Prospects for Bioprocess Development Based on Recent Genome Advances in Lignocellulose Degrading Basidiomycetes

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1 Introduction

Efficient and complete degradation of woody plant cell walls requires the concerted action of hydrolytic and oxidative systems possessed by a relatively small group of filamentous basidiomycetous fungi. Among these wood decay species, ***Phanerochaete chrysosporium*** was the first to be sequenced (Martinez et al. 2004). In the intervening 10 years, over 100 related saprophytes have been sequenced. There genomes have revealed impressive sequence diversity. and recent functional analyses are providing a deeper understanding of their roles in the deconstruction of plant cell walls and the transformation of xenobiotics.

Wood cell walls are primarily composed of cellulose, hemicellulose and lignin. Many microbes are capable of hydrolyzing the linkages in cellulose and hemicellulose, even though crystalline regions within cellulose can be rather challenging substrates (reviewed in Baldrian and Lopez-Mondejar 2014; van den Brink and de Vries 2011). In contrast, few microbes possess the oxidative enzymes required to efficiently degrade the recalcitrant lignin, a complex, amorphous, and insoluble phenylpropanoid polymer (Higuchi 1990; Ralph et al. 2004). These unusual wood decay fungi secrete extracellular peroxidases with impressive oxidative potential. Potential applications have focused primarily on lignocellulosic bioconversions to

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M. Schmoll, C. Dattenböck (eds.). *Gene Expression Systems in Fungi: Advancements and Applications*, Fungal Biology,
DOI 10.1007/978-3-319-27951-0_6

high-value. low molecular weight products and on organopollutant transformations Voluminous literature covers the physiology and genetics of wood decay (for reviews see Cullen 2013: Cullen and Kersten 2004; Eriksson et al. 1990; Hatakka and Hammel 2010: Kersten and Cullen 2013; Kirk and Farrell 1987). This chapter does not provide a comprehensive treatment of the field. but rather highlights recent genome progress relevant to bioprocess development.

2 Diversity and Microbiology of Wood Decay Fungi

Two distinct forms of wood decay were generally recognized. White rot fungi degrade cellulose. hemicellulose. and lignin, although the patterns vary depending on the wood species and fungal strain (Blanchette 1991; Daniel 1994: Eriksson et al. 1990; Schwarze 2007). White rot fungi such as *P. chrysosporium*, simultaneously degrade all cell wall polymers, while *Ceriporiopsis subvermispora* selectively degrades lignin ahead of cellulose and hemicellulose (Akhtar et al. 1992; Behrendt and Blanchette 1997: Srebotnik and Messner 1994). A few white rot fungi, including *Phlebiopsis gigantea*, are able to rapidly colonize freshly cut conifers by metabolizing and tolerating resins. triglycerides and fatty acids. In contrast to white rot, brown rot fungi modify, but do not remove. lignin. Instead, a polymeric residue is left (Niemenmaa et al. 2007; Yelle et al. 2008, 2011) and the cellulose is rapidly depolymerized. Seemingly consistent with decay patterns, initial genome analyses revealed multiple genes encoding extracellular peroxidases in white rot fungi, but none in brown rot. In addition to wood decay fungi, certain litter decomposers also degrade lignin (reviewed in Eriksson et al. 1990: Hatakka 2001). and these saprophytes may play an essential role in the transformation and degradation of humic substances (Kluczek-Turpeinen et al. 2005; Snajdr et al. 2010; Steffen et al. 2002). The white rot fungus *Trametes* sp. degrades humic substances in a process likely involving Fenton-based mechanisms (Grinhut et al. 2011 a, b).

Importantly, brown rot depolymerization of cellulose (Gilbertson 1981; Kirk et al. 1991; Kleman-Leyer et al. 1992; Worrall et al. 1997) proceeds rapidly in advance of extensive colonization and substrate weight loss. This observation and the limited porosity of cell walls strongly argue for the involvement of diffusible, small molecular weight oxidants (Blanchette et al. 1997; Cowling 1961; Flournoy et al. 1993; Srebotnik et al. 1988; Srebotnik and Messner 1991). Highly reactive hydroxyl radical. generated via the Fenton reaction ($H_2O_2+Fe^{2+} + H^+ \rightarrow H_2O+Fe^{3+} + \cdot OH$) has been implicated in this process (Akhtar et al. 1992; Cohen et al. 2002 2004; Xu and Goodell 2001). The precise mechanisms supporting sustainable hydroxyl radical production remain unclear although plausible redox systems have been proposed (reviewed in Arantes et al. 2012; Goodell 2003). and considerable evidence supports hydroquinone redox cycling (Arantes et al. 2011; Paszczynski et al. 1999; Suzuki et al. 2006) (Fig. la).

