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Review

Systems biology-guided understanding of white-rot fungi for biotechnological applications: A review

Teeratas Kijpornyongpan,[1,3] Alexa Schwartz,[1,2,3] Allison Yaguchi,[1,3] and Davinia Salvachúa[1,*]

SUMMARY

Plant-derived biomass is the most abundant biogenic carbon source on Earth. Despite this, only a small clade of organisms known as white-rot fungi (WRF) can efficiently break down both the polysaccharide and lignin components of plant cell walls. This unique ability imparts a key role for WRF in global carbon cycling and highlights their potential utilization in diverse biotechnological applications. To date, research on WRF has primarily focused on their extracellular 'digestive enzymes' whereas knowledge of their intracellular metabolism remains underexplored. Systems biology is a powerful approach to elucidate biological processes in numerous organisms, including WRF. Thus, here we review systems biology methods applied to WRF to date, highlight observations related to their intracellular metabolism, and conduct comparative extracellular proteomic analyses to establish further correlations between WRF species, enzymes, and cultivation conditions. Lastly, we discuss biotechnological opportunities of WRF as well as challenges and future research directions.

INTRODUCTION

Carbon from terrestrial plants accounts for 450 gigatons of the total 550 gigatons of bio-based carbon on the planet, with the latter including all kingdoms of life (Bar-On et al., 2018). Most of the plant-derived mass resides in their cell walls, which primarily comprise two polysaccharides, cellulose and hemicellulose, and an aromatic polymer, lignin, with minor components such as pectin, suberin, and cutin. The dry weight concentration of each component varies between plant types but, in general, cellulose is the most abundant biopolymer (~50%), followed by lignin (~15–40%) and hemicellulose (~10–35%) (Chundawat et al., 2011; Martínez et al., 2005; Ragauskas et al., 2014). Cellulose is a homopolysaccharide consisting of β-1,4 linked D-glucose monomers whereas hemicellulose is a heteropolymer composed of pentose and hexose sugars, which are often acetylated. Lignin is a heterogeneous aromatic polymer which includes phenylpropanoid units that differ in their extent of ring methoxylation and are linked by a variety of C–C and C–O bonds, along with other phenolic compounds (e.g., tricin) that have been also recently described as lignin monomers (del Río et al., 2020). The structural complexity of lignin confers defense against pathogens and mechanical strength to the plant cell walls (Martínez et al., 2005). Although many bacteria and fungi are able to depolymerize cellulose and hemicellulose in nature, white-rot fungi (WRF) are the only clade of known organisms that have evolved to efficiently depolymerize and mineralize the lignin polymer to CO_2 and H_2O (Floudas et al., 2012).

WRF are saprotrophic organisms that are classified in the fungal kingdom, Dikarya subkingdom, Basidiomycota phylum, and Agaricomycotina subphylum (Figure 1A). This subphylum is divided into four classes and 18 orders (Grigoriev et al., 2011, 2014). WRF can be found within the orders Agaricales, Auriculariales, Hymenochaetales, and Russulales, among others (Figure 1A). However, most WRF belong to the order Polyporales (Martínez et al., 2018). All of these orders include a variety of fungi apart from WRF, some of which are also involved in the degradation of plant-derived biomass (lignocellulose), namely brown-rot fungi (BRF). WRF and BRF utilize different mechanisms to degrade lignocellulose (Riley et al., 2014; Schilling et al., 2020). WRF degrade all plant cell wall components via extracellular, enzymatic, and non-enzymatic (e.g., reactive oxygen species) mechanisms. Subsequently, WRF obtain carbon from sugars and, as recently suggested, from lignin-derived aromatic compounds (del Cerro et al., 2021). This type of degradation usually causes an enrichment in cellulose and generates a white decay residue (Martínez et al., 2005).

[1]Renewable Resources and Enabling Sciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA

[2]Advanced Energy Systems Graduate Program, Colorado School of Mines, Golden, CO 80401, USA

[3]These authors contributed equally

*Correspondence: davinia.salvachua@nrel.gov

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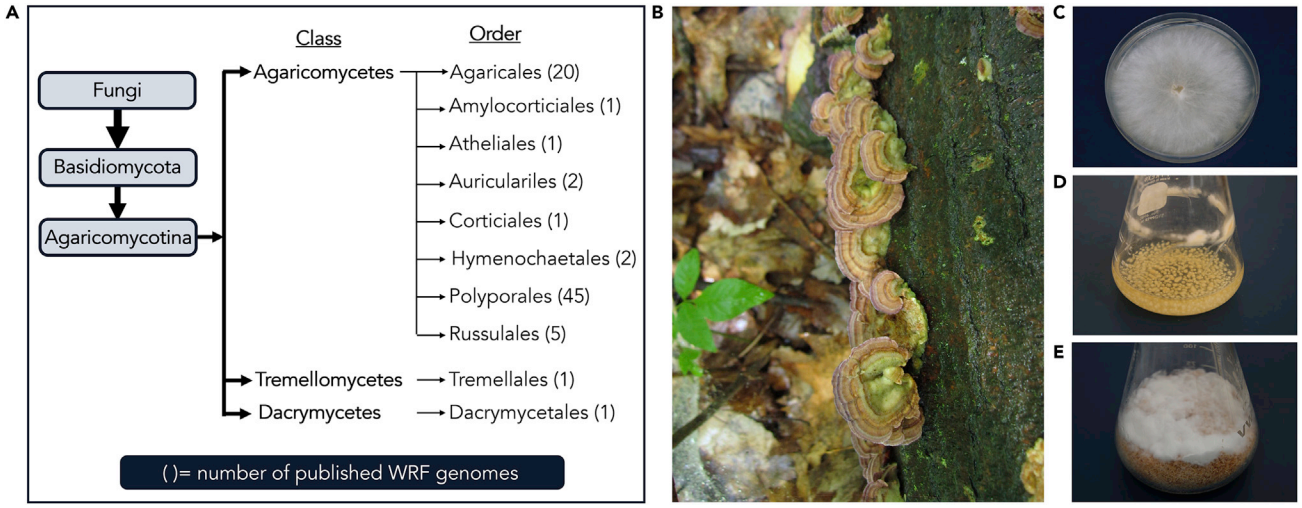

Figure 1. White-rot fungi (WRF) distribution in the fungal kingdom and examples of a WRF growing in different environments and experimental conditions

(A) Simplified diagram of the fungal kingdom with branches that include WRF with the number of published genomes in parentheses at the time of writing (the species are detailed in Table S1).

(B) Fruiting bodies of *Trametes versicolor* (commonly known as turkey tail) growing on woody biomass.

(C–E) Mycelia of *T. versicolor* growing (C) on a yeast extract-peptone-dextrose (YPD) agar plate, (D) in YPD broth under submerged-state and agitation cultivation conditions, and (E) on milled poplar in solid-state cultivation conditions (defined as cultivations in the absence of free water).

Conversely, BRF modify, but do not degrade, the lignin polymer via extracellular, non-enzymatic chemistries to increase the exposure of polysaccharides for successful enzymatic depolymerization and utilization of sugars as carbon sources. The enrichment of lignin results in a brown decay residue (Martínez et al., 2018; Schilling et al., 2020). Regarding the enzymatic mechanisms, carbohydrate active enzymes (CAZymes) are typically secreted by both WRF and BRF to cleave glycosidic bonds (Drula et al., 2022). However, WRF produce additional enzymes that belong to the class of 'auxiliary activities (AA)', which act in conjunction with CAZymes and are involved in redox processes and lignin breakdown, such as laccases (subfamily AA1_1) and class II heme-containing peroxidases (a subset of the AA2 subfamily) (Drula et al., 2022). WRF can be further divided into two groups based on the degradation patterns – those that selectively degrade lignin and those that degrade all lignocellulosic components simultaneously (Fernández-Fueyo et al., 2012).

Based on the unique features of WRF, these organisms and their extracellular enzymes have long been realized to exhibit promise in biotechnological applications involving, among others, conversion of both lignocellulose and lignin-rich substrates (Camarero et al., 2014). However, the full potential of WRF as biocatalysts for the conversion of carbohydrates and lignin-derived aromatic compounds to value-added fuel and chemical precursors – as commonly demonstrated in the metabolic engineering field for bacteria, yeasts, and filamentous ascomycetes (Lee et al., 2019) – is still uncertain. This is, in part, due to the lack of efficient genetic tools in WRF and the limited information about their intracellular metabolism. Indeed, neither a comprehensive metabolic map or carbon flux data have been described yet, in either native environments or in varied laboratory conditions, where fungi can display a diversity of morphologies (Figures 1B–1E). To that end, systems biology methods can substantially accelerate the development of biocatalysts via (1) the elucidation of metabolic pathways, regulatory mechanisms, and metabolic fluxes, (2) the discovery of enzymes, (3) the construction of metabolic networks, and (4) the generation of computational models to predict biological outputs.

Modern systems biology comprises a large number of methods (e.g., genomics, transcriptomics, proteomics, metabolomics, lipidomics, fluxomics, glycomics, epigenomics, etc.) (Veenstra, 2021), but to date, the main approaches utilized to study WRF have been primarily limited to genomics, transcriptomics, and extracellular proteomics. Very few systems biology studies of WRF have incorporated intracellular proteomics or metabolomics (Figure 2). Reviews or comparative-omic analyses that include WRF have specifically focused on genomics in basidiomycetes (Lundell et al., 2014), genomics in the order Agaricales

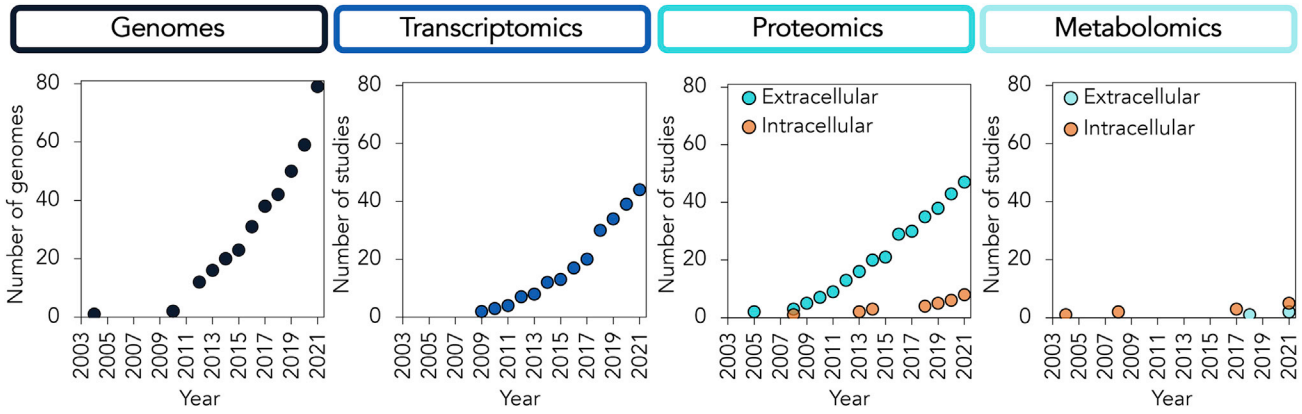

Figure 2. Cumulative number of WRF genomes published over time and transcriptomic, proteomic, and metabolomic studies of WRF conducted on lignocellulose or lignocellulosic-derived substrates

The references used to generate these graphs are shown in Tables S1 and S2.

