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Multifunctional cellulases are potent, versatile tools for a renewable bioeconomy

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Abstract

Enzyme performance is critical to the future bioeconomy based on renewable plant materials. Plant biomass can be efficiently hydrolyzed by multifunctional cellulases (MFCs) into sugars suitable for conversion into fuels and chemicals, and MFCs fall into three functional categories. Recent work revealed MFCs with broad substrate specificity, dual exo-activity/endo-activity on cellulose, and intramolecular synergy, among other novel characteristics. Binding modules and accessory catalytic domains amplify MFC and xylanase activity in a wide variety of ways, and processive endoglucanases achieve autosynergy on cellulose. Multidomain MFCs from *Caldicellulosiruptor* are heat-tolerant, adaptable to variable cellulose crystallinity, and may provide interchangeable scaffolds for recombinant design. Further studies of MFC properties and their reactivity with plant biomass are recommended for increasing biorefinery yields.

Introduction

Global efforts to reduce carbon emissions are spurring technological innovations worldwide. As part of an integrated bioeconomy, the synthesis of fuels and bioproducts from renewable plant biomass may supplant petroleum-derived products, reduce net carbon emissions and promote energy security. Inedible lignocellulosic biomass (LCB) contains vast reserves of convertible carbohydrates and aromatics for next-generation fuel and product synthesis but recovering these molecules from the polymeric matrix of a plant cell wall remains a significant challenge.

Physical and chemical pretreatments render LCB susceptible to cellulases, which hydrolyze the exposed polysaccharides into small, fermentable sugars. The complex polymeric

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CRediT authorship contribution statement

Evan Glasgow: Conceptualization, Investigation, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Kirk Vander Meulen:** Conceptualization, Investigation, Data curation, Formal analysis, Software, Visualization, Writing - original draft, Writing - review & editing. **Nate Kuch:** Conceptualization, Investigation, Writing - review & editing. **Brian G Fox:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Conflict of interest statement

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composition of LCB (Figure 1) requires several distinct enzyme activities to liberate as much sugar as possible. While enzymatic deconstruction has several advantages over chemical hydrolysis, enzyme cocktails are a major (up to 30%) operational expense for biorefineries [1•]. Reducing the cost of LCB deconstruction is imperative for a viable bioeconomy to produce renewable fuels and bioproducts at competitive prices, and enzymes are a critical cost-reduction target.

Recent investigations identified glycoside hydrolases (GH) with multiple catalytic functions. Here, we define multifunctional cellulases (MFCs) as a subset of GHs that possess, at minimum, β-(1,4)-endoglucanase or cellobiohydrolase activity with two or more polysaccharide types. Multifunctional cellulases (MFCs) may provide a route to lower enzyme costs by reducing the complexity of enzyme cocktails while increasing efficiency of enzyme hydrolysis. This review highlights recent discoveries, mechanistic investigations, and laboratory-scale applications of MFCs and recommends several considerations for future technology development. We also note that many trends in MFC research are occurring in parallel on xylanases, spurred by the abundance of xylan in LCB and the special utilities of pentose sugars in biosynthetic pathways [2].

Better understanding of MFCs may simplify the composition of enzyme cocktails, lower the amount of enzyme needed, and, along with organic co-solvents, may enable the as-yet unmet goal of biomass-agnostic deconstruction, in which a single chemoenzymatic process efficiently deconstructs a variety of LCB feedstocks. Catalytic versatility can thus enable a more robust bioeconomy operable across a variety of agricultural regions and climatic zones [3].

A multi-tiered view of MFCs

We propose a novel, three-tier functional classification system (Figure 2) to help group MFCs by complexity and to distinguish among the different factors contributing to multifunctionality. Tier 1 MFCs *consolidate* breadth of substrate specificities into a single GH active site, while Tier 2 MFCs incorporate additional non-GH domains or reaction mechanisms that further *amplify* the native activity. A Tier 3 MFC brings two or more GH domains that catalyze closely associated steps in LCB deconstruction and may *synergize* in reaction on the same substrate. Tier 3 MFCs may be built from Tier 1 and 2 components, just as the GH domains in Tier 2 MFCs may have Tier 1 broad specificity.