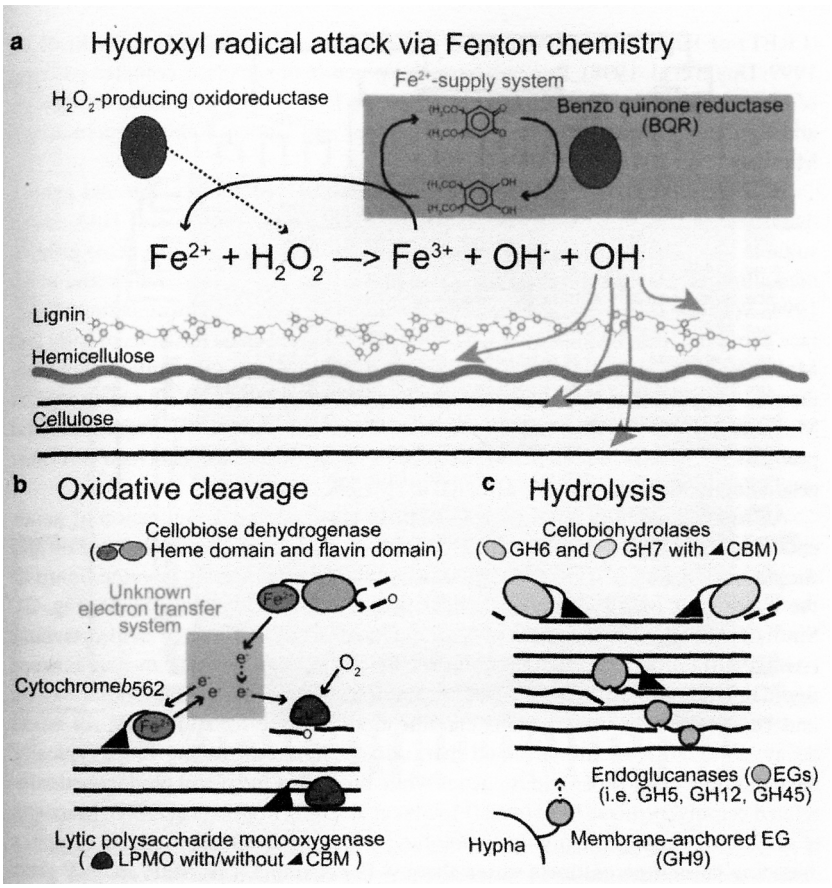

Fig. 1 Simple diagram of cellulose attack by wood decay fungi with hydroxyl radical (a), oxidative enzymes (b) and glycoside hydrolases (c)

Enzymes and Oxidative Processes Involved in Lignocellulose Conversions

3.1 Lignin Degradation

Owing to their high oxidation potential, the Class II peroxidases lignin peroxidase (LiP), versatile peroxidase (VP) and manganese peroxidases (MnP) modify lignin and related aromatic molecules. LiP and VP have redox potentials of approximately 1.5 V, and are able to directly oxidize non-phenolic lignin model compounds by a single electron (Kersten et al. 1985; Kirk et al. 1986; Miki et al. 1986). LiP and VP feature a surface tryptophan mediating long-range electron transfer

(LRET) of larger sterically-hindered non-phenolic substrates (Choinowski et al. 1999; Doyle et al. 1998). Depending on the structure of substrate, complex patterns of intermediates are formed (Miki et al. 1986; Tien and Kirk 1983). Peroxidases and ligninolysis have been reviewed (Hammel and Cullen 2008; Higuchi 1990; Martinez et al. 2014).

In contrast to LiPs and VPs, MnPs lack a conserved surface Trp and cannot directly oxidize non-phenolic aromatics. However. in the presence of H_2O_2 and a suitable Mn^{3+} chelator, conserved Mn-binding sites in the vicinity of a heme propionate allow MnP to catalyze the oxidation of Mn^{2+} to Mn^{3+} (Sundaramoorthy et al. 1994; Wariishi et al. 1992). These Mn-binding sites and the abovementioned surface Trp are conserved in VPs which thereby have hybrid characteristics of LiPs and MnPs (Ruiz-Dueñas et al. 2009). Diffusible Mn^{3+} chelates will oxidize phenolics directly or generate other oxidizing species, and oxalate is a likely physiological Mn^{3+} chelator for the MnP and VP reactions (Kuan and Tien 1993). Superoxide and perhydroxyl radicals might, in the presence of lipids, initiate radical chain reactions producing ligninolytic radicals (Kapich et al. 1999).

