The importance of sourcing enzymes from non-conventional fungi for metabolic engineering & biomass breakdown

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Abstract

A wealth of fungal enzymes has been identified from nature, which continue to drive strain engineering and bioprocessing for a range of industries. However, while a number of clades have been investigated, the vast majority of the fungal kingdom remains unexplored for industrial applications. Here, we discuss selected classes of fungal enzymes that are currently in biotechnological use, and explore more basal, non-conventional fungi and their underexploited biomass-degrading mechanisms as promising agents in the transition towards a bio-based society. Of special interest are anaerobic fungi like the *Necocallimasticomycota*, which were recently found to harbor the largest diversity of biomass-degrading enzymes among the fungal kingdom. Enzymes sourced from these basal fungi have been used to metabolically engineer substrate utilization in yeast, and may offer new paths to lignin breakdown and tunneled biocatalysis. We also contrast classic enzymology approaches with emerging 'omics'-based tools to decipher function within novel fungal isolates and identify new promising enzymes. Recent developments in genome editing are expected to accelerate discovery and metabolic engineering within these systems, yet are still limited by a lack of high-resolution genomes, gene regulatory regions, and even appropriate culture conditions. Finally, we present new opportunities to harness the biomass-degrading potential of undercharacterized fungi via heterologous expression and engineered microbial consortia.

Keywords: Fungi, lignocellulose, enzyme, cellulase, cellulosome, anaerobic fungi

1. Introduction

Modern biotechnology uses enzymes and engineered microbes to produce a wide variety of fuels, materials and chemicals from renewable feedstocks (Otero and Nielsen, 2010). In contrast, current commodity- and fine-chemical production relies on non-renewable petroleum feedstocks. Given the dwindling resources and the heavy carbon footprint of oil, the demand for environmentally friendly alternatives is urgent and ever increasing.

The so-called first generation biofuels were derived from crops that are rich in starch and sugars, such as corn and sugarcane (Saini et al., 2015). As the world's population is predicted to increase to ~10 billion by the year 2050, this approach is not sustainable because it competes with food resources and for agricultural land (Bothast and Schlicher, 2005; Rogers et al., 2017). Current efforts seek to convert lignocellulosic energy crops and residues from agriculture and forestry into hexose and pentose sugars (Sanderson, 2011; U.S. Department of Energy, 2017, 2016). Given the impetus of the European Union's goal to develop a bio-economy by 2050, as well as the estimated €2 trillion bio-market size in 2012, there are significant political and financial drivers to pursue these endeavors (Scarlat et al., 2015). To fully realize the potential of sustainable bioproduction platforms, there is a great need to identify novel organisms, enzymes and molecules with activities that can be harnessed for a range of breakdown and conversion applications (Adrio and Demain, 2014; Curran and Alper, 2012; Monciardini et al., 2014; Rocha-Martin et al., 2014; Thies et al., 2016). In particular, we need to be able to cost-effectively convert diverse, underutilized plant biomass into tailor-made value-added compounds.

Lignocellulosic biomass, available worldwide in plant cell walls, is arguably the most promising feedstock for the sustainable production of bio-based chemicals and value-added products (Himmel et al., 2007; Rogers et al., 2017; U.S. Department of Energy, 2017). Underutilized lignocellulosic feedstock is abundant – it is estimated that 1.3 billion tons of agricultural waste is generated on an annual basis worldwide (Saini et al., 2015; Sarkar et al., 2012). It has been suggested that the US alone could sustainably produce as much biomass that could be funneled into bioprocessing applications on an annual basis (Himmel et al., 2007; Rogers et al., 2017). However, the inherent recalcitrance of plant cell walls presents a formidable challenge for biotechnological applications. Few organisms can fully degrade the highly heterogeneous and recalcitrant structures found in plant cell walls. Therefore, biomass-degrading organisms are highly sought after, as their enzymes can be directly harvested or produced for consolidated bioprocessing (Hess et al., 2011; Piao et al., 2014; Zhang et al., 2016).

Fungi play a major role in nutrient cycling and biogeochemical cycles in both aquatic and terrestrial environments (Dighton, 2007; Gadd, 2007; Gessner et al., 2007). Already, most industrial enzymes for lignocellulosic bioprocessing are sourced from fungi but the fungal kingdom is vast, largely hidden, and

exhibits a wide range of interesting bioactivities that remain underexploited (Banerjee et al., 2010; Falade et al., 2017; Payne et al., 2015; Ramanjaneyulu and Rajasekhar Reddy, 2016). This review describes selected examples of fungal enzymes that are currently in commercial use, and those that could further transform biotechnology, sourced from under-characterized clades of fungi. We provide examples of how fungal enzymes and pathways have been used to enhance the performance of microbial production strains with particular focus on biomass degradation, and discuss challenges and future opportunities.

2. Current approaches & challenges to biomass breakdown

The industrial application of biomass-degrading enzymes is a multi-billion dollar market (Mathew et al., 2008). While biomass-degrading enzymes can be found in both bacteria and fungi, nearly all industrial enzymes are sourced from fungi. This is likely because the fungal enzymes are often stabilized by glycosylation and they have been shown to be active in the presence of proteases and surfactants, and at high temperature (Beckham et al., 2012; Hong et al., 2001; Ilmberger, 2013). Fungi excel at biomass degradation in nature and possess a wide variety of enzymes that depolymerize plant biomass with high efficiency (Dighton, 2007). As shown in Table 1, fungal biomass-degrading enzymes are heavily used for the processing of paper and pulp; for the production of food, feed, pharmaceuticals and cosmetics; as well as for bioremediation.

Table 1: Industrial applications of fungal enzymes in current use

Enzyme	EC Number	Key reaction(s)	Main Applications	References
Hydrolases				
Cellulase	3.2.1.4	Hydrolysing the β-1,4-glycosidic bonds in cellulose	Food industry, textile manufacturing, detergent industry, paper and pulp industry, bioremediation, biofuel production	(Kuhad et al., 2011; Payne et al., 2015)
Xylanase (Hemicellulase)	3.2.1.8	Hydrolysing the β 1,4-glycosidic bonds in xylan	Food industry, biofuel production, paper and pulp industry, deinking, production of animal feed	(Ramanjaneyulu and Rajasekhar Reddy, 2016)
Alpha-amylase	3.2.1.1	Hydrolysing the α-1,4-glycosidic bonds in starch	Food industry, starch conversion, biofuel production, detergent industry, paper and pulp industry	(Souza and Magalhães, 2010)
Invertase	3.2.1.26	Hydrolysing sucrose into glucose and fructose	Food industry, cosmetics, pharmaceutical industry, paper industry	(Kotwal and Shankar, 2009)
Beta-galactosidase; Lactase	3.2.1.23	Hydrolysing lactose into glucose and galactose	Food industry	(Husain, 2010)
Lipases	3.1.1.3	Total or partial hydrolysis of fats and oils	Food industry, biofuel production, spills, detergent industry, paper and pulp industry, pharmaceutical industry	(Gupta et al., 2015; Jaeger and Reetz, 1998; Takó et al., 2017)
Phytase	3.1.3.8	Catalyzing phosphate monoester hydrolysis of phytic acid	Food industry, agriculture, production of animal feed	(Haefner et al., 2005)
Oxidases				
Laccase	1.10.3.2	Catalyzing the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular O_2 to H_2O	Nanotechnology, synthetic chemistry, bioremediation, cosmetics	(Rodríguez Couto and Toca Herrera, 2006; Singh Arora and Kumar Sharma, 2010)
Peroxidases				
Lignin peroxidase	1.11.1.14	Catalyzing the oxidation of various organic and inorganic substrates in the presence of H_2O_2 as electron acceptor via long-range electron transfer (LRET)	Paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, biomass conversion, cosmetics	(Falade et al., 2017; Hofrichter et al., 2010)
Manganese peroxidase	1.11.1.13	Catalyzing the oxidation of Mn (II) to Mn (III), as well as a variety of low redox potential organic substrates, in the presence of H_2O_2 as electron acceptor	Paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, biomass conversion	(Hofrichter et al., 2010; Mendonça Maciel et al., 2010)
Versatile peroxidase	1.11.1.16	Catalyzing the oxidation of various high and low redox	Paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, biomass	(Busse and Czermak, 2016; Hofrichter et al., 2010)