Tier 1 MFCs: broad substrate specificity in single domains

The simplest MFCs are single-domain GHs with broad substrate specificity. According to the Carbohydrate-Active Enzymes Database (CAZy, http://www.cazy.org/), families enriched with MFCs include GH5, 26, 44, 45, and 74 [4], with GH5 being most enriched in broad-specificity endoglucanases. GH5 is divided into subfamilies to help map its many specificities into sequence-similar groups [5]. GH5 endoglucanase activity often co-occurs with endoxylanase [6–12] or endomannanase [13••,14•] activity, in some cases with both [15••,16], as in subfamily 4 (GH5_4). The glucanase efficiency is typically highest (Figure 3), although xylanase activity sometimes exceeds it [7], mimicking the specificity of GH10

xylanases (see ACM60945 [17] and ACY69972 [18] in Figure 3). The identification of gluco-mannanase [19], β-(1,3)(1,4)-mixed-linkage glucanase [20,21] and xyloglucanase [22] activities demonstrates that MFCs accept variations in sugar monomers and linkages, and also tolerate branching. Interestingly, Tier 1 MFCs from GH9 have been found in herbivorous insect genomes, with high expression in the digestive tract and cross-reactivity on cellulose and either xylan or xyloglucan [23,24]. Critically, some Tier 1 MFCs are as efficientas 'specialized' monospecific GHs on each of their multiple substrates [25], illustrating that broad specificity and high reactivity are not mutually exclusive. The comparable reactivity of MFCs and monospecific GHs supports the promise of MFCs in designed enzyme cocktails.

Tier 1 MFCs with more open binding clefts can tolerate a diversity of polysaccharides, including some with non-β-(1,4) linkages or charged sugar residues [18]. For example, a dual cellulase/xylanase from GH26 [with an $(\alpha\beta)_8$-barrel fold like that observed in GH5] [26] also hydrolyzed anionic β-(1,4)-polyglucuronic acid in a Ca^{2+}-independent fashion distinct from pectate lyases. In another example, GH131 MFCs (with a β-jelly roll fold) have a widely accessible cleft that can hydrolyze β-(1,4) and β-(1,3) bonds equally well [27]. Activity on chitosan (the cationic, deacetylated form of chitin) is also observed in cellulases encompassing several distinct folds and mechanisms [28]. Thus, MFCs are widely distributed among GH families, offering many options for design of customized enzyme cocktails.

Tier 2 MFCs: multi-domain amplification of reactivity

The combination of a GH and a carbohydrate-binding module (CBM) is the most abundant example of a Tier 2 MFC and provides a mechanism to direct the reactivity of an MFC onto different polysaccharides [29]. Various combinations of MFC and CBM can give ~10^5 distribution of the specificity ratio (k_{cat}/K_M) across a breadth of hexose and pentose polysaccharides (Figure 3).

CelE, a cellulosome-associated MFC from *Hungateiclostridium thermocellum*, is a multidomain enzyme consisting of a GH5 endoglucanase domain, a dockerin domain, and a CE2 domain. The GH5 domain is a Tier 1 MFC that degrades cellulose, mannan, xylan, and several heteropolysaccharides [15••,29,30], while the CE domain deacetylates xylan [31] and further exposes the hemicellulose to the GH5 active site, demonstrating a Tier 2 functionality.

GH amplification can also be achieved by fusion of carbohydrate esterase (CE) domains to xylanases from GH10 and GH11 (Figure 4a). When added in excess as separate proteins, CEs can potentiate xylanases up to 20-fold on some plant biomass [32•]. Non-covalent 'xylanosome' complexes can confer over threefold synergy by keeping xylanase and acetylxylan esterase in close proximity [33]. Although some of these fusions bind to amorphous cellulose [34], their cellulase activities have only been sparsely explored.

