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Minireview

Enzymatic Breakdown of Biomass: Enzyme Active Sites, Immobilization, and Biofuel Production

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Saikat Dutta,[a] Kevin C.-W. Wu[a]*

A bacterial enzyme efficiently breaks down cellulose and hemicellulose without the cooperation of other enzymes. An emerging trend in the versatile development of biomass breakdown techniques involves multidomain cellulase enzymes of bacteria, which efficiently hydrolyze microcrystalline cellulose by outperforming enzyme cocktails typically used commercially to break down biomass produced by the fungus *Hypocrea jecorina*. This article presents a review of current developments in the understanding of the microstructure of plant biomass, treatment of biomass by using bacterial hydrolase enzymes, active site structures of hydrolytic and oxidative enzymes, and their overall impact on the biomass degradation process. This article addresses the nanoscale features of a biomass surface during enzymatic reactions, the implication of enzyme-based biorefinery in biofuel production, and the mechanism of action of cellulases and other enzymes in the degradation of insoluble biomass substrates. The environment and roles of the active sites of the hydrolytic and oxidative enzymes are also discussed. The concept of immobilized cellulase on a solid surface is emphasized, which is an effective alternative for developing biorefineries for biofuel production driven by enzyme function.

Introduction

Biopolymers, such as cellulose, chitin, and diverse marine polysaccharides, are abundant primary feedstocks used in the production of biofuels and chemicals.[1] Since the economic growth of biorefineries became apparent, a detailed understanding of the processes underlying biomass degradation occurring on cellulose surfaces is essential. Plant cell walls represent a vast, renewable carbon source in the biosphere that requires several enzymatic strategies to deconstruct structural polysaccharides.[2,3] Enzymatic cellulose decomposition relies primarily on glycoside hydrolases (GHs) and oxidative enzymes. Various organisms secrete "free enzyme" cocktails, wherein various proteins diffuse independently of one another and work synergistically to degrade biomass.[4] However, the degradation of biomass into fermentable sugars clearly requires the concerted action of GHs and protein accessories. Cellulose and chitin are the two most abundant biopolymer resources.[5] Enzyme action involving three-dimensional (3D) protein arrangement and the chemical biology of enzymes are an emerging field. However, the physicochemical recalcitrance of cellulose and chitin limits rapid and cost-effective degradation.[6] Cellulolytic and hemicellulolytic enzymes that can deconstruct cellulose into fermentable sugars facilitate the use of a plentiful source of renewable carbon. Thus, surveying rich source of GHs and others enzymes that play critical roles in plant cell wall degradation is crucial. GHs catalyze the cleavage of glycosidic linkages located between adjacent carbohydrate residues, generally by using either a configuration-inverting or configuration-retaining acid-catalyzed mechanism. Approximately 115 families of GHs are currently known, based on similarities in their amino acid sequences and 3D folds.[7] GHs and cellobiohydrolases (CBHs) are the primary targets for the product inhibition of the synergistic hydrolysis of cellulose. In nature, cellulolytic microorganisms produce enzymes that function synergistically with microorganisms (cellulosome)[2,8] or act independently (fungal and bacterial cellulases).[9] Three categories of enzymes are essential for hydrolyzing native cell walls: cellulases, hemicellulases, and accessory enzymes (*e.g.*, hemicellulose debranching, phenolic acid esterase, and lignin degrading enzymes). Generally, the hemicellulose barrier of cell-wall microfibrils is exposed by chemical pretreatments, whereas cellulase enzymes hydrolyze the crystalline cellulose cores.

Most cellulolytic enzymes comprise two types: one type includes noncomplexed cellulases and hemicellulases, and the other involves polysaccharides that self-assemble onto a protein scaffold to form macromolecular assemblies of cellulosome (Fig. 1).[2] Few bacteria synthesize cellulosomes, that is, large multi-enzyme complexes containing multiple catalytic units.[10] The multi-enzyme cellulose complex of anaerobic cellulolytic bacteria provides enhanced synergistic activity among the various resident enzymes to hydrolyze cellulosic and hemicellulosic substrates of the plant cell wall efficiently. In this case, a pivotal noncatalytic subunit called scaffoldin secures the various enzymatic subunits into the complex through a cohesin-dockerin interaction. This occurs when cellulosomes physically separate individual cellulose microfibrils from larger particles, resulting in enhanced access to cellulose surfaces with consequent synergistic deconstruction, as revealed using transmission electron microscopy (TEM) analysis (Fig. 2). This indicates the changes in the cellulose surface and internal

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structure of Avicel PH101 particles treated with CelA, in which the individual cellulose microfibrils are identified within the particles.

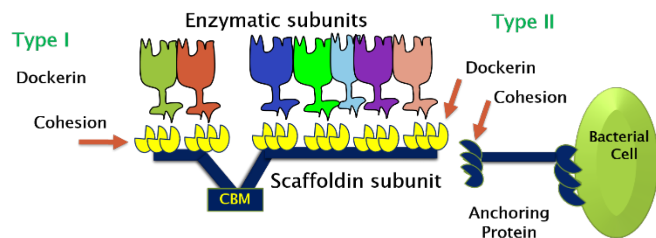

Fig. 1 Bacerial cellulosomal system consisting of macromolecular assemblies of cellulosomes.

The Avicel PH101 particle digested by the Cel7A-containing formulation indicated that one end of the particle was finely tapered to a narrow point (Figs. 2A and B) and the opposite end displayed a blunt edge, exhibiting a slight angle from the long axis (Figs. 2A' and B'); this resulted in a notable digestion mechanism. A comparative analysis of the digestion of crystalline cellulose by free enzymes or cellulosomes, including morphological analysis, revealed that free enzymes ablate the surface of cellulose microfibril bundles preferentially on one end; however, this is distinct from the ablative mechanism of free cellulases in which they separate individual cellulose microfibrils from crystalline cellulose particles for a localized attack. This implies that free enzymes containing single catalytic units per protein molecule and multi-enzyme cellulosomes operate using different mechanisms to deconstruct recalcitrant cell wall polysaccahrides, despite employing similar component enzymes and carbohydrate binding modules (CBMs).

Most commercial enzymes are of fungal origin. In addition to Ce1A (large secreted multidomain cellulase from the thermophilic bacterium), other bacterial enzymes play a crucial role in the enzymatic degradation of plant polysaccharides when combined with commercial fungal enzymes. Bacterial cellulosomes have been observed to substantially enhance the hydrolytic activity of a fungal cellulase. Cellulosomes are multi-enzyme complexes of GHs anchored to noncatalytic subunits (scafoldins); they are mainly observed in bacteria. Methods for producing cellulosic liquid biofuels by using enzymatic hydrolysis have developed since the late 1990s. Brunecky *et al*. reported that new enzyme classes and new modes of actions, such as multifunctional cavity-forming Ce1A, have been discovered.[11]

This review addresses enzymatic cellulose degradation from the perspective of the action mode of cellulase and polysaccharide monooxygenase enzymes. Crystalline biopolymers (cellulose and lignin) and their ability to construct plant cell walls cause biomass recalcitrance. This article emphasizes a model of cellulose degradation and an active site of hydrolase enzymes for glycoside bond cleavage. The enzyme action of polysaccharide monooxygenase and the features it exhibits on a biomass surface during its action is described. The nanoscale morphological change that occurred during enzymatic hydrolysis was observed using high-resolution imaging techniques. The critical role of nanomaterial-enzyme composites in eliminating the difficulties of cellulose hydrolysis through an enzyme-based biocatalytic process and its impact on biofuel production are emphasized, including their critical consequences. This article provides implications for future strategies of enzymatic biomass degradation and its relevance to biofuel production.