	potential organic substrates in the presence of H_2O_2 as electron acceptor in either a manganese-mediated reaction or a manganese-independent reaction via LRET	conversion	87 88

2.1. Plant biomass vs. microbial enzymes

Plant cell walls are complex and dynamic structures made mainly of cellulose (40-50%), hemicellulose (20-40%) and lignin (20-35%) (Houston et al., 2016; Liao et al., 2016), which together form a formidable barrier against chemical and enzymatic degradation (Figure 1). Cellulose is an unbranched polymer of D-glucose moieties that are linked by β(1→4) bonds; the cellulose chains may contain thousands of glucose units and aggregate into crystalline microfibrils. In contrast, hemicelluloses are a heterogeneous group of branched polysaccharides composed of various 5- and 6-carbon sugars *e.g.* xylose, mannose, arabinose and galactose (Rubin, 2008). In plant cell walls, cellulose microfibrils are surrounded by a network of hemicelluloses (Figure 1). Further, the energy-rich cellulose and hemicellulose are encapsulated by lignin, which is a complex aromatic polymer resulting from the oxidative combinatorial coupling of p-coumaryl-, coniferlyl-, and sinapyl alcohols (Haghighi Mood et al., 2013). In addition to providing structural support to the plant, the chemically recalcitrant lignin protects the cellulose and hemicellulose polymers from enzymatic hydrolysis and most microbial invaders.

Despite its recalcitrance, fungi have a natural advantage against crude biomass – they break it down both physically and enzymatically. For example, fungi may burrow into the biomass, increasing its surface area and making it more accessible to biomass-degrading enzymes from fungi as well as from other neighboring microbes (Figure 2). As fungi cannot take up all polymeric compounds from their environment, they secrete extracellular enzymes that degrade the polymers to short oligomers and monomers that are imported through targeted transporters and metabolized in the cells (Seppälä et al., 2016). In order to accomplish this complex task, fungi harbor a variety of biomass degrading enzymes, where each enzyme excels in the biocatalysis of one (or more) specific compounds within the lignocellulosic structure (Dashtban et al., 2009). Cellulose is degraded by glycoside hydrolases (GHs) that are either endocellulases or exocellulases: endocellulases bind anywhere along the length of the cellulose molecule and hydrolyze the β-1,4 glycosidic linkage, whereas exocellulases -cellodextrinases and cellobiohydrolases- bind at the ends of the cellulose polymer and release glucose or unit-length oligosaccharide products (Li et al., 1997). β-glucosidases break down cellooligosaccharides and cellobiose into glucose monomers (Sørensen et al., 2013). Hemicellulose breakdown requires the added action of hemicellulases such as xylanases and mannanases (Bhattacharya et al., 2015). Known mechanisms of fungal lignin degradation require laccases that couple the oxidation of substrates to the reduction of oxygen (Singh Arora and Kumar Sharma, 2010) and peroxidases that couple the oxidation of substrates to the reduction of hydrogen peroxide (Hofrichter et al., 2010). The fungal biomass-degrading machinery typically consists of a cocktail of powerful cellulases, β-glucosidases, hemicellulases, and lignin-modifying enzymes that act synergistically.

Given the ubiquitous interwoven enzymatic activities required for lignocellulose hydrolysis, the CAZy (carbohydrate-active enzymes) database (www.cazy.org) serves as an excellent resource that classifies (and updates) all known enzyme families involved in cellulolysis, hemicellulolysis, and, by a recent addition, the degradation of lignin (Levasseur et al., 2013). Apart from fungi, various bacterial genera have been observed to metabolize lignin and shown to be competent to release C^{14}-CO_2 from labeled lignin (Brown and Chang, 2014; Chen et al., 2012; Kern and Kirk, 1987; Kerr et al., 1983). Although the bacterial catabolism of lignin is not as complete compared to fungal systems, it seems clear that bacteria can react with lignin and possibly produce smaller aromatics that can be imported into the cell for aromatic catabolism (Chakraborty and Coates, 2004; Kanaly and Harayama, 2000).

2.2. Current fungal workhorses for biomass breakdown and their limitations

Industrial biomass conversion typically requires physical and chemical pretreatment to separate individual biopolymer constituents followed by enzymatic processing (Galbe and Zacchi, 2012). Physical pretreatment is resource intensive, and generally involves milling, or the utilization of hot steam to increase the surface area of the lignocellulosic biomass (Chandra et al., 2007). Chemical pretreatment involves incubating the biomass with an acid or alkali (Davis et al., 2013; Sanderson, 2011). After pretreatment, the biomass is hydrolyzed either by cellulolytic microbes or purified enzyme cocktails. As it has been estimated that the cost of enzymes make up a significant part of the cost of bioethanol production there is great interest to identify enzymes with increased degradation activity that can be produced at high titers (Dashtban et al., 2009; Klein-Marcuschamer et al., 2012; Liu et al., 2016).