Some xylanases, such as XynY and XynZ from *H. thermocellum* and Xyn10A/Fae1A from a soil consortium [35], harbor a *p*-feruloyl esterase domain (CE1), which cleaves linkages to

lignin and releases ferulic acid. To our knowledge, this lignin-targeting module has not been found in cellulases, suggesting selective evolution of the most appropriate pairs of catalytic domains in Tier 2 MFCs.

Disruption of substrate superstructure is another Tier 2 mechanism, exemplified by the action of swollenins. Swollenins can increase the activity of both xylanases and cellulases [36,37] up to ~3-fold by weakening the non-covalent interactions between polysaccharide chains. Swollenin fusions to CEs [38] and xylanases [39] have shown promise, but fusions to cellulases have been limited to expansins, the bacterial counterpart [40], and show less impact on activity than swollenins.

Processivity is yet another Tier 2 function that amplifies the native ability of exocellulases to hydrolyze β-(1,4)-glucosidic linkages by threading the substrate polymer into the active site without dissociation [41•]. The effect is limited to crystalline cellulose and impacts k_{cat} rather than K_M [42]. Processivity is often assisted by a tunnel-shaped active site and an intimately associated CBM. Different exocellulase families exhibit strict specificity for reducing (GH7 and 48) or non-reducing (GH6) ends.

Processivity is not limited to exocellulases. Processive endoglucanases (PEGs) are less understood, but they have been found in microbes lacking a canonical exocellulase [43••]. PEGs are fascinating because they first create new chain ends by endocellulase action, and then processively depolymerize the polysaccharide by exocellulase action (Figure 1, *green*). PEGs found in GH5 may possess CBMs from families 1, 3, or 6, although their precise function in processivity is less clear and may even be inhibitory [44]. PEGs found in GH9 show close association between the GH domain and a CBM3. One case was found in GH48, demonstrating PEGs may evolve in both endoglucanase and exoglucanase families.

Tier 3 MFCs: multi-domain intramolecular synergy

Two or more GH domains of differing (or similar) reactivity can be encoded by a single gene (Figure 4b). GH5, GH26, and GH44 are three families often found fused to additional GH domains. Fusion of endocellulase and exocellulase (or β-glucosidase) motifs may create intramolecular synergy [45•,46], but this is not a general rule: additional CBMs are often required [47]. Synergy in these multidomain enzymes is a complex property, and the position of CBM relative to GH is critical [48].

Bacteria in the genus *Caldicellulosiruptor* are the top sources of Tier 3 MFCs [49•]. Their MFCs are composed typically of two GH domains, interspersed with one to four CBMs, mostly from family 3 [49•,50•]. While they do not assemble into cellulosomes (which are complex assemblies of enzymes noncovalently bound to scaffoldin proteins by dockerin/cohesion pairs), the arrangements of GHs and CBMs in *Caldicellulosiruptor* MFCs recapitulate the function of cellulosomes with superior stability and specific activity without the noncovalent assembly domains. These MFCs also deconstruct LCB more effectively than mixtures of free fungal enzymes, and some, like CelA from *Caldicellulosiruptor bescii* [51••], are agnostic to the crystallinity index of cellulose [52]. CelA connects a GH9 PEG to a GH48 exocellulase, and the enzyme is optimally active at 75°C. The modular

assembly, thermostability, and advances in native host expression [53] suggest considerable technological promise for *Caldicellulosiruptor* MFCs.

MFC reactivity is not confined to LCB polysaccharides. Recently, a cellulase was discovered in hot spring bacteria [54] possessing activity on agar and carrageenan (found in red algae) and amylose (from starch). The enzyme is composed of three GH70 domains, and its ability to degrade polycyclic, heterogeneous, and sulfated polysaccharides (in addition to both α-glycosidic (1,4)-glycosidic bonds and β-(1,4)-glycosidic bonds) offers promise for processing marine biomass and enzymatic synthesis of bioactive compounds [55].

