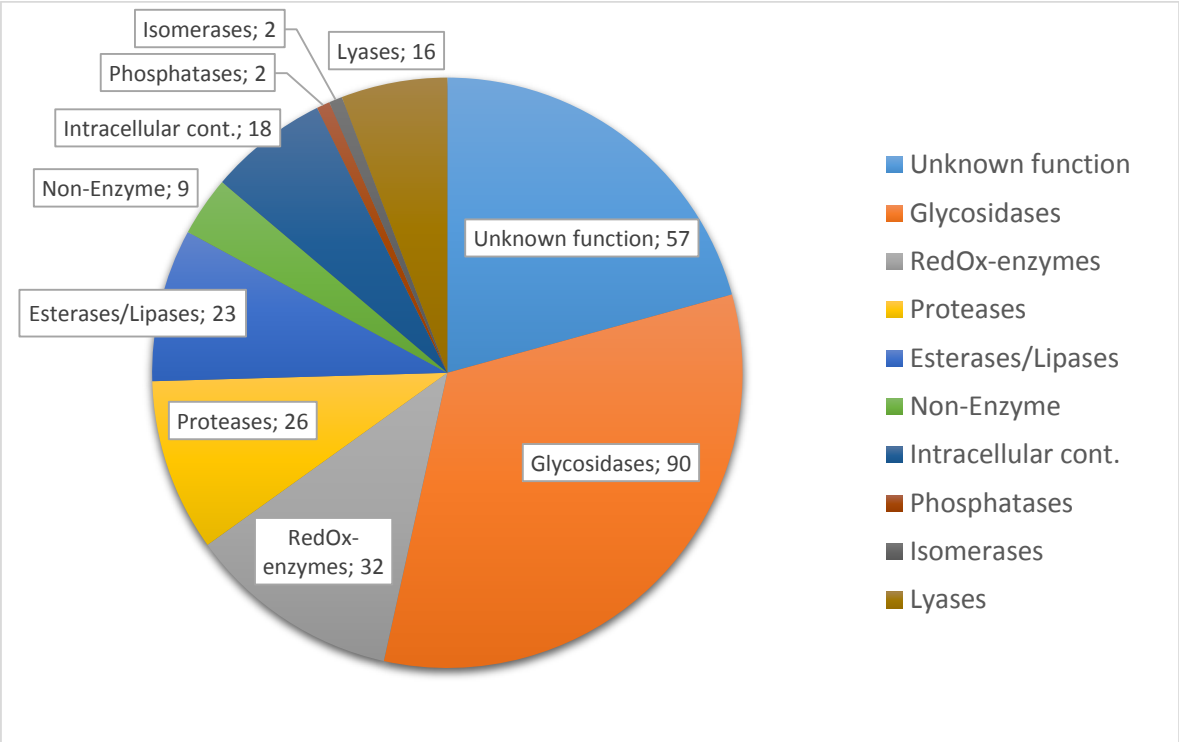

Fig 3. Functional classification of the proteins identified in all samples

Figure 4 shows the functional classification of the secreted proteins recovered from the three strains in the different substrates studied. Panels A, B and C permit the comparison of the behavior of each strain in each of the culture conditions. The main groups of proteins recovered when cultures had glucose as sole carbon source were glycosyl hydrolases (GH), proteases, RedOx and proteins with unknown function (panel A). In these cultures, mkPC15 differed from mkPC9 and dkN001 in that it produced more GH and less proteases and RedOx enzymes. In the cultures performed in the presence of wood (panel B), the production of GH increased in mkPC9 and dkN001, but not in mkPC15; there was an increase in the production of esterases/lipases and lyases in the three strains and a reduction of the presence of proteases in mkPC9. Finally, in the cultures that used glucose and wood as carbon source (panel C), the increase in the production of GH, esterases/lipases and lyases seen in the wood cultures was reversed. In summary, the presence of wood induces the production of glycosyl-hydrolases and, in a smaller extent, of esterases/lipases and lyases.

If we compare the secretomes for each strain in the three culture conditions, the induction of the production of esterases/lipases, GH and lyases in dkN001 (panel D) and mkPC9 (panel E) was clearly visible, whereas there was not a wood induction of GH in PC15 (panel F).

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Moreover, the basal production of these enzymes in mkPC15 was higher than in the other two strains. In mkPC9 and dkN001 the wood induction of GH was reversed by the presence of glucose in the culture medium. This effect was also partially seen in the cultures of mkPC15. Also, the small induction of esterases/lipases and lyases seen in the wood cultures seemed to be reversed by the presence of glucose in the WG cultures. Additionally, the production of RedOx enzymes by mkPC9 seemed to be repressed in the presence of wood. Finally, mkPC15 seemed to be a poor producer of RedOx enzymes in all the conditions tested.

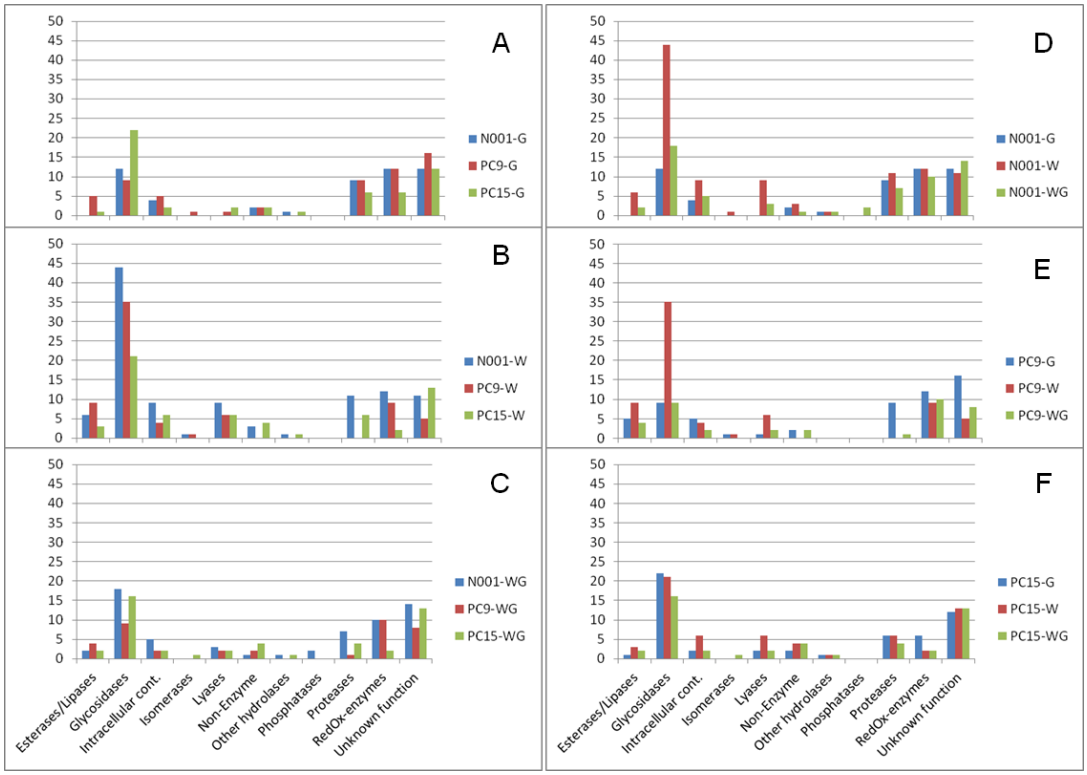

Fig 4. Functional classification of the proteins identified per fungal strain.

In summary, these qualitative results suggest that the glucose-dependent regulatory mechanisms controlling the production of glycosyl-hydrolases differ in mkPC9/dkN001 and in mkPC15. Even though it was not seen an induction of GH by wood in mkPC15, this protein group is the more abundant in the secretome of this strain indicating that the basal production of these enzymes in this strain is higher than in dkN001 and mkPC9.

Consequently, mkPC15 shows two peculiar characteristics: (1) a higher level of basal production of GH and (2) a lack of wood-dependent induction of GH production.

General quantitative analysis

In order to add quantitative data of protein abundance, we determined the emPai (Ishihama et al., 2005) values for every protein identified in these analyses. EmPai quantitation gives us an insight about which proteins are found in high concentration in our samples.

The emPai quantitative secretome profiles were largely similar to those based on the number of different proteins identified (**Figures 4 and 5**)).