Currently, a handful of fungi are directly utilized on an industrial level for biomass hydrolysis. Foremost in this regard is the filamentous fungus *Trichoderma reesei*; other notable species include the filamentous *Aspergillus niger* and the thermophilic *Humicola insolens* (Bischof et al., 2016; Paloheimo et al., 2016; Sukumaran et al., 2005). Typically, these organisms are employed because they are natural hypersecreters of cellulolytic enzymes, and the secretion system of *T. reesei* has been subject to extensive investigation for decades (for recent reviews, see *e.g.* (Bischof et al., 2016; Paloheimo et al., 2016)). A complication is that fungal enzyme production is typically subject to carbon catabolite repression and the molecular mechanisms behind these processes are complex and difficult to engineer (Amore et al., 2013). Current enzyme yields stand at around 100 g/L, and it has been suggested that further improvements are likely to be modest (Banerjee et al., 2010). An alternative to improving enzyme yields is to improve the performance of the saccharolytic enzymes directly though protein engineering. For example, through directed evolution, the cellulases of *Hypocrea jecorina*, a teleomorph of *T. reesei*, were evolved to function optimally at 70°C instead of 60°C (Wu and Arnold, 2013). Also, recently the AA9 (formerly GH61) family was shown to greatly increase the cellulolytic capabilities of the cellulases secreted by *T.*

reesei (Langston et al., 2011). Although the enzymes of *T. reesei* - and moreover its ability to secrete astonishing quantities of enzymes - remain useful for industrial applications, there are limits to what we can expect from this organism. Notably, it has become apparent that the genome of *T. reesei* encodes for the smallest diversity of cellulases and hemicellulases of any sequenced fungus capable of plant cell wall degradation (Martinez et al., 2008) (Figure 3). Further, these enzymes cannot act on lignin, which is generally separated from most industrial substrates and burned as an energy source (Kuhad et al., 2011). Therefore, bioprospecting in other fungal clades for novel proteins that can be utilized for biomass breakdown is an appealing path forward.

2.3. The potential for biomass deconstruction within unexplored fungal branches

Most fungi degrade cellulose and some, such as the white-rot fungus *Phanerochaete chrysosporium*, are able to efficiently depolymerize lignin (for a review, see (Chandel et al., 2015). Given the paucity of enzymes available, ligninolytic enzymes are an especially keen target for biotechnology and metabolic engineering (Floudas et al., 2012; Riley et al., 2014), as aromatic breakout products derived from lignin would open up a new class of bio-based commodity chemicals. The lignin degradation system of the aerobic fungi involves laccases: multicopper oxidases that couple the oxidation of substrates to the four-electron reduction of O_2 to H_2O (Singh Arora and Kumar Sharma, 2010). In addition, there are high-oxidation potential class II peroxidases that, on the basis of conserved catalytic sites, are classified mainly as lignin peroxidase, manganese peroxidase, and versatile peroxidase (Floudas et al., 2012) (Table 1).

Notably, many of the lignin degrading enzymes are nonspecific and enable the degradation of a large range of natural and anthropogenic compounds that have structural and chemical similarities to the lignin substructure (Knop et al., 2015). These include dyes, polycyclic aromatic hydrocarbons and various pharmaceuticals (Hadar and Cullen, 2013; Martinez et al., 2004). Attempts have been made to express ligninolytic genes both homologously (Salame et al., 2012) and heterologously (Zhang et al., 2012), in order to enhance natural and novel microbial biomass-degrading abilities. For example, the production of biomass-degrading enzymes by *P. chrysosporium* was recently enhanced by overexpression of endogeneous genes encoding a manganese peroxidase and a lignin peroxidase, and the heterologous expression of a versatile peroxidase-encoding gene from *Pleurotus eryngii* (Coconi-Linares et al., 2014). The yield and activity of the ligninolytic enzymes was up to four times higher, in comparison to previously published reports.

In recent years the advent of new biotechnological tools related to next-generation sequencing and 'omics' techniques enabled the discovery of new biomass degradation enzymes in the more basal clades (Floudas et al., 2012; Haitjema et al., 2017; Riley et al., 2014; Youssef et al., 2013). It has become apparent that anaerobic fungi, in the early diverging phylum *Neocallimastigomycota*, possess a largely untapped source

of biomass degrading enzymes (Haitjema et al., 2017; Solomon et al., 2016). A recent comprehensive look at transcriptomic data collected from three gut fungal strains suggested that the anaerobic fungi are prodigious producers of glycoside hydrolases (Solomon et al., 2016), and further may possess novel receptors and transporters for carbohydrates that hold biotechnological promise (Seppälä et al., 2016) (Figure 3).

3. Early diverging anaerobic fungi: an untapped source of biomass-degrading enzymes

An inviting alternative to current methods of biomass breakdown is to find superior biomass-degrading enzymes from natural sources where crude biomass degradation is abundant. Anaerobic gut fungi inhabit the intestines of a range of large herbivores, from cows, horses and sheep to elephants, camels and even iguanas (Hibbett et al., 2007; Liggenstoffer et al., 2010). In spite of their key role in conversion and recycling of photosynthetically fixed plant biomass, research on anaerobic gut fungi did not gain steam until the late 1970s, when Colin Orpin established that the 'protozoan flagellates' that were found in rumen fluid are in fact fungi (Orpin, 1977, 1975) (Figure 4). Even today, anaerobic gut fungi remain understudied, primarily owing to challenges in isolation and in maintaining the fungi in culture (Haitjema et al., 2014). However, powerful integrated 'omics' approaches are rapidly filling the knowledge gaps (Mondo et al., 2017; Youssef et al., 2013; Solomon et al., 2016; Haitjema et al., 2017).

Recent advances in next generation sequencing have revolutionized the study of non-conventional fungi, and anaerobic gut fungi in particular. In the past four years, researchers have increased the inventory of known anaerobic fungal enzymes from a mere handful to thousands (Youssef et al., 2013; Solomon et al., 2016). These enzymes have been validated with proteomics, and biochemical characterization has revealed that anaerobic fungal enzymes exhibit little substrate preference, in contrast to their later-diverging fungal cousins that strongly favor cellulose-rich substrates (Solomon et al., 2016). This ability is a consequence of extensive horizontal gene transfer where the anaerobic fungi have complemented their cellulolytic capabilities with hemicellulases derived from bacteria (Haitjema et al., 2017). Thus, anaerobic fungi possess the largest and most diverse inventory of biomass-degrading enzymes among fungi sequenced to date (Figure 3), and their enzyme setup is seemingly regulated by growth substrate so that the optimal enzyme cocktail is produced at all times (Solomon et al., 2016). Similarly, sequencing-enabled discovery recently revealed the elusive fungal cellulosomal complex that synergistically combines diverse biomass-degrading enzymes for efficient biomass degradation (Haitjema et al., 2017). With these rich and growing databases of characterized anaerobic fungal enzymes, we are now poised to enter a new phase of innovation where anaerobic fungal enzymes may be harnessed for bioengineering applications.

3.1. Heterologous production of gut fungal enzymes in model systems

While large-scale cultivation of anaerobic organisms remains challenging, and while most of them are still genetically intractable, it has been shown that gut fungal enzymes can be successfully produced in engineered microbes. Early studies on the enzymatic activities of gut fungal culture fractions revealed that these organisms greatly contribute to biomass degradation in the animal host, and possess powerful enzymes that efficiently degrade α- and β-glucans, β-galatans, galactomannans, and arabinoxylans (Lowe et al., 1987; Mountfort and Asher, 1985; Pearce and Bauchop, 1985; Williams and Orpin, 1987; Wood et al., 1986) (Figure 4). It was soon discovered that the majority of the gut fungal enzymatic activities are extracellular or confined to the membrane fraction (Pearce and Bauchop, 1985), and a number of enzymes have been purified and characterized from gut fungal cultures since (see *e.g.* (Borneman et al., 1991; Garcia-Campayo and Wood, 1993; Hebraud and Fevre, 1990a, 1990b; Li and Calza, 1991; Teunissen et al., 1992; Vardakou et al., 2008; Wang et al., 2014)).