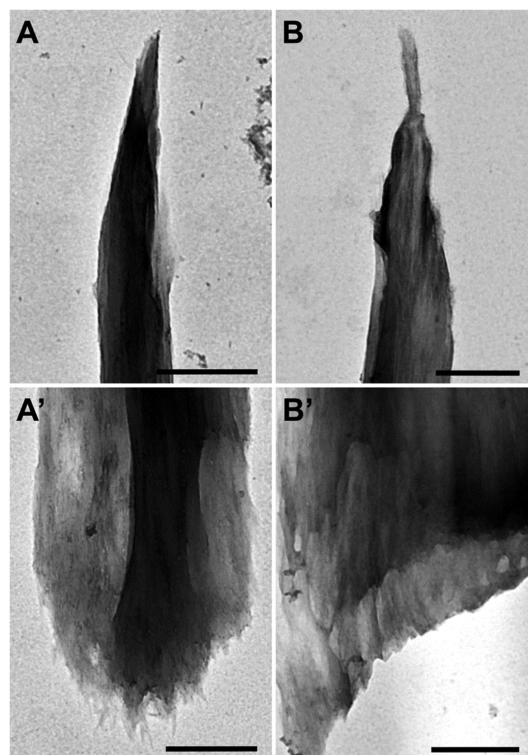

Fig. 2 TEM-micrograph based identification of individual cellulose microfibrils within the electron-translucent particles recovered from digestions carried out to ~65% cellulose conversion when compared to Avicel PH101 (cellulose particles digested to ~60% conversion using CTec2 (composed primarily of Cel7A). (A, B): Particles digested by the Ce17A- displayed morphology where one of the particle is finely tapered to a narrow point. (A', B'): Opposite end of the particle displayed a blunt edge with a slight angle from long axis. (Reproduced from reference 11 with permission from AAAS publishers, copyright 2013).

Recalcitrance of Biomass

The recalcitrant plant cell walls are solid biological substrates composed of cellulose, hemicellulose, and lignin polymers, which can be degraded using hydrolytic enzymes such as hydrolases and redox enzymes.[12] The structure of the crystalline cellulose core of cell-wall microfibrils is constructed by precisely arranged chains of cellodextrins.[13] The chair conformation of the glucose residues in the cellulose forces the hydroxyl groups into a radial (equatorial) orientation and the aliphatic hydrogen atoms into axial positions, resulting in strong interchain hydrogen bonding between adjacent chains in a cellulose sheet and weak hydrophobic interactions between the cellulose sheets (Fig. 3). The hydrophobic face of cellulose is resistant to acid hydrolysis because it forms a dense aqueous layer near the hydrated cellulose surface.[14]

However, hemicellulose and amorphous cellulose are readily digestible. The primary component of plant cell walls, cellulose, is composed of thousands of glucose units that contain both amorphous and highly recalcitrant crystalline regions with O-glycosidic bonds with which glucose units are connected (Fig. 4). Unlike cellulose, which is a homopolymer of β-D-glucopyranose, units linked through deconstructable β-glycosidic bonds, hemicellulose comprises amorphous branched polymers exhibiting a low degree of polymerization. This allows for hemicellulose to be removed under mild reaction conditions. Another major component of

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lignocellulosic biomass is lignin, which is an amorphous polymer that exhibits structural variation in the source.[15] The physicochemical recalcitrance of cellulose and chitin limits their cost-effective degradation. Higher-order structures in plants also contribute to biomass recalcitrance, which is evident because of the restricted access to the crystalline cores of microfibrils caused by a coating of amorphous cellulose and hemicellulose.[16]

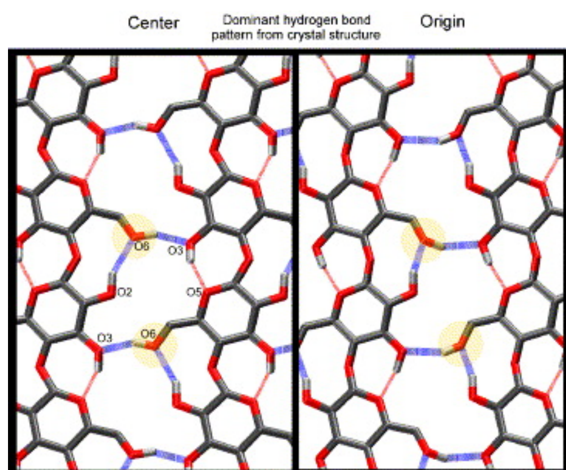

Fig. 3 H-bonding patterns for the cellulose 1β crystal; the left image is the center chain pattern and the right image is the origin chain pattern. The oxygen atoms are labeled to facilitate the identification of hydrogen bonds. (Reproduced from reference 14 with permission, copyright Elsevier, 2006).

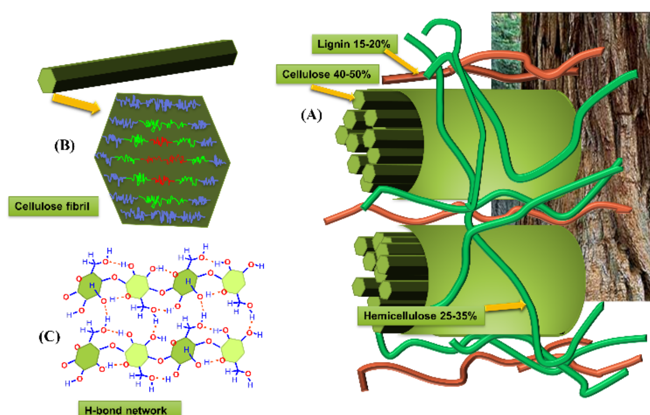

Fig. 4 A) A simplified model showing how the hemicelluloses are closely associated with the surface of the rigid cellulose crystallite forming the microfibril network. B) The 36-chain model of cellulose elementary fibril based on the X-ray structure of cellulose 1β. C) The intra- and interchain hydrogen-bond network in cellulose 1β.

Crystalline structures, consisting of tightly packed linear glycan polymers are insusceptible to attack from GHs because the active site is located in a pocket, cleft, or tunnel of the protein (Fig. 5).[17] For example, a comparison of the active sites of PbFucA with those of family 5 and 51 glycosidases revealed that the essential catalytic framework is identical between these enzymes, whereas the steric contours of the respective catalytic site clefts are distinct and likely account for substrate discrimination. The results of structural and functional analysis indicated that members of this cluster of orthologous group (COG) 5520 demonstrate β-D-fucosidase activities, despite exhibiting an overall sequence and structural similarity to GH-5 xylanases.