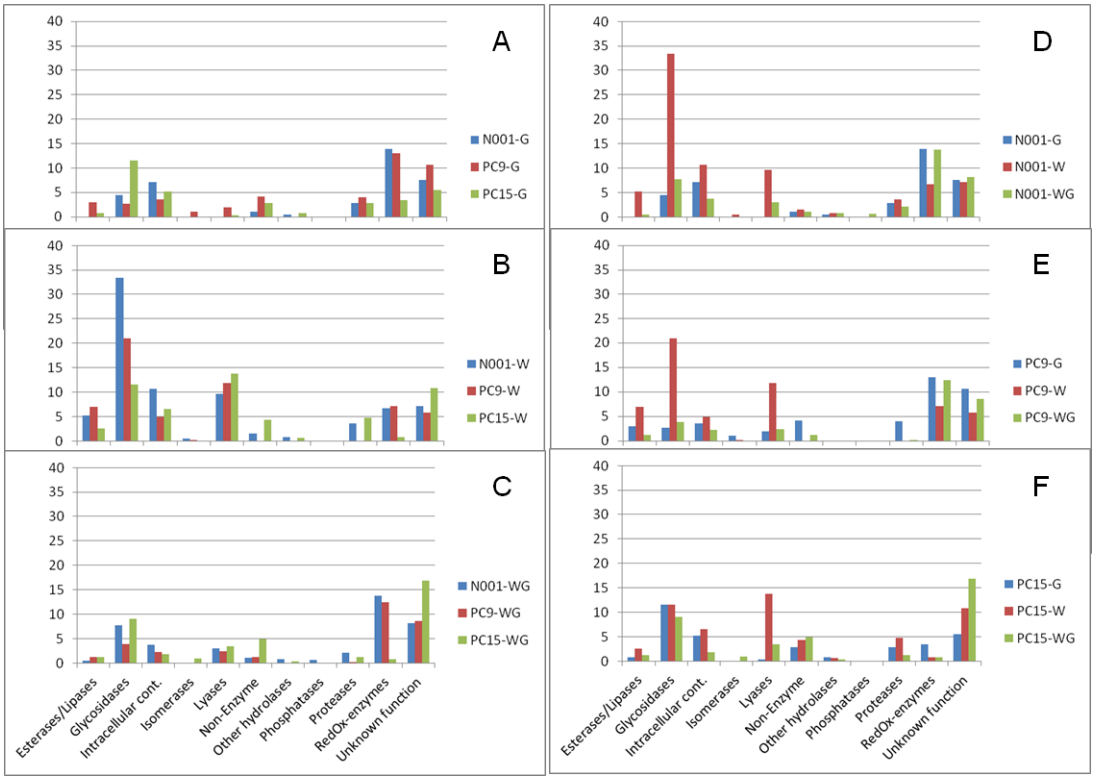

Fig 5. Quantitative (emPai) secretome profile of dikaryon dkN001 and monokaryons mkPC9 and mkPC15 cultivated using glucose (G), wood (W) or glucose plus wood (WG) as carbon source).

If we compare the emPai accumulation of the different protein families in the different conditions, the results discussed in the preceding section were more clearly seen. Panel A in Fig 5 shows that, in the sole presence of glucose, mkPC9 and dkN001 accumulated RedOx enzymes, whereas mkPC15 accumulated GH. When the cultures were made in the sole

presence of wood (panel B), the production of glycosidases was induced in mkPC9 and dkN001, the production of lyases was induced and that of RedOx enzymes was repressed in the three strains. Interestingly, in the cultures carried out in the presence of glucose and wood, the induction of glycosidases and of lyases and the repression of RedOx observed in the sole presence of wood appeared to be abolished(Fig 6).

When the results were compared strain by strain, the wood induction of GH (mkPC9 and dkN001) and lyases (the three strains), and repression of RedOx (three strains) production was clearly seen (panels D, E and F). As observed in the qualitative analysis, the basal production of GH was higher in mkPC15 than in the other two strains and it was insensitive to the induction by wood. The wood dependent induction of lyases and GH would be presumably mediated by different mechanisms since there is a clear wood dependent induction of lyases in mkPC15 whereas wood is not a GH inducer in this strain.

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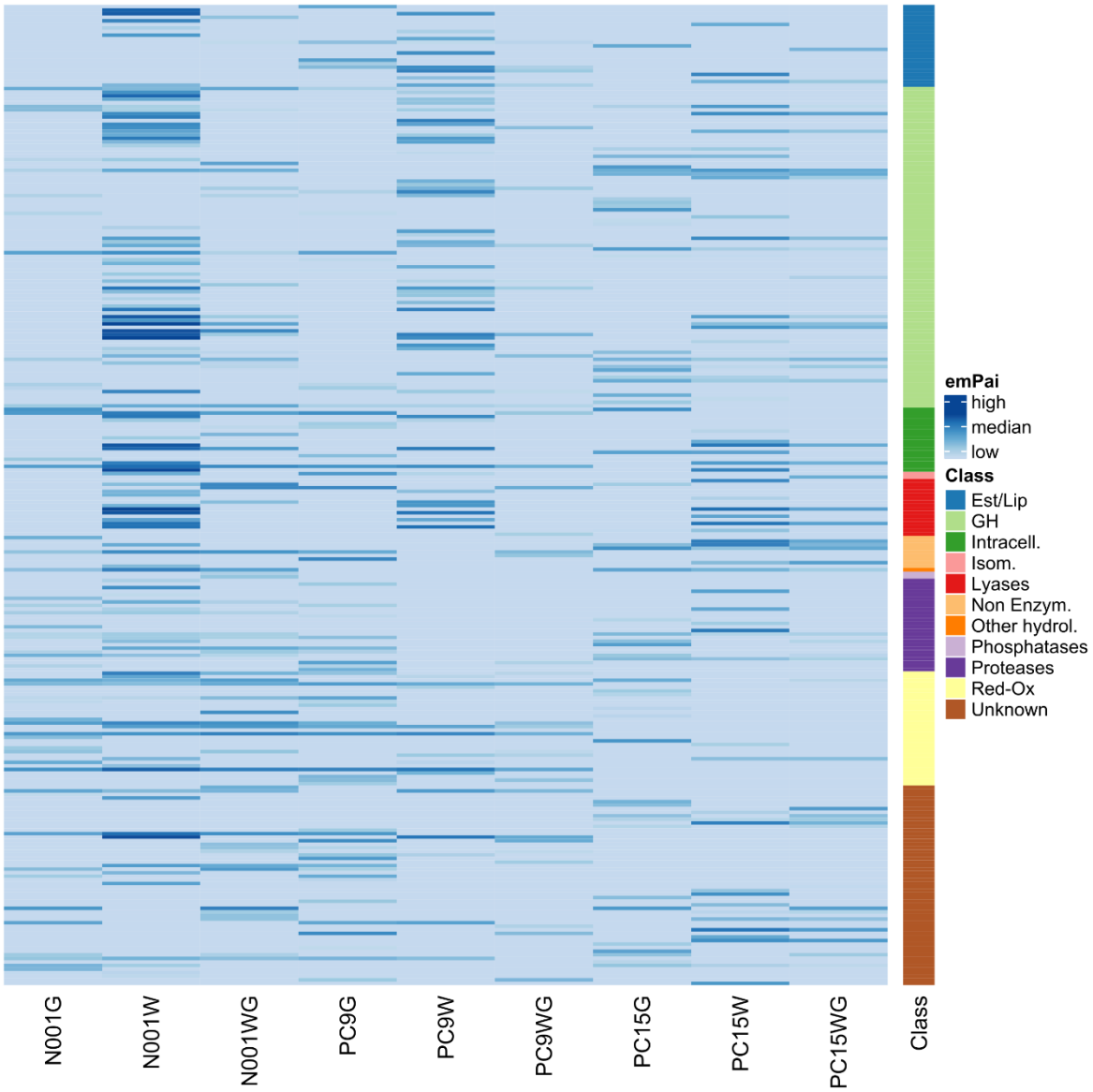

Fig.6. EmPAI of proteins identified in P. ostreatus secretomes

Figure 6 shows general view of EmPAI level of proteins identified in all the samples, and shows the higher protein concentration in dkN001 cultured on wood samples

Focus on the main secreted protein families

Glycosidases

The five GH families more represented in the studied secretomes were: GH7 (putative reducing-end cellobiohydrolase, seven proteins), GH10 (endoxylanase, three proteins), GH5 (endoglucanase, four proteins,), GH28 (polygalacturonase, four proteins), GH61/AA9 (copper-dependent lytic polysaccharide monooxigenase, LPMO, three proteins) and GH79 (glucuronidase, three proteins). GH7 and GH61/AA9 are among the largest glycosylhydrolase families found in the genome of *P. ostreatus* (16 and 29 gene models, respectively, in PC15 genome).

	dkN001	mkPC9	mkPC15
Total GH	54	42	33
Found in G	12	9	22
Found in W	44	35	21
Found in WG	18	9	16
All three	5	1	7
Glucose-repressed	31	25	3
Wood-induced	6	6	7
Glucose-induced	3	5	

Table 2. GH detected in the three strains by carbon source

54 GH were identified in the secretomes of dkN001: 12 in the G cultures, 44 in the W cultures and 18 in the WG cultures containing. Five of these proteins were detected in the three cultures (putatively constitutive). 31 of these proteins were found in the wood (W) but absent in the glucose containing cultures (WG and G, putative glucose repression), six appeared only in cultures containing wood (W and WG, putative wood induction), and three only in cultures with glucose as sole carbon source (G). The GH involved in cellulose degradation displayed either glucose repression (GH5, GH10 and GH61/AA9) or wood induction (GH7).

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In mkPC9, 42 GH were identified: nine in G, 33 in W, and nine in WG cultures. Only one protein was putatively constitutive, 25 seemed to be glucose repressed, six putatively wood induced, and five only present in cultures with glucose as sole carbon source. As for dkN001, the GH families involved in cellulose degradation displayed either glucose inhibition (GH45) or of wood induction (GH7).

Cultures with wood as carbon source (W and WG), in particular cultures without sugar (W), had a higher number of GH identified (Fig X). This was true for mkPC9 and dkN001, but mkPC15 seemed not to have this ability to secrete more GH when cultured on wood. dkN001 and mkPC9 have shown to behave in a similar way in many culture conditions whereas mkPC15 is a slow grower in all the substrates used in our lab, especially on lignocellulosic substrates (L M Larraya et al., 2001; A. Parenti et al., 2013). In opposition to dkN001 and mkPC9, the largest number of GH was found in mkPC15 glucose samples: 33 GH were identified: 22 in G cultures, 21 in W, and 16 in WG.