Conclusions

Tier 1 broad specificity enzymes add to a growing parts list for cellulase design, yet their ability to degrade LCB is typically limited, despite their broad reactivity. Although improvements in stability are common [56], it has been difficult to surpass modest improvements in catalytic efficiency or achieve broad specificity by sequence optimization of single domains [57], highlighting the importance of bioprospecting for novel Tier 1 MFCs [58,59] and the need for additional screening methods specifically for *endo*-acting MFCs.

Engineered modularity offers a different approach and greater promise for generating new MFCs by taking advantage of Tier 2 and Tier 3 functionality. Tier 2 phenomena require deeper mechanistic insights, including structural dynamics and other constraints on the interacting domains. In Tier 2, fusion to CBMs and other non-GH domains can expand potential by adding new substrate binding and kinetic mechanisms.

Tier 1 and Tier 2 MFCs are building blocks for multidomain, potentially synergistic Tier 3 enzymes. Combinatorial assembly of multi-domain Tier 3 MFCs can leverage Golden Gate assembly [60] and other rapid domain-swapping techniques [61]. The most compatible domains may come from families with high natural frequency of multidomain fusions, such as GH5, 9, 26, 44, and 48 (Figure 4b). Substituting these domains into the scaffolds of existing Tier 3 cellulases may be a promising strategy, since inter-domain interactions are sensitive to linker length [62], and linker glycosylation can be important [63•]. Focusing on MFCs with thermal stability and salt tolerance will facilitate future integration with thermochemical biomass pretreatments, which are critical to obtaining maximum sugar yields in the biorefinery.

Finally, LCB structure itself deserves equal attention if yields of monomer sugars are to be maximized, since LCB composition varies greatly between plant species, growth, and harvest conditions. Enzymatic deconstruction changes the properties of LCB, as do the myriad physical and chemical treatments preceding enzyme addition. These changes impact the amount and quality of lignin residue, the valorization of which is another key component of a sustainable bioeconomy [64•]. Experiments in LCB imaging, simulation, and engineering can also reveal bottlenecks in enzymatic hydrolysis and inform the best use of MFCs in an innovative deconstruction pipeline.

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• of special interest

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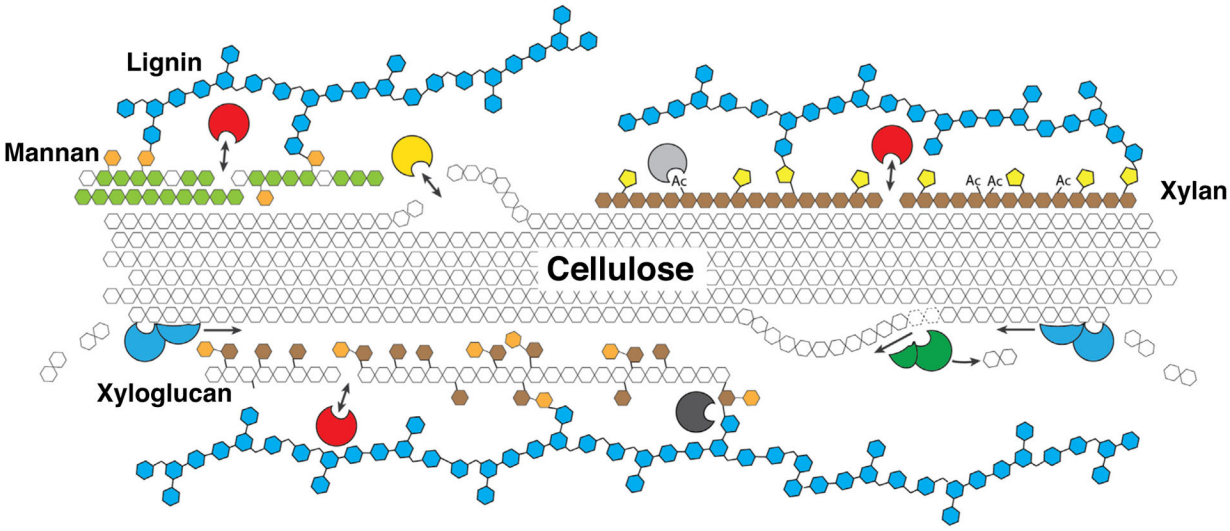

Figure 1.

