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REVIEW

Designing cellulolytic enzyme systems for biorefinery: From nature to application

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Cellulolytic enzymes play a key role on conversion of lignocellulosic plant biomass to biofuels and biochemicals in sugar platform biorefineries. In this review, we survey composite carbohydrate-active enzymes (CAZymes) among groups of cellulolytic fungi and bacteria that exist under aerobic and anaerobic conditions. Recent advances in designing effective cellulase mixtures are described, starting from the most complex microbial consortium-based enzyme preparations, to single-origin enzymes derived from intensively studied cellulase producers such as *Trichoderma reesei*, *Talaromyces cellulolyticus*, and *Penicellium funiculosum*, and the simplest minimal enzyme systems comprising selected sets of mono-component enzymes tailor-made for specific lignocellulosic substrates. We provide a comprehensive update on studies in developing high-performance cellulases for biorefineries.

[**Keywords:** Biorefinery; Cellulolytic enzyme; Enzyme synergy; Glycosyl hydrolase; Lignocellulose]

The rapid rise in greenhouse gases and fluctuating oil price have driven the search for alternatives to fossil resources for the production of liquid fuels and platform chemicals. Plant biomass is a sustainable alternative resource which can be utilized through biorefinery. It is completely renewable and carbon-neutral in nature and represents the ideal carbon feedstock for conversion to a variety of commodity products, used as drop-in, smart green drop-in alternative or specialty products in various applications. Biorefinery is expected to constitute a significant market share of the global energy and commodity product markets within the next decade (1).

Although varying in their detailed physical structure and chemical architecture, lignocelluloses from different plant origins comprise three biopolymers as their main components (2). Cellulose, the linear homopolymer of D-glucose linked by β-glycosidic bonds, represents the major component of the plant cell wall (30–50% of the total dry matter). It is organized into a highly ordered crystalline structure, in which the polysaccharide layers are held together by H-bonds. The parallel cellulose chains are formed into microfibrils and are linked to a network of hemicellulose, the second most abundant polysaccharide (20–40% by weight). Hemicellulose consists of pentoses (D-xylose and L-arabinose), a minor proportion of hexoses (D-mannose, D-glucose, and D-galactose) and sugar acids which are formed into a highly branched, amorphous and more easily hydrolysable structure. Hemicellulose is inter-linked through ester and ether bonds to an intricate three-dimensional network of lignin, a protective shield polymer constituting 15–25% of lignocellulose by weight. Lignin is a heteropolymer of phenylpropanoid subunits (guaiacylpropane, syringylpropane, and *p*-hydroxyphenylpropane) that are held together mainly by ether and carbon-carbon linkages. Pectin, proteins, lipids and minerals are present as the minor components in lignocellulose. Lignocellulose is highly recalcitrant to external physical, chemical, and biological attacks. Moreover, lignocelluloses derived from different sources vary in their physicochemical properties, and hence methods for their processing in biorefinery must be optimized for each type of lignocellulose.

SYNERGY OF ENZYMES AND AUXILIARY FACTORS ON LIGNOCELLULOSE DEPOLYMERIZATION

Depolymerization of lignocelluloses to sugars is the main barrier for utilization of lignocellulose feedstock in biorefinery. A pretreatment step typically carried out by various physical, chemical, and biological methods is generally required in order to destroy the lignin shield, which provides access of hydrolytic enzymes to the holocellulose fraction to generate streams of cellulose-derived glucose and a mixture of hemicellulose-derived pentoses and hexoses (3). According to the Carbohydrate-Active enZYmes Database (CAZy), a repertoire of carbohydrate-active enzymes (CAZymes) from diverse taxa of fungi, bacteria, and archaea is classified into various CAZy families (4) act synergistically on complete hydrolysis of lignocelluloses through hydrolytic