The advent of molecular tools opened up new possibilities for enzyme discovery and production, and in the early 1990s, gut fungal cDNA libraries expressed in *Escherichia coli* were successfully used to screen for and identify novel cellulolytic enzymes (Reymond et al., 1992, 1991; Xue et al., 1992b) (Figure 4). Today, a handful of gut fungal proteins have been successfully produced in a number of heterologous hosts, firmly establishing that these enzymes can be transferred to a wide variety of biotechnologically interesting organisms for downstream applications, including bacteria (*E. coli, Butyrivibrio fibrisolvens, Bacillus subtilis, Lactobacillus reuteri, Clostridium beijerinckii*) (Elliott et al., 1999; Gilbert et al., 1992; Lee et al., 1993; Liu et al., 2005a; Smidt et al., 2001; Xue et al., 1997, 1992a); yeasts (*Saccharomyces cerevisiae, Pichia pastoris, Kluveromyces lactis, Hansenula polymorpha*) (Durand et al., 1999; Harhangi et al., 2002; Li et al., 2004; O'Malley et al., 2012; van der Giezen et al., 1998); filamentous fungi (*Penicillium roqueforti, Trichoderma reesei, Hypocrea japonica*) (Durand et al., 1999; Li et al., 2007; Poidevin et al., 2009); and plants (*Brassica napus, Nicotiana tabacum*) (Liu et al., 1997; Obembe et al., 2007).

Several gut fungal cellulases and β-glucosidases have been produced heterologously in metabolically engineered hosts (Denman et al., 1996; Hung et al., 2012; Li et al., 2004; Morrison et al., 2016a; Qiu et al., 2000; Tseng et al., 2011; G.-P. Xue et al., 1992b; Zhou et al., 1994). These enzymes often exhibit numerous activities, sometimes catalyzed from the same site. It has been shown that the gut fungi acquired many of these proteins through horizontal gene transfer from bacteria, and that the genes have undergone subsequent duplications and evolution in gut fungi (Garcia-Campayo and Wood, 1993; Gilbert et al., 1992; Haitjema et al., 2017; Wood et al., 1986). Further, several gut fungal xylanases, mannanases, and esterases have been cloned for heterologous expression (Blum et al., 1999; Cybinski et al., 1999;

Dalrymple et al., 1997; Fanutti et al., 1995; Lee et al., 1993; Millward-Sadler et al., 1996; Pai et al., 2010; Xue et al., 1995). In several cases, fungal esterases work *in concert* with xylanases and some enzymes carry both activities on different domains (Blum et al., 1999; Cybinski et al., 1999; Pai et al., 2010). Among hemicellulases, glycoside hydrolase family 11 (GH11) xylanases are particularly interesting, as they have been shown to be exceptionally active. Engineering of Xylanase C from *N. patriciarum* has generated an alkalophilic and thermostable version of the protein, coined XynCDBFV (Chen et al., 2001; You et al., 2010). The stability of XynCDBFV at 60°C and pH 10 makes the protein a suitable candidate for *e.g.* paper pulp biobleaching. Moreover, it has been shown that an oleosin-tagged XynCDBFV that is immobilized on artificial oil bodies retains its activity, showing promise for the numerous biotechnological applications that utilize immobilized enzymes (Hung et al., 2008). Finally, XynCDBFV has been used to generate a *L. reuteri* strain that is capable of breaking down carboxymethyl cellulose, β-glucan and xylan, by co-expression with a β-glucanase from *Fibrobacter sp.*(Liu et al., 2007, 2005b). Recently, XynCDBFV was crystallized at high resolution, which facilitates further protein engineering (Cheng et al., 2015, 2014).

Although *S. cerevisiae* is a popular bioethanol producer, owing to its high ethanol tolerance and high resistance to inhibitors that are present during the hydrolysis of lignocellulosic biomass, it cannot natively ferment pentose sugars that comprise a substantial portion of the substrate (Matsushika et al., 2009; Young et al., 2010). In theory, this could be remedied by the heterologous production of the bacterial xylose isomerase pathway, but many attempts have failed because of the low activity of the bacterial isomerases in yeast (Matsushika et al., 2009). The discovery that anaerobic gut fungi possess a xylose isomerase that can be functionally produced in *S. cerevisiae*, thereby allowing the yeast to anaerobically ferment xylose, was a biotechnological breakthrough (Harhangi et al., 2003; Kuyper et al., 2004, 2003). Since then, the xylose utilizing yeast strain has been enhanced by the overexpression of endogenenous xylulokinase; by engineering of the endogeneous oxidative pentose phosphate pathway; and by the addition of heterologous xylose transporters (Bamba et al., 2016; Kuyper et al., 2005a; Madhavan et al., 2009b; van Maris et al., 2007; Zhou et al., 2012). Adaptive evolution has proven an effective tool to improve on these strains further (Diao et al., 2013; Kuyper et al., 2005b; Madhavan et al., 2009a; Shen et al., 2012). Currently, efforts are underway to identify novel xylose isomerases from natural sources, and to elucidate how yeast should best be engineered into an optimal xylose converter (Hou et al., 2016; Parachin and Gorwa-Grauslund, 2011; Usher et al., 2011). Directed evolution of proteins is a promising approach for tailoring proteins that require refined functions, such as higher stability, tolerance, substrate specificity, and product selectivity of the protein. For example, in 2012, the performance of xylose isomerase from *Piromyces sp*. was improved by 77% by three rounds of mutagenesis and selection (Lee et al., 2012). Gut fungal xylose isomerases have also been successfully produced in other industrially

important fungi, such as *Kluveromyces sp.* (R. Wang et al., 2013), *Candida sp.* (Koivuranta et al., 2014), and *Pichia sp.* (Li et al., 2015). In conclusion, fungal enzymes hold great promise for the engineering of fast growing yeast strains that efficiently convert lignocellulosic biomass into biofuels and other valuable compounds (Alper and Stephanopoulos, 2009).

3.2. The fungal cellulosome: a eukaryotic biomass-degrading supermachine with borrowed bacterial parts

Cellulosomes are multi-enzyme complexes, first described in the anaerobic bacterium *Clostridium thermocellum* (Lamed et al., 1985), that tether plant biomass-degrading enzymes together for improved hydrolysis. While aerobic fungi release their biomass degrading enzymes to the external *milieu*, it was recently discovered that anaerobic gut fungi secrete fungal cellulosomes that bear some architectural resemblance to the bacterial cellulosomes (Guerriero et al., 2015; Haitjema et al., 2017). The power of the cellulosomal system lies in its modular ability to host a variety of enzymes that can break down unpretreated lignocellulosic plant biomass synergistically (Resch et al., 2013).