Simplified structure of LCB reacting with different classes of enzymes. Processive exocellulases (*blue*) depolymerize cellulose from reducing or non-reducing ends. Endocellulases (*yellow*) create new chain ends in amorphous regions. Hemicellulases (*red*) hydrolyze non-cellulose polysaccharides. Processive endocellulases (*green*) create new ends and continue to depolymerize. Carbohydrate-targeting and lignin-targeting esterases (*light gray* and *dark gray*, resp.) remove acetyl side chains and cleave linkages to lignin. Glucose (*white hexagon*), mannose (*green hexagon*), xylose (*brown hexagon*), galactose (*orange hexagon*), arabinose (*yellow pentagon*), lignin (*blue hexagon*).

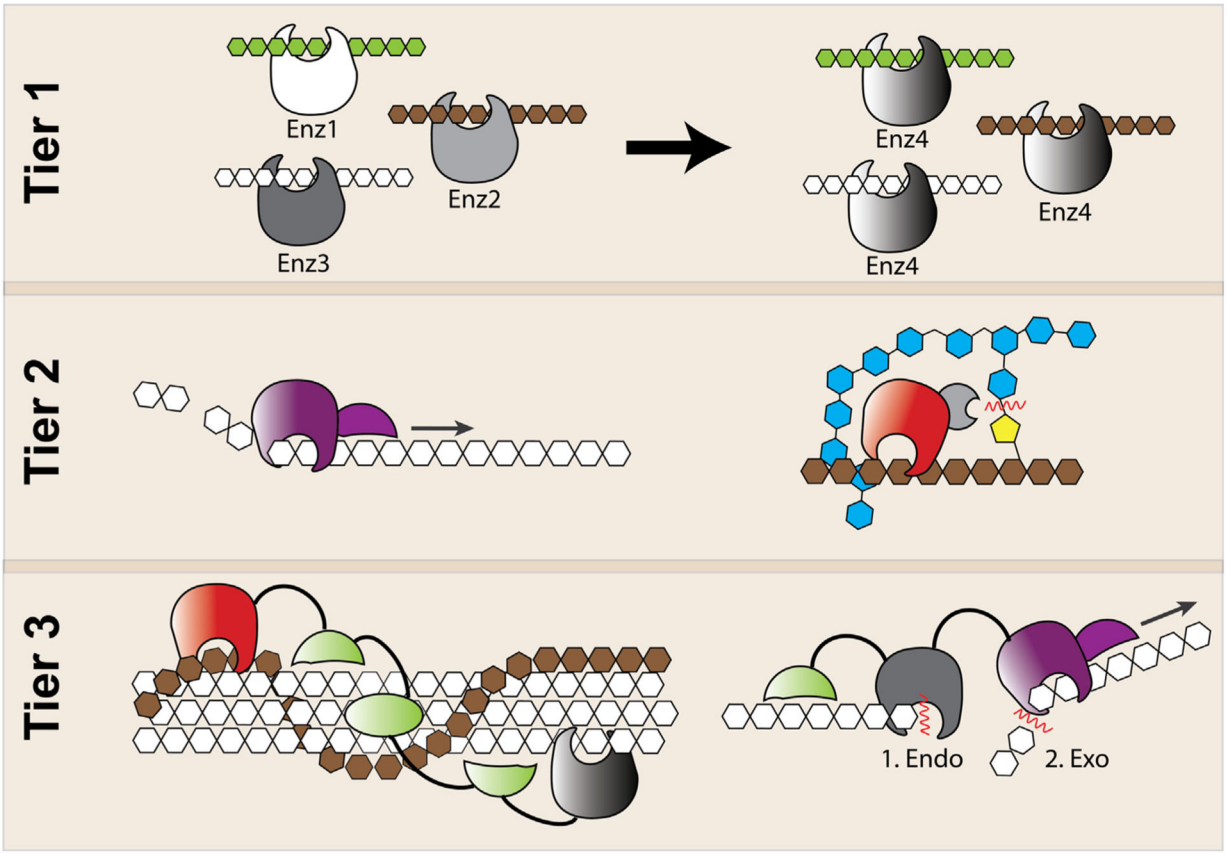

Figure 2.

