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The importance of sourcing enzymes from non-conventional fungi for metabolic engineering and 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 *Neocallimastigomycota*, 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.

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

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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.

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 (Fig. 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 (Fig. 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 (Fig. 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

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 Bottom of Form	(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 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	Paper and pulp industry, textile industry, pharmaceutical industry, bioremediation, biomass conversion	(Busse and Czermak, 2016; Hofrichter et al., 2010)

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