Although both fungal and bacterial cellulosomes digest plant cell wall material, there are significant differences between the kingdom-specific complexes. In addition to both the structural differences between the so-called dockerin and cohesin units, as well as their relative position on the cellulosome (Haitjema et al., 2017), the two cellulosome types differ in the diversity of glycoside hydrolase (GH) families present as catalytic components (Artzi et al., 2017; Haitjema et al., 2017). In contrast to the bacterial cellulosomes, the fungal scaffoldin system is broadly conserved across the anaerobic fungal clade, allowing interspecies cross-linking binding activity. The most profound biochemical difference between the bacterial and fungal cellulosomes is the end products produced during the hydrolysis of crystalline cellulose. Degradation of cellulose by Clostridial cellulosomes results in the production of cellobiose, which is taken up by the cell, hydrolyzed to glucose and metabolized (Schwarz, 2001). However, fungal cellulosomes produce glucose during cellulose degradation (Gilmore et al., 2015). Taken together, these differences may make fungal cellulosomes a superior candidate for metabolic engineering. Hence, we foresee that anaerobic fungal cellulosomes will provide important inspiration for the design of industrially feasible biomass-degrading bioplatforms. In an encouraging study along these lines, gut fungal cellulases were heterologously expressed and fused with bacterial dockerin-domains that allowed them to bind to the cellulosome of *Clostridium cellulolyticum* (Mingardon et al., 2007) through cohesin/dockerin interactions.

Recently, a comprehensive set of proteins that is critical to the assembly of fungal cellulosomes was described for the first time (Haitjema et al., 2017). This advancement is bound to lead to improvements in the "designer cellulosome" field (Gilmore et al., 2015), especially since the "parts" of the fungal

cellulosome (i.e. cohesin, dockerin, scaffoldin) are markedly different from their bacterial counterparts. For example, fungal cohesion/dockerin units, which tend to be promiscuous by nature, could be used to engineer an extracellular production platform. By making use of a consortium of engineered organisms, each producing different enzymes required for a specific function, it is possible to assemble a complex *bona fide* cell factory line for almost limitless applications.

While the immediate application of designer cellulosomes is the rational design of more efficient cellulose degrading machinery (Gunnoo et al., 2016), cellulosomes could also be used as scaffolds to localize other enzymes for efficient substrate channeling. Indeed, synthetic metabolons, inspired by nature, have been applied to a host of metabolic engineering applications, see (Wheeldon et al., 2016) for a recent review. Recently a group co-localized three glycolytic enzymes, using bacterial cohesin/dockerin components, to improve the initial reaction rate of the system by 27% (You et al., 2012; You and Zhang, 2014, 2013). Thus, fungal cohesion/dockerin components expand the parts list available for such approaches. This is important for developing modular non-interacting metabolon systems within the same organism that could be directly exploited in metabolic engineering applications.

4. Discovery of novel enzymes from fungi

Fungi have played an important role in human activities for millennia, and continue to lend themselves well to diverse applications. Great effort has been made to identify and characterize novel fungal species and enzymatic activities. However, although ~1,200 new fungal species are described each year, it has been estimated that the ~100,000 described species represent a mere 2-10% of the total number (Blackwell, 2011; Tedersoo et al., 2014), and so it seems that we have barely begun to unlock the hidden biotechnological potential of this extensive kingdom.

4.1. Hunting for interesting enzyme activities in nature

The discovery of fungal enzymes can be traced back to the origins of enzymology itself. Living systems were long believed to possess a 'vital' spark responsible for its 'magical' ability to chemically transform organic compounds. However, Eduard Büchner demonstrated the presence of a biochemical catalyst in yeast extracts that was able to ferment sugars into alcohol, which he called 'zymase' from 'zym' for yeast ('leaven') and 'ase' the now generic suffix for enzyme (Buchner, 1897; Buchner and Rapp, 1899). This breakthrough sparked a revolution for which Büchner was awarded the Nobel Prize in Chemistry in 1907 (Buchner, 1907). Work quickly progressed to the screening of fungal secretions and lysates for desired activities, which lead to the discovery of many enzymes that were soon harnessed for industrial applications. Among early discoveries were pectinases from saprophytic fungi such as *Aspergillus sp., Penicillium sp.*, and *Rhizopus sp.* that clarify fruit juices (Mantovani et al., 2005; Zoltan and John, 1933),

rennet from *Aspergillus sp.* and *Mucor sp.* that clot milk for cheese production (Arima et al., 1967), manganese peroxidases from *Phanerochaete sp.* that decolor paper pulp effluents and degrade xenobiotics (Bajpai et al., 1993; Field et al., 1993; Glenn and Gold, 1983), and cellulases from *Trichoderma sp.* (Allen et al., 2009) and anaerobic fungi (Orpin, 1975; Solomon et al., 2016; Youssef et al., 2013) that degrade plant biomass. Once a target enzyme system is identified, enzymes may be purified to homogeneity before they are characterized in detail (Kester and Visser, 1990; Tien and Kirk, 1984; Wood and McCrae, 1978). While screening, biochemical purification, and activity evaluation are central components of fungal enzyme discovery, they can be slow to identify specific fungal enzymes responsible for a chemical transformation and restrict discovery to easily assayed enzymatic activities.

4.2. Classic molecular approaches to identify fungal enzymes and pathways

Molecular biology, and recombinant DNA methods in particular, have greatly expanded fungal enzyme discovery through a number of approaches. A common technique is to clone a cDNA library from fungi grown under conditions hypothesized or proven to produce a desired enzyme activity into a vector for heterologous expression. The library is then expressed and the resulting proteins screened for activity. This approach has successfully identified more than a 100 cellulases from *N. patriciarum* (Xue et al., 1992b) and *H. insolens* (Dalbøge, 1997). Despite the utility of this method, it can easily generate hundreds of clones that do not contain the desired enzyme. More sophisticated methods employ techniques such as complementation to enrich the function of the resulting library through the use of hosts with deleterious phenotypes that must be 'complemented' with targeted cDNA for survival (Saloheimo et al., 1997). Similarly, techniques such as differential hybridization (Reymond et al., 1991) and suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) have been used to enrich cDNA unique to conditions that produce a given enzyme. In these methods, mRNA from cells grown under inducing and non-inducing conditions are converted to cDNA and uniquely labelled. These libraries are then hybridized against mRNA from the opposite condition leaving unique transcripts with a single label, which may then be selectively amplified (SSH) or purified (differential hybridization) before cloning. For example, SSH was used to identify an elusive polyketide synthase from *A. ochraceus* responsible for the production of a mycotoxin (O'Callaghan et al., 2003), while differential hybridization revealed a new exo-cellobiohydralase from *N. frontalis* (Reymond et al., 1991). Molecular library cloning approaches have also been used to discover fungal metabolic pathway genes. While eukaryotic genomes do not contain operons, fungal systems regularly cluster genes for biosynthetic pathways related to secondary metabolite production (Brakhage, 1998; Kämper et al., 2006; Keller et al., 2005; Nierman et al., 2005). Exploiting this fact, researchers have created genomic libraries consisting of 30-80 kb DNA fragments in fosmids and artificial chromosomes for expression in bacteria and other fungal systems. These powerful

approaches have reconstituted pathways containing several fungal enzymes that synthesize a wide array of pharmaceutical backbones (Bok et al., 2015; So et al., 2012).