(Ruiz-Dueñas et al., 2020), genomics in the order Polyporales (Hage et al., 2021a), transcriptomics in basidiomycetes (Peng et al., 2018), extracellular proteomics in basidiomycetes (Alfaro et al., 2014), and extracellular proteomics in lignocellulose-degrading bacteria and fungi (Guo et al., 2018). Here, we aim to review the breadth of systems biology studies conducted exclusively in WRF to date. First, we highlight observations that relate to both the extracellular and intracellular metabolism of WRF from a genomic, transcriptomic, and proteomic perspective. We note that most of the -omic studies in WRF utilize lignocellulose or lignocellulose-derived compounds in the cultivation media and thus, we will focus on these publications. However, it is worth mentioning that several-omic studies have been also conducted for other purposes in WRF (e.g., to describe the degradation of xenobiotic dyes (Sun et al., 2017) or interspecific fungal interactions (Luo et al., 2017)). This review does not include a metabolomics section because metabolomic analyses are emerging and are scarce in research with WRF (Daly et al., 2018; del Cerro et al., 2021; Matsuzaki et al., 2008; Miura et al., 2004). Second, within the proteomics section, we show a comparative analysis from over 90 independent extracellular proteomic datasets to establish correlations among fungal species, secreted enzymes, and cultivation conditions. Third, we provide an overview of the biotechnological opportunities of WRF in addition to lignocellulose bioconversion and lastly, we present a series of conclusions and perspectives related to future research in WRF.

CAN WE PREDICT LIGNOCELLULOSE DEGRADATION PHENOTYPES FROM WRF GENOMES?

The availability of WRF genomes has increased substantially in the last decade (Figure 2). Since the release of the *Phanerochaete chrysosporium* genome (Martínez et al., 2004), 79 WRF genomes have been published (Tables 1 and S1), and a total of 151 have been sequenced and included in MycoCosm – an online fungal genome portal operated by the United States Department of Energy Joint Genome Institute (Grigoriev et al., 2014). This increase has been due to extensive sequencing initiatives such as the 1000 Fungal Genomes Project (Grigoriev et al., 2011). This information has unveiled the size variance of WRF genomes (i.e., from ~8,000 genes in *Tremella mesenterica* to ~33,000 genes in *Fibulorhizoctonia psychrophile* (Table S1)) as compared to model bacteria (e.g., *Escherichia coli* has ~4,000 genes) or yeasts (e.g., *Saccharomyces cerevisiae* has ~6,000 genes). Five years after the publication of the *P. chrysosporium* genome, the genome of *Schizophyllum commune* was published (Ohm et al., 2010). Although this fungus was initially considered to be a WRF, its lignin-degrading capacity is limited (Zhu et al., 2016) and it lacks lignin-degrading peroxidases (Riley et al., 2014), which are defining features of WRF. In 2012, six more WRF genomes were published (Floudas et al., 2012). This study conducted one of the first comparative genomics and molecular clock analyses in WRF and found that the origin of lignin-degrading peroxidases coincided with the end of the Carboniferous period. In the same year, two more studies conducted comparative genome analyses between *P. chrysosporium* and *Ceriporiopsis subvermispora* (Fernández-Fueyo et al., 2012) and between *P. chrysosporium* and *Phanerochaete carnosa* (Suzuki et al., 2012). The former study began to illuminate the genotypic differences that produce the two lignocellulose degradation phenotypes in WRF (selective lignin degradation or simultaneous degradation of polysaccharides and lignin). The latter study

Table 1. WRF species with genomes currently published, categorized, and deposited in Mycocosm (as of December 2021; https://mycocosm.jgi.doe.gov/White_rot_fungi/White_rot_fungi.info.html)

Lineages (Class/Order)	Species	Systems Biology studies			Lineages (Class/Order)	Species	Systems Biology studies		
		T	P	M			T	P	M
DACRYMYCETES					Polyporales (Continuation)	*Dichomitus squalens*	x	x	x
Dacrymycetales	*Calocera viscosa*					*Earliella scabrosa*			
TREMELLOMYCETES						*Epithele typhae*			
Tremellales	*Tremella mesenterica*					*Fomes fomentarius*			
AGARICOMYCETES						*Ganoderma* spp.	x	x	
Amylocorticiales	*Plicaturopsis crispa*					*Hexagonia nitida*			
Atheliales	*Fibulorhizoctonia psychrophila*					*Irpex lacteus*	x	x	
Auriculariales	*Auricularia subglabra*		x			*Leiotrametes lactinea*	x		
	Exidia glandulosa					*Leiotrametes menziesii*			
Agaricales	*Armillaria cepistipes*	x	x			*Leiotrametes* sp.			
	Armillaria gallica					*Lentinus tigrinus*	x		
	Armillaria mellea	x	x			*Obba rivulosa*	x		
	Armillaria ostoyae	x	x			*Panus rudis*			
	Armillaria solidipes	x				*Phanerochaete carnosa*	x	x	
	Crepidotus variabilis					*Phanerochaete chrysosporium*	x	x	x
	Crucibulum laeve					*Phlebia brevispora*			
	Cyathus striatus					*Phlebia centrifuga*			
	Galerina marginata					*Phlebia radiata*	x	x	
	Gymnopilus junonius					*Phlebiopsis gigantea*	x	x	x
	Hypholoma sublateritium					*Polyporus arcularius*			
	Lentinula edodes	x	x			*Polyporus brumalis*	x	x	
	Oudemansiella mucida					*Polyporus squamosus*			
	Pleurotus eryngii	x	x			*Pycnoporus cinnabarinus*	x	x	
	Pleurotus ostreatus	x	x	x		*Pycnoporus coccineus*	x	x	
	Pluteus cervinus					*Pycnoporus puniceus*			
	*Schizophyllum commune**	x	x	x		*Pycnoporus sanguineus*	x	x	
Corticiales	*Punctularia strigosozonata*		x			*Rigidoporus microporus*	x	x	x
Hymenochaetales	*Fomitiporia mediterranea*		x			*Trametes betulina*			
	Schizopora paradoxa					*Trametes cingulata*			
Russulales	*Dentipellis* sp. KUC8613					*Trametes gibbosa*			
	Heterobasidion annosum	x				*Trametes ljubarskyi*	x	x	
	Peniophora spp.	x				*Trametes maxima*			

(*Continued on next page*)

Table 1. *Continued*

Lineages (Class/Order)	Species	Systems Biology studies T	P	M	Lineages (Class/Order)	Species	Systems Biology studies T	P	M
	Stereum hirsutum FP-91666		x			*Trametes meyenii*			
Polyporales	*Abortiporus biennis*	x				*Trametes polyzona*			
	Artolenzites elegans	x	x			*Trametes pubescens*			
	Bjerkandera adusta		x			*Trametes versicolor*	x	x	x
	Ceriporiopsis (Gelatoporia) subvermispora	x	x	x		*Trametopsis cervina*			

The Table is organized by fungal Class (in capital letters and bold) and fungal Order (bold). The 'Systems Biology' columns indicate if the species of WRF has been utilized for transcriptome (T), proteome (P) or metabolome (M) analyses. Note that we exclude studies that utilize transcriptomic sequences only as evidence for gene prediction and gene annotation, but not for differential gene expression analyses. **S. commune* is not currently classified as WRF.

correlated soft wood and hard wood degradation phenotypes with strain genotypes. Since 2012, numerous individual WRF genomes have been released, such as those from *Pycnoporus cinnabarinus* (Levasseur et al., 2014), *Lentinula edodes* (Chen et al., 2016), *Trametes pubescens* FBCC735 (Granchi et al., 2017), *Irpex lacteus* CD2 (Qin et al., 2018), *Phlebia centrifuga* (Mäkelä et al., 2018), and *Dichomitus squalens* (Casado López et al., 2019) (Table S1). Furthermore, comparative genome analyses have been conducted to explore the distribution of certain enzyme families (i.e., CAZymes) in fungal groups such as white-, brown-, and soft-rot fungi (Hori et al., 2013; Sista Kameshwar and Qin, 2018), to decipher the evolutionary origin of WRF (Nagy et al., 2016), and to describe the lignocellulose-degrading machinery in specific fungal clades (i.e., the Polyporales phleboid clade (Mäkinen et al., 2019)). Recently, two major comparative genome efforts have been also published and focused on the orders Agaricales (Ruiz-Dueñas et al., 2020) and Polyporales (Hage et al., 2021a), which analyzed 52 and 50 genomes, respectively, and released over 20 new WRF genomes in total. Based on comparative genomic data and evolutionary analyses in certain enzyme families, Ruiz-Dueñas et al. (2020) showed a detailed analysis of Agaricomycetes lifestyles and unraveled how Agaricales have developed specific sets of enzymes to degrade a variety of lignocellulosic sources. Hage et al. (2021a) studied the genomic basis of evolutionary and functional adaptations in Polyporales related to wood degradation and described a series of genome rearrangements as well as gene family expansion and contraction histories for various enzymes.

All publications mentioned above primarily focused on enzymes related to lignocellulose degradation. However, some studies also conducted additional analyses that are relevant to further understand the overall metabolism of WRF, which will be critical to develop WRF as biocatalysts for a variety of biotechnological applications. For instance, Suzuki et al. (2012) mapped central carbon metabolism from sugars and identified potential sugar transporters. Levasseur et al. (2014) searched for genes involved in protein secretion and glycosylation. Ohm et al. (2010) and Chen et al. (2016) pinpointed a series of transcription factors and regulatory mechanisms for the formation of fertile dikaryons. Mäkinen et al. (2019) reported a series of ABC transporters and genes involved in secondary metabolism such as polyketide synthases, adenylate-forming reductases, terpene cyclases, and AMP-dependent acyl-CoA synthetases, and both Mäkinen et al. (2019) and Hage et al. (2021a) introduced the importance and quantity of genes predicted to encode small-secreted proteins, such as hydrophobins. Overall, genomic studies have been the foundation to elucidate traits that differentiate WRF from other fungi. However, these datasets are not sufficient to predict lignin-degrading ability (Riley et al., 2014). Phenotypically, there is a spectrum of 'gray' options between BRF and WRF regarding the degradation of the different lignocellulosic components (Schilling et al., 2020). We propose that combining genomics with phenotypes during lignocellulose degradation remains critical to classify fungi. Furthermore, as described in the proteomics section below, complementing genomics with proteomics is also a more direct strategy to examine the actual degradative capabilities of WRF than using only genomics.

TOWARDS UNDERSTANDING INTRACELLULAR METABOLISM AND GENE REGULATION IN WRF VIA TRANSCRIPTOMICS

Often, the investigation of lignocellulose degradation by WRF via transcriptomics focuses largely on extracellular degradation processes. Within these studies, temporal dynamics of the regulation of gene-coding

CAZymes and ligninolytic enzymes have been broadly studied, leading to differentiation between WRF that simultaneously degrade lignin and polysaccharides and those that selectively degrade lignin (Fernández-Fueyo et al., 2012; Kowalczyk et al., 2019; Kuuskeri et al., 2016; Miyauchi et al., 2017, 2018; Qin et al., 2018; Zhou et al., 2018). Transcriptomic analyses in WRF have steadily increased over time (Figure 2). However, a wealth of information related to the intracellular metabolism of WRF is ready to be discovered using this methodology. Due to the limitation of studies focusing on intracellular metabolism and because recent reviews have included the expression of extracellular gene-coding CAZymes and ligninolytic enzymes in WRF (deVries and Mäkelä, 2020; Hage and Rosso, 2021; Okal et al., 2020; Zhang and Yamaura, 2020), this review will highlight the major findings from transcriptomic analyses related to regulatory mechanisms of central carbon metabolism, detoxification mechanisms, and transcription factors during lignocellulose utilization by WRF.

