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Enzymatic deconstruction of plant biomass by fungal enzymes

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Lignocellulosic plant biomass is the world's most abundant carbon source and has consequently attracted attention as a renewable resource for production of biofuels and commodity chemicals. Still the process is economically not fit enough to compete with then use of fossil resources, and the costs associated with enzymatic hydrolysis and product recovery are the major obstacle. The discovery of the role of non-hydrolytic enzymes in lignocellulose hydrolysis has recently contributed significant improvements to hydrolysis but also added new challenges to the biomass to ethanol process. Transfer of the new insights to the industrial scale and shaping the enzymes to tolerate associated adverse conditions has now shown first success, thus optimizing the economy of cellulosic ethanol (or other biofuel) production.

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Introduction

Lignocellulosic plant biomass is the world's most abundant carbon source and has consequently attracted attention as a renewable resource for production of biofuels and commodity chemicals that could replace fossil resources [1]. It typically consists of a three-dimensional network of cellulose (40–50%), hemicellulose (25–30%), and lignin (15–20%). Therein, cellulose forms highly organized microfibers that are intimately associated with hemicelluloses. This microstructure is covered by lignin, a heteropolymer of phenolic substance, which acts as a protective shield. This structure makes lignocellulose highly recalcitrant to biological degradation [2].

Nevertheless, lignocellulose is completely recycled in nature [3•], suggesting that there are organisms that possess the appropriate enzymes for degradation of this complex structure. Fungi are on top of this list, and have developed several strategies to break the recalcitrance and feed on the polysaccharides [4]. Enzymes used in industry today are therefore almost entirely of fungal origin [5]. Most of them are produced by the ascomycete *Trichoderma reesei*, whose biology and application has recently been reviewed [6•].

Despite of the availability of strains producing the enzymes that degrade plant biomass to monosaccharides in sufficient quantities, the costs for enzyme production and enzymatic hydrolysis still represent the limiting step in the lignocellulose bioconversion process (Table 1). Here, we will review the recent progress in understanding the reasons for this and how these bottlenecks may be overcome, thereby mainly emphasizing on cellulases and auxiliary enzymes. A broader survey dealing also with hemicellulases and ligninases has most recently been published [7].

What limits cellulase activity?

The 'cellulases' used in lignocellulose depolymerisation are enzyme cocktails comprising various glycoside hydrolases with synergistic functions that efficiently cleave the respective glycosidic linkages. The canonical view of cellulose depolymerization was that endo-β-1,4-glucanases (EG; enzymes that are believed to randomly hydrolyse β-1,4-glucosidic linkages primarily in amorphous regions of polymer fibres; EC 3.2.1.4); and cellobiohydrolases (CBH; enzymes that attach to carbohydrate chains and processively hydrolyse disaccharide units from the end of a chain without dissociation after each catalytic event; EC 3.2.1.-). They act in synergy on cellulose and the arising cellobiose units are finally hydrolyzed to D-glucose by β-glucosidases (EC 3.2.1.21). In the overall process, the processivity (i.e. the enzyme's ability to catalyse consecutive reactions without releasing its substrate) of the CBHs is the major power in the hydrolytic potential of cellulases, and they therefore make up for more than 70% of protein in fungal cellulolytic secretomes [8••]. Consequently, the mechanism of catalysis and its rate limiting steps have been subject to many detailed investigations (for review see [8••]). Jalak *et al.* [9••] showed that the overall rate of lignocellulose hydrolysis was limited by the rate of CBH1 (=CEL7A) processivity (in average 52 steps), and the factors influencing their processivity of CBHs has consequently become a major focus of research [8••,9••,10,11]. Using optical tweezers to monitor single-molecule motility for tracking