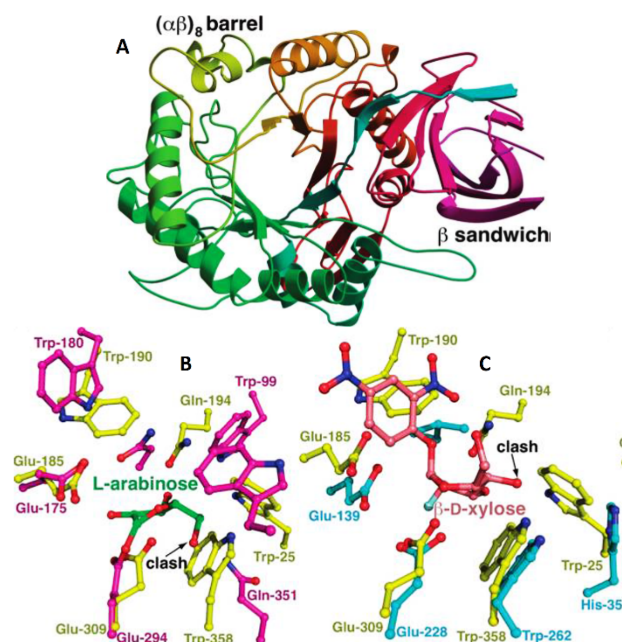

Fig. 5 A) Overall structure of PbFucA (ribbon diagram derived from the 2.2-A resolution crystal structure of PbFucA exhibiting the disposition of the (α/β) 8 barrel and the β sandwich domains. A comparison of the active site of PbFucA (yellow) with that of (A) the GH-51 L-∞-arabinofuranosidase AbfA (purple) in a complex with l-arbinose (in green) and (B) GH-5 β-glucanase Ce15A (in cyan) in a complex with β-D-xylose (pink) revealed the basis for the substrate specificity of PbFucA. (Reproduced from reference 17, copyright American Chemical Society, 2011).

Models for Enzymatic Degradation of Cellulose

Advanced industrial biomass enzymes have been developed based on the complementary hydrolytic action of cellulases (Fig. 6A) and hemicellulases (Fig. 6B) on cellulose and hemicellulose.[18] Recent discovery of other types of enzymatic synergies has facilitated the development of additional efficient industrial biomass enzymes. These novel catalytic enhancers differ dramatically in mode of action from that of classic cellulases and hemicellulases. Certain lignin oxidase, such as laccases, can enhance cellulase activity, possibly by releasing cellulases from their nonproductive binding sites on lignin, thereby increasing the effective concentration of free cellulases in the solution. Figure 6A shows the enzymatic degradation of cellulose involving the joint action of exoglucanases or CBHs, endoglucanases (EGs), and β-glucosidases. CBHs cleave cellulose in a manner that involves releasing primarily cellobiose from the ends of the cellulose chain. The degradation of cellulose caused by the joint action of CBHs and EGs is the foundation of commercial cellulase. The removal of the hemicellulose barrier enhances cellulase activity, thereby increasing the accessible surface area. The concerted action of numerous hemicellulases efficiently hydrolyzes hemicellulose. Degradation also involves the synergistic action of diverse enzymes. CelA can hydrolyze both cellulose and hemicellulose in raw biomass to create fermentable sugars. During biomass hydrolysis, fragments of Ce1A are released, allowing small enzyme fragments to reach regions of the substrate that the

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complete protein would not be able to access. Supplementing Ce1A with a β-glucosidase causes the complete hydrolysis of the recalcitrant form of cellulose in 7 days. The recognition of enzyme classification has indicated substantial interest in oxidative biomass decomposition, ranging from basic research to biotechnological applications. In-depth studies are required to expand the knowledge of enzymatic tools.

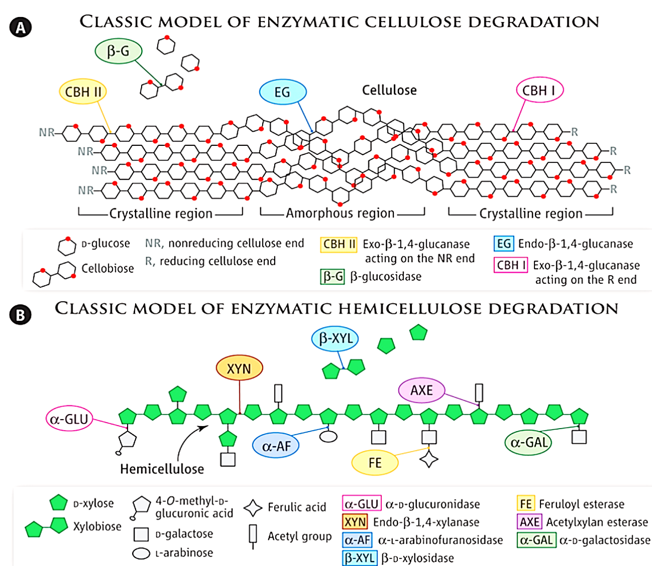

Fig. 6 Classic models of enzyme cellulose (A) and hemicellulose (B) degradation show how plant polysaccharides are broken down. (Reproduced from reference 18, copyright AAAS, 2013).

Cellulase Action

Two types of enzyme systems are involved in cellulose hydrolysis. One system includes noncomplex cellulases and hemicellulases produced by aerobic fungi and bacteria, whereas the other includes those in which polysaccharides have self-assembled onto a common protein scaffold to form large macromolecular assemblies called cellulosomes. A recent pioneering study on the digestion mechanism of *Caldicellulosiruptor bescii* CelA investigated the roles of exo- and endoglucanase (Cel7A and C2l5A), which mimic the two cellulolytic (endo- and exoglucanase) activities that occur in Ce1A when using Avicel as a model. The results indicated that, when acting on Avicel, Ce1A is a considerably more active single enzyme than the dominant enzyme in current commercial cellulose formulations is, such as *T. reesei* Ce17A.[11] The levels of glucan conversion achieved by Ce1A deconstructing crystalline cellulose (Avicel) are considerably higher than those of *T. reesei* Ce17A. The overall performance of Ce1A on witchgrass and corn stover is low, even when compared with that of β-D-glucosidase. The difference in the digestion mechanism is illustrated in Fig. 7, which shows that the small enzyme Cel7A may be more effective at disrupting plant cell walls, even after pretreatment, whereas large Ce1A, which has multiple CBMs, may be too large and more prone to nonproductive binding. The diagrams in Fig. 7 contrast the surface ablation and reducing-end-oriented mechanism of Ce17A (left) with the surface-ablation and cavity-forming mechanism of Ce1A (right). This representation suggests how this deconstruction processes is synergistic in terms of different aspects of the nanoscale architecture of exposed surface of biomass substrate. Comparison of dimensions of the cavities (Fig. 7) created by CelA (calculated from TEM micrographs) and calculated from a 40-ns molecular dynamic simulation, revealed that CelA with effective size between 10-35 nm fits into these cavities. These spatial dimensions for Ce1A are highly correlated with smaller cavity diameters in the range of 15 to 30 nm. The digestion mechanism of Ce1A suggests that the specific mode of action of Ce1A causes cavity formation.