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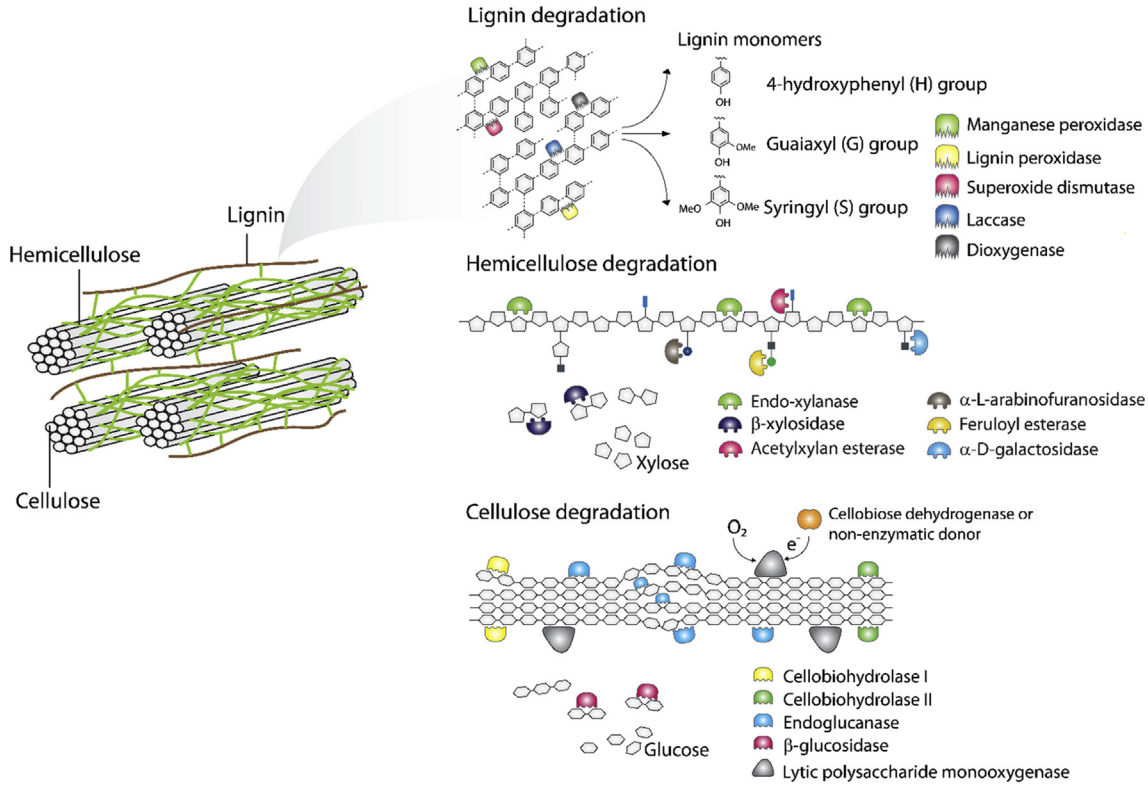

FIG. 1. Schematic diagrams of lignocellulose degradation by the synergistic actions of glycosyl hydrolases and auxiliary activities. Auxiliary non-enzymatic components work to create more accessible sites for core cellulases with accessory hydrolytic glycosyl hydrolases and de-branching enzymes, which act cooperatively for depolymerization of lignocellulose (covered lignin, hemicellulose and cellulose polymers).

and non-hydrolytic mechanisms (Fig. 1). Three cellulases, namely endo-glucanases (EG), exo-glucanases (cellobiohydrolases: CBH), and β-glucosidases (β-GLU) represent the core set of enzymes for hydrolysis of cellulose. Synergism among the cellulases and non-cellulolytic partners can be basically based on variations in their substrate specificities, the endo-/exo-catalytic mechanisms, and removal of the inhibitory products generated by the upstream enzymes by the downstream enzymes, which altogether result in increasing enzyme's accessibility or catalytic efficiency (5). From the outside in, ligninase enzymes work by peeling away the lignin shield existing as the lignin-carbohydrate complex covered the cellulose fibers. Ligninases comprising a set of non-hydrolytic enzymes (laccases, lignin-peroxidases, and manganese peroxidases) attack the lignin shield via generation of highly-reactive and nonspecific free radicals that cleave carbon-carbon and ether inter-unit bonds in the lignin structure (6). The heterogeneous hemicellulose network is degraded by hemicellulases, which constitute a large group of hydrolytic and non-hydrolytic enzymes with different specificities that function cooperatively (7). They comprise various endo- and exo-acting enzymes that attack the main chain of hemicellulose, in addition to a variety of debranching enzymes, e.g., α-arabinofuranosidase, ferulic acid esterase, acetyl xylan esterase and α-glucuronidase which cleave different decorated groups attached to the main chain. Actions of pectinases (polygalacturonase, pectin esterase, and pectate lyase) on removal of the pectic substances associated with cellulose in the cell wall structure are required in some biomass rich in pectin.

In addition to the classical synergism among the conventional enzymes, synergy by the C1-Cx cellulose degradation model first proposed in the 1950s (8) has gained increasing attention due to recent discoveries of new enzymes and proteins through omics- and meta-omics studies. In this model, cellulose depolymerization occurs through the co-operative action of C1 factors referring to non-hydrolytic proteins or enzymes, and Cx factors, i.e., classical cellulases directly involved in the hydrolysis of cellulose. C1 factors generate little or no soluble sugars by themselves, but act by inducing structural modifications of cellulose. This C1-mediated physical alteration process can be termed amorphogenesis, which involves combined structural changes relevant to loosening of the inaccessible crystalline regions of cellulose, thus allowing improved accessibility of the Cx factor to the cellulose (9). Among the C1 factors, the non-catalytic cellulose-loosening expansin proteins, and more importantly the lytic polysaccharide monooxygenase (LPMO) oxidative enzymes have gained interest as promising enhancers in enzyme formulations for bioconversion of lignocellulose. The synergistic roles of expansins and LPMO with conventional cellulases in enzyme mixture formulations on depolymerization of cellulose and other polysaccharides have been recently reviewed (10,11).

EXPLORATION OF CAZYME PROFILES OF AEROBIC VS. ANAEROBIC CELLULOLYTIC MICROBES

Lignocelluloses are decomposed in nature by microbial communities composed of various groups of microbes that act synergistically by employing different sets of catalytic machineries. Their enzyme systems have been explored using various strategies. Molecular approaches of cellulolytic microbes have been expedited by the rapid development of next-generation sequencing of metagenomes and transcriptomes of cellulolytic microbes (12). This is complemented by advancement in biochemical methods using high-performance proteomic tools based on advanced chromatographic and mass spectrometry techniques to reveal patterns of cellulolytic systems employed by different microbial taxa (13) and

by improvement in microbiological methods on isolation and identification of novel cellulase producing strains.