Central carbon metabolism

Differential transcriptomic analyses have revealed that central metabolism is complexly regulated by the source and composition of substrates. For example, genes involved in glycolysis and those in the pentose phosphate pathway (PPP) were upregulated compared to those that funnel pentoses to the PPP (Marinović et al., 2018). This was shown in cultivations of *Obba rivulosa* grown on solid spruce wood relative to liquid low nitrogen-asparagine-succinate media, and this result is presumably due to a preference for hexoses over pentoses. The glyoxylate shunt, which is an alternative route within the tricarboxylic acid (TCA) cycle that uses cytosolic or peroxisomal acetyl-CoA to create oxaloacetate, has been also reported to be upregulated during lignocellulose utilization by WRF (Hori et al., 2014b; Iwata et al., 2021; Jurak et al., 2018; Marinović et al., 2018; Qin et al., 2018). The acetyl-CoA funneled to the glyoxylate shunt is typically derived from the β-oxidation pathway, as described in *Phlebiopsis gigantea* (Hori et al., 2014b; Iwata et al., 2021); however, in *Phlebia radiata*, it is thought to come from the PPP (Mattila et al., 2020). Glyoxal oxidase (GLOX) produces glyoxylate with hydrogen peroxide as a by-product (Figure 3); it is simultaneously upregulated alongside extracellular peroxidases in (1) *P. chrysosporium* in nitrogen and carbon-limited media relative to complete media, (2) *P. carnosa* on spruce and aspen compared to rich (yeast malt peptone glucose) media, and (3) *I. lacteus* CD2 on corn stover compared to glucose (Jurak et al., 2018; Qin et al., 2018; Vanden Wymelenberg et al., 2009). Hydrogen peroxide is a necessary cofactor for peroxidases, which highlights and reinforces a close relationship between central carbon metabolism and lignin degradation. Oxaloacetase (OXA), an enzyme that generates oxalate from oxaloacetate (Figure 3), was upregulated in *O. rivulosa* when cultivated on solid spruce wood compared to low nitrogen-asparagine-succinate media (Marinović et al., 2018). Oxalate production by *O. rivulosa* was previously demonstrated in cultivations containing spruce wood chips (Hakala et al., 2005). Apart from chelating manganese, oxalate acidifies the extracellular fungal environment, which further enhances lignin degradation, as most laccases and manganese peroxidases (MnPs) require acidic conditions for optimal activity. In addition, formation of oxalate from glyoxylate also generates acetate. Acetate regeneration to acetyl-CoA would reinforce the glyoxylate shunt. Glyoxylate and oxalate are known manganese chelators and have been shown to positively affect the activity of ligninolytic enzymes (Urzúa et al., 1998) and, as discussed above, other glyoxylate cycle by-products generate favorable conditions and cofactors for biomass degrading enzymes. Based on these observations, the expression of non-ligninolytic enzymes that favor degradative cultivation conditions should be considered when engineering microbial systems for efficient lignocellulose degradation.

Transcription factors

Identifying transcription factors in WRF is essential to understand and control gene regulation for further genetic engineering efforts. Though the development of efficient genetic tools in WRF is still in its infancy, a series of knockout strains from *Pleurotus ostreatus* have been recently built to probe the role of putative transcription factors in lignin degradation more precisely (Okuda et al., 2021; Wu et al., 2020). Specifically, two putative transcription factors, *wrt1* and *gat*-1, and one chromatin remodeler, *chd1*, were knocked out or mutated (Wu et al., 2020). In these three strains, genes encoding ligninolytic enzymes and other oxidoreductases (i.e., AA2 and AA5 CAZyme families) and genes with roles in polysaccharide hydrolysis and oxidative reaction processes (i.e. AA9 and GHs families) were downregulated compared to the corresponding parental strains. In addition, the *gat-1* and *chd1*mutants upregulated cellulolytic and xylanolytic genes, whereas pectinolytic genes were downregulated. Together, these results indicate that the three putative transcription factors may have a role in regulating both ligninolytic and cellulolytic enzymes. Co-expression data revealed another zinc-finger transcription factor that might also play a role in regulation. A follow-up study implies a different mechanism involving a putative mitogen-activated protein kinase (MAPK) cascade (Okuda et al., 2021). Disruption of the MAPK cascade – whether through deletion of a gene encoding for a protein kinase, *rho1b*, or its downstream acting kinase,

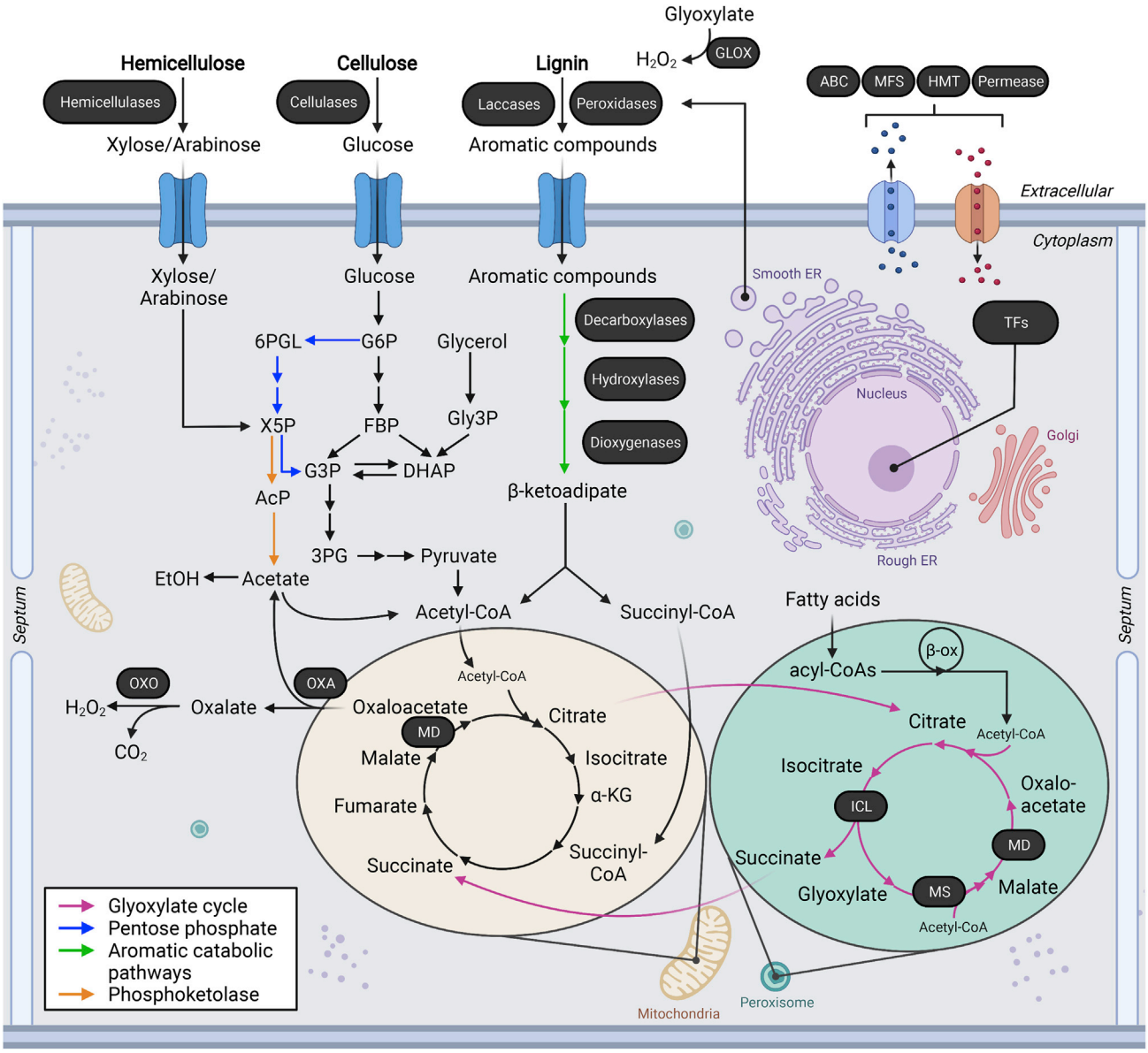

Figure 3. Summary of proposed intracellular catabolic pathways during lignocellulose degradation in WRF

Extracellular cellulose-, hemicellulose-, and lignin-degrading enzymes break down biopolymers from lignocellulose into low molecular weight products (e.g., glucose, xylose, aromatic compounds) which are subsequently funneled to the tricarboxylic acid and glyoxylate shunt pathways. Solid arrows represent enzymatic reactions and faded arrows represent transport in or out of organelles. Key proteins and enzymes highlighted in this review are shown in dark gray ovals. Metabolic compound abbreviations: 3PG, 3-phosphoglycerate; 6PGL, 6-phosphogluconolactone; AcP, acetyl phosphate; DHAP, dihydroxyacetone phosphate; EtOH, ethanol; FBP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose-6-phosphate; GLOX, glyoxal oxidase; X5P, xylulose-5-phosphate; Gly3P, glycerol-3-phosphate. Enzyme and protein abbreviations: ABC, ATP-binding cassette efflux transporters; HMT, heavy metal translocation protein; ICL, isocitrate lyase; MD, malate dehydrogenase; MFS, major facilitator superfamily; MS, malate synthase; OXA, oxaloacetase; OXO, oxalate oxidase; TFs, transcription factors. Figure created with BioRender.com.

mpk1 – resulted in disrupted upregulation of several CAZyme-encoding genes in the AA9 or GH7 families compared to parental strains grown in the same conditions or compared to a *rho1b*-overexpressing strain. The role of the MAPK cascade in oligosaccharide degradation remains vague; however, this study points to the importance of intracellular signaling for gene regulation of enzymes that degrade lignin and carbohydrate containing biopolymers. Similarly, solid-state cultivation on wheat straw induced 14 genes encoding for protein kinases and 5 putative transcription factors in *Polyporus brumalis* relative to liquid culture on malt extract (Miyauchi et al., 2018). Changing environmental conditions is another strategy to identify key transcription

factors. For instance, *P. radiata* may use a transcription factor called Adr1 to control primary carbon catabolism in hypoxic conditions relative to fully aerobic conditions (Mattila et al., 2020). Adr1 binding motifs were found in promoters of genes in the xylose reductive, pentose phosphate, phosphoketolase, glyoxylate shunt, and glycerol catabolic pathways (Figure 3). It has been proposed that the shift in primary metabolism balances energy molecules and cofactors. Understanding the regulation of central carbon metabolism in WRF will be crucial to maximize carbon flux to targeted products if WRF can be used as biocatalysts to make products derived from lignocellulosic sugars.

