White-rot fungi: the key to sustainable biofuel production?

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> "…it is clear that the biofuels industry has an opportunity to exploit the innate abilities of white-rot fungi through biochemical understanding, biotechnological creativity and innovative engineering, as a means to maximize conversion efficiency, decrease biofuel cost and encourage sustainable biofuel production."

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Pretreatment of biomass is required in order to efficiently access the energy potential of the substrate. Many pretreatment strategies have been described, which are typically thermochemical or thermomechanical processes that are major contributors to the costs of producing energy from biomass [1]. Pretreatment strategies that overcome this key barrier to biofuel production can therefore be very beneficial. One approach is to exploit the natural ability of saprophytic fungi to access the energy-rich components of this difficult substrate. These fungi can be applied to many different lignocellulosic substrates and, through their natural growth at ambient temperatures, these organisms will readily degrade and modify the lignin component. This 'biological' pretreatment is known to decrease the severity of subsequent thermochemical or thermomechanical pretreatments, or even eliminate the necessity of these energy-intensive processes [2].

Biochemical mechanism of lignocellulose degradation

Wood-degrading fungi can access densely-packed lignocellulose by producing chemicals, primarily reactive oxygen species, that are small enough to penetrate the substrate that is generally too compact for enzymes to access. The production of hydrogen peroxide by fungal redox enzymes results in the production of highly reactive hydroxyl radicals ($OH^{\bullet}$), which are oxidizing species that cause downstream reactions, leading to covalent bond cleavage in both lignin and cellulose. Other notable redox enzymes, such as manganese peroxidase and laccase, catalyze similar nonspecific breakage of covalent bonds in lignocellulose. Cellobiose dehydrogenase (CDH) oxidizes cellobiose rather than lignin, and transfers two electrons to many different substrates, including ferric and cupric ions, metalloproteins and other substrates [3]. Although the substrates

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reduced by CDH can theoretically participate in reactions leading to the cleavage of both lignin and cellulose, it is becoming increasingly clear that the primary role of CDH is in facilitating access to carbohydrate polymers within lignocellulose [3,4].

Hydrolytic enzymes, unlike redox enzymes, recognize and catalyze the cleavage of specific glycosidic bonds within lignocellulose to release metabolizable sugar molecules. An array of carbohydrate-degrading enzymes is produced that act synergistically, which include endo-(1,4)-β-glucanase (endocellulase), cellobiohydrolase (exocellulase) and β-glucosidase. An analogous armada of enzymes recognize and act upon the glycosidic linkages found within hemicellulose, including endo-xylanases, endo-α-L-arabinase, endo-mannanase, β-galactosidase and corresponding β-glucosidases. Esterases (e.g., feruloyl esterase and glucuronoyl esterase) support the activity of these enzymes by cleaving covalent bonds between lignin and hemicellulose.

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Most of these enzyme families (e.g., oxidases, hydrolases and esterases) can be found in all three groups of fungi: soft rot, brown rot and white rot. Soft-rot fungi preferentially degrade plant polysaccharides, whereas brown-rot fungi produce enzymes for cellulose and hemicellulose degradation with minimal modification to the lignin [5]. White-rot fungi (WRF) are a unique group that degrade cellulose, hemicellulose and lignin approximately equally. The biomass resulting from the attack becomes white and very light, because WRF typically degrade lignin before cellulose.

One of the best known industrial enzyme cocktails used to produce fermentable sugars from plant biomass is produced from the soft-rot fungi *Trichoderma reesei*, a large producer of hydrolases. However, the *T. reesei* genome contains a limited set of cellulases and hemicellulases, and several approaches need to be combined to improve the hydrolysis capacity of the cocktail and decrease the final cost of the bioethanol produced [6]. One of the pertinent strategies is to find, within the fungal biodiversity, complementary or synergistic enzyme activities necessary for the *T. reesei* cocktail to more efficiently release sugar from lignocellulosic substrates. This objective could be achieved either by comparing enzyme profiling and proteome analysis of several filamentous fungi, including *T. reesei* [7], and/or by comparing genome data using dedicated databases from the large number of available fungal genomes [8]. Candidate enzymes from other organisms can be selected from enzyme families that are poorly represented in *T. reesei* when compared with other fungi, or among enzyme families that are entirely absent in the *T. reesei* genome. Finally, selected enzymes could be produced in an appropriate fungal host, recovered and tested in combination with the *T. reesei* secretome [9].