CAZymes with different functions and specificities are distributed among all forms of life with a high proportion in microbial genomes (14). They are classified into glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharides lyases (PL), carbohydrate esterases (CE), and the auxiliary activity (AA), based on amino acid sequence similarity, secondary and tertiary fold conservation, stereochemical architecture and catalytic mechanisms. Some of them may contain carbohydrate binding modules (CBMs) which frequently associate with the catalytic domains of extracellular degradative enzymes and function in enhancing their binding and activity towards the target substrates. The distribution of CAZymes among widely studied cellulolytic genera of bacteria and fungi is shown in Fig. 2. Differentiation in their CAZyme profiles thus reflect the differences in their enzyme's molecular architectures and catalytic mechanisms and hence, varying efficiencies of the employed cellulolytic machineries adapted to work under different environmental conditions. Owing to their prominent roles in lignocellulose depolymerization, only GH and AA activities are discussed in this review.

Aerobic fungi are considered the most active plant biomass degraders in open environments. Cellulolytic fungi involve various fungal taxa from the phyla Basidiomycota and Ascomycota. A systematic survey of 218 genome sequences revealed genes and proteins involved in polysaccharide degradation in fungi (15). Fungal enzymes involved in plant polysaccharide degradation are assigned to at least 35 GH families, 3 CE families and 6 PL families (16). Twenty of the 22 critical GH families required for plant biomass decomposition (17) are found in filamentous fungi, underlining the importance of aerobic fungi as the major plant biomass degraders in nature. The ability of fungi to degrade polysaccharides is proposed to rely on apparent redundancy in functional traits and the high frequency of LPMO and other auxiliary activities, in addition to other physiological adaptation such as hyphal growth which assist in penetrating the lignocellulose structure (15). Most aerobic cellulolytic fungi are generalists possessing several enzymes with a wide range of specificities for polysaccharide deconstruction. Their

		Aerobic bacteria							Anaerobic bacteria				Aerobic Fungi									Anaerobic Fungi			
		Actinobacteria				Bacteroidetes	Firmicutes				Fibrobacteres	Thermotogae	Agaricomycetes			Eurotiomycetes			Sordariomycetes			Neocallimastigomycetes			
		Cellulomonas	Streptomyces	Micromonospora	Thermobifida	Cytophaga	Bacillus	Paenibacillus	Clostridium	Ruminococcus	Fibrobacter	Thermotoga	Ganoderma	Phanerochaete	Pleurotus	Aspergillus	Penicillium	Talaromyces	Fusarium	Humicola	Trichoderma	Anaeromyces	Neocallimastix	Orpinomyces	Piromyces
Cellulases	GH5	11	451	77	13	5	335	253	102	54	16	19	1	4	0	79	29	8	53	4	20	1	6	10	7
	GH6	16	386	46	4	0	0	27	0	0	0	0	0	2	0	7	5	7	5	3	12	1	15	13	17
	GH7	0	0	0	0	0	0	0	0	0	0	0	3	6	8	34	27	9	34	6	48	0	0	0	0
	GH8	0	15	0	0	0	195	59	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	GH9	15	82	39	4	7	74	52	60	25	11	0	0	1	0	0	0	0	0	0	0	0	2	0	3
	GH12	1	118	21	0	0	30	10	8	0	0	24	0	1	0	31	7	1	20	1	8	0	0	0	0
	GH44	0	2	6	0	0	0	22	11	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	GH45	0	0	0	0	0	0	0	0	0	4	0	0	1	0	1	2	0	8	6	3	0	0	0	1
	GH48	4	68	19	1	0	60	34	26	23	0	0	0	0	0	0	0	0	0	0	0	0	2	1	3
Endohemi-cellulases	GH10	37	218	80	4	3	52	150	29	10	8	24	0	3	1	32	20	7	33	5	4	1	1	1	0
	GH11	10	83	16	4	1	366	66	26	17	6	0	0	2	1	70	20	15	21	4	26	0	23	5	5
	GH26	11	65	46	0	1	318	189	48	18	5	1	0	0	0	5	1	0	0	1	0	0	0	1	6
	GH28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	101	22	2	291	0	3	0	1	1	0
	GH53	3	56	8	0	0	266	50	32	1	2	8	0	0	0	7	2	2	3	1	0	0	0	0	0
Debranching enzymes	GH51	9	133	35	0	2	494	183	38	5	2	13	0	0	2	15	7	1	6	0	0	0	0	0	0
	GH54	0	50	9	0	0	0	0	0	0	1	0	0	0	0	10	4	6	6	0	4	0	0	0	0
	GH62	7	113	41	0	0	0	0	4	0	0	0	0	0	0	15	5	1	6	0	2	0	0	0	0
	GH67	3	31	17	0	0	8	57	7	2	0	10	0	0	0	8	2	0	4	0	1	1	0	0	0
	GH74	4	113	7	1	1	0	59	13	6	1	4	0	1	0	6	0	0	2	0	1	0	0	0	0
	GH78	1	102	6	0	0	39	128	5	2	0	2	0	0	0	32	7	0	25	0	0	0	0	0	0
	GH93	0	44	0	0	0	0	15	0	0	0	0	0	0	0	5	3	1	10	0	0	0	0	0	0
	GH115	1	28	0	0	0	1	49	4	0	0	0	0	0	0	6	0	0	9	0	0	0	0	0	0
Oligosaccharide-degrading enzymes	GH1	10	470	106	3	1	1438	352	413	3	0	12	0	2	0	12	4	5	13	2	13	0	1	3	5
	GH2	5	475	35	2	0	62	436	122	13	2	33	0	0	0	33	8	0	35	0	3	0	0	0	0
	GH3	30	798	119	2	6	376	380	122	19	3	34	0	2	0	114	26	19	70	5	17	0	2	1	1
	GH29	0	162	3	0	0	8	107	40	2	0	10	0	0	0	1	0	0	20	0	0	0	0	0	0
	GH35	4	179	4	0	0	11	86	10	0	0	0	0	0	0	30	7	1	9	0	1	0	1	0	0
	GH38	0	202	10	0	0	51	180	63	0	0	20	0	0	0	5	1	0	3	0	0	0	0	0	0
	GH39	5	63	19	0	0	17	46	38	1	1	3	0	0	0	1	2	0	9	0	0	0	4	1	1
	GH42	12	193	37	1	0	337	212	53	2	0	21	0	0	0	0	1	0	0	0	0	0	0	0	0
	GH43	24	568	150	3	2	1038	725	136	18	14	5	0	4	0	61	27	4	79	6	0	0	2	0	1
	GH52	0	0	0	0	0	2	48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Auxiliary Activities (AAs)	AA1	0	0	0	0	0	0	0	1	0	0	0	57	6	53	31	2	1	58	0	2	0	0	0	0
	AA2	0	0	0	0	0	0	0	0	0	0	0	33	49	0	0	0	0	2	0	0	0	0	0	0
	AA3	0	1	0	0	0	0	0	1	0	0	0	0	4	4	89	8	1	68	0	0	0	0	0	0
	AA5	0	14	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	22	0	0	0	0	0	0
	AA6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	4	0	0	0	0	0	0
	AA7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	11	0	0	0	0	0	0
	AA8	0	0	0	0	0	0	0	0	0	0	0	0	3	0	7	0	0	11	0	0	0	0	0	0
	AA9	0	0	0	0	0	0	0	0	0	0	0	0	2	0	29	6	1	42	2	10	0	0	0	0
	AA10	8	540	80	2	0	582	58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	AA11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	9	0	12	0	0	0	0	0	0
	AA12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0	0	0	0
	AA13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	3	0	0	0	0	0	0