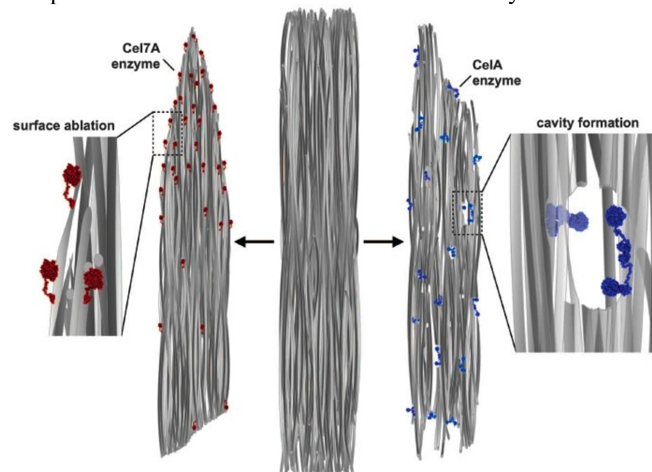

Fig. 7 Schematic representation of digested cellulose microfibril bundles. Surface ablation and the reducing-end-oriented mechanism of Ce17A (left) and cavity-forming mechanism of Ce1A (right). (Reproduced from reference 11, copyright AAAS, 2013).

Active Site of Hydrolase Enzymes

Molecular determinants that control the activity of GHs require determining the crystal structure of the protein that exhibits xylanase activity to deconstruct the hemicellulose and cellulose. Biochemical analysis of the GHs discovered in the genome of bacterium that deconstructs the hemicellulose indicated that each protein between the two endoxylanases functions as a β-fucosidase instead of demonstrating the predicted endoxylanase activity. To characterize the molecular basis for the substrate specificity, the protein crystal structure in which the overall topology of the Pb280 consists of a central catalytic (α/β) 8 barrel flanked by amino- and carboxy-terminal extensions that consist of sheets that form an independent β-sandwich domain (Fig. 8a). The small β domain was associated with the backside of the catalytic domain. The two domains were associated through hydrophobic interactions. GHs catalyzed cellulose and hemicellulose hydrolysis, and were classified based on the measurement of weak endo-1,4-β-glucanase activity. Certain GH61 proteins do not demonstrate measurable hydrolytic activity, but in the presence of various divalent metal ions, they can substantially reduce the total protein loading required to hydrolyze the lignocellulosic biomass. The structure of the GH61 protein was devoid of conserved, closely juxtaposed acidic side chains that could serve as proton donors or a nucleophilic base for hydrolytic reactions. The 3D active site structure of GH61E revealed a compact single-domain β-sandwich, consisting of two sheets in a variation of a fibronectin type III fold (Fig. 8a). This revealed the absence of large surface clefts, crevices, or holes that could indicate a possible binding pocket for soluble polysaccharides. The most highly conserved side-chain residues in the GH61 family are located in the core β-sandwich and participate in a large buried ionic network.[19] No clustering of commonly found conserved catalytic acidic residues were evident.[20,21] The structure of another GH61 protein, GH61B,

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was revealed (Fig. 8b), which shared only 29% of its structure-based sequence identity with GH61E.[11]

Structural studies on hydrolytic enzymes, such as GH61A, have revealed that metal ion binding is essential for stimulating pretreated corn stover (PCS) hydrolysis. The results indicated a binding pocket for a soluble polysaccharide. The most highly conserved side chain residues exist in the GH61. The results clearly indicated that the enhancement of PCS hydrolysis by GH61A is metal ion-dependent and that several divalent metal ions are functional in this regard. Similar results were obtained using GH61E. These results revealed a metal ion-binding site with considerable plasticity, which was suggested by the structural data. The importance of the metal ion-binding site was further investigated by mutagenizing the residues that were directly or indirectly involved in metal binding (Fig. 8). Mutations of the directly interacting His-1 to Asn or His-68 to Ala resulted in a completely inactive protein. The mutation of the closely interacting Tyr-153 to Phe substantially reduced, but did not eliminate activity. The mutation of Gln-151 (H-bonded to Tyr-153) to Leu was not apparent, whereas the more conservative substitutions Asn or Glu retained little residual activity. Mutation also occurred in one of three solvent-exposed tyrosines (Tyr-192) that form a relatively flat planar surface on GH61E, resembling the polysaccharide binding surface present in family 1 CBMs (Fig. 8c). The Ala mutation reduced activity substantially, but not completely. This suggests the crucial role of this residue, which is conserved in TrGH61B and is moderately well conserved in other GH61 proteins.

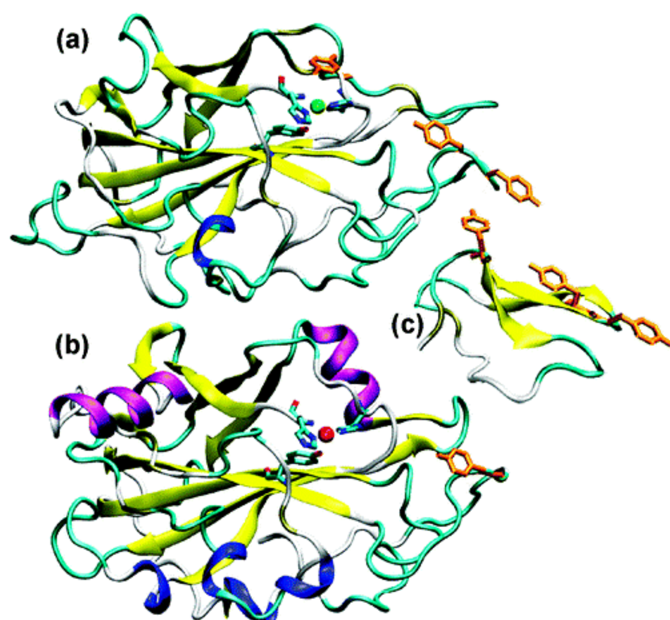

Fig. 8 Illustration of (a) GH61E, (b) TrGH61B (PDB ID2VTC), and (c) the family 1 cellulose-binding module (PDB ID 1CBH). β-strands are depicted in yellow, R-helices are shown in purple, 310-helices are blue, turns are cyan, and random coils are white, as determined by the STRIDE algorithm (68). Near the N-terminus of both structures is a bound metal ion, Mg^{+2} (green sphere) in GH61E and Ni^{+2} (red sphere) in TrGH61B. The metal ions are in close proximity to two histidines and a tyrosine conserved in GH61, which are shown in stick form. Three solvent-exposed surface tyrosines (orange) in GH61E (Tyr-67, -191, -192) that form a relatively flat planar surface potentially suitable for polysaccharide binding adjacent to the metal ion are also shown in stick form. For comparison, the similarly solvent-exposed planar tyrosines in a known cellulose-binding domain are shown in (c). Only one of the three GH61E tyrosines (structural equivalent of Tyr-191) was conserved in TrGH61B. (Reproduced from reference 19, copyright American Chemical Society, 2010).

This report indicated that relatively a small amount of these proteins can dramatically stimulate the hydrolysis of lignocellulosic substrates such as PCSs. The co-expression of highly active GH61 proteins in the cellulase producer *T. reesei* can increase the apparent specific activity of the Trichoderma cellulases by a factor of approximately two to achieve high cellulose conversion. *T. reesei* expresses its own GH61 proteins, but none of the three present in the genome sequence were expressed at high levels in our strain under the employed culture conditions. Current models for the economic conversion of lignocellulosic biomass into biofuels typically offers 80–90% conversion with respect to lowering the selling price of the product which depends on the relative cost of enzymes and biomass.[22] At the 80%–90% conversion level, the reduction in protein loading enabled by GH61 translates directly to a 1.7–1.9-fold cost reduction and further enables the enzymatic saccharification platform to produce cheap sugars and biofuels from abundant and renewable lignocellulosic biomass.