Table 1

Major parameters negatively affecting lignocellulose hydrolysis

Parameter	Consequences
Fibrous nature of substrate	Impedes mass transfer at high substrate loadings
Lignin	Binding of cellulases; impairs cellulase recycling
Lignin degradation products (vanillin, tannic acid, syringaldehyde)	Inhibition of cellulases
Byproducts of pretreatment (formic acid, furfural)	Inhibitory to cellulases and hemicellulases

of CBH1 showed that it acts at distinct steps on the scale of 1 nm, and is not mechanically limited [12••]. Their analysis also revealed that the catalytic domain alone is sufficient for processive motion and the rate-limiting steps in a cycle are of biochemical and not mechanical nature. A comparison of several fungal CBHs showed that a high moving velocity of CBH1 on the cellulose surface correlated with a weak interaction between enzyme and substrate, whereas slow movement resulted in higher processivity associated with stronger interaction [13]. CBH1 acts via a retaining two-step mechanism that includes the formation of a glycosyl-enzyme intermediate and the glycosylation product (cellobiose) subsequently positioned a water molecule for nucleophilic attack on the anomeric carbon atom of the glycosyl-enzyme intermediate [14], and results from molecular modelling suggest that this glycosylation rather than chain processivity is the actual rate limiting step in CBH1 activity [15,16••,17].

In contrast, CBH2 (=CEL6A) operates by a single step, inverting mechanism that does not involve a glycosylated intermediate, and is less processive than CEL7A [9••]. The differences in the catalytic cycle between CEL6A and CEL7A are listed in Table 2.

'Non-hydrolytic' accessory proteins

In addition to hydrolytic enzymes, there is now compelling evidence that also non-hydrolytic proteins play pivotal roles in lignocellulose degradation (cf. Figure 1).

Lytic polysaccharide monooxygenases (LPMOs)

The discovery of the LPMOs (also termed AA, auxiliary activities) was a major breakthrough in research on lignocellulose degradation, and has been subject of several excellent reviews [18–20]. They catalyse the oxidation of one of the carbons in the β-1,4-glycosidic bonds, either on C1 or C4, by a mechanism that involves cycling of a copper atom between Cu (I) and Cu (II) to activate molecular oxygen, whereby a copper-oxyl radical abstracts a hydrogen and then hydroxylates the substrate via an oxygen-rebound mechanism involving a type-2 Cu site, molecular oxygen and an electron donor. *In vitro*, L-ascorbate is frequently chosen as external electron donor. Yet there is evidence that the heme group of cellobiose dehydrogenase (CDH) is the physiological donor [21•]. LPMOs degrade cellulose nano-fibrils exposed on the surface into shorter and thinner insoluble fragments. The prior action of LPMO enables cellulases to attack otherwise highly resistant crystalline substrate areas and promotes an overall faster and more complete surface degradation [22]. Consequently, supplementation of the enzymatic cocktail Cellic CTec1 (Novozymes) with an LPMO (also termed auxiliary activity 9, AA9) improved the hydrolysis of lignocellulose [23], and AA9 has now been added as a new component to result in the advanced cellulase preparations Cellic CTec2 and Cellic CTec3. Interestingly, an LPMO from *N. crassa* (NcLPMO9C) was also able to attack xyloglucans, β-glucans, and glucomannan [24]. The crystal structure of the catalytic domain of NcLPMO9C revealed an extended, highly polar substrate-binding surface well suited to interact with a variety of sugar substrates [25•].

Table 2

Enzymatic mechanism and catalytic cycle of the two cellobiohydrolases CEL6A and CEL7A*

	Step	CEL7A	CEL6A
Substrate		Reducing end	Non-reducing end
Mechanism		Two steps, retaining	One step, inverting
Enzyme conformation		Stable	Changes
Substrate conformation		Changes	Stable
Catalytic cycle:	1	Product site vacant	Product site vacant
	2	Cellulose chain processed over the catalytic site	Cellulose chain processed over the catalytic site
	3	Rotation/translation of the cellulose chain and distortion of the −1 sugar ring to form the Michaelis complex	Closing of the catalytic center loop wherein the Michaelis complex is formed
	4	Forming the glycosyl–enzyme intermediate (GEI) with the cellobiose product in 'Unprimed' mode	Hydrolysis starts to form the Substrate–Product complex with an α-cellobiose unit in the product site
	5	Translation of the product towards the tunnel exit producing the 'primed' GEI	Opening of the catalytic center loop and product expulsion
	6	Deglycosylation breaks the glycosyl–enzyme covalent bond, and allows product expulsion	

* Summarized from [8••].