No. proteins in CAZY: 0 | 1 | 2-10 | 11-20 | 21-50 | 51-100 | 101-200 | 201-500 | 501-1000 | >1000

FIG. 2. Distribution of key lignocellulose degrading CAZymes in the four groups of cellulolytic microbes: aerobic fungi, aerobic bacteria, anaerobic fungi and anaerobic bacteria. The data were collected from the CAZy database (http://www.cazy.org/; accessed January 2019).

enzymes are mostly extracellular small proteins with simple domain organization and secreted upon induction through a complicated regulatory system at multiple levels from transcription, translation, secretion, to the epi-genetic mechanism (18). Their EGs are classified into several GH families with variation in their structures (e.g., $(\beta/\alpha)_8$ or β-jelly roll), catalytic mechanisms (retaining or inverting), and catalytic amino acid residues (Glu or Asp). Most are grouped in GH5, 7, and 12 with minor members in GH6 and 45. The internally-cleaved cellulose is subsequently hydrolyzed by the exo-acting cellobiohydrolases attacking the non-reducing (GH6) or reducing (GH7) end of the cellulose chain while β-GLU (GH1 and GH3) cleaves cellobiose into glucose. Some species of basidiomycetes lack CBH but possess a multi-purpose processive EG which combines the properties of exo-acting enzymes (19). The major enzymes attacking the backbone hemicellulose are endo-xylanases (GH10 and GH11) that cleave xylose glycoside linkages in xylan in addition to other backbone attacking enzymes, e.g., endo-galactanase (GH53). Various debranching enzymes exist for cleaving the linkages at the branching points including: α-arabinofuranosidases (GH3, GH51, GH54, and GH62), which catalyze hydrolysis of α-arabinofuranosidic bonds in arabinose-containing hemicelluloses; α-glucuronidases (GH67 and GH115), which hydrolyze the ester linkages between the 4-*O*-methyl-D-glucuronic acid of glucoronoxylan and lignin alcohols; xyloglucanases, which cleave glycosidic bonds of branched (GH74) or unbranched (GH12) glucose residues of xyloglucan; arabinanases (GH43 and GH93), which catalyze hydrolysis of α-L-arabinofuranoside linkages of α-1,5-L-arabinan from the non-reducing end and α-L-rhamnosidase (GH78), which catalyzes cleavages of terminal rhamnose residues. The xylo-oligomers are then hydrolyzed by β-xylosidases (GH3 and GH43), which release successive xylose residues from the non-reducing end whereas manno-oligomers are processed by GH2 β-mannosidases. The majority of the pectin hydrolyzing enzymes belong to the GH28 family, which include endo-/exo-acting polygalacturoronases and rhamnogalacturonases that act together with enzymes in CE on pectin depolymerization.