Detoxification mechanisms

Genes encoding for lignocellulose-degrading enzymes are not the sole contributor to the efficient utilization of lignocellulose by WRF. Genes related to detoxification are essential because lignin degradation processes produce free radical-containing compounds, peroxides, low molecular weight aromatic compounds, and extractives (i.e., terpenoids, resins, and tannins) that may be toxic to the organisms. Common mechanisms to detoxify lignin-derived aromatic compounds include transporter and permease activity, upregulation of signal transduction pathways, oxidative stress response, and catabolic processes related to degrading toxic compounds (Hori et al., 2014b; Iwata et al., 2021; Miyauchi et al., 2017, 2018, 2020a; Thuillier et al., 2014). Free radicals and peroxides produced from ligninolytic enzymes can trigger oxidative stress. Glutathione accumulation is a common symptom of oxidative stress, and increased cytochrome P450, glutathione S-transferase (GST), and glutathione reductase expression are all indicative of antioxidant responses. GSTs from class GTT2 were upregulated in *P. chrysosporium*, *Pycnoporus sanguineus*, and *P. cinnabarinus* relative to control conditions (Miyauchi et al., 2020b; Thuillier et al., 2014). In a comparative genomics study across all available genomes in MycoCosm, all fungi reported to grow on hardwood harbored a GTT2.1 isoform (Miyauchi et al., 2020b). Other GSTs were also upregulated in *P. gigantea* (Hori et al., 2014b; Iwata et al., 2021), *P. chrysosporium* (Thuillier et al., 2014), *P. brumalis* (Miyauchi et al., 2018), *P. sanguineus*, *P. cinnabarinus* and *Pycnoporus coccineus* (Miyauchi et al., 2020a) in the presence of lignocellulosic biomass-derived media compared to control conditions, and a GST was upregulated in *Ganoderma lucidum* MDU-7 cells in response to copper induction (Jain et al., 2020). Glutathione reductase upregulation was only seen in *P. chrysosporium* grown in acetone extractives from oak relative to minimal media without extractives, although this could be due to the concentration of extractives supplemented in the media compared to a natural biomass-derived substrate (Thuillier et al., 2014). Many WRF grown on lignocellulosic biomass show upregulation of various cytochromes P450, which are diverse in their function; however, there are several families that were upregulated across various species. The most commonly upregulated families of cytochromes P450 seemed to address toxicity by degrading xenobiotics through oxidation of plant compounds (i.e., flavins and flavonoids) and lipophilic lignin groups (Hori et al., 2014b; Iwata et al., 2021; Jurak et al., 2018; Miyauchi et al., 2017, 2018, 2020a; Thuillier et al., 2014).

Transporters play a key role in removing toxic compounds from the intracellular environment. In *P. gigantea* grown in pine extractives, an ATP binding cassette (ABC) efflux transporter that may contribute to the tolerance to terpenes was upregulated up to 20-fold relative to control conditions in two separate studies (Hori et al., 2014b; Iwata et al., 2021). In *P. chrysosporium* grown in acetone extractives from oak, transporters belonging to the major facilitator superfamily (MFS) were upregulated up to 25-fold relative to minimal media without extractives (Thuillier et al., 2014). Similar upregulation was shown in *P. coccineus* grown on solid cereal straw, softwood, and hardwood relative to liquid maltose media (Miyauchi et al., 2017). *P. brumalis* grown on solid wheat straw showed high constitutive expression of 14 permease and MFS transporters compared to cultures grown in liquid malt extract (Miyauchi et al., 2018). Heavy metals such as copper and iron also require transport out of the cell; simultaneously, laccases and peroxidases use them as cofactors. *G. lucidum* MDU-7 cells induced with copper ions show induction of heavy metal translocation (HMT) protein, ABC transporters, and a zinc transporter (Jain et al., 2020). The authors also propose an increased cell membrane permeability because of the downregulation of hydrophobin proteins, causing a higher secretion of laccases and peroxidases. In doing so, these proteins could synergistically utilize extracellular copper ions and ultimately enhance biomass degradation.

SPATIOTEMPORAL METABOLISM OF WRF FROM A PROTEOMICS PERSPECTIVE

Extracellular proteomic analyses

The development of advanced analytical techniques has accelerated the growth of proteomic studies in WRF in the last decade (Figure 2). The methodology utilized in initial proteomic analyses of WRF involved collecting extracellular proteins from the culture media, separating them by one-dimensional (1D) or

two-dimensional (2D) gel electrophoresis, digesting protein bands or spots from the gels, and identifying peptides through mass spectrometry and proteins via sequence homology searches (Alfaro et al., 2014). Due to challenges in protein separation, this approach only identified dozens of proteins per sample (Abbas et al., 2005; Ravalason et al., 2008; Salvachúa et al., 2013a; Vasina et al., 2016; Xiao et al., 2017, 2019; Zorn et al., 2005). The utilization of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for shotgun or bottom-up proteomic studies – which aim to identify proteins from the overall pool of peptides in one sample – as well as the recent release of many WRF genomes, has increased the throughput of proteomic analyses and expanded the number of identified proteins to hundreds per sample (del Cerro et al., 2021; Fernández-Fueyo et al., 2012; Fernández-Fueyo et al., 2016; Floudas et al., 2012; Miyauchi et al., 2020a; Miyauchi et al., 2017; Xie et al., 2021). Other modern proteomic approaches include quantitative and top-down proteomics – which provide additional information on exact protein abundances and post-translational modifications, respectively (Toby et al., 2016) – and will further expand the breadth of proteomic research in WRF (Alfaro et al., 2014). However, to our knowledge, the latter techniques have not been yet applied to research with WRF. This proteomics section focuses on bottom-up proteomic studies that aim to understand the diversity, temporal dynamics, and effect of abiotic factors on the secretion of lignocellulose-degrading enzymes by WRF.

Since the first profiling of the extracellular proteome (hereafter referred to as the secretome) in *P. chrysosporium* in 2005 (Abbas et al., 2005), the majority of the proteomic studies in WRF have aimed to describe the enzymatic arsenals released by individual fungi while growing on lignocellulosic substrates. Manavalan et al. (2011) observed an association between the production of certain enzyme families and the type of substrate in *P. chrysosporium* cultivations. Specifically, cellulases, hemicellulases, and pectinases were abundant in cellulose-containing substrates whereas the production of oxidoreductases (i.e., copper radical oxidase, quinone oxidoreductase, GST, and aryl alcohol oxidase) was enhanced in lignin-containing substrates. Xiao et al. (2019) also observed different enzymatic profiles in *P. ostreatus* BP2 depending on the substrate. Ligninolytic enzymes were found abundantly in cultivations containing sawdust (hardwood), whereas CAZymes were more abundant in corncob and cottonseed hull (non-woody plants). Conversely, Couturier et al. (2015) revealed that the production of certain enzymes is independent of the substrate. For example, MnPs and glucose-methanol-choline (GMC) oxidoreductases were commonly secreted by *P. coccineus* when cultivated on both pine (softwood) and aspen (hardwood) and laccases and peroxidases were common in *P. ostreatus* secretomes when cultivated on poplar (hardwood) and wheat straw (non-woody) (Fernández-Fueyo et al., 2016). Rytioja et al. (2017) also observed that the abundance of ligninolytic enzymes secreted by *D. squalens* was not significantly different between hardwood, softwood, and non-woody substrates (Rytioja et al., 2017). However, the abundance of oxidoreductases, such as cytochromes P450, and lytic polysaccharide monooxygenases (LPMOs) was different under various types of lignocellulosic substrates (hardwood, softwood, and non-woody) in *Pycnoporus* spp. cultivations (Couturier et al., 2015; Levasseur et al., 2014). Another interesting observation was that both *Pycnoporus* spp. (Miyauchi et al., 2017, 2020a) and *P. ostreatus* PC9 (Fernández-Fueyo et al., 2016) produced a higher number of exclusively secreted enzymes in non-woody substrates compared to hardwoods, which is notable considering the relatively higher lignin content and recalcitrance of woody substrates.

In the section above, we described studies that conduct proteomic analysis in a single white-rot fungus. However, comparative analysis of a diversity of WRF secretomes within the same study is a powerful approach to avoid experimental and technical variability and establish direct correlations among organisms and/or cultivations conditions. For instance, Xie et al. (2021) showed a correlation between WRF species and lignocellulose degradation patterns and showed that two selective lignin-degrading WRF, *P. ostreatus* and *Pleurotus eryngii*, secrete higher abundances of ligninolytic and lower abundances of cellulolytic enzymes compared to *P. chrysosporium* and *I. lacteus,* WRF that simultaneously degrade lignocellulose, in ramie stalks-containing cultivations. Some studies also investigate the conservation of secreted enzymes among organisms, either focusing on the overall enzyme machinery or specific enzymes. For instance, Miyauchi et al. (2020a) revealed that the enzyme pools released for the degradation of cellulose, hemicellulose, and pectin were conserved in three different *Pycnoporus* spp. growing on cellulose, wheat straw, and aspen, whereas Machado et al. (2020) showed that the specific production of orthologous enzymes of cellobiohydrolases Cel7C and Cel7D in *P. chrysosporium* and *Trametes versicolor* was conserved in cellulose-containing cultivations.

Temporal dynamics of the lignocellulolytic enzyme repertoire is another common theme of secretome studies in WRF. In the selective lignin-degrading WRF *C. subvermispora*, MnPs and aryl-alcohol oxidases were detected in aspen wood cultures at 3 days, whereas CAZymes for cellulose and xylan degradation

were detected at 5 and 7 days (Hori et al., 2014a). The detection of enzymes involved in lignin degradation was as early as 7 days in *P. radiata*, which was cultured on spruce wood for 6 weeks (Kuuskeri et al., 2016). Lignin peroxidases and lignin-modifying oxidoreductases were detected at 7 days in cultivations of *P. coccineus* on various lignocellulose substrates (aspen, pine and wheat straw) (Miyauchi et al., 2017) and at 10 and 15 days in cultivations of *P. brumalis* on wheat straw (Miyauchi et al., 2018). Peña et al. (2021) further showed that *P. eryngii* – another selective lignin-degrading WRF – produced an increased number of secreted enzymes over time and a higher abundance of LPMOs and MnPs at 14 and 43 days compared to earlier cultivation stages (6 days) on wheat straw whereas a higher abundance of hydrogen peroxide-producing aryl-alcohol oxidases was found at 6 days compared to 14 and 43 days. In *D. squalens*, secretomic profiles from cultures containing woody substrates (aspen and spruce) incorporated a higher number of secreted enzymes at 16 days than at 9 days, whereas there was no major change in cultures growing on non-woody substrates (Rytioja et al., 2017). Presley et al. (2018) conducted a unique secretomic study by matching degradation zones in aspen wood wafers with different WRF growth stages. This study revealed a delayed production of GHs and pectinases in two WRF, *T. versicolor* and *Stereum hirsutum*, compared to two BRF, *Postia placenta* and *G. trabeum*. The presence of MnPs in the secretome of older mycelia of *S. hirsutum* indicated that its ligninolytic activity was also delayed. In contrast, *T. versicolor* did not demonstrate delayed activity and MnPs, dye-colorizing peroxidases (DyPs), and laccases were detected in all early degrading zones. Collectively, these studies indicate that temporal dynamics of WRF secretomes depend on the WRF species and type of substrates.