4.3. Fungal enzyme discovery in a post-genomic era

The past decade has seen extraordinary advances in sequencing technology that have rapidly sequenced the genomes of many previously uncharacterized fungal systems, including elusive anaerobic fungi (Dean et al., 2005; Haitjema et al., 2017; Kämper et al., 2006; Martinez et al., 2008; Nierman et al., 2005; Solomon et al., 2016; Youssef et al., 2013). These sequences are annotated and curated in fungal databases such as the Saccharomyces Genome Database (Cherry et al., 2012), the Aspergillus Genome Database (Arnaud et al., 2010), and the JGI Mycocosm, which is a repository for the 1000 fungal genome project covering all branches of fungi (Grigoriev et al., 2014). The pace of sequencing has greatly overtaken discovery efforts, resulting in a growing catalog of unexplored fungal enzymes. However, bioinformatic tools and approaches readily analyze these growing datasets to provide new insight. Classical bioinformatics approaches include homology and Hidden Markov Model (HMM) searches that analyze new sequences for conserved signatures found in well-documented proteins (Karplus et al., 1998; McGinnis and Madden, 2004). These approaches were recently applied to both genomic and transcriptomic sequences to discover hundreds of new cell wall degrading enzymes in gut fungi *P. finnis* and *Orpinomyces sp.* C1A (Solomon et al., 2016; Youssef et al., 2013). Like its molecular analog, the sample space of genes to be examined may be reduced by gene set enrichment analysis (Clark and Ma'ayan, 2011; Subramanian et al., 2005) and differential expression analysis (Anders and Huber, 2010) of the transcriptome to identify enzymes whose production is enhanced under specific conditions (Solomon et al., 2016). These genomics approaches are increasingly complemented with proteomics methods that map individual proteins back to the genes that produce them to identify new enzymes from fungal secretions and lysates (Doyle, 2011; Solomon et al., 2016).

A particularly interesting recent study shows the power of combining transcriptomic and proteomic based approaches to identify novel biomass degrading enzymes in gut fungi (Wang et al., 2011). Here, transcriptomic and secretomic analyses were used to identify 219 putative glycoside hydrolase contigs in *N. patriciarum W5* that were subsequently classified into 25 different glycoside hydrolase families. Subsequently, 19 of the genes were expressed and screened for activity in yeast. One β-glucosidase and one exocellulase showed particularly strong activities and may lend themselves to commercialization. Another study that highlights how 'omics' based approaches are a powerful tool in enzyme discovery is the transcriptomics-guided construction of an enzyme cocktail composed of various biomass-degrading enzymes from *Orpinomyces sp. C1A* (Morrison et al., 2016b). The enzymes were produced and purified

from *E. coli*, mixed in different ratios, and the activity of the cocktail on various substrates was assayed, resulting in the identification of several promising enzyme candidates for lignocellulosic bioprocessing.

5. Challenges and potential solutions

Two of the greatest challenges facing the further development of anaerobic, and other non-conventional, fungi for metabolic engineering applications are their unique growth requirements and their genetic intractability. Furthermore, owing to the difficulty in maintaining cultures of isolates (Haitjema et al., 2014) it is not surprising that so few basal clades (like the anaerobic fungi) have been characterized. Similar to "unculturable" prokaryotes (Cowan et al., 2005), the construction of viable culture collections is limited by the lack of information on the nutritional requirements of non-conventional fungi, *e.g.* dependence on another species to provide a key resource. Furthermore, the slow pace of discovery and biotech translation from these fungal clades is likely the result of the relatively small number of researchers dedicated to studying non-model fungi.

5.1. Challenges to exploiting novel isolates

Apart from difficulties in isolating and maintaining cultures of slow-growing non-conventional fungi in a laboratory setting, the fungal isolates may be remarkably resistant to further genetic manipulations. Currently, only one report describes the transformation of anaerobic fungi with a plasmid that encodes a heterologous gene (Durand et al., 1997). In the 1997 study, *N. frontalis* was biolistically transformed with a plasmid containing the bacterial β-glucuronidase gene, downstream of a promoter sequence that was previously derived from the fungus (Fischer et al., 1995). While activity of the reporter protein was detected, gene expression was transient and lost after only a few generations, possibly due to the lack of a selection marker on the plasmid. The authors suggest that for improved stability, the reporter gene should be coupled to a selection marker such as a gene encoding resistance to an antibiotic, which would minimize the risk of plasmid loss over time (Durand et al., 1997). However, there are additional considerations, such as the mechanisms by which a plasmid is maintained in the host organism. A prerequisite for stability of the transformed strain is that the host is able to replicate and distribute the plasmid to progeny, and currently the gut fungal mechanisms for plasmid replication and maintenance remain unknown. Further, given the complicated life cycle of gut fungi that involves both a single-celled motile zoospore-stage and a stage where a maturing sessile sporangium encapsulates hundreds of novel zoospores (Orpin, 1975), it is difficult to predict when, and how, the fungi should be transformed for optimal stability. These challenges may be overcome by more permanent genome engineering, which necessitates high-resolution genomic information and suggested regions of integration.

Recently, several versatile genome-editing tools have been developed, based on zinc finger nucleases (ZFN) (Porteus and Carroll, 2005), transcription activator-like effector nucleases (TALENs) (Arazoe et

al., 2015; Boch et al., 2009; Christian et al., 2010), recombination-mediated multiplex automated genomic engineering (MAGE) (Wang et al., 2009), and the increasingly popular clustered regularly interspaced short palindromic repeats/CRISPR associated protein (CRISPR/Cas) system (Cong et al., 2013; Deltcheva et al., 2011; Fuller et al., 2015; Gasiunas et al., 2012; Jinek et al., 2012; Liu et al., 2017, 2015; Mali et al., 2013; Nødvig et al., 2015). Nonetheless, lagging development of a well-defined genetic toolkit for non-conventional fungi, such as promoters, transcriptional terminators and ribosomal binding sites, limits direct manipulation efforts. Also, in order to edit genomes using these methods, it is necessary to get DNA across the gut fungal cell wall without compromising viability, and again the intricate and largely anaerobic life cycle of gut fungi serve as an example of the complications that should be taken into consideration. Moreover, compared to most model organisms, the gut fungal genomes are remarkably AT-rich (Haitjema et al., 2017; Nicholson et al., 2005; Youssef et al., 2013), and that may affect how the organism handles DNA that does not adhere to the apparent codon preference (Komar, 2016). Last, virtually nothing is known about the post-translational modifications or folding behavior of gut fungal proteins, and that should be taken into account when one wants to use a heterologous selection/reporter protein.