Only three proteins were found in mkPC15 growing in wood media (W) that were absent in glucose containing cultures (WG and G). This number is much lower than that found in dkN001 (31 proteins) or mkPC9 (25 proteins). This result suggests that the wood degrading mechanism was not working in mkPC15 as well as it was in mkPC9 and dkN001, and that the wood degrading mkPC9 behavior is dominant over that of mkPC15 as the observed phenotype in dkN001 is closer to that observed in mkPC9.

Additionally, there were seven proteins that appeared only in wood-containing cultures (W and WG), and which can be supposed to be induced by wood, and 11 proteins that were present solely in cultures made using glucose as sole carbon source.

Interestingly, 10 and 49 out of 90 GH protein models identified in these experiments were not predicted as secreted by SignalP software and Secretool pipeline respectively, due to alternative secretion pathways and the high stringency of the bioinformatics pipeline used.

- *Cellulose*

Cellulose-degrading enzymes found in P. ostreatus secretomes.			
Endoglucanase	**Cellobiohydrolases**	**B-glucosidase**	**LPMO**
GH5	GH6	GH3	GH61/AA9
GH12	GH7	GH5	
GH45			
GH7			

Table 3.(Note that several of this GH classes have different enzymatic activities, and therefore are included in several of this classes).

Glycosyl-hydrolases are the enzymes responsible of cellulose degradation process that requires the action of cellobiohydrolases (CBH), endoglucanases (EGL) and β-glucosidases (BGL). These enzymatic activities are carried out by GH classified in different families in the carbohydrate-active enzyme database (CAZy).

The endoglucanases (GH12 and GH45) and cellobiohydrolases (GH6, GH7) required for the degradation of cellulose were only detected in the cultures that contained wood as carbon source (W and WG). GH3 β-glucosidases (BGL, GH3) were not detected in these cultures and peptides for this enzyme were only recovered from cultures of mkPC15 made using glucose as sole carbon source. GH5 endoglucanases is one of the largest CAZy GH families and possesses a huge variety of enzymatic activities, including β-glucosidases and endoglucanases. According to this broad activity range, these enzymes were detected in all the different carbon source cultures, but mainly in wood containing cultures.

The GH7 enzymes were the predominant GH detected in the wood containing media in mkPC9 and dkN001: five out of the top ten more detected proteins in dkN001 growing in W cultures (one in mkPC9W and none in mkPC15W) were GH7, whereas only one was present in the top ten in dkN001 and in mkPC9 growing in WG (none in mkPC15), and no peptide for this enzyme was detected among the more abundant in the glucose based cultures in the three strains.

Consequently, we can hypothesize that the production of enzymes of GH7 family is induced by wood and that this induction is partially repressed by glucose. GH7 enzymes were present in every wood containing media from the three strains used here. Furthermore, this enzyme

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was absent in G media. The presence of cellulose justifies the identification of this protein here and supports the presence of wood as the main reason of the enhanced presence of GH in W media.

LPMOs function is proposed to be the creation of access points to allow classical hydrolytic enzymes such as cellulases a massive degradation of cellulose (among other polysaccharides) (Kracher et al., 2016). Four GH61/LPMO proteins were identified here, only in W cultures, confirming their involvement in lignocellulose degradation. Other proteins participate in LPMO degradation mechanism, including CDH, AA3 and Aldose epimerase; their role in lignocellulose degradation is discussed below.

As expected, cellulase encoding genes were mainly found in cultures with wood as the sole carbon source, without added sugar (W cultures).

- EmPai values of cellulose-degrading enzymes found in *P. ostreatus* secretomes:

Gene Model	GH Family	G	W	WG	Strain
PoPC9FM84996	GH12		0,7		N001
PoPC9FM84996	GH12			0,53	PC9
PoPC15FM1108932	GH12		0,5		N001
PoPC15FM1108932	GH12		0,66	0,5	PC15
PoPC15FM41613	GH3	0,25			PC15
PoPC15FM1035754	GH3	0,32			PC15
PoPC15FM1049518	GH3	0,27			PC15
PoPC9FM54135	GH45		0,47		N001
PoPC9FM54135	GH45		0,47	0,33	PC9
PoPC15FM42791	GH5	0,11	0,07		PC15
PoPC9FM75659	GH5		0,26		N001
PoPC9FM75659	GH5	0,2			PC9
PoPC9FM116228	GH5		0,41		N001
PoPC9FM125269	GH5		0,6		PC9
PoPC9FM114400	GH5	0,07	0,07		PC9
PoPC15FM1041397	GH5			0,3	PC15
PoPC9FM85079	GH5		0,34		N001
PoPC9FM85079	GH5		0,26	0,12	PC9
PoPC9FM45206	GH6		0,35		N001
PoPC9FM45206	GH6		0,35		PC9
PoPC9FM43698	GH6		0,35		PC9
PoPC9FM87701	GH61/AA9		0,16		N001
PoPC9FM59310	GH61/AA9		0,44		PC9

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PoPC9FM46220	GH61/AA9		0,29		N001
PoPC9FM122311	GH61/AA9		0,95		N001
PoPC9FM122311	GH61/AA9		1,44		PC9
PoPC15FM27620	GH7		1,91	0,31	N001
PoPC15FM27620	GH7		0,8	0,38	PC15
PoPC15FM1038048	GH7		0,67		N001
PoPC15FM1039504	GH7		4,12	0,76	N001
PoPC15FM1039504	GH7		0,48	0,57	PC15
PoPC15FM1092970	GH7		1	0,82	PC15
PoPC9FM114771	GH7		2,47	1,33	N001
PoPC9FM83320	GH7		1,91	0,31	N001
PoPC9FM83320	GH7		1,48	1,23	PC9
PoPC9FM83849	GH7		4,12		N001
PoPC9FM83849	GH7		1,2		PC9
PoPC15FM1105963	GH7		0,22		PC15
PoPC9FM100398	GH7		1,01		PC9
PoPC9FM47295	GH7		0,23		N001
PoPC9FM47295	GH7		0,58		PC9

(One gene model can be included several times in this table if it was identified in several strains)

- *Hemicelluloses*

Hemicelluloses include xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan and several monosaccharides. In accordance with this huge variety of compounds, fungi can use a broad portfolio of enzymes to break down this cell wall component. Enzymes with hemicellulolytic activities identified in these analyses are classified into the GH5, GH10, GH11, GH27, GH31, GH35, GH44, GH74 and GH115 CAZy families. Carbohydrate esterases are also involved in the degradation of hemicelluloses, especially acetyl xylan esterases. CE families 1, 4, 12, 15, 16 were found in *P. ostreatus* secretome.

In accordance to the secretion of cellulolytic enzymes, peptides belonging to hemicelluloses were found mainly in W media. 22 times (out of 33) hemicellulases were found only in W media, and five times more in W and/or WG media, suggesting that the production of these enzymes was induced by wood and partially repressed by glucose.

Galactose residues connected to mannan can be cleaved by α-galactosidases. We identified one GH27 in *P. ostreatus* secretomes, but its behavior was not the expected; GH27 was found

in all the different carbon source media. Interestingly, enzymes of GH27 family also showed α-N-acetylgalactosaminidase activity in *Aspergillus niger* (Kulik et al., 2010) an enzyme usually found in lysosomes whose deficiency leads to a human disease (Clark & Garman, 2009). It is therefore argued that some GH27 α-galactosidases are not only involved in hemicellulose degradation (Kulik et al., 2010; van den Brink & de Vries, 2011), a thesis supported by our findings.

GH35 enzymes also act in a non-expected way; they were found only in G media, without contact with lignocellulosic materials, even when they are predicted to be a β-galactosidase. The presence of terminal β-linked D-galactose residues in some hemicelluloses, e.g., xylan, xyloglucan, and galactoglucomannans, suggested that β-galactosidases (GH35) could play a role in the degradation of hemicelluloses (Sims et al., 1997).

A GH5 family gene model (PoPC9FM123701) similar to other fungal mannanases (van den Brink & de Vries, 2011) was identified in the analyses described here, as well as a GH44 family enzyme characterized as a xyloglucan hydrolase (Ye et al., 2012). GH115, α-glucuronidase, was demonstrated to be a crucial in the *Schizophyllum commune* deconstruction of softwood glucuronoarabinoxylan (McKee et al., 2016)

In conclusion, hemicellulases are, as expected, more abundant in wood containing cultures, and show a broad diversity necessary target the hemicellulose complexity.