Recent secretomic studies have also investigated the effect of various abiotic factors (i.e., metal traces, temperature, pH, free water content) and genetic regulation on the composition of the lignocellulolytic machineries. Vasina et al. (2016) tested the effect of copper concentration on the production of extracellular enzymes by *Trametes hirsuta*. Their enzyme assays showed that higher copper concentrations increased laccase and aryl-alcohol oxidase activities and decreased LiP and versatile peroxidase activities. They also found more isoforms of laccases under copper-supplemented media compared to regular media. Small changes in temperature (20 °C versus 24 °C) were demonstrated to cause major differences in secretomic profiles related to overall lignocellulose degradation, sugar utilization, and xenobiotic detoxification in *Bjerkandera adusta* (Moody et al., 2018). Fernández-Fueyo et al. (2014) combined intra and extracellular proteomics and transcriptomics of *P. ostreatus* to better understand the effect of temperature and pH on the production and regulation of lignin-degrading peroxidases. They found that a pH of 5.5 and a temperature of 25 °C was the optimal condition for the expression of peroxidases, though temperature exhibited less of an influence relative to pH (Fernández-Fueyo et al., 2014). In general, non-optimal cultivation conditions (i.e., temperatures of 37 °C and 10 °C and pHs of 8 and 3) caused a decreased expression of peroxidase genes. Liu et al. (2020) further investigated the effect of water content on the secretomic profiles of *P. chrysosporium*. They detected higher relative abundances of cellulases and hemicellulases in solid-state cultivations compared to submerged-state cultivations, and enzymes with carbohydrate-binding modules (CBMs) were more abundant in submerged-state cultivations, providing an advantage for polysaccharide hydrolysis at lower substrate loadings. Regarding the genetic regulation of the secretome, Yoav et al. (2018) showed that the genetic manipulation (either knockout or overexpression) of the transcriptional regulator gene *CRE1* affects the secretion of cellulose specific CAZymes. Daly et al. (2019) identified catabolite repression of CAZymes in *D. squalens* in both transcriptomic and secretomic studies when glucose was added in polysaccharide-containing media. Alfaro et al. (2020) also revealed catabolite repression of CAZymes in *P. ostreatus* via differential proteomic analyses, specifically when monomeric glucose was supplemented on a woody substrate. However, secretomic profiles of *P. ostreatus* were not different among ploidy statuses (Alfaro et al., 2020). Overall, the secretome studies described above highlight that the enzymatic machinery released by each WRF is highly specific, dynamic, and environment dependent. This implies that predicting the composition of WRF enzyme cocktails remains a challenge.

Intracellular proteomic analyses

Secretomic studies have been the preferred type of proteomic analyses utilized to understand the performance of WRF during lignocellulose degradation, and very few studies to date have focused on the intracellular proteome (Figure 2). Intracellular proteomics is not only critical to better understand the overall metabolism of WRF, but also to validate observations from transcriptomic analyses. For instance, Xiao et al. (2017) found that intracellular proteins with functional classifications in carbohydrate and energy metabolism (including enzymes from central carbon metabolism) are less abundant in *P. ostreatus* cultures growing on minimal media supplemented with lignin than in cultures containing media supplemented

with xylan or carboxymethyl cellulose. Further, Xiao et al. (2019) found that the level of enzymes involved in glucose metabolism are higher when growing *P. ostreatus* on cottonseed hull and corncob (polysaccharide-rich substrate) compared to hardwood sawdust (lignin-rich substrate). As expected, these results indicate how intracellular responses also depend on the substrate. Regarding the modification and catabolism of lignin-derived aromatic compounds, Matsuzuki et al. conducted a differential proteomic study in *P. chrysosporium* in cultivations with and without benzoic acid (Matsuzaki et al., 2008). In benzoic acid-containing cultivations, cells produced a higher protein abundance of aryl-alcohol oxidases, aryl-aldehyde dehydrogenases, and cytochromes P450, which collectively suggest a potential biochemical conversion of this aromatic compound. Recently, intracellular proteomic studies were also conducted in *T. versicolor* and *C. subvermispora* to identify enzymes (e.g., decarboxylases, hydroxylases, and dioxygenases) involved in the catabolism of lignin-derived aromatic compounds in cultivations containing 4-hydroxybenzoic acid, syringic acid, lignin isolated from poplar, and poplar chips (del Cerro et al., 2021). This proteomics study was coupled to ^{13}C-isotopic labeling, transcriptomics, and metabolomics analyses to demonstrate that WRF utilize lignin-derived aromatic compounds as carbon sources and to initiate the elucidation of novel aromatic catabolic pathways in these organisms.

COMPARATIVE ANALYSES OF WRF EXTRACELLULAR PROTEOMES: FINDINGS AND CHALLENGES

Based on the diversity of WRF secretomes, we concluded that predicting secretome composition is currently a difficult task. Comparative -omic analyses may be a useful approach to make stronger conclusions on enzyme cocktail composition by establishing correlations among different variables (e.g., fungal species, enzyme families, growth media composition) and/or by identifying secreted enzymes that are conserved among species. This information can be essential for biotechnological applications that aim to produce targeted enzyme cocktails or discover new enzyme functions. A comparative analysis across a variety of published transcriptomic studies has been recently conducted for basidiomycetes (Peng et al., 2018); however, to the best of our knowledge, the integration of multiple proteomic studies has not been yet carried out. In this review, we therefore conducted an additional analysis to identify trends of secreted enzymes across different types of substrates and WRF. We collected 92 secretomic datasets from 31 publications and spanning 19 WRF species, all of which have published genomes (Couturier et al., 2015; del Cerro et al., 2021; Fernández-Fueyo et al., 2012; Fernández-Fueyo et al., 2016; Floudas et al., 2012; Hori et al., 2014a; Hori et al., 2014b; Iwata et al., 2021; Kuuskeri et al., 2016; Levasseur et al., 2014; Liu et al., 2020; Machado et al., 2020; Mahajan and Master, 2010; Manavalan et al., 2011; Miyauchi et al., 2020a; Miyauchi et al., 2017; Miyauchi et al., 2018; Moody et al., 2018; Peña et al., 2021; Presley et al., 2018; Ravalason et al., 2008; Rytioja et al., 2017; Salvachúa et al., 2016; Salvachúa et al., 2013a; Vanden Wymelenberg et al., 2009; Vanden Wymelenberg et al., 2010; Vanden Wymelenberg et al., 2011; Xiao et al., 2019; Xie et al., 2021; Yoav et al., 2018; Zhu et al., 2016). Metadata for each dataset can be found in Table S3, and detailed analytical pipelines can be found in Transparent Methods Supplemental File. First, we determined whether there is any difference in secreted CAZymes across different substrates: cellulose, lignin, hardwood, softwood, and non-woody substrates. For each of the analyzed secretomic datasets analyzed, we examined a list of secreted CAZyme families that are considered as plant cell wall-degrading enzymes based on a previous study (Miyauchi et al., 2020b; Table S4). We then calculated the prevalence of each CAZyme family for each substrate type (calculated as the coefficient between the number of datasets in which the family is present divided by the total number of datasets for a specific substrate). We used the calculated prevalence coefficients to construct a heatmap (Figure 4). Based on the clustering patterns, there are distinct CAZyme prevalence profiles between single macromolecular substrates (cellulose and lignin) and plant biomass substrates (softwood, hardwood, and non-woody). Cultivations containing cellulose as the sole carbon source have the least overall prevalence of secreted CAZymes. Some CAZyme families are universally detected in secretomes regardless of substrate types (i.e., overall prevalence more than 75%): GH5_5, GH7, GH51, AA3, and AA5_1, as well as some accessory domains covalently attached to CAZymes, such as CBMs from Family 1, denoted CBM1. Some others are rarely detected such as GH11, GH62, polysaccharide lyase (PL) PL3, PL14_4, AA14 and CBM67. In addition, several CAZyme families are more prevalent in certain substrates: GH43, GH44, GH74, GH131, CE8 and PL4 in softwood; AA1_1 in hardwood; GH10 and GH88 in hardwood and non-woody; GH28 in hardwood and softwood; GH5_22 in softwood and non-woody. Lastly, CAZyme families for laccases (AA1_1), lignin-modifying peroxidases (AA2), and copper radical oxidases (AA5_1) are the least represented in cellulose substrates (Figure 4), suggesting a specificity of these families on lignin-containing substrates. Considering the prevalence patterns from our analyses, these listed CAZymes would be interesting candidates for designing enzyme cocktails to increase lignocellulose degradation in specific types of substrates.

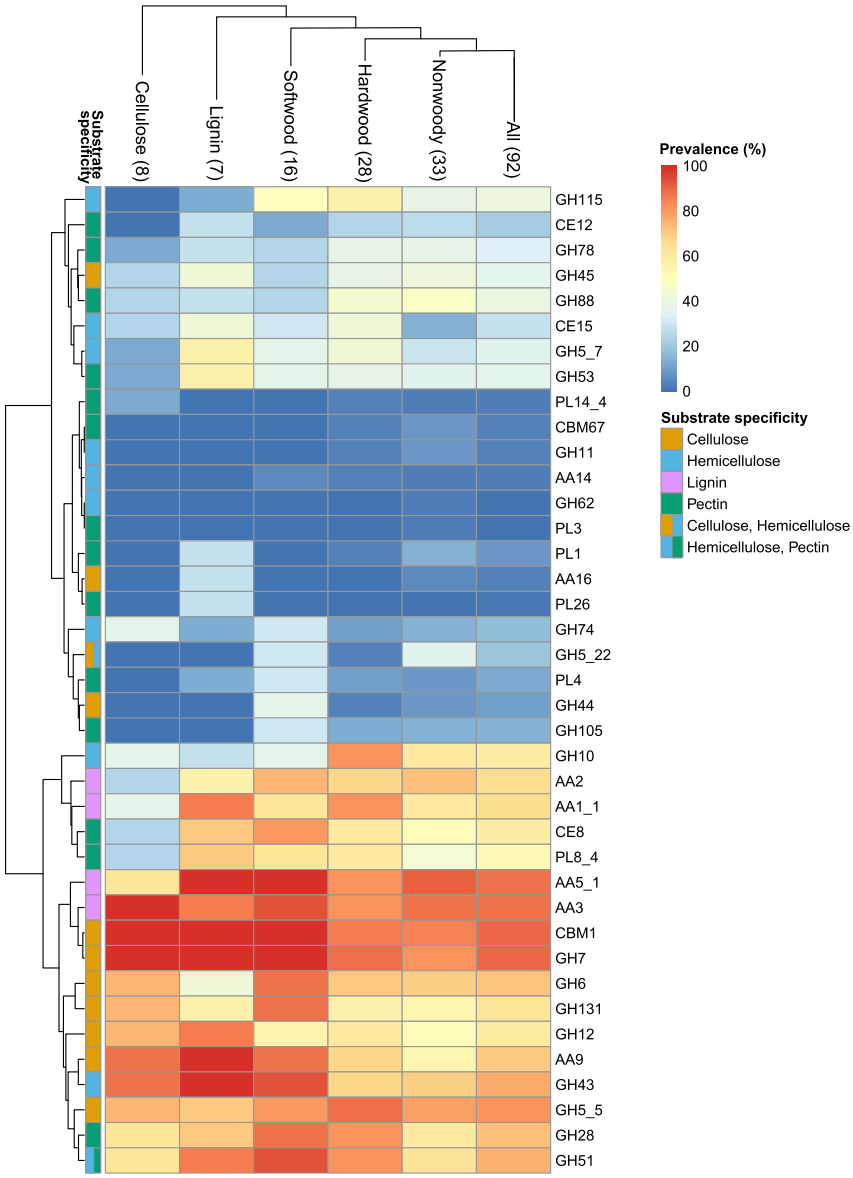

Figure 4. Prevalence of detected CAZymes families in WRF secretomes from cultivations in different substrates

The presence of CAZyme families was examined in 92 secretomic datasets from 19 WRF. Colors in each cell indicate the prevalence of a CAZyme family detected in secretomes for each substrate type. Color keys in the leftmost column indicate substrate specificity of CAZyme families, which has been retrieved from a recent publication (Miyauchi et al., 2020b). Values in parentheses on the top indicate the number of examined datasets for each substrate type and 'All' includes the datasets containing all the substrates. Euclidean distance method was used for clustering. A full list of secreted CAZyme families in each dataset can be found in Table S4. Detailed information about the examined datasets can be found in Table S3.