The major AA enzymes in aerobic fungi involve LPMOs in the AA9 family, in addition to those in AA11, 12, and 13 besides various redox enzymes attacking lignin (AA1, 2, 4, 6) and carbohydrate (AA3, 5, 7, 8). *Aspergillus* and *Fusarium* are rich in most AA families. LPMOs are copper-dependent enzymes that are capable of catalyzing the cleavage of β-1,4 glycosidic bonds in cellulose via the oxidation of the C1- or C4 atom with supply of electrons from co-substrates such as ascorbic acid, or naturally occurring electron donors such as lignin (20,21). They induce oxidative cleavages in the crystalline cellulose region, which creates more accessible sites for other enzymes to hydrolyze cellulose, as well as other substrates such as xylan (22). Their action is coupled to the actions of AA3 flavin-dependent oxidoreductases and AA12 pyrroloquinoline-quinone (PQQ)-dependent pyranose dehydrogenase (PDH) (23). Agaricomycetes lack LPMOs and have less diverse GH enzymes but are highly rich in lignolytic enzymes particularly AA1 and AA2 (24,25). These fungi use the hydroxyl radicals generated by cellobiose dehydrogenase, quinone redox cycling or glycopeptide-based Fenton reaction on depolymerization of plant cell wall components, including cellulose and lignin (19).

Some fungal genomes, particularly various species of *Trichoderma*, *Aspergillus* and *Penicillium*, contain genes encoding for expansin-like proteins called swollenins. These non-catalytic proteins contain a GH45-like domain (10), and act on loosening the densely packed crystalline cellulose region via disruption of the non-covalent H-bonds between the layers, which results in increased accessibility of cellulases to the cellulose. However, their enhancing effects have only been shown under certain conditions with low cellulase dosages (26).

Aerobic bacteria (including facultative anaerobic members) are another key group of lignocellulose degrader in open environments (27). Although a large number of them are capable of producing some plant biomass degrading enzymes, only about 4% of bacteria with a cellulase gene(s) are truly cellulolytic (28), which includes selected species of Firmicutes (e.g., *Bacillus* and related genera), Actinobacteria (e.g., *Streptomyces*, *Cellomonas*, *Thermobifida*, and *Micromonospora*), Bacteroidetes (e.g., *Cytophaga*), and Proteobacteria (e.g., *Cellvibrio*). Their enzymes are mainly in secreted forms with the exception of a few members which also produce enzymes in complex xylanosomal systems, e.g., *Paenibacillus curlanolyticus* (29) and *Streptomyces olivaceoviridis* (30). According to data in CAZy, cellulases from several GH families are represented among both aerobic bacteria and fungi (GH5, 6, and 12 for EG) whereas GH8 (chitosanase with some EGs), 9, 44, and 48 are found only in bacteria. CBH-like cellulases are found in GH6 and GH48. Cellulases of the GH7 and GH45 families are present in fungi, but not bacteria. The main chain and debranching hemicellulases of aerobic bacteria are distributed into GH families similar to aerobic fungi, with the exception of GH28 which is only found in fungi. However, the levels of glycoside hydrolase activities, especially cellulolytic enzymes were generally low compared to the aerobic fungi counterpart which limit their use as cellulase producers in industry. Compared with the diversity of AA enzymes in fungi, far fewer AA families are represented among aerobic bacteria. Bacterial AAs include LPMOs in the AA10 family, which share structural homology but no significant sequence similarity to fungal homologs, and copper radical oxidases in the AA5 family. A few bacterial AA10 LPMOs have been characterized revealing unique properties in their molecular mechanisms and catalysis, including those acting on cellulose and chitin (31).

Expansins have also been identified in various aerobic bacteria, including *Bacillus* and some plant pathogens, e.g., *Clavibacter* and *Pectobacterium* (32). These proteins are potent cellulase enhancers for hydrolysis of cellulosic substrates under certain experimental conditions, particularly at a relatively low cellulase dosage (<1–5% cellulose hydrolysis) (33). Bacterial expansins bind to other polysaccharides with different chemical structures and levels of crystallinity, e.g., arabinoxylan and pectin (34,35). Expansins synergize with hemicellulases for hydrolysis of hemicellulose, but the mechanism is unknown (35).