All lignin degrading fungi were thought to possess some combination of genes encoding class II peroxidases, but recent analysis of *Botrybasidium botryosum* and *Jaapia argillacea* has shown that these wood decay fungi slowly degrade lignin in the absence of high oxidation potential peroxidases (Riley et al. 2014) (Fig. 2). Similarly, no class II peroxidases were detected in the genome of *Schizophyllum commune* (Ohm et al. 2010), a white rot fungus exhibiting weak to non-existent lignin degradation (Boyle et al. 1992; Schmidt and Liese 1980). These observations undermine the strict dichotomous classification of white rot and brown rot wood decay, although it should be noted that efficient lignin degrading fungi typically have multiple class II peroxidase genes while brown rot fungi and phylogenetically related ectomycorrhizae have none (Floudas et al. 2012; Martin et al. 2008; Martinez et al. 2009). Among the highly ligninolytic white rot fungi, the number of penes encoding class II peroxidases varies sharply. For example, LiP, MnP, and VP gene numbers are 10, 5, and 0, respectively in *P. chrysosporium*, but 0, 9, and 3 in *Dichomitus squalens* (summarized in Cullen 2013). The role of this genetic multiplicity is uncertain, but differential regulation in response to substrate composition was observed prior to the 'genomics era' (Holzbaur and Tien 1988; Stewart and Cullen 1999) and, of particular relevance here, transcripts corresponding to specific *P. chrysosporium* LiP and MnP penes were identified in colonized soil containing polycyclic aromatic hydrocarbons (PAHs) (Bogan et al. 1996a, b). *Pleurotus ostreatus* lacks LiP-encoding genes, but transcript levels of MnP and VP are influenced by media composition (Knop et al. 2014) and the VP4 dominates under Mn deficient conditions.

The degradation of PAHs and other organopollutants has been attributed to the striking oxidation potential and low substrate specificity of the class II peroxidases (reviewed in Cullen 2002; Hadar and Cullen 2013; Hammel 1995a, b; Higson 1991; Pointing 2001). PAHs such as benzopyrene, pyrene and anthracene have ionization potentials below 7.6 eV and serve as substrates for LiP (Hammel 1995a; Hammel et al. 1986). LiPs will also transform chlorinated phenols (Hammel and Tardone 1988;