Because of the limited datasets belonging to individual species, we did not find any association between CAZyme presence, substrate types, and fungal species. In addition, secretomic profiles were more dependent on protein detection and identification methods from different studies than the substrate type (Figures S1–S11). Thus, we took a different approach and pooled all the datasets from each species to examine how many plant cell wall-degrading CAZymes were detected in the secretomes relative to the predicted CAZymes-expressing genes in their respective genomes (Figure 5, Tables S5 and S6). For this analysis, we only selected species that had at least three datasets, all of which included at least 50 detected proteins to have a good coverage of protein detection and identification (Table S3). This analysis results

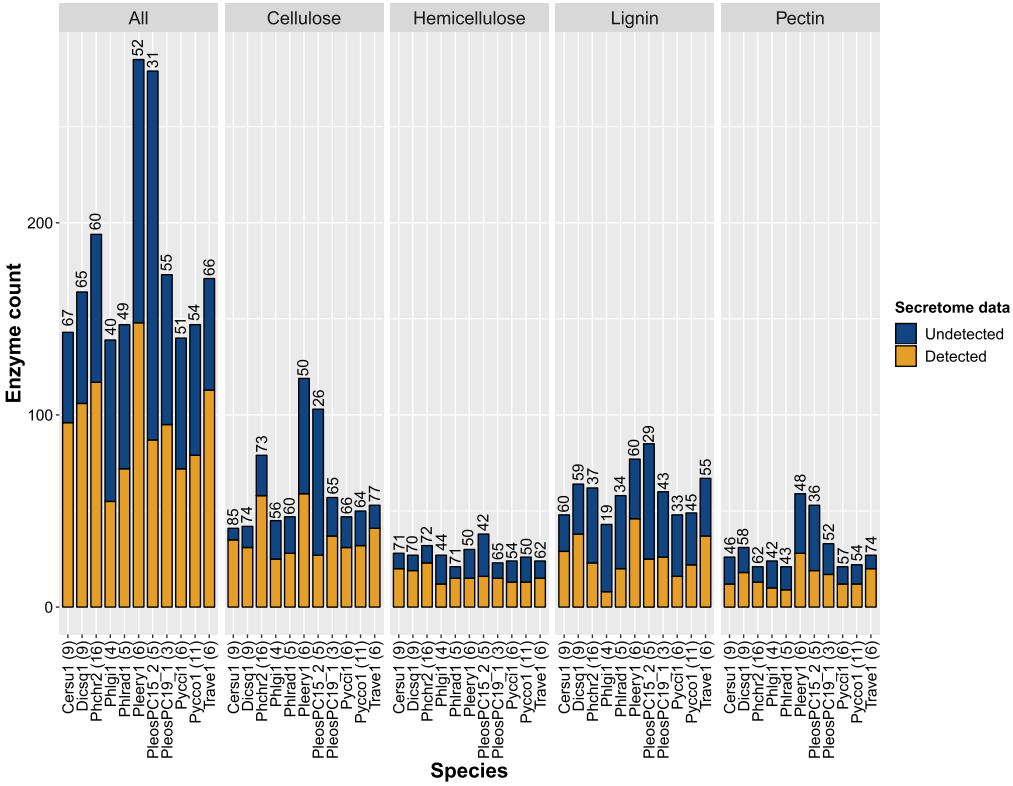

Figure 5. Number of detected and undetected CAZymes in WRF secretomes based on predicted CAZymes in the reference genomes

To have a reasonable coverage of secreted proteins for these analyses, we selected fungal species that have at least three corresponding datasets, with each dataset containing at least 50 detected proteins. This results in 80 secretomic datasets from 11 WRF. CAZyme counts were based on substrate specificity of CAZyme families: cellulose, hemicellulose, pectin, lignin, and all substrates. Numbers on bar plots indicate the proportion of detected CAZymes compared to total predicted genes. Values in parentheses on the X-axis indicate numbers of examined datasets for each species. The full list of enzyme counts for each CAZyme family can be found in Tables S3 and S4. Abbreviations: Cersu1, *Ceriporiopsis (Gelatoporia) subvermispora*; Dicsq1, *Dichomitus squalens*; Phchr2, *Phanerochaete chrysosporium*; Phlgi1, *Phlebiopsis gigantea*; Phlrad1, *Phlebia radiata*; Pleery1, *Pleurotus eryngii*; PleosPC15_2, *Pleurotus ostreatus* PC15; PleosPC9_1, *Pleurotus ostreatus* PC9; Pycci1, *Pycnoporus cinnabarinus*; Pycco1, *Pycnoporus coccineus*; Trave1, *Trametes versicolor*.

in 80 datasets which encompass 11 species. We found that less than three-quarters of predicted CAZymes were detected in most of the WRF secretomes. *T. versicolor* and *C. subvermispora* were the species with the highest number of detected CAZymes in the secretomes at 67 and 66%, respectively. Among these, CAZymes for cellulose and hemicellulose degradation were generally more prevalent than CAZymes for pectin and lignin degradation. The proportion of known secreted CAZymes may increase with increasing available datasets. However, it is worth noting that *P. chrysosporium* and *P. coccineus*, which contain 16 and 11 datasets in the analyses, respectively, still have fewer proportions of CAZymes than *T. versicolor* (6 datasets) and *C. subvermispora* (9 datasets). Considering this, we suggest that CAZymes gene count from genomic analyses may not be an adequate proxy to determine capabilities for lignocellulosic degradation. This is because the gene count may not be directly translated to the number of functional enzymes due to improper gene annotation, the presence of pseudogenes, and gene silencing. Numbers of CAZymes that have been characterized in biochemical studies and detected in secretomes can be an alternative for comparison.

Besides CAZyme family analyses, we also compared datasets within individual species to identify proteins that are commonly detected in the secretomes of at least 75% of the datasets (Table S7). This approach may enable the discovery of proteins or enzymes that are constitutively involved in lignocellulose degradation and in other metabolic processes in the extracellular milieu. Because the number of proteins from this study is very large (over 2,000 proteins), and knowledge on the catabolism of lignin-derived aromatic compounds

in WRF is still limited (del Cerro et al., 2021), we targeted our analyses to potential enzymes involved in the degradation and/or modification of aromatic compounds. For that purpose, we down-selected enzymes that contained signal peptides and CAZymes belonging to families of auxiliary enzymes – AA1_1 and AA2 (which include laccases and peroxidases), AA3 and AA5 (which include alcohol oxidases), AA4 (which include vanillyl-alcohol oxidases), and AA12 (which include pyrroloquinoline quinone-dependent oxidoreductases) (Drula et al., 2022) – and excluded proteins annotated as proteases/peptidases, esterases/lipases, nucleases, and hypothetical/unknown proteins (Table S8). In this pool of enzymes, apart from oxidoreductases, we identified a variety of hydrolases, amidases, and oxalate decarboxylases (the latter in 5 of 11 WRF species). Regarding oxidoreductases, we identified an extracellular dioxygenase from *P. eryngii*, which may be potentially involved in the extracellular catabolism (cleavage) of aromatic compounds (Table S8). We also found laccases (AA1_1) as commonly secreted enzymes in most analyzed species (9 of 11 species), peroxidases (AA2) in 5 of the 11 species, and a dye-decolorizing peroxidase (DyP) in *P. gigantea*. The limited number of oxidoreductases in the extracellular fraction suggests that intracellular proteomics is necessary for the discovery of enzymes related to the catabolism of aromatic compounds.

Overall, we faced some challenges when analyzing the secretomic datasets. First, not all the proteins detected in the extracellular fractions are true secreted proteins. Some detected proteins can be the result from fungal cell lysis. For instance, Kuuskeri et al. (2016) detected several transcription factors and translation elongation factors in the secretomes, which very likely function intracellularly. The cytoskeletal protein actin is also commonly detected in secretomes of several species including *T. versicolor*, *P. eryngii*, *P. radiata*, and *P. gigantea* (Table S7). The presence of putative intracellular proteins in the secretomes can be also the result of the production of extracellular vesicles (Bielska and May, 2019; Salvachúa et al., 2020). However, vesicular production and the enzyme content in these vesicles have not been studied in WRF to date. Having complementary pieces of information such as signal peptides, transmembrane domains, and annotation data are important to justify whether the detected proteins are secreted or potentially functional in the extracellular milieu of WRF. For instance, we also found few membrane-bound oxidoreductases such as aldehyde reductases and FAD-dependent oxidoreductases that could potentially have an effect on aromatic compounds (Table S7). Another challenge is the lack of consensus in analyzing proteomics data. Some publications utilize relative abundance of total peptide count in each sample for protein quantitation (Kuuskeri et al., 2016; Machado et al., 2020; Rytioja et al., 2017; Yoav et al., 2018). Some others use peptide spectral count as a proxy for relative quantitation (Fernández-Fueyo et al., 2012, 2016; Peña et al., 2021; Presley et al., 2018). Few studies adopted more accurate quantitation such as emPAI calculation or iTRAQ labeling (Alfaro et al., 2014; Liu et al., 2020; Manavalan et al., 2011; Xie et al., 2021), whereas several others used only qualitative presence-absence values. Standardizing analytical approaches will be crucial for comparison across studies and meta-analyses.

BIOTECHNOLOGICAL OPPORTUNITIES OF WRF

WRF offer many opportunities toward reducing carbon emissions and building a bio-based circular economy. Due to their intrinsic capabilities, WRF have been employed in applications that involve the degradation of lignocellulosic biomass. For instance, WRF or their enzymes may serve as biocatalysts for biological pretreatments of lignocellulose, or WRF cultivated on lignocellulose can produce edible mycelia or fruiting bodies. Additionally, WRF are capable of degrading xenobiotic compounds, which is a promising feature for bioremediation purposes. Another potential and emerging application is the generation of materials from fungal mycelia. In general, these applications have not yet required a comprehensive picture of the intracellular metabolism of WRF. However, future applications will require a deeper understanding of their metabolism to improve their performance. Furthermore, the elucidation of intracellular mechanisms can promote novel biotechnological opportunities in WRF such as lignin bioconversion to value-added compounds or the discovery and generation of fungal natural products. These applications are detailed below (Figure 6).