Anaerobic bacteria, particularly *Clostridium* and *Ruminococcus* employ multi-component cellulolytic complexes, so called cellulosomes located on the cell surface for cellulose degradation (36), whereas some *Fibrobacter* utilize a unique cellulase enzyme system with enhanced activity through cell-enzyme synergy (37). The enzyme complex comprises a set of catalytic enzyme units with different specificities attached to the core scaffoldin via a dockerin domain. Cellulosomes are highly efficient for degradation of the whole lignocellulose structure owing to synergy among the catalytic units, which is enhanced by their proximity to one another in the complex. The cellulase domains in the catalytic units of cellulosomes are mostly classified into GH5, 9, and 44 for EGs, GH48 for CBHs, and GH1 and 3 for β-GLU. The distribution of main chain and debranching hemicellulases, together with the oligosaccharide processing activities are similar to those of aerobic bacteria, except for the absence of GH52 in anaerobic bacteria. The advances in cellulosome researches particularly on their molecular design (so called artificial or designer cellulosomes) are recently reviewed (38). Expansin-like proteins CclEXL1 and 2 have been recently identified in the cellulosomal complex of *C. clariflavum*, which function on loosening cellulose to enhance cellulase activity (39). Only two AA enzymes have been found in anaerobic bacteria and no LPMO has been identified. The deficiencies of natural bacterial cellulosomes can be overcome by protein engineering, in which new enzymatic parts fused to dockerin can be grafted onto native

scaffoldin or cohesins to create so called artificial or designer cellulosomes (38).

Anaerobic fungi have started receiving attention on their role in degradation of lignocellulosic materials in recent years, although they are poorly characterized owing to difficulties in their isolation and culturing (40). So far, only a few cellulolytic anaerobic fungi have been reported with roles on decomposition of plant materials in different ecological niches, e.g., fiber degradation in guts. These include genera *Anaeromyces*, *Neocallimastix*, *Orpinomyces*, and *Piromyces*, which produce cellulases arranged in cellulosomes with shared architecture to those of bacterial origins. Fungal cellulosomes evolved independently, although some catalytic domains appear to have been acquired horizontally from gut bacteria (41). According to the currently limited CAZy data, anaerobic fungal cellulases are classified into GH6, 9, and 45 for EGs, GH1, GH3, GH5, GH9 for CBHs, and GH1, GH9, GH3, GH5 for BGLs. Their hemicellulases are distributed mostly in GH11 with only few in GH10, 26, and 28 for main chain hydrolases. Very few side chain cleaving and oligosaccharide processing enzymes have been identified, and no AA enzymes from anaerobic fungi have been reported so far.

DESIGNING CELLULASE ENZYME SYSTEMS: FROM COMPLEX TO MINIMAL SYSTEM

The development of cellulase, or more correctly, cellulolytic enzyme blends is a key factor for biorefinery. Enzymes can constitute up to 30% of the cost structure for producing biofuel from cellulosic sugar (42). In order to achieve economic feasibility, the cost of enzyme for biomass depolymerization should not exceed US$ 0.10/L of ethanol product (43). A variety of cellulase blends for biorefineries have been developed by a number of companies. The market is currently dominated by several global enzyme suppliers, including Novozymes (Bagsværd, Denmark), Genencor (Leiden, the Netherlands; merged with Dupont), Solvay Enzymes (Nienburg/Weser, Germany), DSM (Heerlen, the Netherlands), and Dyadic (Jupiter, FL, USA). The cost of producing cellulases has decreased markedly during the past decade, making cellulase more economically viable for production of second-generation biofuels made from cellulosic sugar feedstock. Enzymes used in biorefinery for the production of second-generation biofuels have been recently reviewed by Binod et al. (44).

In general, a single microbial enzyme cannot degrade biomass efficiently enough for biorefinery; hence, a cocktail of enzymes is needed. Similarly, one enzyme cocktail will not show optimal performance for every type of biomass owing to compositional variability. All these factors need to be addressed in order to develop an enzyme or enzyme blend that can overcome this key technical bottleneck. Improvements in enzyme formulation have arisen through the accelerated knowledge on synergy among CAZymes gathered from omics studies of individual microbes and meta-omic studies of microbiomes. Cellulase mixtures can be obtained by several strategies, including the use of complex, microbiome or consortium-derived enzyme mixtures containing a crude multi-species enzyme preparation, to the widely used formulated blends using a selected core enzyme from a single cellulolytic microbial origin with synergized partners, and the simplest synergistic minimal enzyme systems comprising a specified set of mono-component enzymes tailor-made to a specific substrate of interest (Fig. 3).

Consortium-based enzyme systems In nature, lignocellulosic materials are degraded by complex microbial communities composed of multiple microbial taxa and different cellulolytic machineries. Intra-species and inter-species interactions among the microbial members (cellulolytic and non-cellulolytic microbes) in the microbiomes relevant to catabolic capability of the individual composite microbes and at the mechanistic enzyme synergism level. Culture-independent meta-omics techniques based on meta-genomics, meta-transcriptomics, meta-proteomics and meta-secretomics (45,46) have been used to reveal the complexity of cellulolytic systems and investigate mechanisms of lignocellulose degradation used by microbiomes residing in various ecological niches. Metagenomic approaches are the main strategy to identify enzymes from uncultured microbes involved in lignocellulose breakdown through cataloging the diversity of genes with identifiable functions by high-throughput sequencing of microbial communities residing in normal and extreme environments. Metagenomic surveys of