- EmPai values of hemicellulose-degrading enzymes found in *P. ostreatus* secretomes:

Gene model	CAZy Family	G	W	WG	Strain
PoPC15FM1097054	CE1			0,38	N001
PoPC9FM117351	CE1		0,77		N001
PoPC15FM1078467	CE4		0,66		N001
PoPC9FM125794	CE4		0,66		PC9
PoPC15FM1102068	CE12		0,69		PC15
PoPC15FM1086797	CE15		0,2		N001
PoPC9FM89668	CE15		0,28		PC9
PoPC9FM82810	CE16	0,74			PC9
PoPC15FM1075485	CE16		1,53		N001
PoPC9FM96445	CE16		1,72		N001
PoPC9FM96445	CE16		1,12		PC9
PoPC9FM123701	GH5		0,27		N001

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PoPC9FM125911	GH10		0,69		N001
PoPC9FM125911	GH10		0,3		PC9
PoPC9FM81650	GH10		1,41		N001
PoPC9FM96691	GH10		0,48		N001
PoPC9FM96691	GH10		0,37		PC9
PoPC15FM1079521	GH11		0,71		N001
PoPC15FM1079521	GH11		1,12	1,12	PC15
PoPC9FM110996	GH11		0,73		N001
PoPC9FM110996	GH11		0,73		PC9
PoPC15FM1035175	GH27	0,21	0,47	0,57	N001
PoPC15FM1035175	GH27	0,67	0,9	1,02	PC15
PoPC15FM1088219	GH27	0,6	0,6	1,02	PC15
PoPC15FM51341	GH27		0,69	0,39	PC15
PoPC15FM1105441	GH35	0,11			PC15
PoPC15FM1066752	GH35	0,13			PC15
PoPC9FM54923	GH44		0,28		N001
PoPC9FM54923	GH44		0,52		PC9
PoPC15FM1111407	GH74		0,3	0,15	N001
PoPC15FM162095	GH115		0,87		N001
PoPC9FM127463	GH115			0,05	N001
PoPC9FM127463	GH115		1,56		PC9

(One gene model can be included several times in this table if it was identified in several strains)

- *Fungal cell wall related enzymes*

Chitin, composed of β-1,4 linked N-acetylglucosamine (GlcNAc) units, is present in the fungal cell wall. Enzymatic degradation of chitin is catalyzed by a two-component chitinolytic enzyme system. One component is chitinases (EC 3.2.1.14, GH18), which hydrolyze chitin polymers, and release chitooligosaccharides as chitobiose The other is β-N-acetylhexosaminidases (EC 3.2.1.52, GH20), which degrade chitooligosaccharides into monomers (Konno, Takahashi, Nakajima, Takeda, & Sakamoto, 2012). We identified both enzymes in *P. ostreatus* secretomes, and we assume that they could be likely involved in the modification of fungal cell wall during growth (together with β-glucans related enzymes)

The GH71 (α1,3 glucanase) enzymes are able to degrade cell walls of some phytopathogenic fungi degrading (S-glucans), which represent the major cell wall matrix polysaccharides for most fungi; in some instances, like in *Aspergillus nidulans*, S-glucans account for approximately 25 % of the dry weight of the cell wall.(Ait-Lahsen et al., 2001).

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- *Other GH families (cannot degrade cellulose, hemicellulose or pectin) identified in P. ostreatus*

GH13 and GH15 are αamylases that cleave α–1,4 glycosidic bonds, therefore can degrade starch but not hemicellulose or cellulose, formed by β -1,4 or β-1,3 glycosidic bonds. Both enzymes were found only in W media, on the contrary to other αamylase GH31, which was found only in G media.

GH131 is a relatively new GH family demonstrated to be a β-glucanase with exo-β-1,3/1,6- and endo-β-1,4-glucanase activity, therefore, it could act on several glucans including cellulose, but also on other fungal compounds as β-glucans linked by 1,3 glycosidic bonds with 1,6 branches. Two proteins from this class were identified in secretomes of *Podospora anserina* and *Aspergillus niger* (Lafond, Navarro, Haon, Couturier, & Berrin, 2012; Poidevin et al., 2014), growing on cultures with plant compounds as carbon sources. In *P. ostreatus*, this protein was found only in cultures using wood as a sole carbon source, giving an insight about a role in lignocellulose degradation. Nevertheless, more research is needed to decipher the role of this enzyme.

GH47 and GH92 α-mannosidases are Ca^{2+}-dependent enzymes related with fungal metabolism, usually related with Golgi apparatus. Out of six GH92 α-1,2 mannosidases present in *P. ostreatus* genome (in both PC9 and PC15) we identified three in the secretomes. One of them has no signal peptide for secretion, and the other two show a signal peptide using SignalP but the more restrictive Secretool pipeline discard them as secreted. In the ascomycete *Magnaporthe orizae,* α1,2 mannanase is also encoded by a multi-gene family with nine genes, including four that are predicted to encode secreted proteins. (Zhou et al., 2009).

These enzymes are known to be important in protein glycosylation, and are localized in the endoplasmic reticulum and Golgi complex. In eukaryotes, proteins translocated across the ER membrane (the secretion signal peptide drives the protein to the endoplasmic reticulum) are, by default, transported through the Golgi apparatus and exported by secretory vesicles, but some proteins have specific signals (poorly characterized) and are retained in the ER or

the Golgi or are targeted to lysosomes (Olof Emanuelsson et al., 2007). The web based pipeline Secretool (Cortázar et al., 2014) includes tools able to identify proteins targeted to stay at Golgi/ER, like TargetP or PSORT(Paul Horton et al., 2007).

Fungal GH72 and GH76 enzymes have been speculated to be involved in cross-linking of GPI-anchored proteins into the cell wall, where they are proposed to act as transglycosylases that elongate and remodel the 1,3-β-glucan of the cell wall(Kitagaki, Wu, Shimoi, & Ito, 2002).

Lyases

Polysaccharide lyases (PL) were also found more abundantly when wood was used as a sole carbon source (W cultures), whereas cultures made in WG media do not show such abundance. Polysaccharide lyases PL4 (rhamnogalacturonase), PL1 and PL3 (pectate lyases) were identified in W media, being the most abundant proteins in these media (Supp Table X). The lignocellulosic carbon source can explain this high recovery of PL in these experiments.

- *Pectin*

In primary cell walls, the matrix in which the cellulose network is embedded is composed of pectin, a highly hydrated network of polysaccharides rich in galacturonic acid (Alberts et al., 2002) Polysaccharide lyases (PL) are the main enzymes that degrade these components. *P. ostreatus* secretome presents PL1, 3, 4, 8 family enzymes, which are mainly pectate and rhamnnogalacturonan lyases. GH16, 28, 43, 53, 62, 78, 79, 88 and 105 and CE 8, 12 are also involved in pectin degradation and were identified in *P. ostreatus* secretomes.

Some of these enzymes are required for the hydrolysis of the pectin backbone as α-rhamnosidases (GH78) and unsaturated glucuronyl hydrolases (GH88)(van den Brink & de Vries, 2011).

- EmPai values of pectin-degrading enzymes found in *P. ostreatus* secretomes:

Gene Model	CAZy Family	G	W	WG	Strain
PoPC15FM1044335	CE8	0,7			PC15
PoPC15FM1061918	CE8			0,74	PC15

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PoPC9FM116926	CE8		1,2		PC9
PoPC15FM1113799	CE12		1,19		PC15
PoPC9FM78619	CE12		0,39		PC9
PoPC9FM128131	GH105		0,37		PC9
PoPC15FM1063776	GH105	0,5	0,22		N001
PoPC15FM1063776	GH105	0,22	0,83	0,14	PC15
PoPC9FM47522	GH105	0,5	0,22	0,14	N001
PoPC9FM47522	GH105		0,31		PC9
PoPC9FM132563	GH16		0,22		N001
PoPC15FM40942	GH16	0,23	0,39		PC15
PoPC9FM87194	GH16		0,13		PC9
PoPC15FM1076482	GH16	0,51	0,51		PC15
PoPC9FM82945	GH16	0,17	0,27		N001
PoPC9FM90953	GH28		0,52		PC9
PoPC9FM59334	GH28		0,34		PC9
PoPC9FM85018	GH28			0,26	N001
PoPC9FM85018	GH28		0,48	0,37	PC9
PoPC9FM51760	GH28	0,26	1,32		PC9
PoPC9FM89478	GH28	0,21		0,21	N001
PoPC9FM89478	GH28		0,46		PC9
PoPC9FM97623	GH43		0,18		N001
PoPC9FM45547	GH43		0,75		PC9
PoPC9FM127963	GH43		0,26		PC9
PoPC15FM1074766	GH43		0,6		N001
PoPC15FM1074766	GH43		1,15	0,6	PC15
PoPC15FM1061994	GH53			0,35	N001
PoPC9FM116896	GH53		0,95	0,35	PC9
PoPC9FM116896	GH53		0,95		N001
PoPC9FM121125	GH 62		0,14		N001
PoPC9FM58710	GH78		0,61		PC9
PoPC15FM13903	GH78	0,24	0,24		PC15
PoPC15FM1067626	GH79	0,68	0,38	0,48	PC15
PoPC9FM116181	GH79	0,23			N001
PoPC9FM116181	GH79	0,23			PC9
PoPC9FM116292	GH79	0,16			N001
PoPC9FM116292	GH79	0,34			PC9
PoPC9FM48699	GH79		0,76		N001
PoPC9FM48699	GH79		0,33	0,12	PC9
PoPC15FM1031712	GH88	0,67			PC15
PoPC9FM100039	PL1		0,4		N001
PoPC9FM100039	PL1		0,4		PC9
PoPC15FM1075634	PL1		0,41		N001

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PoPC15FM199583	PL1		0,23		PC15
PoPC9FM123331	PL1			0,42	N001
PoPC9FM123331	PL1		1,01		PC9
PoPC9FM83989	PL1		0,41		N001
PoPC9FM83989	PL1		0,98		PC9
PoPC15FM1113870	PL3		3,15		N001
PoPC15FM1113870	PL3		6,64	1,5	PC15
PoPC9FM116196	PL3		2,42		N001
PoPC9FM116196	PL3		3,65		PC9
PoPC15FM1044820	PL4		0,75		PC15
PoPC9FM58117	PL4		0,51		N001
PoPC9FM58117	PL4		0,67		PC9
PoPC15FM1109346	PL4		0,96		N001
PoPC9FM128966	PL4		0,96		N001
PoPC9FM128966	PL4		5,16		PC9
PoPC15FM1109346	PL4		4,54	1,97	PC15
PoPC15FM1111478	PL8	0,07	0,42		PC15
PoPC9FM53101	PL8			0,32	PC9

(One gene model can be included several times in this table if it was identified in several strains)

- Oxalate decarboxylase

Oxalic acid is a common fungal metabolite that is synthesized as a waste compound by tricarboxylic acid cycle in mitochondria, and by glyoxylate cycle in glyoxysomes and peroxisomes. Oxalic acid is toxic; therefore the regulation of its concentration is crucial. To achieve this, fungi express specific oxalate-degrading enzymes such as oxalate decarboxylase (Mäkelä et al., 2014). The proteins of the two alleles of this gene have been identified in the *P. ostreatus* secretomes (PC9FM55739 and PC15FM1078793). Both genes code for proteins which carry a signal peptide for secretion, and were found in all the different carbon source media.