Biological pretreatment of lignocellulose

The pretreatment of lignocellulose is a key step to reduce the recalcitrance of the plant cell wall to ultimately release sugars from plant biomass before they can be converted to biofuels, biochemicals, or biomaterials. Cellulose and hemicellulose can be chemically or enzymatically hydrolyzed into lowmolecular weight sugars (e.g., glucose and xylose), which can be converted to biofuel or biochemical precursors (Mosier et al., 2005; Salvachúa et al., 2021). Common methods of biomass pretreatment include acidic

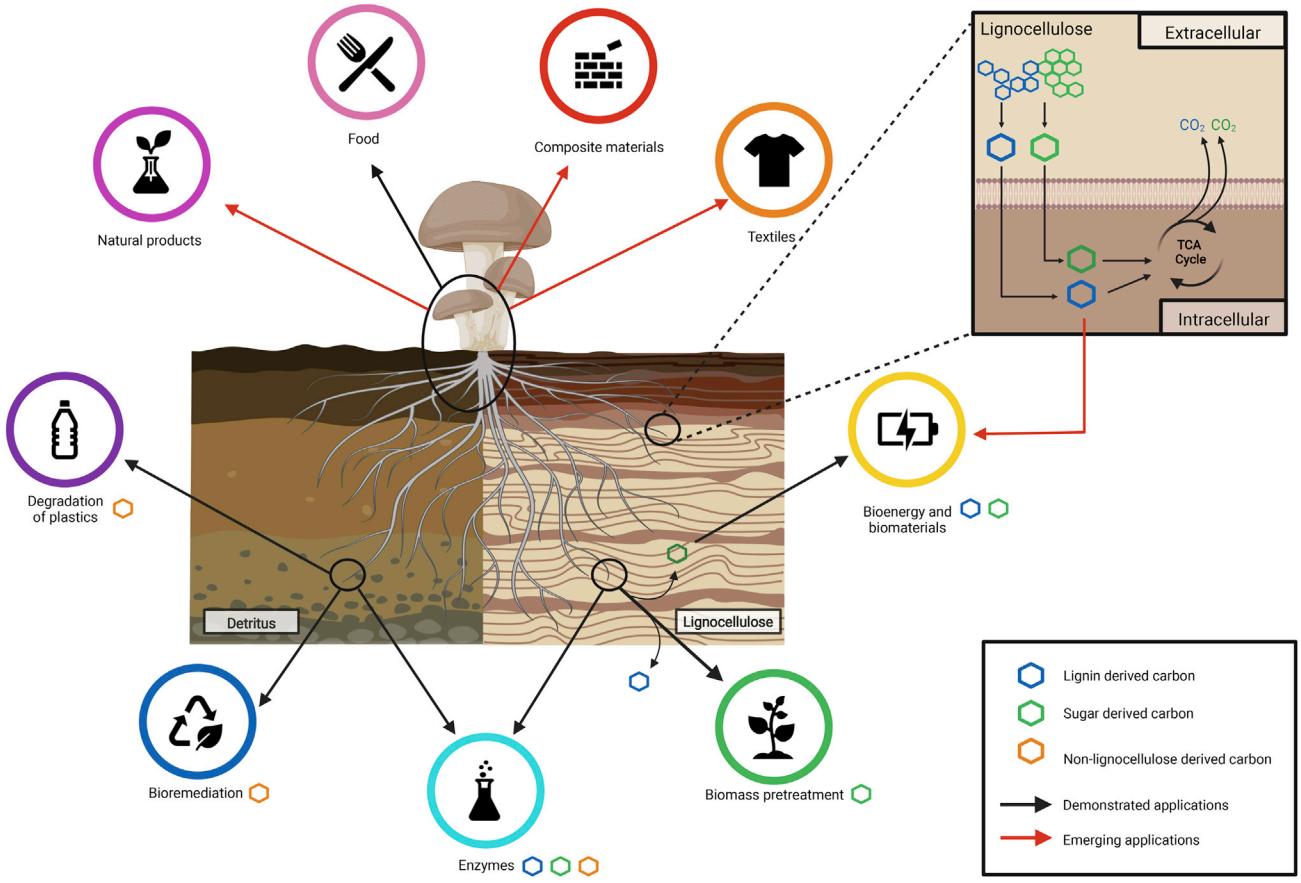

Figure 6. Biotechnological applications of WRF

WRF offer a variety of biotechnological opportunities. The metabolic capabilities of WRF to degrade lignocellulose and xenobiotic compounds can be utilized for the treatment of plant derived biomass and plastics, bioremediation and production of bioenergy (bioconversion to fuels, chemicals, or materials precursors) or natural products. The fruiting bodies or fungal mycelia can be processed into food or biomaterials. Figure created with BioRender.com.

or alkaline pretreatments that, in many cases, produce by-products that can inhibit downstream biological upgrading processes and lower product yields. Steam explosion is another industrially useful method of biomass pretreatment (Alvira et al., 2010) but presents energetic challenges at an industrial scale. Biological pretreatments, such as with WRF (e.g., *I. lacteus* and *C. subvermispora*) (Salvachúa et al., 2011), offer an alternative to efficiently remove lignin from plant biomass and increase yields in bioprocessing while improving process safety. However, increasing the biodegradation rates and decreasing the biodegradation ratio of sugars to lignin is still necessary to enhance the efficiency of biological pretreatments.

Applications of enzymes from WRF

To efficiently degrade lignocellulose, WRF have evolved an assortment of extracellular enzymes that function to depolymerize and modify sugar and lignin oligomers that are not readily bioavailable to the organisms. These enzymes have great potential to be used in biotechnological applications from biomass pretreatment to bioremediation. Furthermore, WRF have a high level of adaptation when grown on different media, a trait that can be utilized to produce enzyme cocktails tailored to specific substrates (deVries and Mäkelä, 2020). Fungal and bacterial CAZymes, such as cellulases and hemicellulases, which depolymerize cellulose and hemicellulose, respectively, are already prevalent in industrial applications including enhancing detergent effectiveness, processing of food, bleaching of paper and textiles, and enhancing the germination of seeds for agricultural uses (Singh et al., 2007). Regarding enzymes related to lignin depolymerization, research began in the 1880s with the discovery of laccases (Yoshida, 1983). These oxidoreductase enzymes oxidize phenolic compounds. However, laccases can also oxidize non-phenolic aromatics in the presence of mediators and cleave Cα-Cβ lignin linkages (Cañas and Camarero, 2010; Camarero et al., 2014). These enzymes sparked interest for biotechnological applications, as they provided a safe and effective way to detoxify streams from steam explosion pretreatments and

bleach paper pulp without chlorine (Camarero et al., 2014; Zhu et al., 2020). Furthermore, laccase-rich enzyme cocktails from WRF have been demonstrated to reduce the molecular weight of solid lignin-rich biorefinery streams, which is another relevant outcome for lignin valorization purposes (Salvachúa et al., 2016). Lignin peroxidase enzymes (LiPs) were discovered in the 1980s and demonstrated high redox potential, allowing them to oxidize phenolic and non-phenolic aromatic compounds as well as cleaving Cα-Cβ and β-*O*-4 ether bonds in the absence of mediators (Martínez et al., 2005; Tien and Kirk, 1983). Since then, considerable research effort has been invested into discovering other WRF peroxidases such as MnPs, versatile peroxidases (VPs), and DyPs which have been tested for lignin cleavage, industrial oxidations and redox conversion processes, as additive for a more efficient enzymatic hydrolysis of wheat straw, and dye decolorization (Hofrichter et al., 2010; Salvachúa et al., 2013b; Linde et al., 2015). Some challenges for the application of these oxidoreductases have been nicely described by Martínez et al. (2017) and include the lack of required selectivity, commercial availability, and the compatibility with certain process conditions (i.e., high substrate concentrations and strong oxidative conditions). For example, a limiting factor of lignin depolymerization reactions is that laccases and peroxidases nonspecifically oxidize aromatic compounds, which can lead to repolymerization reactions that naturally occur in lignin biosynthesis in plants (Ragauskas et al., 2014). However, this functionality has been also utilized to polymerize aromatic compounds for the generation of novel structures or materials (Moya et al., 2011; Salvachúa et al., 2013c). Other types of enzymes recently described to modify lignin-derived aromatic compounds are unspecific peroxygenases (UPOs) and cytochromes P450 (Črešnar and Petrič, 2011; Hofrichter et al., 2010; Martínez et al., 2017). These two classes of enzymes have recently arisen as biocatalysts of great biotechnological interest due to their capacity catalyzing in many cases the stereoselective and/or regioselective oxyfunctionalization of both aliphatic and aromatic compounds (Aranda et al., 2021; Martínez et al., 2017).

Lignin valorization

Petroleum and natural gas are by far the most prevalent sources of organic molecules for energy, chemicals, and materials in the industrial sector. Aromatic compounds are used in plastics, textiles, adhesives, fertilizers, food products, fuels, and more. Lignin is the single largest renewable resource of aromatic molecules and, if properly utilized, could provide an environmentally sustainable source of valuable aromatic molecules that could reduce the need for fossil carbon-derived chemicals (Ragauskas et al., 2014; Schutyser et al., 2018; Wong et al., 2020). Despite the abundance of lignin, it is highly underutilized due to its amorphous, heterogeneous, and recalcitrant structure, and it is only used in pulp and paper mills for heat production, yielding very little value (Banu et al., 2019). If lignin was alternatively used for chemical production, it could contribute roughly $13 billion in revenue annually (Troiano et al., 2020).

Lignin valorization is one of the most significant challenges in modern biorefining. To produce chemicals, lignin must first be depolymerized, and the monolignol subunits must subsequently be transformed to the desired products. Chemo-catalytic strategies for lignin depolymerization are being pursued by the large global community working on this topic (Rinaldi et al., 2016; Schutyser et al., 2018; Sun et al., 2018) but still presents some challenges that need to be overcome before adopting this strategy for lignin refining industrially. Biological lignin valorization utilizing various soil bacteria has been successful in modifying lignin-derived aromatic subunits into desired chemicals (Johnson et al., 2019; Lubbers et al., 2019; Salvachúa et al., 2020); however, bacterial catabolism of oligomeric lignin remains quite nascent in understanding, and rigorous studies of bacterial lignin conversion (Rouches et al., 2021; Salvachúa et al., 2018, 2020) have not yet elucidated molecular pathways for the conversion of lignin oligomers larger than dimers (Beckham et al., 2016; Kamimura et al., 2017). WRF are the most efficient lignin-degrading organism in nature, and, therefore, offer unique opportunities for lignin valorization in the biorefining industry. As previously mentioned, WRF have been researched for their ability to mineralize lignin to CO_2 and H_2O (Camarero et al., 2014), but only recently have lignin-derived aromatic catabolic pathways been proposed based on systems biology results (del Cerro et al., 2021). These pathways unlock the potential to utilize WRF for consolidated bioprocessing (CBP) of lignin: the simultaneous lignin depolymerization, conversion, and upgrading to value-added chemicals. Despite this opportunity, WRF are not yet effectively used in biorefining as a biocatalyst due to limited genetic engineering tools and an incomplete understanding of their full metabolic pathways, both of which are necessary to maximize carbon flux from substrate to product.

Bioremediation and plastic degradation

Many industrial human activities leach high levels of chemical contamination into terrestrial and aquatic ecosystems. Bioremediation, the utilization of microbial metabolism to degrade harmful chemicals, is a

promising field of discovery. WRF are potential candidates in bioremediation because their extracellular lignin-degrading enzymes (i.e., laccases and peroxidases) are also efficient in the xenobiotic detoxification of various recalcitrant aromatic molecules (Cañas and Camarero, 2010). Although these enzymes evolved to degrade lignin from plants, WRF such as *T. versicolor, P. chrysosporium, Irpex flavus, D. squalens*, have successfully been used to degrade synthetic chemicals such as aromatic dyes, which are toxic and carcinogenic (Asgher et al., 2008; Chander and Arora, 2007). Another subset of WRF such as *Phanerochaete sordida, S. hirsutum,* and *Coriolopsis polyzona* has been also demonstrated to degrade polycyclic aromatic hydrocarbons from petroleum, which are known to disrupt the endocrine system (Asgher et al., 2008; Cabana et al., 2007; Tamagawa et al., 2005). Even enzyme cocktails of MnPs and laccases have been shown to eliminate the estrogenic activities of bisphenol A and nonylphenol (Asgher et al., 2008). Once degraded, the products from these toxic chemicals may become more bioavailable to other microorganisms, allowing the carbon to cycle through the biosphere.