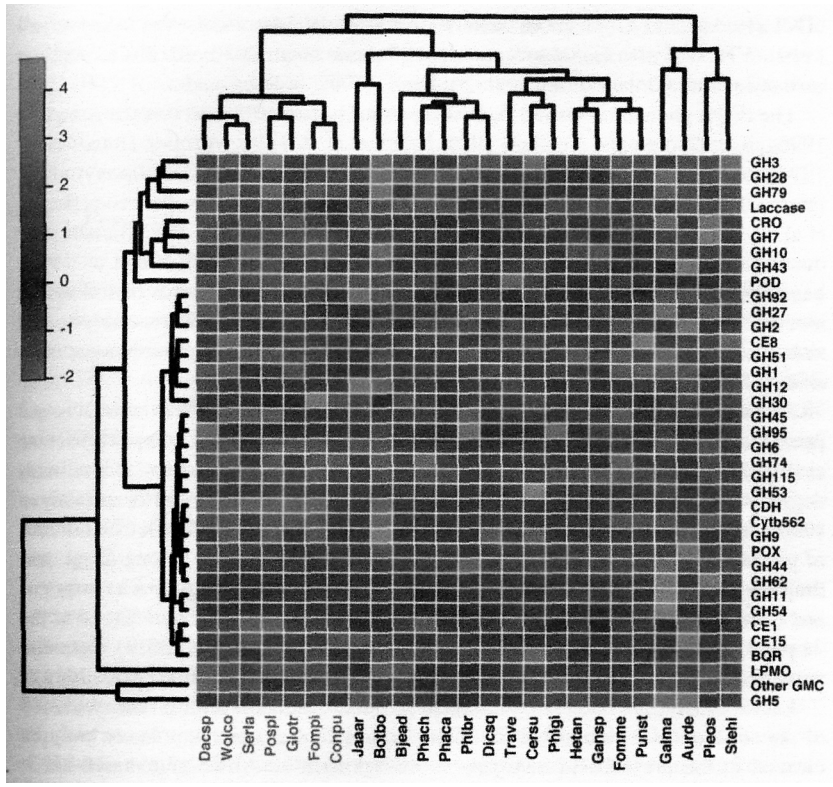

Fig. 2 The numbers of lignocellulose-degrading enzymes predicted in 25 wood decay fungal genomes. Heatmap color scale shown on *upper left.* Cluster dendrogram was performed by R using gene numbers and clearly separates seven brown-rot fungi and sixteen white-rot fungi. Unsusual white rot fungi, *Jaapia argillacea* (Jaaar) and *Botrybasidium botryosum* (Botbo), which lack POD genes, cannot be easily assigned to white- or brown-rot categories (Riley et al. 2014). Seven species of brown-rot fungi include *Dacryopinax* spp. (Dacsp), *Wolfiporia cocos* (Wolco), *Serpula lacrimans* (Serla), *Postia placenta* (Pospl), *Gloeophyllum trabeum* (Glotr), *Fomitopsis pinicola* (Fompi) and *Coniophora puteana* (Conpu). The 16 species of white-rot fungi are: Bjead, *Bjerkandera adusta*; Phach, *Phanerochaete chrysosporium*; Phaca, *Phanerochaete carnosa*; *Phlbr, Phlebia brevispora*; Dicsq, *Dichomitus squalens*; Trave, *Trametes versicolor*; Cersu, *Ceriporiopsis subvermispora*; Phlgi, *Phlebiopsis gigantea*; Hetan, *Hererobasidion annosum*; Gansp, *Ganoderma* spp.: Fomme, *Fomitiporia mediterranea*; Punst, *Punctularia strigosozonata*; Galma, *Galerina marginata*; Aurde, *Auricularia delicate*; Pleos, *Pleurotus ostreatus*; Stehi, *Stereum hirsutum.* This aggregated data set was derived from (Floudas et al. 2012: Hori et al. 2013, 2014c; Riley et al. 2014).

(Mileski et al. 1988; Reddy and Gold 2000: Valli and Gold 1991), tetrahydrofurans (Vazquez-Duhalt et al. 1994), dioxins (Hammel et al. 1986; Valli et al. 1992b), methoxybenzenes (Kersten et al. 1985), and chloro- and nitro-methoxybenzenes (Teunissen et al. 1998; Valli et al. 1992a, b; Valli and Gold 1991). MnP can decolorize a70 dyes (Heinfline et al. 1998) and oxidize pentachlorophenol and 2, 4, 6-trinitrotoluene

(TNT) (Reddy and Gold 2000; Scheibner and Hofrichter 1998; Van Aken et al. 1999). VPs will also transform azo dyes (Salame et al. 2010, 2012b) as well as carbamazepine (Golan-Rozen et al. 2011).

The PAHs phenanthrene and flourene are not MnP or LiP substrates (Bogan et al. 1996a, b, c; George and Neufield 1989; Hammel et al. 1992; Vazquez-Duhalt et al. 1994), but peroxidation of unsaturated lipids can generate transient lipoxyradical intermediates that oxidize non-phenolic lignin model compounds, flourene (Bogan et al. 1996a, b, c) and phenanthrene (Moen and Hammel 1994). The efficient production of class II peroxidases in *E. coli* (Doyle and Smith 1996: Nie et al. 1998) has substantially advanced understanding of the catalytic properties of individual isozymes (Martinez et al. 2014), and directed evolution using a *S. cerevisiae* expression system has been used to alter temperature-, peroxide-, and pH-tolerance of *P. eryngii* VP (Garcia-Ruiz et al. 2012).

Although not class II peroxidases, recent studies also suggest that heme thiolate peroxidases (HTPs) and the dye decolorization peroxidases (DyPs) (Hofrichter et al. 2010; Lundell et al. 2010) may serve as useful biocatalysts for organopollutant degradation. HTPs include chloroperoxidases and peroxygenases and these catalyze various reactions (Gutierrez et al. 2011; Ullrich and Hofrichter 2005). The number of genes predicted to encode these enzymes varies substantially among fungi, and they are particularly abundant in the genomes of the saprotrophs *Agaricus bisporus* and *Coprinopsis cinereus.* In the case of the button mushroom *A. bisporus,* 16 of the 24 putative HTP genes were upregulated in compost (Morin et al. 2012). Potential applications for fungal peroxidases were recently reviewed (Martinez et al. 2014).

Laccases have also been implicated in lignin degradation through the oxidation of phenolic units in lignin, but the major non-phenolic substructures can only be cleaved in the presence of auxiliary substrates such as ABTS (2,2'-azino-bis-3-ethylthiazoline-6-sulfonate) (Bourbonnais et al. 1997, 1998; Collins et al. 1999) (reviewed in Giardina et al. 2010). Similarly, organophosphorous insecticides are degraded by *P. ostreutus* laccase (Amitai et al. 1998), and high ionization potential PAHs are oxidized by *Coriolopsis gallica* and *T. versicolor* laccases (Johannes et al. 1996; Pickard et al. 1999) in the presence of synthetic mediators. Laccase activity and stability were improved using *S. cerevisiae*-based directed evolution (Mate et al. 2010). The genomes of most white-rot fungi feature multiple laccase genes but some, notably *P. chrysosporium* and *Phlebiopsis gigantea,* contain none (Fig. 2).