In brown rot fungi, oxalate decarboxylase has been proposed to have a role in Fenton mechanisms: reactive hydrogen species have no substrate specificity, and a spatial separation between oxidants and GH (as well as fungal hyphae) seems to be necessary to avoid damage. Fe^{3+} near the hyphae is strongly chelated by secreted oxalate, hindering its reduction to the Fe^{2+} required for Fenton chemistry (Zhang et al., 2016).

RedOx

Dikaryon dkN001 and monokaryon mkPC9 present a higher number and also a higher sum of emPai values for RedOx enzymes when cultured in glucose containing media (G and WG). mkPC15 cultures have a higher number of redox enzymes when glucose is used as a carbon source (G) but the secretome profile of WG is more similar to W than to G media.

Some of these enzymes have a clear relationship with the presence of glucose, as it is the case for glucose oxidase (PoPC15FM154703, PoPC9FM91123) or FAD-oxidase (putative. glucooligosaccharide oxidase, PoPC15FM1101230, PoPC9FM90315) that catalyze the oxidation of glucose (Lee et al., 2005) and were found in mkPC15 and mkPC9G cultivated in G media.

A cupredoxin domain containing protein (PoPC9FM87572) corresponding to a protein predicted to be secreted was the most abundantly found protein among redox enzymes in *P. ostreatus* secretomes, being present in all media despite the carbon source present. Cupredoxin domains are involved in inter-molecular electron transfer reactions and are present in multicopper oxidases and laccases, which contain three of these domains on their sequence. Many other proteins can include this domain, making the information available on this gene model not enough to infer an enzymatic function. (Phyre2 99.9% similarity to plastocyanin/azurin).

- *Lignin*

Several enzymes belonging to this RedOx classification, such as those belonging to the AA1 and AA2 CAZy classes, are known to have the ability to degrade or modify lignin.. The AA1 enzymes are multicopper oxidases including laccases (EC 1.10.3.2), ferroxidases (EC 1.10.3.-), and multicopper oxidase (EC 1.10.3.-). Family AA2 contains class II lignin-modifying peroxidases, including manganese peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16), lignin peroxidases (EC 1.11.1.14), and peroxidases (EC 1.11.1.).

Only two laccases (AA1_1) have been identified in these analyses, and have been found in all the different carbon source media. No laccases have been found in cultures of strain mkPC15. The mkPC15 genome includes 11 laccases and one ferroxidase (AA1_2)

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(Castanera et al., 2012; Palmieri et al., 2000) while mkPC9 codes for 10 laccases and one ferroxidase; only mkPC9 laccases Lacc2 (PoPC9FM116143) and Lacc10 (PoPC9FM81117) and have been identified here, confirming previous findings on the importance of these two genes for the laccase activity (Castanera et al., 2012).

In the *Phlebia radiata* (Kuuskeri et al., 2016) secretome, laccases were also found regardless the carbon source, suggesting the importance of laccases besides lignin degradation. Proposed functions for laccases range from mushroom development to fungus/host interactions (Kües & Rühl, 2011; Mate & Alcalde, 2016).

Only DyP2 (Faraco et al., 2007) has been identified with enough confidence in these analyses among the Heme peroxidase group (Ruiz-Dueñas, Fernández, Martínez, & Martínez, 2011). DyP4, that is reported to be able to oxidize Mn(Fernández-Fueyo et al., 2015), was also detected, but without enough confidence level.

Copper radical oxidases (AA5) were found in high concentrations in nearly all the media analyzed here. Glyoxal oxidase, a peroxide-producing enzyme, is the most intensively studied representative of this CAZy class (Kersten and Cullen, 2014). Together with glucose methanol choline aryl-alcohol oxidases (AA3), these enzymes may supply extracellular hydrogen peroxide for the fungal wood decay carried out by class-II peroxidases(Ferreira, Carro, Serrano, & Martinez, 2015). AA5 enzymes are enzymes very common in fungal cultures, and are recently reported to exhibit a broad substrate range (Yin et al., 2015) explaining the ubiquity of this CAZy class in the *P. ostreatus* secretomes.

Cellobiose dehydrogenase CDH (AA3_1/AA8) is a key enzyme in the recently discovered pathway of cellulose degradation by LPMO (Kracher et al., 2016; Phillips, Beeson, Cate, & Marletta, 2011). It binds to the cellulose surface despite the absence of a CBM (Henriksson, Salumets, Divne, & Pettersson, 1997) and oxidizes cellobiose (Tan et al., 2015) The only gene present in *P. ostreatus* genome has a signal peptide for secretion and corresponding peptides have been found only in wood containing media (W), in agreement with their function.

The two alleles of the unique gene in *P. ostreatus* genome coding for a soluble quinoprotein glucose/sorbosone dehydrogenase has been identified in the *P. ostreatus* secretomes. A new CAZy family (AA12) was created after the discovery of the oxidation activity toward

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monosaccharides of this proteins (Matsumura et al., 2014). Like CDH, AA12 enzymes contain a cytochrome domain that could transfer electrons to an LPMO (Kracher et al., 2016) to allow cellulose degradation, and also have a CBM1 domain that could be related with their binding affinity for insoluble cellulose (Matsumura et al., 2014) suggesting a role similar to CDH in the extracellular oxidative degradation of cellulose.

- All together, these findings highlight the connections between polysaccharide and lignin biodegradation in plant materials (Martínez, 2016). Products from lignin degradation can act as electron donors for cellulose degrading LPMOs, linking the whole lignocellulose fungal degrading process. EmPai values of Red-Ox enzymes found in *P. ostreatus* secretomes

Gene Model	CAZy Family	G	W	WG	Strain
PoPC9FM116143	AA1_1		0,8	0,56	N001
PoPC9FM116143	AA1_1	0,41		0,22	PC9
PoPC9FM81117	AA1_1		0,49		N001
PoPC9FM81117	AA1_1		0,22		PC9
PoPC9FM115057	DyP		0,49		PC9
PoPC9FM62103	AA3_1		0,29		PC9
PoPC15FM1087553	AA3_2	0,3			PC15
PoPC15FM154703	AA3_2	0,18			PC15
PoPC9FM116309	AA3_2	0,14	0,14	0,43	N001
PoPC9FM116309	AA3_2	0,86			PC9
PoPC9FM59433	AA3_2	0,14			N001
PoPC9FM59433	AA3_2	0,24			PC9
PoPC9FM93955	AA3_2	0,37			PC9
PoPC15FM1098737	AA3_2	0,14			PC15
PoPC15FM1081617	AA5_1			1,16	N001
PoPC15FM1109334	AA5_1	0,14			PC15
PoPC15FM1114640	AA5_1	0,55			N001
PoPC9FM101121	AA5_1	1,08	0,75	1,08	N001
PoPC9FM101121	AA5_1	0,69		0,47	PC9
PoPC9FM134564	AA5_1		0,6	1,02	N001
PoPC9FM134564	AA5_1	0,73	0,68	0,37	PC9
PoPC15FM1079389	AA5_1		0,07		N001
PoPC9FM62166	AA5_1	1,22	0,68	1,51	N001

PoPC9FM62166	AA5_1	1,22	1,31	1,61	PC9
PoPC9FM94009	AA5_1	0,48			N001
PoPC15FM1101230	AA7	2,04			PC15
PoPC15FM1114567	AA12	0,69	0,42	0,9	N001
PoPC15FM1114567	AA12	0,69		0,26	PC15
PoPC9FM90832	AA12	0,51	0,34	0,6	N001
PoPC9FM90832	AA12	1,02	0,6	1,27	PC9
PoPC15FM1064574	Amino acid oxidase		0,33		PC15
PoPC9FM114605	Amino acid oxidase	0,38			N001
PoPC9FM88952	Copper radical oxidase	0,43		0,37	N001
PoPC9FM88952	Copper radical oxidase			0,13	PC9
PoPC9FM43770	Cupredoxin domain		0,33	0,24	PC9
PoPC15FM1062660	Cupredoxin domain		0,38		N001
PoPC15FM1062660	Cupredoxin domain		0,47	0,57	PC15
PoPC9FM71620	Cupredoxin domain	0,67			N001
PoPC9FM71620	Cupredoxin domain		0,21		PC9
PoPC9FM79407	Cupredoxin domain		0,31		N001
PoPC9FM87572	Cupredoxin domain	7,6	1,66	6,07	N001
PoPC9FM87572	Cupredoxin domain	6,07	2,93	7,6	PC9
PoPC9FM100586	FAD-linked oxidase	0,57		0,11	PC9
PoPC9FM90315	FAD-oxidase	0,53		0,45	PC9
PoPC9FM91123	Glucose oxidase	0,34			PC9

(One gene model can be included several times in this table if it was identified in several strains)

Proteases

Many proteases have been identified in the *P. ostreatus* secretomes. Proteases have a role on the fungal cell wall reorganization and degradation during hyphal growth, to recycle essential nutrients (Kuuskeri et al., 2016). Another function of these enzymes could be nitrogen acquisition from decomposing organic matter proteins in the nitrogen-limited wood environment (Wymelenberg et al., 2005).