Action of Polysaccharide Monooxygenase

The lytic polysaccharide monooxygenase (LPMO) enzyme (*e.g.*, GH61) can substantially enhance the hydrolytic performance of a cellulase enzyme mixture on cellulosic substrates.[19] Generally, canonical cellulase enzymes have been demonstrated to cleave cellulose by using a hydrolytic mechanism involving conserved carboxylic acid residues within either channel- or cleft-type substrate loading sites. However, GH61, which contains a divalent metal ion, is presumed to cleave cellulose chains by using an oxidative mechanism at the planar active site.[23-25] LPMOs provide a solution to the problem of accessing the active site of GHs. In LPMOs, the active site is located on a planar surface of approximately 1200 $Å^2$ to facilitate the metal-dependent oxidative cleavage of the glycan chains.[26] Therefore, the action of the LPMO creates an abrasion (Fig. 9) on the biomass surface to produce an entry point for GHs, such as CBHs, EGs, and chitinases.[27,28] Because LPMO supplementation exerts a strong effect in combination with GHs, they can be used to enhance recalcitrant biomass degradation. However, only two families of LPMOs, cellulose (AA9) and chitin (AA10) in the carbohydrate-active enzyme (CAZy) database, have been described thus far.

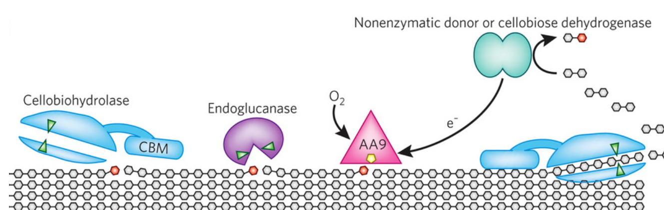

Fig. 9 Mode of action and structure of cellulolytic LPMOs. Fungal enzymatic degradation system of cellulose. The initial attacks of an AA9 LPMO may generate partially amorphous sections with oxidized chain ends that become points of attachment for hydrolytic enzymes with a tunnel or cleft-like active site such as cellobiodehydrogenase and endoglucanase (Reproduced from reference 25, copyright McMillan Publishers, 2014).

Hemsworth *et al*. reported a chitin active family (AA11) of LPMOs that moves through a small conserved module in a genomic database of fungi, suggesting new possibilities for synergistic biomass degradation.[26] Proteins in this LPMO

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family secrete cellulolytic fungi and chitinolytic bacteria, and exert synergistic effects on biomass degradation. The proteins are also structurally distinct from their original CAZy cousins, because both GH61 and CBM33 family members adopt an immunoglobulin-like-β-sandwich fold and use a planar surface to coordinate a metal ion by using the imidazole and main-chain amino group of the N-terminal histidine and a histidine side chain (Fig. 10). LPMOs as oxidative enzymes are dependent on a copper ion and electron donor, such as ascorbic acid or reduced glutathione. Therefore, the proteins were renamed from GH61 and CBM33 to auxiliary activities (AA) family 9 and 10, respectively. AA is a newly established class of redox enzymes acting with carbohydrate active enzymes. For example, cellobiose dehydrogenases, which catalyze the oxidation of cellobiose, are classified as AA3 and AA8.

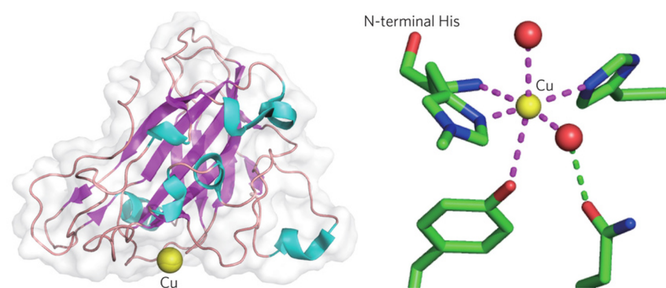

Fig. 10 Illustrations of the surface structure (left) and the copper active site (right) of the AA9 LPMO from *Thermoascus aurantacus*. The flat surface binds crystalline cellulose, and the metal center catalyzes oxidative cleavage glycosidic bonds. (Reproduced from reference 25, copyright McMillan Publishers, 2014).

The overall fold and metal coordination were similar to the known LPMOs; however, a slightly convex surface on which the copper active site sits is a unique feature of the new family. Hemsworth confirmed that certain LPMOs are multimodular proteins that carry a small conserved domain of an unknown function called X278.[26] An extensive search in which a "module walking" approach was used to identify proteins containing the X278 domain produced approximately 450 sequences of a possible new enzyme family of fungal genomes. Although this recent discovery elucidates the mechanism of LPMOs for the oxidative cleavage of structural biopolymers and biochemical foundations, certain critical questions remain unanswered, including 1) what is the electron donor of LPMOs in the natural environment, 2) which route do the electrons take to reach the copper active center, 3) where is the binding site for electron donors, and 4) how does a monooxygenase reaction occur at the active site of these unusual enzymes. The copper active site of LPMOs is notably similar to that of copper methane monooxygenase, which can act on highly oxidation-resistant methane. The discovery of a new LPMO is crucial for the development of a new enzymatic biomass decomposition technique involving abundant microbial genome information.

Determining the synergistic action of cellulases is critical for the effective saccharification of cellulosic biomass based on quantitative analysis conducted at the level of single molecules. Cellulases engage in "work sharing" among cellulolytic enzymes based on the various adsorption specificities of their CBMs. Typical cellulases hydrolyze insoluble substrates to produce cellobiose, a soluble β 1,4-linked glucose dimer. The reaction occurs at a solid-liquid interface, rendering the interpretation of the biochemical results difficult, such as the speed of product formation, substrate decomposition, or both. For example, the hypercellulolytic fungus *Trichoderman reesei* produces two major cellulases, Ce16A and Ce17A, to degrade crystalline cellulose synergistically.[30] According to structural analysis, these enzymes are considered to act on opposite ends of the cellulose polymer; however, how they orient themselves relative to each other on the cellulosic matrix is unclear. Because they do not interact with each other, determining how they cooperate during crystalline cellulose degradation is worth investigating. A real-time observation of morphological changes that occur during the enzymatic decomposition of plant cell walls[31] and the single-molecule analysis of cellulases[32,33] have facilitated the identification of the molecular mechanisms of individual enzymes. Fox *et al*. provided a direct visualization of the cellulose degradation process and outlined the method for preparing effective enzyme cocktails to further develop enzyme biorefineries. They used a quantitative technique called photo-activated localization microscopy (PALM) to visualize individual photo-activable fluorescent protein molecules fused with six CBMs, as classified in the CAZy database.[26]

PALM, a recently developed fluorescence-based microscopic method, enables single-protein molecules to be observed using photo-switchable fluorescent probes with nanometer-scale spatial resolution.[34] The utility of this technique was first tested by observing the localization of several fusion proteins, incorporating mEos2 as the fluorescent protein, and using dewaxed cotton as a substrate. Based on the variable patterns observed in the various proteins, the authors defined a "CBM order parameter," W, as an indicator of the preference of these modules for absorbing CBM in crystalline or amorphous regions, in which a low W indicates that the CBM is adsorbed randomly, and a high W indicates that the adsorption of the CBM occurs in an ordered manner. The authors interpreted a low Ω as indicating a preference for the amorphous regions of the substrate, and considered a high Ω as indicating a preference for the crystalline regions of the substrate (Fig. 11).[35] This evaluation considerably advances the current knowledge on the subject because previous classical single-molecule techniques could not be used to identify the preferred binding regions of CBMs. Fox *et al.* used the Ω values to design fusion cellulases with different CBMs, and compared the degrees of synergism for all combinations of the fusion proteins. An apparent optimal value of ΔΩ exists (the difference between the Ω values of two CBMs) for obtaining maximal synergy, indicating that high synergy is obtained when enzymes work on tasks that are separate but not overly dissimilar.