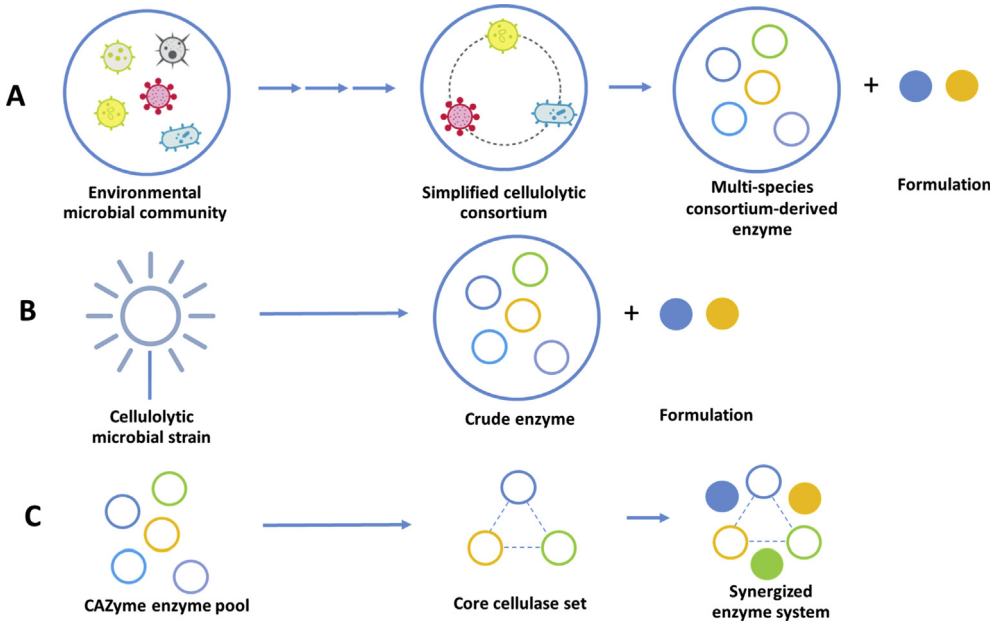

FIG. 3. Cellulase system designs. (A) Microbial consortia-based multi-enzyme; (B) single microbial origin enzyme with synergistic partners; (C) minimal enzyme systems based on mono-component enzymes.

lignocellulose-degrading microbiomes have been reviewed recently (47).

Because lignocellulose degrading microbial communities in nature comprise such diverse collections of microbes, it is often advantageous to enrich for organisms of interest to reduce the taxonomic complexity. This can be achieved by successive culturing from environmental samples rich in cellulolytic microbes and isolating microbial consortia capable of effectively degrading cellulosic substrates of interest. The first work on isolation of cellulolytic consortia was demonstrated by Haruta et al. (48) using successive dilution to stimulation cycles. Consortia from soils, compost, and other sources of potent cellulase-degrading microbes, e.g., cow rumen and activated sludge have been reported. These consortia are capable of degrading various agricultural wastes under different conditions of oxygen tension and temperature. The cellulolytic consortia are enriched for genes encoding CAZymes compared with the unenriched environmental microbial communities (49). The cellulolytic consortia possess high degradation activity on different substrates (50–52). These consortia can be applied for direct conversion of biomass to desirable bioproducts by consolidated bioprocessing (53), as inoculum in bioaugmentation for accelerating cellulose depolymerization in anaerobic digestion (52,54), or as a source for crude extracellular microbiome-based enzyme for lignocellulose degradation. A consortium can be further engineered by introducing and acclimating additional members for alteration of the consortium's structure and functions (55). The design of minimal active synthetic microbial consortia with nearly identical degradation potential to the complex consortia with complete members based on systematic combination of isolated members according to their metabolic function has been recently demonstrated (56).

The taxonomic composition and enzyme patterns of cellulolytic consortia vary according to the inoculum sources and the isolation conditions (49,57). Under aerated enrichment conditions, facultative anaerobic Firmicutes, particularly *Paenibacillus* were predominant taxa in cellulolytic consortia. In contrast, cellulolytic consortia obtained under self-generated (facultative) anoxic or anaerobic conditions typically comprised anaerobic cellulolytic clostridia mainly in phylum Firmicutes such as genera *Clostridium* and *Ruminococcus* (49,50,57,58), which co-existed with various non-cellulolytic facultative members in a symbiotic fashion. The non-cellulolytic facultative partners may produce enzymes attacking the hemicellulose and in some cases the lignin fractions or function to generate the anoxic environment, control pH and remove inhibitory metabolites (59). Enzyme activity profiles and specificities of cellulolytic consortia are shaped by the chemical nature of the target substrate used for enrichment (49). Taxonomic distributions of composite microbial members in previously reported cellulolytic consortia are shown in Fig. 4. The identified activities and derived enzyme profiles of the consortia characterized by shotgun sequencing and proteomics are summarized in Table 1.

Cellulolytic consortia are not yet widely used in industry owing to challenges in large-scale culturing and instability (change in microbial composition over time). Instead of culturing microbes to obtain consortia, cellulolytic enzymes can be obtained directly from cellulolytic microbiomes residing in environments where lignocellulose decomposition occurs. The crude protein extract from environmental samples can contain a variety of enzymes that act synergistically with one other, and/or with recombinantly-produced enzymes. The earliest report of a whole community-derived enzyme demonstrated that endogenous enzymes obtained from cow rumen act synergistically with one another and with exogenous fungal enzymes added to feed, leading to markedly increased hydrolysis of soluble cellulose, xylan, and corn silage (60).