Tiers of function in MFCs. Several monospecific enzymes (*left*) can be replaced by a single *Tier 1* MFC (*right*) possessing broad substrate specificity. *Tier 2* MFCs possess additional domains that confer increased activity on native substrates by promoting processivity (*left*), cleaving crosslinks or branches (*right*), and so on. *Tier 3* enzymes combine several GH and binding domains to achieve intramolecular synergy, for example, by xylanase-cellulase fusion (*left*) or endoglucanase-exoglucanase fusion (*right*). Polysaccharide colors and shapes are as in Figure 1.

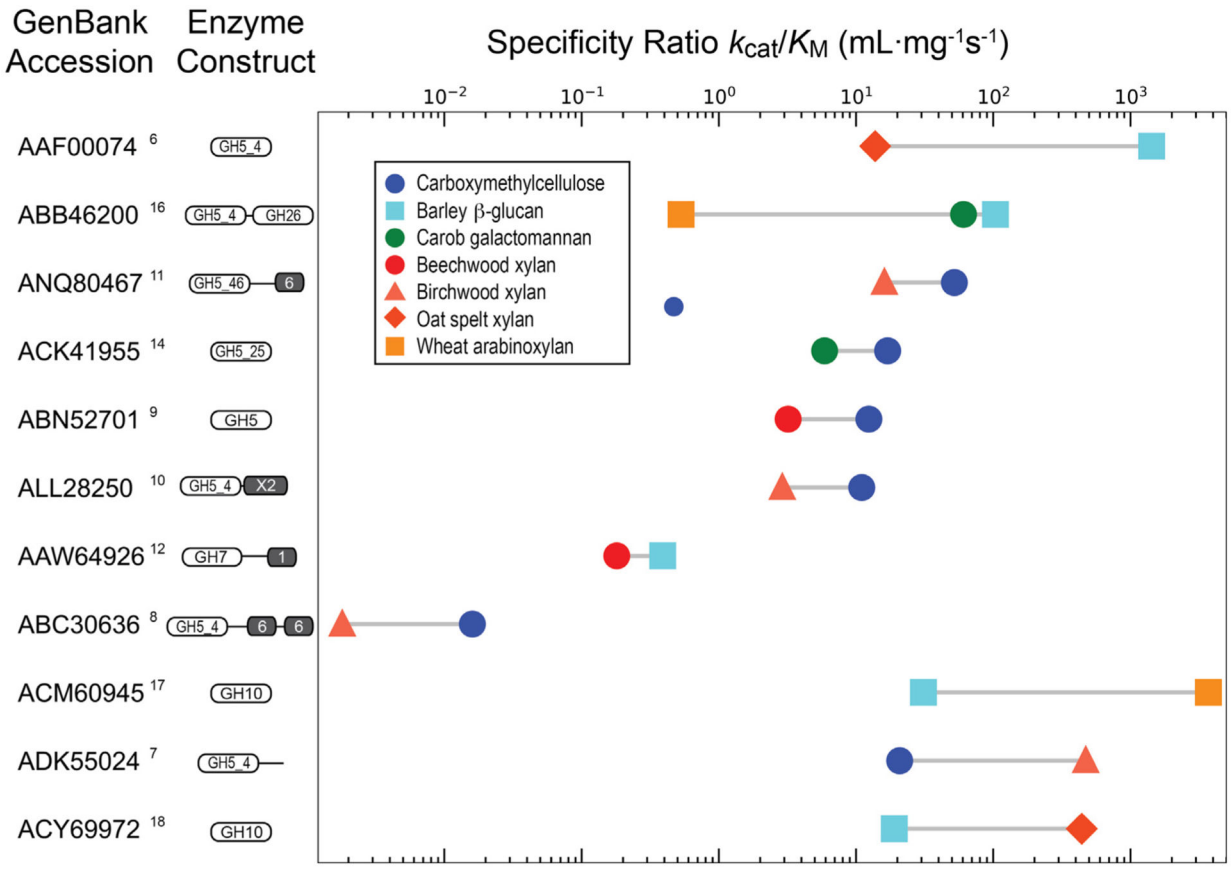

Figure 3.