An extreme threat to ecological health is plastic pollution (Borrelle et al., 2020). Plastics are universally used for their versatility, durability, and inexpensive production costs. However, they are extremely stable, allowing them to persist for hundreds of years in the environment (Ward and Reddy, 2020). Plastics are made from a wide variety of organic polymers for an even wider variety of uses, yet this makes recycling expensive and difficult. Roughly 380 million metric tons of synthetic polymers are produced every year Nicholson et al. (2021); up to 13 million metric tons accumulate in marine environments annually (Jambeck et al., 2015). Although little is still known about biological degradation of petroleum-derived plastics, basidiomycetes have been shown to break down naturally occurring substituted aromatic biopolymers such as lignin (Meyer et al., 2020), which have similar structures to some plastics such as polyethylene terephthalate (PET). Clades of basidiomycetes (e.g., *Pycnoporus* sp.(Pathak, 2017)) and their characteristic enzymes are being researched to degrade PET and other similar plastics (Singh et al., 2021).

Food

Over 120,000 species of fungi have been characterized, with more being discovered every year (Willis, 2018; Wu et al., 2019); however, only about 350 species are regularly eaten (Hyde et al., 2019). The fruiting bodies of fungi have been consumed as food for thousands of years (Hyde et al., 2019), and new cultivation advancements have focused on growing WRF mycelia, such as those from *Pleurotus* spp. (some species are commonly known as oyster mushroom and king trumpet mushroom) and *L.edodes* (commonly known as shiitake mushroom), for food. At present, fungi offer a sustainable solution to reducing food insecurity globally. The global market for edible fungi is massive, at roughly 48 million tons of production in 2017, most of which (75%) was produced in China. Of that, 7.7 million tons (~16%) consisted of WRF *L. edodes* and 5.9 million tons (~12%) of WRF *Pleurotus* spp. (Hyde et al., 2019). The global mushroom industry was valued at $46.1 billion (USD) in 2020, and is expected to grow at an annual growth rate of almost 10% (compound annual growth rate) over the next 10 years (Grand View Research, 2021).

Fungi provide a nutritious, vegan alternative to meat due to their high protein content (20-30% dry mass), high fiber, low cholesterol, and complete essential amino acid content (Moore and Chiu, 2001). The fruiting bodies of fungi have diverse tastes and textures, some of which are known to mimic meat. Fungi can be cultivated with a considerably smaller resource footprint compared to meat, consuming half the water and a fraction of the land required to raise chicken (Meyer et al., 2020). Indoor fungal cultivations (i.e., *Pleurotus* spp.) are highly successful, allowing for the mass production of food without the risk of crop loss to environmental disasters (Moore and Chiu, 2001). Edible WRF can be grown on raw plant biomass (e.g., corn stover) that would normally be considered a waste product. Large-scale WRF cultivations can therefore offer avenues for up-cycling waste from other agricultural industries.

Materials

In nature, fungi are responsive to environmental stimuli and can modify their growth to adapt to environmental factors such as pH, temperature, moisture, and chemical availability. Artificially controlling these factors while growing fungi can modify the physical properties of mycelial growth. In addition, once the mycelia have been harvested, treatment with heat, pressure, and chemicals can further differentiate their physical traits. For example, mycelia can be grown into structures with rigidity and strength for building materials that mimic the properties of wood (Appels et al., 2019) or into supple and elastic structures that can be used as a leather replacement (Meyer et al., 2020). Fungi are naturally flame retardant and have good insulation properties to heat and sound, allowing them to be utilized as building insulation alternatives (Meyer et al., 2020; Neffa MycoTEX

https://neffa.nl/mycotex/, accessed August 10, 2021). Importantly, materials made of fungi are easily biodegradable and will not contribute ecologically harmful pollution at their end-of-life. As an area of particular interest within the production of materials, WRF may offer opportunities in the production of textiles. Natural textiles are primarily made from three types of biological polymers: cellulose (i.e., linen, hemp, cotton) from plants, collagen (i.e., leather, fur), and keratin (i.e., wool, fur) from animals. These natural textiles have been used for centuries but can have a detrimental environmental footprint in the industrial world due to high land and water usage (Kumar and Pavithra, 2019). Petroleum-derived textiles have largely replaced natural textiles but are also unsustainable because of the large amounts of pollution generated over the lifecycle of the product (Boucher and Friot, 2017). Though WRF have not historically been used in materials or textiles (or disclosed by the private sector), filamentous fungi are a promising alternative, because they are structurally composed of chitin, a filamentous polymer made of β-1,4-linked N-acetylglucosamine, offering many beneficial physical qualities. To mention an example, recent efforts have focused on treating mycelia from *S. commune* to simulate leather and generate natural polymers and elastomers (Appels et al., 2018, 2019, 2020). Together, fungal materials and textiles can be made with great diversity and tailorable biomechanical properties.

Biosynthesis of natural products

Natural products (or secondary metabolites) are bioactive compounds produced by fungi, bacteria, and archaea that can serve in multiple applications, such as human therapeutics to treat cancerous and infectious diseases (Baltz, 2019; Rodrigues et al., 2016). Their unique structures and biological activities are not only of great interest to the research community that aims to discover natural product-based medicines, but also to chemists, biochemists, microbiologists, and metabolic engineers that aim to understand the mechanisms for the generation of these complex molecules and their ecological role and that search for heterologous hosts to efficiently produce these compounds (Keller, 2019; Mattern et al., 2015; Tsukada et al., 2020). The discovery of novel pathways, gene clusters, and enzymes involved in the biosynthesis of secondary metabolites is still rising due to the progress of various systems biology methodologies from genome sequencing to transcriptomics, proteomics, metabolomics, and bioinformatics (Baltz, 2019; Zhang et al., 2021). A successful example of this approach has been recently published for another clade of fungi (early diverging fungi), in which a series of polyketide synthases – enzymes involved in the biosynthesis of secondary metabolites – and a large diversity of natural products have been identified (Swift et al., 2021). WRF have been reported to have the potential to produce of natural products, but the information to this end is scarce. As described in the genomics section, genes encoding for polyketide synthases were identified in *P. radiata* (Mäkinen et al., 2019), and *G. lucidum* was also reported to produce terpenoids, among other products, with anti-inflammatory and antiviral activity (Paterson, 2006). Overall, this promising biotechnological opportunity together with lignin valorization are perfect examples for the use of systems biology approaches to elucidate novel metabolic pathways in WRF.

CONCLUSIONS AND FUTURE PERSPECTIVES

In nature, WRF are continuously adapting to nutritional availability over their life cycles, which demands that optimal enzyme cocktails are employed to obtain carbon for energy and growth in a variety of environmental conditions, as well as coping with environmental and systemic toxicity. As shown in a variety of examples in this review, the processes used to address each of these issues cannot be investigated as individual modules. To understand the mechanisms that WRF employ to efficiently degrade lignocellulose and xenobiotics, or to produce fruiting bodies, among other applications, requires a holistic systems biology approach and more fundamental knowledge of triggered intracellular mechanisms from regulatory processes to specific pathways and enzymes. Nonetheless, a genetic toolkit for WRF is essential to validate systems biology observations, gene-function relationships, and further improve their performance. Progress on the development of efficient genetic tools (i.e., CRISPR/Cas9) has been recently published for few species of WRF such as *P. ostreatus* (Boontawon et al., 2021), *G. lucidum* (Wang et al., 2020), and *D. squalens* (Kowalczyk et al., 2021), which is a significant advance in fungal biology and proves the suitability of WRF for further metabolic engineering efforts.

Despite the vast benefits of systems biology methods to understand biological processes, there are still some limitations that remain:

- First, gene functional annotation is, in many cases, inaccurate (i.e., prediction of introns in eukaryotic systems), and between 40 and 50% of the proteins are hypothetical and lack functional predictions

(Meyer et al., 2020). Developing high-throughput platforms for enzyme production and evaluation on a diversity of substrates would be essential to improve these annotations.

- Second, to build a robust model for degradation and bioconversion scenarios by WRF, numerous datasets from a higher diversity of systems biology methods would be required due to the nature of some non-specific and non-enzymatic driven reactions. Even though the throughput of -omic analyses has significantly improved over the past few years, the generation of large datasets is still a challenge due to the strong fragmentation in the community studying particular species of filamentous fungi (Meyer et al., 2020). This fragmentation is not only notable on the selection of WRF but also on the substrates (in applications that involve lignocellulose), which are many times selected based on the main biomass sources in each region. Despite these challenges, there is currently a higher awareness on the need of generating detailed metadata with experimental information and sharing raw data with the community, which will be key to continue conducting integrative and comparative -omic analyses.
- Third, metabolomic studies are an essential component in multi-omic studies and have allowed proposing aromatic catabolic pathways in WRF (del Cerro et al., 2021), but as described here, these studies are emerging in research with WRF (Figure 2). Furthermore, metabolomic analyses become particularly complex in laboratory cultivation conditions that mimic those found in nature (i.e., solid-state cultivations, Figures 1B and 1E). Namely, WRF growing on solid materials could not be easily isolated from the material (e.g., lignocellulose, lignin, plastics). Thus, sample collection protocols need to be developed to distinguish intracellular from extracellular metabolites, especially in research studies that aim to elucidate metabolic pathways.
- Lastly, due to the compartmentalization in eukaryotic cells, certain biochemical steps can occur in multiple subcellular locations (Figure 3), which is another constraint to be considered for metabolic modeling. Multi-omic approaches in individual organelles and the compartmentalization of model metabolic networks would further advance the understanding of carbon flux in WRF.

To select robust WRF for biotechnological applications, bioprocess development and scale-up would also need to be investigated in parallel toward building relevant industrial processes and identifying shortcomings both in the process and the fungal species. Each application will need specific cultivation conditions; for instance, if the application involves polymer degradation processes, WRF are more efficient breaking down polymers in the solid-state (Figure 1E) than submerged-state (Figure 1D) cultivation mode. Solid-state cultivation technologies have not been as well developed as submerged cultivations at large scales. Some operational parameters such as substrate and particle size, inoculum, nutrient supplementation, aeration, temperature, moisture content, pH, and mixing are common in both solid-state and submerged cultivations. Nevertheless, solid-state cultivations are highly exothermic, and aeration is essential to not only provide oxygen to the organisms, but also dissipate the heat and moisture generated during growth (Mansour et al., 2016). Despite the aeration, it is likely that temperature gradients will be observed in the cultivation. Thus, WRF able to tolerate different temperature gradients without significant performance variations will be preferred. Solid-state cultivations may be conducted in trays or rotating drums, in the presence or the absence of mixing (Mansour et al., 2016). The incorporation of mixing will also depend on the robustness of the fungal mycelium. An optimal bioreactor design will also need to allow easy diffusion and extraction of metabolites for consolidated bioprocessing (Olson et al., 2012), for which WRF are promising biocatalysts. The slow lignocellulose degradation rates by WRF may be considered detrimental for industrial processes. However, it is worth noting that the volumetric productivity in solid-state cultivation can be significantly higher (~10 times for enzyme production, for example) compared to submerged fermentations (Durand, 2003). Techno-economic analyses and life cycle assessments will be required to assess the feasibility of these processes and inform about cost-effective strategies to scale-up WRF cultivations. In conclusion, this review aims to encourage the global research community to address the open questions presented here to ultimately harness WRF as future biocatalysts.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104640.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science.

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