Biotechnology applications

The capacity of WRF to selectively deconstruct lignocellulose has been exploited for decades, with initial research rooted in pulp and paper technologies [10]. Collectively, this research demonstrated the efficacy of treating wood destined for pulping with WRF, specifically with the goal of reducing or eliminating the negative properties of lignin on paper quality. With the recent resurgence of interest in biofuels, WRF are garnering attention once again. However, instead of modifying wood for pulp and paper production, WRF are being explored as a means to condition a variety of biomass relevant to biofuel production. Although enzyme cocktails for the biochemical treatment of biomass are available, their prohibitive cost has limited their utility in biofuel production. As a result, solid-state WRF fermentation of dedicated crops and agricultural residue suitable for biofuel feedstocks may be a more cost-effective strategy. With the proper environmental conditions, WRF will utilize a suite of specialized enzymes, such as those described above, to deconstruct lignin linkages to access the energy-rich carbohydrates necessary for survival. This is advantageous from an industrial perspective, since lignin removal from biomass is necessary for efficient conversion to biofuels as well. Recent studies have highlighted the efficacy of using WRF to improve biomass-to-biofuel conversion efficiency. For instance, Canam *et al.* demonstrated that canola straw treated with *Trametes versicolor* significantly reduced the pretreatment severity required for efficient lignin removal [3]. In addition, a novel strain of *T. versicolor* (deficient in CDH activity) was shown to not only preserve but enhance the level of fermentable carbohydrates after pretreatment [3].

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An alternative strategy to solid-state fermentation and direct enzyme application is the *in planta* production of fungal enzymes in plants destined for biofuel production. While commercial application has not yet been realized, there have been a number of promising studies indicating the efficacy of this strategy, particularly from non-WRF fungi. Recently, Tsai *et al.* explored the effects of *in planta* overexpression of a

lignocellulytic enzyme (glucuronoyl esterase) from the WRF *Phanerochaete carnosa* within *Arabidopsis thaliana*, which led to an increase in xylose extractability of the biomass [11].

Engineering & processing considerations

To ensure a cellulose-rich but highly delignified biomass for biofuel production, WRF highly selective in lignin degradation are preferred for fungal pretreatment. Selective lignin-degrading WRF degrade larger amounts of lignin relative to cellulose. However, fungal selectivity varies among species and with pretreatment time. In addition, fungal performance on degradation and the resulting digestibility varies with different feedstocks [12]. For example, *Ceriporiopsis subvermispora* effectively degrades lignin in corn stover, switchgrass and wood, but not in soybean straw or wheat straw [13]. Processing parameters, such as moisture, temperature and aeration, are crucial for lignin degradation. Although optimal moisture content varies with strains and substrates, prior studies suggested that fungi can grow well and substantially degrade lignin with an initial moisture content of 60–85% [12]. In general, WRF can grow between 15 and 35°C, and their highest delignification rate is generally obtained within an optimal temperature range of 25–30°C [14,15]. Aeration is necessary to dissipate heat generated by fungal metabolic activity but, more importantly, to provide uniform air/oxygen diffusion throughout the substrate. Oxygen enrichment could increase the delignification rate but may not affect delignification selectivity [15,16]. In general, aeration needs to be controlled to ensure effectiveness of fungal pretreatment.

C. subvermispora was tested recently, and was shown to degrade lignin selectively and preserve cellulose in corn stover and switchgrass during pretreatment at ambient temperature [13,17]. After 35 days of fungal pretreatment with *C. subvermispora*, the optimal glucose yield of corn stover reached 67% (based on the original dry matter of corn stover), which was comparable with that obtained with thermochemical pretreatment [17,18]. Among the three fractions of corn stover (leaves, stalks and cobs), leaves had the least recalcitrance to fungal pretreatment, and the lignin degradation of leaves reached 45% after 30 days of pretreatment [19]. However, stalks had the highest glucose production potential followed by the cobs and then the leaves. This suggests that the leaves may have more value as a soil amendment, while the cobs and stalks would be more suitable for biofuel production [19]. During concurrent wet storage and fungal pretreatment with *C. subvermispora*, the moisture content of corn stover gradually decreased from 75 to 5% during 90 days of pretreatment [20]. The major challenges to scaling up the concurrent wet storage and fungal pretreatment technology are the requirements of sterilization and relatively high moisture content of the feedstock.

Conclusion

Saprophytic fungi, and WRF in particular, have inherent enzymatic mechanisms that are key to providing access to energy-rich carbohydrates locked in lignocellulose. Efficient access to these coveted carbohydrates is also the primary mission of fermentation-based biofuel production facilities. Therefore, it is clear that the biofuels industry has an opportunity to exploit the innate abilities of WRF through biochemical understanding, biotechnological creativity and innovative engineering, as a means to maximize conversion efficiency, decrease biofuel cost and encourage sustainable biofuel production.

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