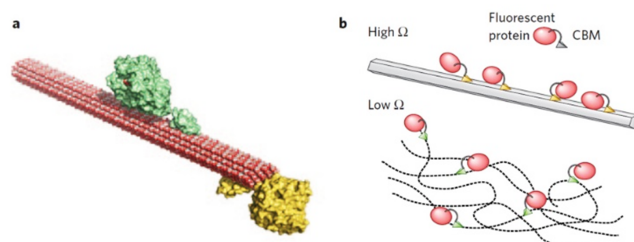

Fig. 11 Cellulase adopt various strategies to break down cellulose together. (a) Two-domain cellulases (Cel6A and Cel7A) from the cellulolytic ascomycete *T. Reesei* bind opposite ends of a crystalline cellulose surface. (b) Schematic representation of high Ω (top, corresponding to a crystalline substrate) and low Ω (bottom, corresponding to an amorphous substrate) situations. (Reproduced from reference 32, copyright AAAS, 2013).

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Cellulose hydrolysis was reported to be initiated by a nonhydrolytic chain-separating enzyme that causes cellulose to swell, thereby facilitating an attack by the hydrolytic enzyme mixture. Thus, the oxidative AA9 enzyme is effective for substantially increasing the accessibility of cellulases to cellulose through the oxidative cleave and the disruption of the crystalline cellulose region.[36] Despite this, the mechanism of synergistic cooperative action between cellulase enzymes (hydrolytic cellulose cleavage) and AA9 (oxidative cellulose cleavage) remains subtle. A thermochemical pretreatment step is required to open the lignocellulosic materials for subsequent enzymatic hydrolysis.[37] Pretreatment produces cellulose-rich substrates suitable for enzymatic hydrolysis. The extent of enzyme synergism is substantially influenced by the nature of the cellulosic substrate, including the overall accessibility of the cellulose, its crystallinity, degree of polymerization, and type, amount, and distribution of residual lignin and hemicellulose.[38]

Scope of Nanomaterial-Enzyme Composites

Three major approaches that have been widely used for cellulose conversion are physical (*e.g.*, high temperature, pressure, microwaves), chemical (*e.g.*, strong acid treatment), and biological processes (*e.g.*, enzymes). Among these, the physical and chemical processes are energy consuming and produce by-products. In certain situations, they are highly effective, such as the recently discovered microwave-mediated cellulose depolymerization method.[39] Enzyme-based biological processes are performed under mild conditions with a high specificity for a single product. Thus, cellulose and chitosan conversion conducted using an enzyme-assisted route is an excellent alternative approach that reduces experimental costs, inhibits by-product formation, and improves process specificity. Two concerns regarding enzyme-assisted strategies are the maintenance of enzyme activity in reactions and recyclability. Immobilizing enzymes on a suitable host material is considered a viable solution because it offers several advantages, including repeated use, ease of separation from the product, the manipulation of enzyme properties, improved stability, and easy storage.[40,41] However, the adsorption of enzymes on a mesoporous solid surface from a mixture is difficult to control because of the varying kinetics of adsorption, the variation in the degree of unfolding, and competitive binding effects.[35] Therefore, plasma techniques are capable of producing a mildly hydrophilic surface that covalently couples to protein molecules and enables the attachment of a uniform monolayer from a cellulase enzyme mixture. Such a phenomena, when tracked by conducting atomic force microscopy (AFM) studies, revealed that the surface layer of the physically adsorbed cellulase layer on the mildly hydrophobic surface consisted of aggregated enzymes that changed conformation during the incubation period. Materials such as amorphous silica or agarose gel have been explored as host materials to immobilize enzymes. Mesoporous silica materials have also been used as potential host materials to immobilize enzymes because of their large surface areas, adjustable pore sizes, diverse surface functionalities, and broad pH range.[13,42] Three major methods of immobilizing enzymes on mesoporous materials are known, which are binding to a support, cross-linking, and encapsulation.[43] Prior to immobilizing the enzymes, magnetic nanoparticles (γ-Fe_2O_3) were grafted onto the mesocellular foam with uniform dispersity, which was subsequently modified using *n*-octyltrimethoxysilane (Fig. 12). This supports the adsorption of more active lipase because of binding with the surface-modified magnetic foam.[44]

Mesoporous silica nanoparticles (MSNs) were used to immobilize cellulase through physical adsorption and chemical binding for cellulose-to-glucose conversion (Fig. 13).[45] This study determined a new biocatalytic route with high efficacy and enhanced stability. MSNs were provided with the host surface and encouraged to form chemical bonds with the enzyme, which improved the stability of the composite. The surface charge of the cellulase and small pore mesoporous silica at pH 4.8 were both negative (*i.e.*, -6.7 and -14.8 mV). Because of the Si-OH and Si-NH_2 groups, the surface charge of the large pore mesoporous silica nanoparticles (LPMSN) was approximately zero. The increased adsorption amount in the cellulase-adsorbed LPMSN resulted from the electrostatic interaction between cellulase and the Si-NH_2 groups. This confirms that the immobilized cellulase was covalently linked with TESP-SA (3-triethoxysilylpropyl succinic acid anhydride)-functionalized LPMSN. The COOH groups of LPMSN were used as linkers to covalently bind cellulase. For the selective conversion of cellulose, such as multistep reactions including cellulose-to-glucose-to-fructose conversion, using more than one enzyme is essential. The individual immobilization of cellulose and isomerase into MSNs instead of simultaneous immobilization was recently reported, which involves Fe_3O_4 nanoparticles in facilitating separation by using magnetic nanoreactors.[46] The Fe_3O_4-MSNs nanocomposite exhibited a worm-like porous structure and narrow pore size distribution that supported the immobilization of cellulase or isomerase. This magnetic enzyme-functionalized material demonstrated a maximum of 51% fructose production from cellulose in a pH-dependent cascade two-step process (Fig. 14).