Secreted proteases can also have a role in modulating the amount of fungal enzymes in extracellular media as suggested in *P. ostreatus* (Palmieri et al., 2000) and *P. radiata* (Kuuskeri et al., 2016)**.**

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26 different proteases have been found here, including serine peptidases S8, S9, S10, S28, S33, S41 and metallopeptidases M28, M35, M36, M43. 21 of them have a signal peptide for secretion, pointing out their targeting to extracellular functions.

- EmPai values of proteases found in *P. ostreatus* secretomes

Gene Model	Merops Family	G	W	WG	Strain
PoPC9FM115140	A1	0,36			PC9
PoPC9FM115424	M28	0,51			N001
PoPC15FM1113156	M28		2,29		PC15
PoPC9FM52745	M35		0,7		N001
PoPC15FM1037634	M35		0,78		PC15
PoPC15FM62198	M36	0,2	0,25	0,25	N001
PoPC15FM62198	M36	0,5	0,2	0,31	PC15
PoPC9FM91073	M36	0,2	0,25	0,25	N001
PoPC9FM91073	M36	0,43			PC9
PoPC15FM1092788	M43		0,1		N001
PoPC15FM1088548	S8	0,44	0,44	0,34	PC15
PoPC9FM71759	S8	0,92		0,24	PC9
PoPC15FM1087304	S9		0,37		PC15
PoPC9FM127085	S10	0,46			N001
PoPC9FM51352	S10		0,43	0,23	N001
PoPC15FM1066015	S10		0,32		N001
PoPC15FM1066015	S10	0,43		0,27	PC15
PoPC15FM175915	S10	0,98			PC15
PoPC9FM83972	S10		0,48	0,63	N001
PoPC9FM83972	S10	0,55			PC9
PoPC9FM88317	S10	0,22		0,32	N001
PoPC9FM88317	S10	0,27			PC9
PoPC9FM85063	S28	0,25			N001
PoPC9FM85063	S28	0,31			PC9
PoPC9FM57949	S28	0,21			N001
PoPC9FM57949	S28	0,26			PC9
PoPC9FM115072	S33		0,2		N001
PoPC15FM1102733	S33		0,2		N001
PoPC15FM1102733	S33		0,72		PC15
PoPC9FM132884	S33	0,27	0,27	0,21	N001
PoPC9FM82641	S41	0,68			PC9

PoPC9FM47034	S53	0,2			PC9
PoPC15FM1064502	S53	0,18			PC15
PoPC15FM1077652	S53	0,56	0,31	0,2	N001
PoPC15FM1077652	S53	0,31		0,25	PC15

(One gene model can be included several times in this table if it was identified in several strains)

Aldose epimerase

Other enzymes have been reported to have a role in lignocellulose degradation. Aldose 1 epimerase (ALE) is present in several media analyzed here. ALE encoding genes are broadly distributed among wood-decay fungi including white-rot and brown-rot Polyporales genomes (Chiaki Hori et al., 2013). ALE role has been connected with cellulose degradation through the generation of cellobiohydrolases (CDH) substrate, β- cellobiose (Higham, Gordon-Smith, Dempsey, & Wood, 1994) and, therefore, could influence the LPMO action (Kracher et al., 2016). ALE enzymes were previously found in secretomes of *P. chrysosporium* (A. Manavalan et al., 2011; Vanden Wymelenberg et al., 2011) and *P. radiata* (Kuuskeri et al., 2016). The two gene models found in *P. ostreatus* secretomes show a signal peptide for secretion, supporting a role outside the cytoplasm besides the classical eukaryotic metabolism function, as noted for *P. chrysosporium* (Wymelenberg et al., 2005).

Unknown proteins

All the proteins without a clear function defined by previous annotation methods (JGI data, Pfam and Blast) were submitted to the Phyre2 Server (Kelley and Sternberg, 2009; Kelley et al., 2015), a web tool to predict and analyze protein structure and function. 28 mkPC15 and 30 mkPC9 models were subjected to analysis and 10 and 12 models, respectively, were found to be very similar (>99% of confidence) to other well know protein structures.

Among them, three proteins from mkPC9 and one from mkPC15 appeared to be very similar to a toxin structure (PDB:4O9X, Crystal Structure of TcdB2-TccC3) that appears in a two-subunits pore forming protein found in *Photorhabdus luminescens* (Meusch et al., 2014). This Gram-negative γ-proteobacteria of the family Enterobacteriaceae is found exclusively in symbiotic association with nematodes of the genus *Heterorhabditis*, widely used as a biological control agent for insect-pests of crops. These nematodes carry the symbiont

bacteria in their gut and release them in insect hemocoel upon infection of new insect host. TcdB2-TccC3 subunits form a strong, dimeric, oval-shaped complex (Gatsogiannis et al., 2013) that harbors the biological activity; TccC3 subunit is an ADP-ribosyltransferase which target the actin cytoskeleton by modification of actin (Sheets et al., 2011).

PoPC15FM110984 appeared to be very similar to an AA10 LPMO structure (PDB ID: 5FTZ) (Chaplin et al., 2016), recently known to be an important enzyme in lignocellulose degradation. AA10 catalyzes cleavage of glycosidic bonds in crystalline chitin, thus opening the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases (Vaaje-Kolstad et al., 2010).

Protein model PoPC9FM116255 was annotated as unknown and appeared to be similar to a Ricin B/Lectin like protein, with a carbohydrate binding module. This protein has a carbohydrate binding module 13 (CBM13), but its function remains unknown. This domain was first identified in several plant lectins such as ricin or agglutinin of *Ricinus communis* which bind galactose residues. These modules have since then been found in a number of other proteins of various functions including glycoside hydrolases and glycosyltransferases, and binding to xylan has been demonstrated in the *Streptomyces lividans* xylanase A and arabinofuranosidase B (CAZy database (Cantarel et al., 2009)), therefore this protein seem to be involved in lignocellulose degradation. In our study, this model was found in all the dkN001 cultures and in mkPC9 grown in G and mkPC9 grown in W media, so this protein seems to be secreted in the presence of lignocellulose but also in media with glucose as sole carbon source. Analyses using Phyre2 program (Kelley et al., 2015) revealed the structure similarity of this model to PDB 3A23 (putative secreted α-galactosidase; c3a23A_), a glycoside hydrolase family 27 (GH27) β-L-arabinopyranosidase identified in *Streptomyces avermitilis* (Ichinose et al., 2009). A BlastP search confirms the presence of homologs of this protein in many other fungi.

Some of the proteins identified with a higher emPai value were proteins of unknown function. In mkPC15 cultured in WG, the three more abundant proteins were unknown proteins (PoPC15FM165420, PoPC15FM1087565, and PoPC15FM1065820).

PoPC15FM165420 model was found to be very similar to the structure PDB: 3L1N crystal structure of mp1p ligand binding domain 2, a cell wall mannoprotein present in the cell wall,

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hyphae, and conidia of *Penicillium marneffei* (Liao et al., 2010). Furthermore, it showed a domain HsbA (Hydrophobic surface binding protein A, pfam:12296) and had BlastP homologs in several basidiomycetes. This mkPC15 model 165420 was among the most abundant proteins recovered in PC15 cultures made in W.

PoPC15FM1065820 was very similar to PDB: 2LIE, nmr structure of the lectin CCL2, a fruiting body lectin from the ink cap mushroom *Coprinopsis cinerea*, toxic towards *Caenorhabditis elegans* and *Drosophila melanogaster* and believed to be part of a defense system against nematodes and insects. The trisaccharide specifically recognized by CCL2 is a key carbohydrate determinant of pollen and insect venom allergens implying this particular glycoepitope is targeted by both fungal defense and mammalian immune systems (Schubert et al., 2012). This protein model was also among the most abundant proteins in PC15W.

Further research will be necessary to confirm the function of this proteins, which are present in many other fungi. Heterologous expression in other organisms and accurate protein structure determination will help to decipher their biological role.

Mass spectrometry identified proteins *versus* predicted to be secreted proteins

Only 18 out of the 275 different proteins identified were annotated as intracellular by functional classification because they have no known function outside the cell. Consequently, enzymes with an extracellular role are considered as secreted even if they have also an intracellular function. Some of these proteins were not predicted to be secreted by *in silico* methods. During fungal cultivation is not possible to avoid cell disruption, releasing intracellular proteins to the extracellular medium. If fungal mycelium is cultured using wood particles as carbon source and under shaking conditions, cell disruption is increased. During the separation of extracellular medium from mycelia, it is also possible to cause cell disruption. Nevertheless, the main cause of differences between predicted and *in vitro* secretome could be the non-conventional pathways of secretion (Rabouille et al., 2012).