Considerable research has examined PCP degradation by ligninolytic fungi and their purified enzymes. Peroxidases and laccases can initiate oxidation via para-quinone formation and release of chlorine. Remediation studies have demonstrated detoxification by humification into organic matter, and LiP, MnP and laccase polymerization of labeled PCP led to soil-bound products (Ruttimann-Johnson and Lamar 1996). Field remediation of PCP-contaminated soils has been demonstrated (Ford et al. 2007a, b; Lamar and Dietrich 1990; Lamar et al. 1990a, b, 1994; Lestan and Lamar 1996).

Numerous investigations have focused on the degradation of azo dyes, the largest class of synthetic dyes (Singh and Arora 2011), and wood decay fungi have shown considerable promise (Kaushik and Malik 2009; Stolz 2001: Wesenberg et al. 2003).

Both class II peroxidases and laccases will oxidize certain azo dyes and site specific mutagenesis verified the importance of Trp 164 in *Pleurotus eryngii* VP (Camarero et al. 2005; Ruiz-Duenas et al. 2008). Random mutagenesis has improved degradative performance of a *P. ostreatus* laccase (Miele et al. 2010).

3.2 Peroxide Generation

Several enzymes have been proposed as the source of extracellular H_2O_2, a key requirement for class II peroxidases and for Fenton chemistry. Considerable evidence supports glyoxal oxidase (GLOX), a radical-copper oxidase (Whittaker et al. 1996) first reported in *P. chrysosporium* cultures (Kersten and Kirk 1987). Considerable evidence supports a close physiological connection between GLOX and LiP (Kersten 1990) and the enzyme is found in most, but not all, white rot fungi (Cullen 2013). Brown rot fungi do not feature GLOX-encoding genes although, as in the case of the white rot fungi *C. subvermispora, H. annosum* and *F. mediterranea*, structurally related copper radical oxidases (Vanden Wymelenberg et al. 2006) may compensate (Cullen 2013).

Other potential sources of extracellular H_2O_2 include glucose-methanol-choline oxidases (GMCs) such as aryl alcohol oxidase (AAO), methanol oxidase and various sugar oxidases (reviewed in Hernandez-Ortega et al. 2012). Methanol oxidase, in particular, is highly expressed by white rot and by the brown rot fungus, *Gloeophyllum trabeum.* In this case, the enzyme is proposed to generate the H_2O_2 required as a Fenton reactant (Daniel et al. 2007) ($H_2O_2+Fe^{2+}+H^+ \rightarrow H_2O+Fe^{3+}+$ ·OH). The biological role of cellobiose dehydrogenase (CDH) remains uncertain (Henriksson et al. 2000; Zamocky et al. 2006), but this multi-domain enzyme (Hallberg et al. 2000) warrants mention because of its ability to enhance cellulose oxidation by lytic polysaccharide monooxygenases (LPMOs) (Harris et al. 2010; Langston et al. 2011). LPMOs were originally classified as 'hydrolases' but subsequently shown to be copper-dependent monooxygenases (Quinlan et al. 2011; Westereng et al. 2011) (Fig. 1b).

3.3 Other Oxidative Enzymes

Beyond secreted peroxidases and laccases, wood decay fungi possess a plethora of intracellular systems for transforming an impressive array of organopollutants. The genomes generally contain approximately 150 or more cytochrome P450 encoding genes (Cullen 2013). but their precise role(s) are uncertain. Nevertheless, advances have been made (Doddapaneni et al. 2013; Syed and Yadav 2012).

White rot P450s have been implicated in the degradation of 4-methyldibenzothiophene (Ichinose et al. 1999), endosulfan (Kullman and Matsumura 1996) and various PAHs (Bezalel et al. 1996a, b; Masapahy et al. 1999; Syed et al. 2010,

2011). In some instances, the combined activities of ligninolytic peroxidases and P450s has been demonstrated (Bezalel et al. 1997). Similarly. the transformation of carbamazepine (CBZ), a widely occurring pharmaceutical pollutant. involves *Pleurotus ostreatus* MnP and P450(s) (Cruz-Morato et al. 2012; Golan-Rozen et al. 2011; Marco-Urrea et al. 2009). In *P. chrysosporium* cultures, 2,4,6-trichlorophenol (Reddy et al. 1998) and PCP (Hammel and Tardone 1988: Mileski et al. 1988: Reddy and Gold 1999, 2000) are degraded by peroxidase-driven dechlorination and then intracellular reductive dechlorination and hydroxylation. The catalytic activity of a *P. chrysosporium* P450 has been improved via mutagenesis (Syed et al. 201 3). Activity of membrane bound P450s against PAHs has been assessed in *Pichia* by coexpression of a reductase partner (Syed et al. 2010).