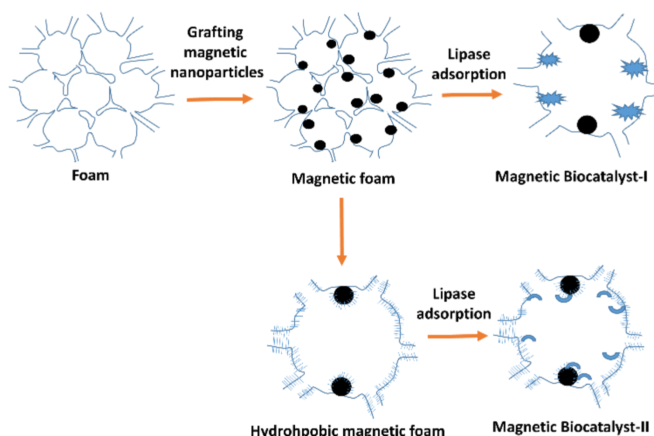

Fig. 12 Enzyme-immobilized cascade conversion of cellulose to fructose.[44]

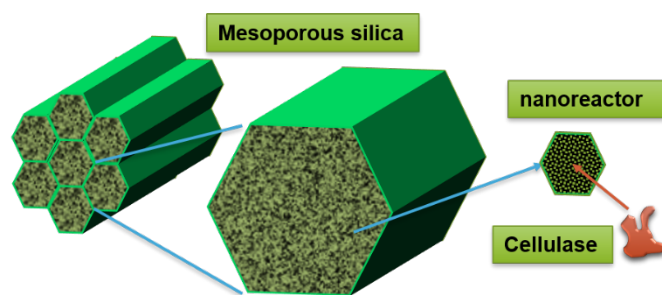

Fig. 13 Diagram of a mesoporous silica nanoreactor for adsorbing enzymes.

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To use the aforementioned strategy successfully, an improved understanding of the reactions involved in a cellulose surface is essential. However, studies on the enzymatic action of solid surfaces have faced numerous technical challenges because most enzymology methodologies are well suited for the soluble substrates. General characterization techniques, such as advanced imaging for interrogating cellulose function on cellulosic surfaces, can be used in the aforementioned processes. Such an approach overcomes the difficulty of interpreting biochemical results obtained from reactions occurring at a solid-liquid interface.

Sakaguchi *et al.* recently studied the encapsulation of cellulase by using mesoporous silica SBA-15 hosts of various pore sizes. They observed that the enzymatic activity of cellulase strongly depends on the pore size of the SBA-15 host. The optimal cellulase performance was achieved when SBA-15 with a pore diameter of approximately 8.9 nm was used.[47] The encapsulation peak of cellulose on mesoporous silica SBA-15 of various pore sizes (8.9 nm, 11 nm, and amorphous) at pH 4 indicated the net positive charge on cellulose and a negatively charged silica surface facilitate electrostatic interaction-driven encapsulation regarding the equilibrium concentration of cellulase.

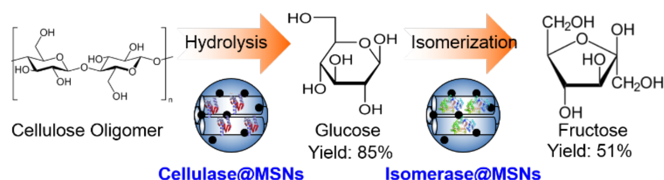

Fig. 14 Enzyme-immobilized cascade conversion of cellulose to fructose.[46]

The SBA-15 structure consists of 2D hexagonal channels measuring several millimeters in length, and inhibits the adsorption of cellulase into the inner surface of the SBA-15, thus decreases the amount of adsorption. Surface modification of the support material could enhance the interaction between the pore walls and the enzyme, thereby substantially affecting the stability, reactivity, and recyclability of enzyme reactors. The covalent enzyme attachment can be enhanced using support materials that exhibit a variety of organic linkers (amine, carboxylate, phenul, alkyl groups) on their surfaces, which enhances the van der Waals interactions. Lu *et al.* studied the effects of the surface functionalities of mesoporous silica FDU-12 (with a pore size of approximately 25.4 nm) on cellulase immobilization.[48] They functionalized FDU-12 with phenyl, thiol, amino, and vinyl groups. Their results indicated that electrostatic and hydrophobic interactions between cellulose and functionalized FDU-12 play crucial roles in the activity and stability of immobilized cellulase. Amine-functionalized FDU-12 adsorbed the largest amount of cellulase, but exhibited the lowest activity. They explained that this was due to the interaction between the amine groups of FDU-12 and the carboxyl groups of the cellulase catalytic site, which subsequently inhibited cellulase activity. By contrast, vinyl-functionalized FDU-12 maintained the activity of cellulase at up to 80% and provided a temporally stable environment, resulting from the existence of hydrophobic groups.[48] This study also revealed that enzyme immobilization efficiency, activity, and stability varied substantially according to organic functionality because of the size exclusion effects exerted at pore entries, electrostatic and hydrophobic interactions between organic-functionalized surfaces and enzymes, and conformation changes of the enzymes occurring on the material surface.

Nevertheless, researchers have not studied the effects of various immobilization methods (*i.e.*, physical adsorption and chemical binding) on cellulase efficiency. For ordered mesoporous silica, hydrophobic groups are necessary to immobilize enzymes such as lipase. By increasing the hydrophobicity of support materials, a favored active conformation of the enzyme is induced, where accesses to active sites in the internal lipase structure is easier for substrates. Enzyme conformation can be affected by the surface functionality and monolayer capacity of enzymes.[49] Overall, the hydrophobic nature of the support matrix is a major factor in adsorbed enzyme conformation and activity.

Impact on Biofuel Production

To determine the benefits of producing liquid fuel enzymatically, researchers must examine various strategies and consider the efficiency of these strategies in the 3D network breaking of biopolymer feedstocks. Current biomass conversion technology primarily comprises four major processes: feedstock harvest and storage, thermochemical pretreatment, enzymatic hydrolysis, and the fermentation of sugar into ethanol. One purpose of pretreatment is to reduce recalcitrance by depolymerizing and solubilizing hemicellulose (approximately 20%–40% of the biomass). The core of crystalline cellulose can be exposed by removing hemicellulose from microfibrils, which is suitable for cellulose enzyme-driven hydrolysis in which the macroscopic rigidity of biomass is broken down and barrier-to-mass transport is reduced. To produce cost-competitive biofuels from biomass, new findings from plant science and carbohydrate chemistry are essential with integrated conversion processes. Engineering new cell walls to be integrated with specially designed enzymes is necessary to overcome biomass recalcitrance. The action of cellulolytic enzymes on cellulose is an example of heterogeneous biocatalysis that has been the focus of considerable research aimed at reducing the cost of lignocellulose-derived sugars for the production of biofuels.[28,50] Cellulolytic enzymes use distinct substrate-binding structural motifs or CBMs to interact preferentially with particular cellulose structures. Increased cellulose-to-glucose conversion efficiencies, and, thus, reduced biofuel production costs, require cellulolytic enzyme cocktails that optimally match the structural organization of biomass substrates and can be used to exploit potential avenues of enzyme synergy.[24,51,52]