In our study, from the 275 proteins identified in the different media, 202 were predicted to be secreted using SignalP (Bendtsen et al., 2004) and 146 using Secretool (that uses more restrictive parameters, see (Cortázar et al., 2014) for details).

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Two proteins identified as a Ribonuclease T2 (PoPC9FM78300, and alleles PoPC9FM110190 and PoPC15FM1062961), were found to have a Signal peptide for secretion, and were found in the extracellular media (dkN001, mkPC9, and PC15 grown in G; and mkPC15 grown in W). A broad range of biological roles for these ribonucleases has been suggested, including scavenging of nucleic acids, degradation of self-RNA, serving as extra- or intracellular cytotoxins, and modulating host immune responses (Luhtala and Parker, 2010). Ribotoxins are fungal extracellular ribonucleases highly toxic due to their ability to enter host cells and their effective ribonucleolytic activity against the ribosome (Olombrada et al., 2014)

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Concluding remarks

This is the first analysis of the lignocellulose degrading capacity of two *P. ostreatus* monokaryons together with the dikaryon formed by the combination of the two nuclei. mkPC15 behave different than mkPC9 and dkN001, showing a lower number of lignocellulose degrading enzymes when cultured using wood as a carbon source. On the contrary, mkPC9 showed to be able to secrete plant cell wall decomposing enzymes in a comparable manner to dkN001. Which mechanisms drive these differences between the two monokaryons is to be deciphered with deeper genetic analyses.

The wood-decay machinery of *P. ostreatus* is typical for a traditional white-rot fungus. The broad repertoire of lignocellulose-attacking enzymes expressed by *P. ostreatus* permit the degradation of all the components of the fungal cell wall and range from red-ox enzymes laccases, dye decolorizing peroxidases, cellobiose dehydrogenases and glyoxal oxidases to cellobiohydrolases GH6 and GH7. All these enzymes are more frequent in white than brown rot fungi, although the separation of rot types is now blurred by the existence of fungal species sharing characteristics from both(Riley et al., 2014b).

P. ostreatus secreted a huge variety of glycosil hidrolases, carbohydrate esterases and polysaccharide lyases when cultured in the presence of wood, and especially when wood is the unique carbon source. As expected, the presence of glucose in addition to wood diminished the need of different enzymes to degrade lignocellulose, because fungi could use sugar as a carbon source. On the contrary, Red-ox enzymes and proteases were not so influenced by wood, probably because they have a broader range of functions in fungal metabolism.

Many of the enzymes detected lacked an enzymatic function or at least a defined role in fungal biology. Other enzymes can degrade several compounds, some of them related and others not related with lignocellulose degradation. Next steps of fungal lignocellulose degradation research must involve linking the whole lignocellulose fungal degrading process. As seen by latest findings, the synergistic action of several enzymes could be necessary to degrade cell wall compounds. Heterologous expression, fungal transformation and accurate protein structure determination will help to decipher their biological role.

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3. - MATERIAL AND METHODS

Fungal strains and culture conditions

Cultures of *P. ostreatus* strains dkN001 (dikaryon), mkPC9 and mkPC15 (monokaryons) were maintained on malt agar medium (20 g/l malt extract and 15 g/l agar) at 24°C in the dark. *Populus alba* wood was chopped to particles of approximately 1-5 x 0.5 x 1 mm and dried for three days at 80°C (moisture content of 4.4% and water content of 4.2%).

Three 10 mm diameter pieces of one week old agar cultures were used for inoculation of 100 ml liquid pre-cultures containing SMY-medium (sucrose 10 g/l, malt extract 10 g/l, yeast extract 4 g/l). After five days of growth in the conditions described below, the mycelium was washed once with sterile water using a nylon filter and resuspended in 200 ml of sterile water. The mycelium was homogenized for 15 seconds at 8000 rpm, (Ultraturrax T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and 3 ml aliquots were used as inoculum for the 150 ml experimental cultures. All experiments were prepared in liquid medium containing 0.1 g/l $Na_2B_4O_7 \cdot H_2O$, 0,07 g/l $ZnSO_4 \cdot 7H_2O$, 0,01g/l $CuSO_4 \cdot 5H_2O$, 0,01g/l $MnSO_4 \cdot 4H_2O$, 0,01g/l $FeSO_4 \cdot 4H_2O$, and 0,01g/l; $(NH_4)_6Mo_7O_2 \cdot 4H_2O$. The culture medium was supplemented with glucose (4 g/l) and/or wood (4 g/l) as required for each experiment. All cultures were incubated for 14 days at 24°C in dark under shaking conditions (130 rpm).

Protein extraction

Culture supernatants containing extracellular proteins were separated from mycelia by filtration through Whatman paper filter and frozen at -20°C overnight. After thawing the samples, insoluble polysaccharides were removed by centrifugation at 13000 rpm for 60 min at 4ºC, and the supernatant was frozen again. The processed supernatants from eight parallel cultures were combined and proteins concentrated by freeze-drying.

Freeze-dried samples were dissolved in 100 ml of distilled water and centrifuged at 4000 rpm for 30 min. After removing the undissolved debris, SDS, sodium chloride, and sodium deoxycholate were added to the samples to final concentrations of 1% (w/vol), 1M and 0.05% (w/vol), respectively (Bensadoun and Weinstein, 1976). The dissolved proteins were precipitated by addition of TCA and phosphotungstic acid (Yeang et al., 1995)(Yeet Yeang et al., 1998). In the first step, TCA was added from a 100% TCA stock solution containing 100 g TCA in 45.4 ml water to obtain a 10% final TCA concentration. After mixing, the samples were allowed to stand on ice for 30 min. Thereafter,

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phosphotungstic acid was added from a 10% (w/vol) stock solution in water to the final concentration of 0.5%. The samples were mixed well and were kept on ice overnight. The precipitated proteins were collected by centrifugation at 25,000xg for 30 min and precipitation agents removed through subsequent washings with ice-cold 20% Tris-buffer (50 mM, pH 7.5) in acetone vol/vol. In most instances, three washing steps were required to remove TCA from the protein pellets. Finally, protein samples were washed with pure, ice-cold acetone, air-dried and then stored at −20°C for further processing through subsequent washings with ice-cold 20% Tris-buffer (50 mM, pH 7.5) in acetone v/v. In most instances, three washing steps were required to remove TCA from the protein pellets (Fragner et al., 2009). The recovered proteins were dissolved in 100 mM ammonium bicarbonate and the total protein amount was determined using Bradford Reagent (Pierce, Germany).

Protein digestion

The proteins were digested with trypsin in two steps as previously described (Eastwood et al., 2011). Briefly, aliquots containing approximately 500 µg of protein from each experiment (three replicates) dissolved in 100 mM ammonium bicarbonate, were digested with sequencing grade trypsin (Promega, Germany) using an enzyme to substrate ratio of 1:40 (w/w) at 37°C for 16 h. Thereafter, the proteins in the samples were reduced using 1,4-DL-dithiothreitol (DTT) (5 mM) and tris-(2-carboxyethyl)phosphine) (TCEP, 5 mM), and alkylated with iodoacetamide (15 mM). After addition of a new amount of trypsin (enzyme to substrate ratio of 1:50), the samples were digested again for 60 min at 58°C (Havlis et al., 2003). The digested peptides were de-salted with a C18 Sep-Pak column (Waters, Milford MA) and dried in a vacuum centrifuge. The samples were dissolved in 20 mM ammonium formate pH 10 and the total peptide amount was determined using BCA Protein Reagent (Pierce, Germany) calibrated with a tryptic bovine serum albumin (BSA) digest.

Shotgun protein identification by LC-MS/MS

The digested peptides were first fractionated at pH 10 (Gilar et al., 2005) using a Reprosil Gold 3µm C18 column (150 x 2 mm; Dr. Maisch GmbH, Ammerbuch, Germany). Samples with 300 µg of peptides in 300 µl ammonium formate were separated using a linear gradient of acetonitrile in 20 mM ammonium formate pH 10. 25 fractions were collected, dried in a vacuum centrifuge and stored at -20°C.

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Peptide analysis by LC-MS/MS was performed using 1100 HPLC (Agilent, Böblingen, Germany) interfaced to an Esquire 3000 ion trap mass spectrometer (Bruker-Daltonic, Germany) *via* an electro spray ionization (ESI) unit. Each one of the 25 collected peptide fractions was dissolved in 10 µl of 5 % (vol/vol) formic acid and 4 µl of the sample were loaded onto a 180 µm i.d. capillary column packed with 3 µm Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany), conditioned with 98% of solvent A (0.1% formic acid in water) and 2% of solvent B (0.1% formic acid in 90% acetonitrile-water). After 20 min isocratic elution at 2 µl/min, the peptides were eluted using a step-gradient of solvent B: 15% in 5 min, 40% in 90 min, 50% in 5 min, and 90% in 5 min.