Specificity ratios (k_{cat}/K_M) for enzymes assayed on substrates with distinct backbone compositions. β-1,4-glucan substrates (carboxymethylcellulose and barley β-1,3-glucan, *blue* shades); β-1,4 mannose backbone (carob galactomannan, *green*); β-1,4 xylose backbone substrates (xylans from beechwood, birchwood and oat spelt, *red* shades); arabinoxylan (*orange*). GenBank accession code (with reference as superscript) and enzyme domain structure are shown on the left. Enzyme and CBM domains are denoted as ovals labeled with black and white text, respectively, and linker regions are also displayed to approximate proportional scale.

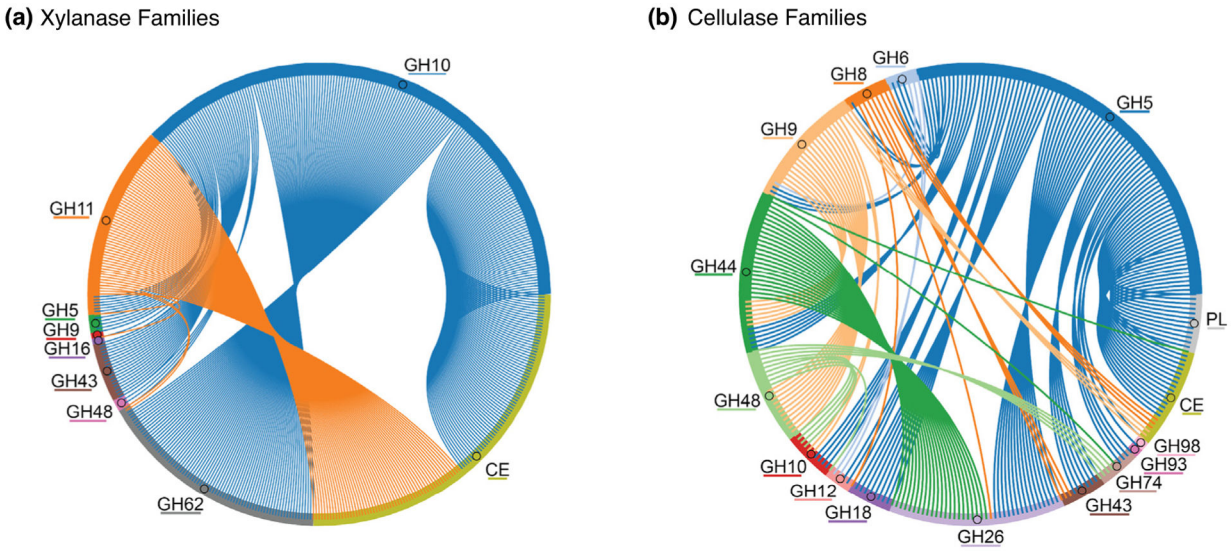

Figure 4.

Chord plot illustration of the connections between GH and other domains in Tier 2 and 3 enzymes. Arcs connect modules present in a single gene according to separate listings in the CAZy database. **(a)** Tier 2 and 3 endoxylanase (GH10, GH11) connections to CE and other GH domains. **(b)** Tier 2 and 3 endoglucanase (GH5, GH6, GH8, GH9, GH44, or GH48) connections to CE, PL, other GH domains. For simplicity, the multiple carbohydrate esterase (CE) and polysaccharide lyase (PL) families have been merged into single composite entries.