In addition to the intensively studied degradative activities of wood decay fungi, certain enzymes hold considerable promise as biosynthetic catalysts. For example, tandemly arranged genes encoding pyranose 2 oxidase and pyranosome dehydratase have been observed in several genomes including *P. gigantea* (Hori et al. 2014b) and *P. chrysosporium* (Martinez et al. 2004). Their precise function remains uncertain, but they can produce the antibiotic cortalcerone (de Koker et al. 2004; Giffhorn 2000). These enzymatic reactions have been coupled to bifunctional catalysts containing Bronsted and Lewis acid sites to produce furylglycolic acid, a compound suitable for copolymerization with lactic acid (Schwartz et al. 2013).

4 Hydrolytic Systems Involved in Cell Wall Degradation

Fungal hydrolases and related carbohydrate active enzymes (CAZys) represent a major portion of the commercial enzyme market. Based on well established fermentation technologies, the ascomycetes *Trichoderma* and *Aspergillus* produce copious quantities of cellulases including exocellobiohydrolase I (CBHI), exocellobiohydrolase II (CBHII), ß-1,4-endoglucanase (EG) and ß-glucosidase (ß-Glu) (Baldrian and Valaskova 2008; Kirk and Cullen 1998). The genetic characterization of the *T. reesei* cellulases was established long before the initial genome analysis (Martinez et al. 2008) and the hydrolases were known to be encoded by relatively few genes within glycoside hydrolase families GH6 (CBHII), GH7 (CBHI, EG), CH12 (EG), GHS (EG) and ß-Glu (GHI, GH3) (Fig. 1c). This stands in sharp contrast to the genetic diversity encountered in wood decay fungi such as *P. chrysosporium* where six CBH1 isozymes are produced. Possibly, this structural diversity reflects functional differences (Munoz et al. 2001). It is now clear that such genetic multiplicity is the norm in white rot fungi (Floudas et al. 2012; Hori et al. 2014b), and this extends to hydrolases, esterases and lyases involved in hemicellulose degradation (Kirk and Cullen 1998; van den Brink and de Vries 2011).

Likely owing to their reliance on oxidative depolymerization of cellulose, brown rot fungi have few, if any, exocellobiohydrolases and endoglucanses. *Serpula lacrymans* appears exceptional in that three GH5s with cellulose binding domains (putative EGs) have been identified in the genome (Eastwood et al. 2011). Several GHS EG-like genes have been identified in other brown rot species but, in the

absence of binding domains and any exocellobiohydrolases (CBH1 or CBH2). it is unclear how such enzymes could efficiently hydrolyze crystalline cellulose.

The repertoire of genes and their regulation vary substantially among wood decay species and the substrate colonized. For example, *P. chrysosporium* transcript and secretome patterns differ markedly when cultured in media containing complex woody substrates, especially those genes encoding a GH92 *a*-1,2-mannosidase, *a*-GH27 *a*-galactosidase, a GH5 1,4 ß-mannan endohydrolase, and two carbohydrate esterases (CE15) (Vanden Wymelenberg et al. 2011). Although closely related to *P. chrysosporium*, the complement of *Phanerochaete carnosa* genes (Suzuki et al. 2012) and their regulation (Macdonald and Master 2012; MacDonald et al. 2012) differ substantially. These patterns are, at least in part, related to a *P. carnosa's* preference for softwood; a characteristic even more pronounced by *P. gigantea*. Development of conversion processes may also be aided by elucidating transcript patterns on conventional forestry feedstocks (Vanden Wymelenberg et al. 2011) as well as gene expression responses to specific synngyl-rich transgenic derivatives of hybrid poplar (Gaskell et al. 2014). These recent studies demonstrate that lignin composition substantially altered gene expression suggesting that commercial enzyme mixtures could be improved by tailoring enzyme components to specific feedstocks. Such studies facilitate development of enzymatic systems for biomass conversions and may help guide *Populus* breeding programs.

In addition to polysaccharide degradation certain wood-inhabiting fungi efficiently remove resinous extractives from freshly harvested and/or wounded pine. Often problematic in paper manufacturing as pitch deposits, these extractives include resin acids, long chain fatty acids and triglycerides. Among white rot fungi, *Phlebiopsis gigantea* is particularly well adapted to colonizing conifer wood and recent genome analyses (Hori et al. 2014b) have identified highly expressed lipases and active ß-oxidation pathways likely involved in the metabolism of the triglycerides (Fig. 3). Such lipolytic systems, particularly the lipases, may be useful for commercial bioprocesses.