In contrast, the plummeting cost of DNA-sequencing and -synthesis, and the development of sophisticated molecular tools have greatly facilitated the discovery of enzymes and the transfer of these enzymes into amenable microorganisms such as bacteria and yeasts. In a way, this abolishes the need to cultivate the original host organism at all: as long as we have the sequence, from *e.g.* a metagenomic library, we can transfer the gene to a workhorse microbe to detect expression and activity. In other words, researchers can select enzymes and biosynthetic pathways from various sources and put them together in a cell factory host of choice. Within the context of directly converting lignocellulose to bioproducts within one organism, this approach is termed consolidated bioprocessing. Unfortunately the "superbug" approach has its own associated drawbacks, such as the high metabolic strain associated with the overexpression of multiple heterologous genes and potential inhibitory or toxic effects of products (Peng et al., 2016).

5.2. Potential for partnering non-model fungi with other organisms

The use of microbial communities for bioprocessing is desirable because it alleviates the metabolic strain associated with expressing an abundance of heterologous pathways in a single organism (see (Wu et al., 2016) for a review of this problem). Although significant progress has been made in this area, heavily modified organisms, a.k.a. "superbugs", typically suffer from very low yields and titers. It has been argued that by splitting pathways into modular subunits across co-operating organisms it is possible to alleviate this problem (Zhang and Wang, 2016). This so-called consortium approach is especially

attractive for the development of biological systems that convert raw plant biomass into sugars, while simultaneously producing some target commodity chemical.

An application of this idea is to design a consortium of different specialist species for an application like biomass deconstruction. For example, recently the model fungus *T. reesei* was co-cultured with *E. coli* to produce isobutanol from pretreated corn stover (Minty et al., 2013). In this example, the use of pretreated biomass is a significant practical limitation inherent to the nature of the cellulolytic specialist. If a cellulolytic species that specializes in the breakdown of raw lignocellulosic plant biomass, such as a non-model anaerobic fungus, were used, it could plausibly reduce the costs associated with biomass pretreatment. This approach also may not require genetic engineering of the non-model host, which can save time to application and potentially open up more rapid avenues to commercialization in the field.

The challenge with this proposition is that the conditions necessary to form a stable consortia are not well understood, even for model microbes (Shong et al., 2012). Furthermore, understanding the metabolic links which would lead to stability within high (more than 2) dimensional consortia is likewise challenging (Ponomarova and Patil, 2015). These limitations need to be overcome to leverage the natural traits of non-conventional fungi for consortia-based bioprocessing. Despite these challenges, the successes achieved by conventional organisms within the consortia paradigm indicate that further research is warranted, see (Kalyani et al., 2013; Zhang et al., 2015; Zhou et al., 2015; Zuroff et al., 2013; Zuroff and Curtis, 2012) for prominent examples. Fortunately, with the plummeting cost and wide spread use of omics-based technologies, it has become feasible to construct genome scale models for non-model organisms that can facilitate the choice of microbial pairings (Song et al., 2014). This could allow researchers to rapidly generate hypotheses concerning metabolic linkages, which could be used to promote stable consortia between non-model biomass-degrading fungi and more conventional microbial cell factory organisms.

6. Conclusions

Fungi are a particularly important and useful class of organisms with significant potential to transform the landscape of a bio-based economy. To fully realize the sustainable potential of bioprocessing and cell factories, there is a compelling need to identify novel enzymes that can be exploited for various biotechnological applications. Although much effort has been spent on characterizing and optimizing a handful of fungi, the vast majority of fungi are significantly under-exploited and we have yet to decipher the hidden potential within their genomes. One motivation for spending engineering resources on non-model species like anaerobic fungi is their remarkable ability to decompose unpretreated lignocellulosic plant biomass. Given that the current industrial titan, *T. reesei*, has likely been engineered to the boundary

of its capabilities, development of organisms like these would be timely. Although this is a daunting task given the scope of the problem, modern 'omics' based tools make discovery and characterization less art and more science. The high throughput revolution and easy access to big biological data invites metabolic engineers to tackle this problem. Whether through heterologous expression of handpicked genes in model organisms, exploiting their natural ability to form mutualistic consortia, or via direct genetic engineering – these strategies can, and should, be leveraged to reclaim the energy trapped in lignocellulosic biomass waste for sustainable bioproduction.

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Figures

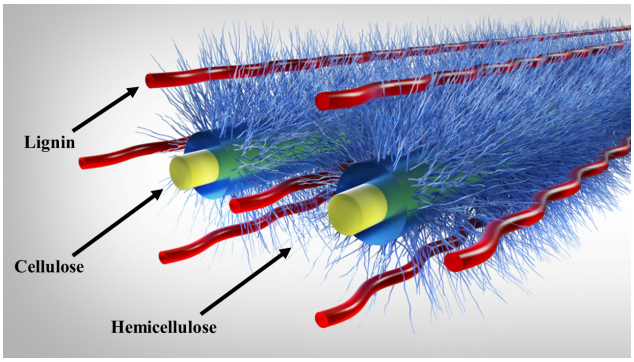


Figure 1: Qualitative diagram of lignocellulose composition. Cellulose is polymer of D-glucose linked by β-1,4-glucosidic bonds with a degree of polymerization of up to 10,000 or higher. Cellulose chains typically aggregate into crystalline fibrils of up to 36 chains. Hemicellulose is much more heterogenous and is composed of a variety of monomers (typically D-xylose or D-mannose). The degree of polymerization is usually significantly less than that of cellulose, around 200 or lower. Lignin is a complex polymer composed of phenyl propane units; it is the most abundant non-polysaccharide in lignocellulosic plant biomass (Jorgensen et al., 2007).

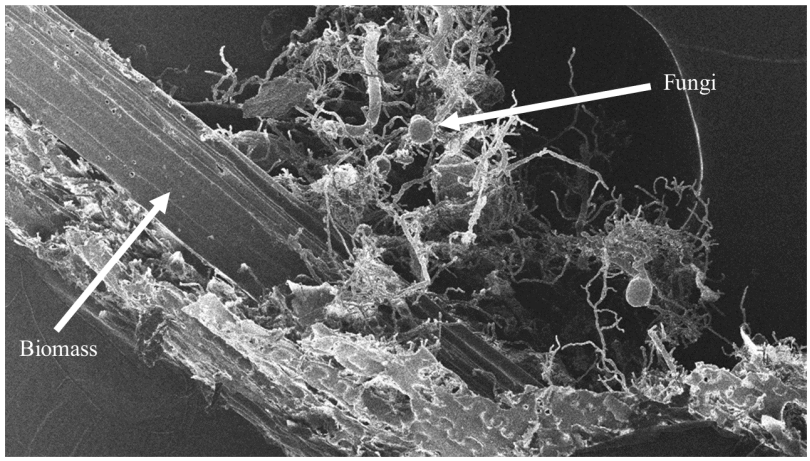
548 

Figure 2: Micrograph showing invasive anaerobic fungal growth on lignocellulosic plant biomass. During the fungal growth cycle a root-like rhizoidal network is formed, which disrupts the biomass structure. The hyphae secrete the cellulolytic enzymes in close proximity to the substrate, greatly enhancing biomass degradation compared to the free diffusing enzyme system of aerobic fungi (Gilmore et al., 2015)**.**