Enzymes for Biodiesel and Liquid Fuel Production

Major focus of the above discussions was about understanding the mechanism of action of hydrolase enzymes such as cellulase for the degradation of insoluble biomass substrates which is crucial to develop efficient process for the cellulose and other biopolymer conversion motored by enzyme function. However, emphasizing the roles of enzymes in biodiesel and liquid fuel production from biomass is equally important as compared to discussions on fundamentals of enzymes and their mechanism of action in the process of biomass degradation. It is envisaged that the experimentally validated model to predict separation resolution of biomass carbohydrate oligomers as a function of system parameters is currently lacking. Application of enzymes (intracellular and extracellular) immobilized on a suitable biomass support can be a good alternative for the production of biofuels. Enzymatic transesterification for the production for biodiesel has been an attractive alternative method that produces high-purity biodiesel with advantage of easy separation from the byproduct glycerol.[53] In this process, Novozyme and vegetable oil has been mostly explored candidates so far by using methanol, ethanol, or butanol as acyl

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acceptors. It is important to note that, scientific insights of enzymatic production of biodiesel is not well-explored however, among all techniques, immobilization of enzyme (e. g. lipase) on the hydrophilic surface was reported as good choice for immobilized enzyme-induced biodiesel production.[54] In addition to the mechanistic understanding of the enzyme actions, unrealized roles of protein families may play a major role in future advances of biofuel production using enzymatic strategies. Toward the production of lignocellulosic biofuels, polysaccharides are hydrolyzed by cellulase enzymes into simple sugars and later fermented to ethanol by microbes. This enzymatic deconstruction for producing fuels faces physical impedance for enzymatic deconstruction due to the crosslinks in the plant cell walls with the hydrophobic network of lignin. This problem can be solved by pretreatments of lignin[55] which is another major area to explore. Even some challenging technical problem may find smart solutions to their end, cost of production of enzyme should not exceed the production cost of biofuels. For example, the cost contribution of enzymes to ethanol production by the conversion of corn stover was calculated to be $0.68/gal if the carbohydrates of biomass could be converted at maximum theoretical yields and it would be &1.47/gal if the yields were based on saccharification as a per a techno-economic model for the production of fungal cellulases.[56] Further such analysis also suggests that significant efforts will be required to lower the contribution of enzymes to biofuel production costs and nevertheless, the situation will be more complicated when involve more sophisticated enzymes and relatively less explored biomass substrates. However, more emphasis to be given on the improved pretreatment of biomass and enzyme technologies for improving activity which would influence in reducing bio-refinery capital costs.

Future of Enzymatic Hydrolysis of Lignocellulose

For adding new insights for advancing the enzyme research with significant further understanding of the fundamentals of enzyme actions on different lignocellulosic materials, it is generally suggested that there can be a large number new areas to explore in the future as described below.

1) Investigation on the biochemical and kinetic properties of enzymes used for lignocellulose biodegradation in relation to the enzyme system produced by the microbial community would be one of the future directions. For these, detail characterization of biopolymer degrading enzymes obtained from the microbial system is essential.

2) Gene coding for biopolymer degrading enzymes is an emerging technique in which a genomic library offers a large number of genes coding. There are many varieties of bacterial cellulase, ligninase, and chitinase which can be employed for such studies and analysis of their impact on the breaking the network of biopolymers.

3) For quantitative large-scale screening of enzyme libraries for biomass hydrolysis and development of energy feedstocks, new techniques for microscale approach is required to be developed.

4) Innovation of new enzyme can offer large scale impacts inspired by the cellulosic ethanol production. Revealing the cellulase binding module involving celluloase/xylanase synergy for continued developments on overall enzymatic degradation of biopolymer network will be essential.[57]

5) Exploring new biopolymers as source of energy and materials requires superior skills of degradation using enzymes and this process might have broad importance beyond biomass transformations such as crop protection and disease control. With regard to this, there is ample scope to study degradation features of chitinolytic enzymes which contains the potential for fully degrading chitin into building block *N*-acetylglucosamine, a potential carbon and nitrogen source.[58] Challenging the rigid chitinous matrix against its degradation in the chitin-containing microorganisms would be of significant future prospects in terms of understanding the mechanism of action of chitinolytic enzymes and production of nitrogeneous carbon source.

Conclusions

A critical understanding of enzyme action exhibiting recalcitrant biomass degradation has been essential to the development of sustainable biorefineries. Numerous approaches to understanding enzyme actions exist. Tightly bound linear glycan polymers, which are not susceptible to attack from GHs, result from the presence of an active site in the inaccessible pocket, cleft, or tunnel of the protein. The recent discovery of the action of LPMOs offers improved performance for degrading cellulose biopolymers because the active site of the enzyme is approximately located on a planar surface and facilitates the metal-dependent oxidative cleavage of the glycan chain. Biomass degradation investigated with PALM techniques has revealed mechanisms of enzyme activity on solid substrates, which facilitates the development of new enzyme-solid composite materials that are capable of efficient glycosidic bond cleavage. Difficulty characterizing the structural heterogeneity of enzyme-substrate interactions and the adsorption steps of enzymes on the surface can be topics of further study. The potential of numerous enzymes, such as endoglucanase, cellobiohydrolase, cellobiose phosphorylase, and glucan phosphorylase,[59] for biomass conversions, as well as the role of active sites, have yet to be investigated in detail. Regarding enzymatic biomass conversions, emerging technology, such as cascading techniques, can be implemented for more efficient biocatalytic conversions. Biohybrid polymersomes are more advantageous as multi-enzyme biocatalysts than soluble enzymes are in the enzymatic cascade process.[60] Enzymatic hydrolysis in which lignocellulosic biomass is converted into fermentable sugars may be the most complex step in this process because of substrate- and enzyme-related effects and their interactions, which are not yet fully understood. However, enzymatic hydrolysis offers the potential for higher yields, higher selectivity, lower energy costs, and milder operating conditions than those of chemical processes. The mechanism of enzymatic hydrolysis and the relationship between the substrate structure and function of various glycosidic hydrolase components require extensive investigation. Consequently, thus far, maximizing sugar yields at a low cost by using immobilized-enzymes on the solid surfaces has achieved limited success. Areas of fundamental interest that require investigation include 1) the synergistic interaction of cellulase and lytic monooxygenase enzymes, 2) the relative amount of accessible crystalline compared to that of amorphous cellulose within a biomass substrate, and 3) the disruption mechanism of the microcrystalline region of a cellulose chain through the synergistic action of hydrolytic and oxidative enzymes. Further research should yield a class of biomass enzymes that are more robust and highly adapted to the specific requirements of the emerging carbohydrate economy.

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Department of Chemical Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan. E-mail: kevinwu@ntu.edu.tw

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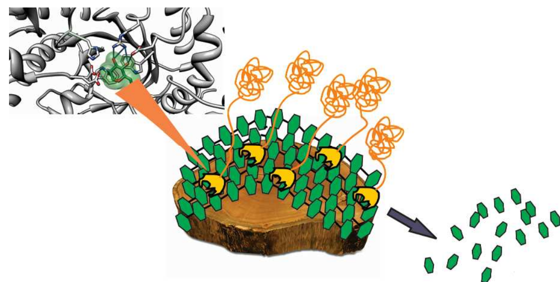
Graphical Abstract

Enzymatic Breakdown of Biomass: Enzyme Active Sites, Immobilization, and Biofuel Production



Saikat Dutta and Kevin C.-W. Wu*

This article presents a review of current developments in the understanding of the microstructure of plant biomass, treatment of biomass by using bacterial hydrolase enzymes, active site structures of hydrolytic and oxidative enzymes, and their overall impact on the biomass degradation process.

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