5 Emerging Experimental Tools and Future Prospects

5.1 Genetic Transformation Systems

Strain improvement and fundamental research on lignocellulose degrading fungi have been hampered by the availability of tenable genetic transformation systems. Auxotroph complementation and drug resistance have been used for the model white rot fungus *P. chrysosporium*. but the efficiency is quite low and multiple, ectopic integration even its are typically obtained. Encouraging advances along these lines include gene silencing via RNA interference (Matityahu et al. 2008) and the uptake of DNA by shock waves (Magana-Ortiz et at. 2013). The latter approach has been employed to generate *P. chrysosporium* transformants producing increased levels of LiP, MnP and a VP (Coconi-Linares et al. 2014). In *P. ostreatus*, enhanced ligninolytic activity was obtained by over expression of the gene encoding VP

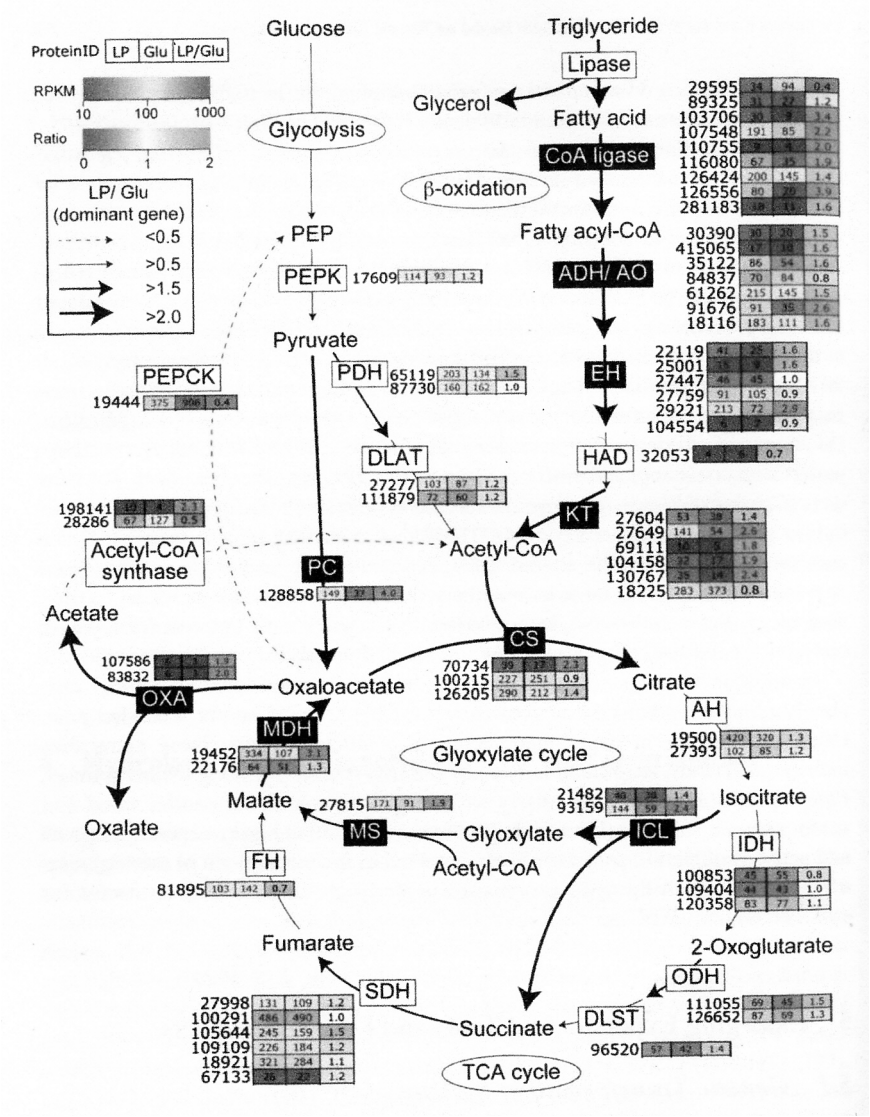

Fig. 3 Adapted From Hori et al. (Hori et al. 2014c). pathways illustrating lipid oxidation and related metabolism when *P. gigantea* is cultivated on medium containing loblolly pine (LP) relative to glucose (Glu) as sole carbon source. Enzymes encoded by upregulated genes are highlighted in *black* and associated with *thickened arrows.* Abbreviations: *ADH/AO* Acyl-CoA dehydrogenase/oxidase, *AH* aconitate hydratase, *CoA ligase* long fatty acid-CoA ligase, DLAT dihydrolipoyllysine-residue acetyltransferase, *DLST* dihydrolipoyllysine-residue succinyltransferase, *EH* enoyl-CoA hydratase; *FH* fumarate hydratase. *KT* ketothiolase (acetyl-CoA C-acyltransferase). *HAD* 3-Hydroxyacyl-CoA dehydrogenase. *ICL* isocitrate lyase, *IDH* isocitrate dehydrogenase, *MDH* malate dehydrogenase, *MS* malate synthase, *ODH* oxoglutarate dehydrogenase, *OXA* oxaloacetase, *PC* pyruvate carboxylase, *PDH* pyruvate dehydrogenase. *PEP* phosphoenolpyruvate, *PEPCK* phosphoenolpyruvate carboxykinase, *PEPK* phosphoenolpyruvate kinase, *SDH* succinate dehydrogenase

mnp4 (Salame et al. 2012a), and peroxidase regulation is influenced by calmodulin is shown by treatment with CaM inhibitors. RNAi suppression and overexpresion (Suetomi et al. 2014).

The inability to target genes has been a longstanding experimental limitation among filamentous basidiomycetes. However, impaired nonhomologous end joining has been demonstrated in Ku knockout strains of non ligninolytic strains of *S. commune* (de Jong et al. 2010) and *C. cinereus* (Nakazawa et al. 2011) and, more recently, in the efficient lignin degrader, *P. ostreatus* (Salame et al. 2012b). The latter $\Delta ku80$ strain allows 100% homologous recombination among the stable transFormants which have been useful for defining the catalytic roles of VP and MnP genes (Knop et al. 2014; Salame et al. 2013, 2014).