The Mass spectrometer was set up to take four averages of MS-spectra (200 to 1500 mu) and four averages of MS/MS-spectra (200 to 3000 mu) of two most abundant precursor ions. The Dynamic Exclusion was set to non-single charged precursor ions and an exclusion time of 1 min. The MS/MS spectra were extracted by DataAnalysis (V. 3.0, Bruker Daltonic) and peptides identified using Mascot (V. 2.4, Matrix Science, UK). The target database was constructed from annotated genomes of *P. ostreatus* mkPC15 and mkPC9 (http://genome.jgi.doe.gov/PleosPC15_2, http://genome.jgi.doe.gov/PleosPC9_1) and the SwissProt database. Peptide identifications were adjusted to 1% false discovery rate (FDR) against a decoy database. All searches were run as a tryptic digest with one missing cleavage allowed, fixed carbamidomethylation of cysteine and variable oxidation of methionine. Mass tolerances were set to 1.4 Da and 0.4 Da for the MS and MS/MS spectra, respectively. Mascot results were extracted from raw DAT-files and transferred to an SQL-database (Microsoft SQL Server 2005). SQL queries were used to extract proteins with at least two peptides with scores higher than the corresponding identity score. emPai values were calculated by MASCOT program using emPai algorithm (Ishihama et al., 2005).

Determination of protein concentration

The total protein concentration was measured by the Bradford assay using a protein determination reagent from Pierce (Coomassie Plus, Thermo Scientific, Bonn Germany). Ultrapure BSA (GERBU Biochemicals, Gaiberg, Germany) was used as a calibration standard. Fresh samples from fungal cultures supernatants were centrifuged for 10 min at 13 000×g before processing. Concentrated protein samples dissolved in electrophoresis buffers were diluted with pure water to fit the measurement range and to reduce the concentrations of buffer components below the interfering limits for protein assays.

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General discussion and concluding remarks

The aim of this thesis is to identify the proteins secreted by the edible and worldwide cultivated basidiomycete fungus *P. ostreatus* to shed light into this essential mechanism for fungal metabolism. *P. ostreatus* secretes large amounts of a broad variety of enzymes to obtain nutrients from the environment. These enzymes include biotechnological useful enzymes such as proteases, laccases and cellulases, broadly used in plant biomass ethanol production or food industry, among other processes. The search for valuable enzymes is one of the main aims of the basidiomycete secretome analyses.

Basidiomycete lifestyles include pathogens, symbionts (mycorrhizas) and saprotrophs (leaf litter degraders, white and brown rots) fungi, although the separation of rot types is now blurred by the existence of fungal species sharing characteristics from both (Riley et al., 2014). All these fungi use secreted proteins to occupy its ecological niche, therefore producing a different secretome. In the first chapter of this thesis, we reviewed several basidiomycete secretome analyses comparing the results obtained using different analytical techniques and discussing some representative examples, with a special attention to the lignocellulolytic enzymes and the different fungal lifestyles. A combination of genomic, transcriptomic and proteomics techniques is still the best approach to analyze fungal secretomes, and allow to identify patterns of secretion by lifestyle, such as the cellulase secretion by white rot fungi, the Fenton mechanism used by brown rot fungi to initiate the wood decay or the diminished presence of carbohydrate active enzymes in symbiotic or pathogenic fungi.

In the second chapter, we screened the two *P. ostreatus* monokaryotic genomes to identify the broad variety of enzymes that this fungus secretes to interact with its environment. Surprisingly, the strains differ in their lignocellulose degrading genomic capabilities. mkPC9 have less annotated CAZy-coding genes, especially in the Glycosyl-hydrolases (GH) class. Nevertheless, mkPC9 grows better than mkPC15 using lignocellulosic substrates and has a higher enzyme secretion capacity when growing in the presence of wood.

In the third chapter, the *P. ostreatus* potentially secreted proteins were identified by bioinformatics methods. Following the previous pattern, less proteins were predicted to be secreted in mkPC9. Afterwards, using transcriptomic analyses to obtain information

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about the actual use of these genes, we compare the number of proteins predicted to be secreted and the number of RPKM by protein function, and we observed a concentrated transcriptional activity in few genes per function and an increased importance of the glycosil hydrolases and proteins without a functional classification.

In *P. ostreatus*, the functional categories found in proteins predicted to be secreted showed that domains associated with lignocellulose degradation were overrepresented compared to the whole proteome, coupling secretome and fungus lifestyle. Indeed, a blind grouping of organisms using the similarity of their secretomes with the secretome of *P. ostreatus* produces a dendrogram in which the fungi are grouped by their lifestyle rather than by their phylogeny. Enzymes classically attributed to white rot (GH6, GH7, laccases and others) contribute to this grouping, but proteins without a functional classification can influence also the lifestyle association in this dendrogram.

Classical genome analyses emphasize the expansion of gene families. However, gene family size does not correlate with an increase in the number of actively expressed genes. Usually, one gene is the responsible for the majority of the expression in a gene family. We suggest that gene family expansions are associated with an increase in potential rather than with an increase in activities in a given environmental condition. Furthermore, functional redundancy has been demonstrated within the *P. ostreatus* MnP gene family by transcriptional and enzymatic compensation after inactivation of one of its members (Salame et al., 2013).

Nowadays, most of the secretome analyses rely on transcriptomics studies to explain fungal rot behavior. Nevertheless, biological processes are mainly mediated by proteins, which can be regulated post-transcriptionally. Genome analyses by bioinformatics methods provide information about the repertoire of genes available for the fungus, transcriptomic analyses provide important information about the actual use of these genes, and mass spectrometry goes a step further on the actual presence of these enzymes acting on the lignocellulosic substrates, demonstrating (or not) the accuracy of transcriptomics and in silico data. This can yield a reciprocal benefit, because the use of proteogenomics methodologies (Nesvizhskii, 2014) can provide protein-level evidence of gene expression to help refine genes annotation.

In the fourth chapter, mass spectrometry analyses were used to confirm the presence of these enzymes acting on the lignocellulosic substrates. Three *P. ostreatus* strains were

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cultured in three different media using with glucose, wood or both (glucose and wood) as a carbon source. As expected, we identify a higher number of lignocellulose degrading enzymes in wood-containing media, especially glycosyl-hydrolases, carbohydrate esterases and polysaccharide lyases. On the contrary, RedOx enzymes and proteases do not showed an increment when cultured in wood medium, probably because they have a broader range of functions in fungal metabolism.

Nevertheless, the importance of each secreted enzyme in a given medium can be highly modulated by fungi. Genes coding enzyme families present in *P. ostreatus* change dramatically its transcripts expression and protein secretion depending on the growing medium. These data emphasize the dynamic and environmentally dependent nature of fungal secretomes.

As said in chapter 4, the wood-decay machinery of *P. ostreatus* is typical for a traditional white-rot fungus. *P. ostreatus* can degrade all the components of the fungal cell wall using a broad repertoire of lignocellulose-attacking enzymes ranging from red-ox enzymes as laccases, dye decolorizing peroxidases, cellobiose dehydrogenases and glyoxal oxidases to cellulases belonging to CAZy families GH6 and GH7. All these enzymes are more frequent in white rot fungi lifestyle (Nagy et al., 2016).

Furthermore, we had the opportunity to compare the behavior of two monokaryons with a complete knowledge of their genomic composition, as well as the dikaryon from which they are derived when cultured in the three previously mentioned media. Interestingly, monokaryons behave in a very different manner; mkPC15 showed a weakest production of lignocellulose degrading enzymes than mkPC9 and dkN001 when cultured using wood as a carbon source. Moreover, dkN001 was able to secrete more plant cell wall decomposing enzymes, correlating with their superior capacity to grow on lignocellulosic substrates. Therefore, the genetic background of each strain is determinant to determine its protein secretion behavior. Future research will focus on the genetic mechanisms driving these differences between the three strains.

The number of secretome analyses is in basidiomycetes is increasing along with the number of sequenced genomes (278, April 2017), and the increasing quality of the genome annotations. Nevertheless, many of the enzymes detected lacked a defined role in fungal biology, giving only coarse pictures of how fungal use secreted proteins to

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More than 20% of *P. ostreatus* secreted proteins that were conserved in other basidiomycetes have an unknown enzymatic function. Transcriptome analysis underlined the importance of these proteins, further confirmed by proteomics. Using domain structure prediction, we were able to give an insight about the possible role of several proteins, including a xylanase and a AA10 LPMO.

Fungal lignocellulose degradation is the result of the synergistic action of several enzymes (Martínez, 2016). Future research must involve the use of heterologous expression, fungal transformation and accurate protein structure determination, added to transcriptomic and proteomics analyses, to decipher their biological role, improving our overall understanding of plant biomass degradation as a step to achieve the goal of using biomass as a sustainable source of energy to support future needs.

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Conclusions

1. This is the first analysis of the lignocellulose degrading capacity of two *P. ostreatus* monokaryons together with the dikaryon from which they are derived.

2. Secretome composition and lifestyle are related in *P. ostreatus*.

3. Gene family expansion does not correlate with an increase in the number of actively expressed genes.

4. The genetic background of each strain is decisive to determine its protein secretion behavior.

5. Among the *P. ostreatus* secreted proteins that were conserved in other basidiomycetes, more than 20% do not have a functional classification.

6. The use of several complementary techniques to analyze secretome samples appears to be the best method for obtaining a broad insight into the complex and highly dynamic mixture of proteins that basidiomycetes use to degrade lignocellulose.

7. Proteomic analyses are essential to demonstrate the accuracy of in silico and transcriptomics data.

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Publications

1. **Comparative and transcriptional analysis of the predicted secretome in the lignocellulose-degrading basidiomycete fungus Pleurotus ostreatus.**

   Alfaro M, Castanera R, Lavín JL, Grigoriev IV, Oguiza JA, Ramírez L, Pisabarro AG.

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2. **Highly expressed captured genes and cross-kingdom domains present in Helitrons create novel diversity in Pleurotus ostreatus and other fungi.**

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