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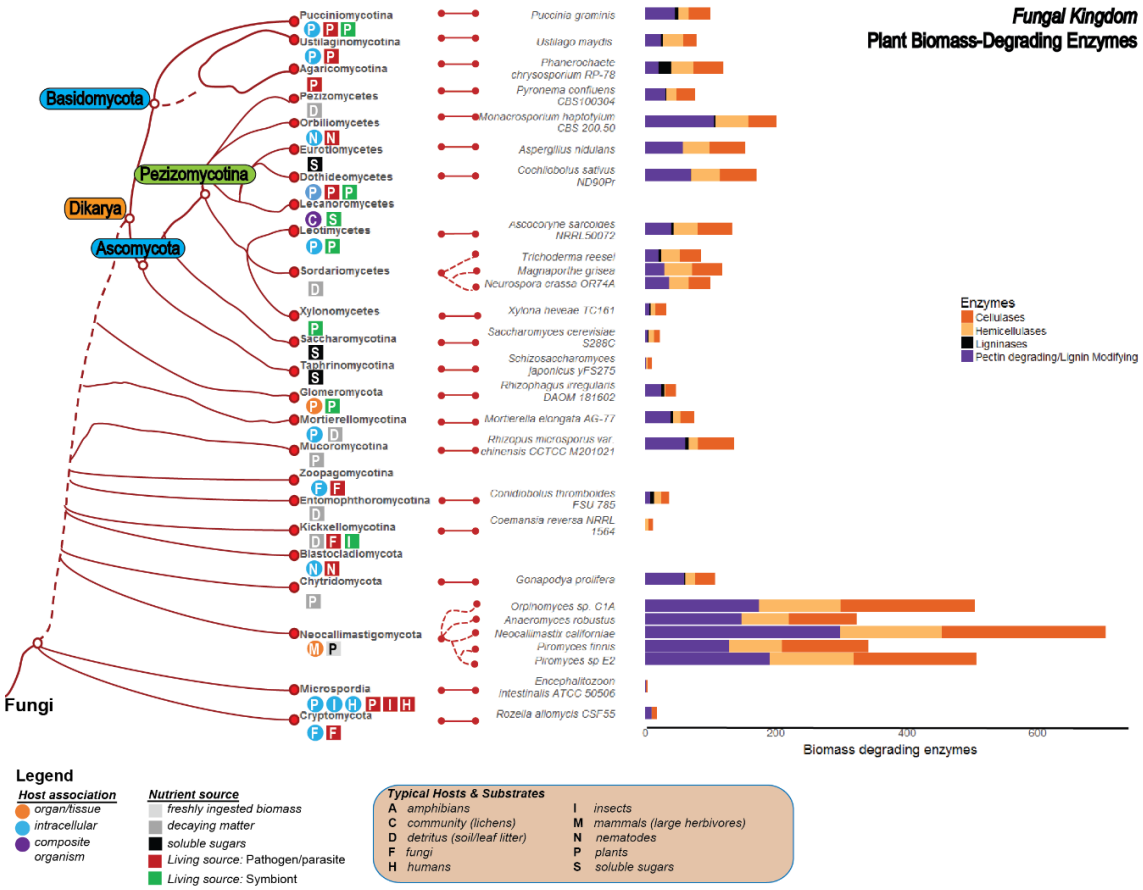

Figure 3: Plant biomass-degrading enzyme content & diversity in the fungal kingdom. Genomes of 27 fungi presenting different divisions of fungal tree of life revealed that the genomes of gut fungi contain the largest number of biomass-degrading enzymes, mainly cellulases (orange), hemicellulases (yellow) and pectin degrading/lignin modifying enzymes (purple) (Arnaud et al., 2010; Chang et al., 2015; Corradi et al., 2010; Crous et al., 2012; Dean et al., 2005; Duplessis et al., 2011; Galagan et al., 2003; Gazis et al., 2016; Gianoulis et al., 2012; Goffeau et al., 1996; Grigoriev et al., 2014; Haitjema et al., 2017; James et al., 2013; Kämper et al., 2006; Martinez et al., 2008; Meerupati et al., 2013; Ohm et al., 2014, 2012; Rhind et al., 2011; Solomon et al., 2016; Tisserant et al., 2013; Traeger et al., 2013; Uehling et al., 2017; D. Wang et al., 2013; Youssef et al., 2013). The tree was modified from Mycocosm (Grigoriev et al., 2014).

2017	First high-resolution gut fungal genomes are published, enabled by PacBio sequencing (Haitjema et al., 2017)
2017	A parts list of gut fungal cellulosomes is published (Haitjema et al., 2017)
2016	Omics-based analyses guide the manufacturing of a gut fungal enzyme cocktail for the efficient degradation of plant biomass (Morrison et al., 2016)
2016	Transcriptomic analyses suggests that gut fungi possess novel sugar receptors and transporters (Seppälä et al., 2016)
2016	Transcriptomic analysis across gut fungal species reveals that anaerobic fungi contain the highest number and diversity of biomass-degrading enzymes (Solomon et al., 2016)
2013	First gut fungal genome is sequenced (Youssef et al., 2013)
2012	Directed evolution of gut fungal xylose isomerase improves its performance (Lee et al., 2012)
2011	Identification of novel gut fungal cellulases through secretomic and transcriptomic analyses (Wang et al., 2011)
2010	Further engineering of gut fungal xylanases (You et al., 2010; Cheng et al., 2015)
2008	Immobilization of a thermostable gut fungal xylanase on artificial oil bodies (Hung et al., 2008)
2007	Incorporation of gut fungal cellulases in bacterial mini-cellulosomes (Mingardon et al., 2007)
2005	Co-expression of rumen microbial β-glucanase, cellulase and xylanase allow *L. reuteri* to break down beta-glucan and xylan (Liu et al., 2005b)
2003	Gut fungal xylose isomerase allows *S. cerevisiae* to ferment xylose anaerobically (Harhangi et al., 2003; Kuyper et al., 2004, 2003)
2001	Heterologous gut fungal glycoside hydrolases enhances the lichenan utilization and solvent production of *C. bijerinckii* (Lopez-Conteras et al., 2001)
2001	An alkalophilic and thermostable gut fungal xylanase produced by protein engineering (Chen et al., 2001)
1997	Heterologous production of gut fungal esterases (Dalrymple et al., 1997)
1992	Heterologous production of gut fungal cellulases and xylanases in model microbes (Xue et al., 1992; Gilbert et al. 1992)
1991	cDNA libraries are used to identify novel cellulolytic enzymes (Reymond et al., 1991; G. Xue et al., 1992)
1975-	Gut fungi are discovered and their powerful biomass degrading capabilities are recognized (Orpin, 1975, 1977; Mountfort and Asher, 1985; Pearce and Bauchop, 1985)

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564 **Figure 4: Timeline of the discovery and utilization of selected enzymes sourced from anaerobic**
565 **fungi.**

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