Such powerful experimental tools offer unprecedented opportunities to establish the function of a wide range of genes and thereby directly contribute to our understanding of lignocellulose conversions. Importantly, this analysis could help resolve the challenging problem of 'hypothetical genes', a common feature of fungal genomes. For perspective, of 11,891 predicted *P. gigantea* genes, 4744 encode 'hypothetical' or 'uncharacterized' proteins. RNA-seq and secretome analyses have shown that many of these genes are regulated and/or encode secreted proteins when cultivated on woody substrates (Hori et al. 2014b). Clearly, these proteins have significant roles, and targeted disruptions/replacements could provide meaningful functional insight along these lines. The regulation or expression levels of promising genes could be altered to improve, for example, bioremediation performance. Additionally, the 'hypothetical' protein of interest might be amenable to production in heterologous systems, in which case detailed biochemical investigations and bioprocess evaluation would be possible.

5.2 Metagenomes and Metatranscriptomes

The availability of an increasing number of fungal genomes and concomitant expansion of databases (Cantarel et al. 2009; Levasseur et al. 2013) facilitate metagenome, and metatranscriptome investigations of complex microbial communities (Baldrian and Lopez-Mondejar 2014). For examples, Fungal transcripts associated with PAH degradation were identified in a soil microcosm amended with the phenanthrene (de Menezes et al. 2012). and eukaryote transcripts in spruce and beech forest soil included diverse fungal CAZys along with metazoan homologs (Damon et al. 2012). Focusing on cellulose degradation in coniferous forests. Baldrian and co-workers showed that fungal decomposition dominated in litter while bacteria were mainly represented in the soil (Baldrian et al. 2012; Stursova et al. 2012). Of relevance to biomass utilization, a diverse array of exocellobiohydrolase (CBHl) genes had been identified (Baldrian et al. 2012).

Recent database expansions, enhanced mass spectrometry performance and improved bioinformatic methods have made possible high throughput identification of extracellular proteins in pure cultures of *P. chrysosporium* (Hori et al. 2011; Manavalan et al. 2011; Ravalason et al. 2008; Sato et al. 2007; Vanden Wymelenberg

et al. 2005, 2009, 2010) and other white rot fungi (Floudas et al. 2012: Hori et al. 2014a, b; Martinez et al. 2009). Metaproteomic analysis of more complex samples will increasingly be used for defining the structure and functional activities within microbial communities (Hettich et al. 2012: Mueller and Pan 2013). For example, Schneider and co-workers (Schneider et al. 2012), have determined that, relative to bacteria, fungi were the main enzyme producers in forest litter samples, and they observed shifts in the relative abundance of ascomycetes to basidiomycetes over time. In these studies, mass spectrometry-identified peptide patterns were associated with activities of hemicellulases and cellulases. Collectively, these studies reveal a rich diversity of microbes and physiological activities in complex substrates.

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Editors
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ISSN 2198-7777 ISSN 2198-7785 (electronic)
Fungal Biology
ISBN 978-3-319-27949-7 ISBN 978-3-319-27951-0 (eBook)
DOI 10.1007/978-3-319-27951-0

Library of Congress Control Number: 2016932377

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

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