Despite the challenges in obtaining consortia, this is still an active area of research because consortia can be studied and

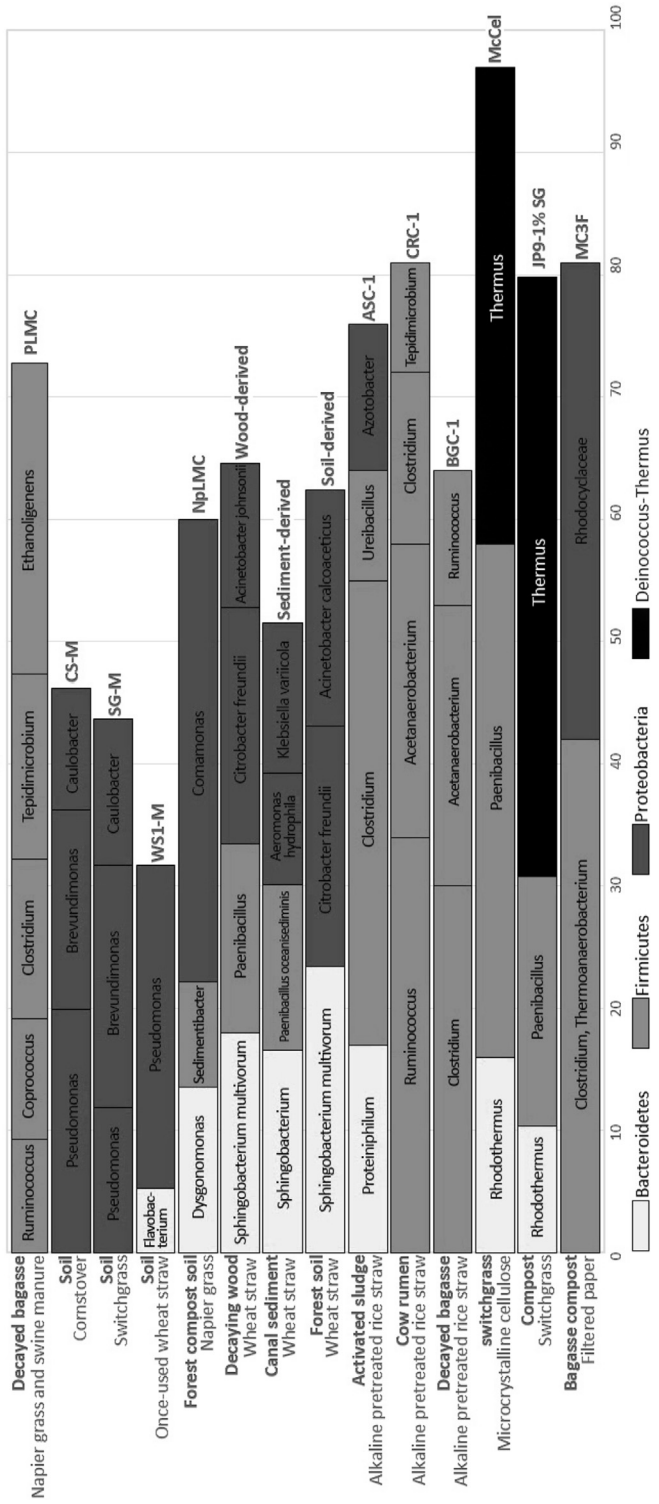

FIG. 4. Taxonomic distribution of enriched cellulolytic microbiomes from different environmental sources on different cellulosic substrates. The source of seed microbes and carbon source using in the subcultivation step are provided. The data are from previous references (49–52, 57, 61–63).

developed systematically into more efficient enzymatic systems. An interesting example is that of a compost-derived, thermophilic, aerobic biomass-degrading consortium containing *Paenibacilli*, *Rhodothermus marinus*, and *Thermus* adapted to switchgrass at 60°C (JP-9) (61). Compared with commercial enzyme preparations, the EG present in the consortium culture supernatant was found to be

TABLE 1. CAZymes and lignocellulose-degrading enzyme activities identified in cellulolytic microbiomes.

Consortium	GH (dominated GH)	AA	Identification method	Enzymatic activities	Reference
MC3F	GH8, 9, 10, 16, 48	nd	Proteomics	Xylanase, β-glucanase, CMCase	50
McCel-adapted consortium	GH5, 9, 10, 48	nd	Proteomics	EG, CBH, mannanase, xylanase, α-arabinofuranosidase	62
BGC-1	Total 26 dominated by GH2, 3, 5, 10, 43	nd	Metagenomics	Xylanase, CMCase, β-glucanase	49
Wood-derived	nd	nd	nd	β-Xylosidase and CBH dominated	57
Soil-derived	nd	nd	nd	β-Xylosidase and β-GLU dominated	57
Sediment-derived	nd	nd	nd	β-Xylosidase dominated with β-GLU, CBH	57
Np-LMC	Total 23 dominated by GH2, 3, 5, 9, 10, 26, 28, and 43	AA1, 3, 4, 6	Metagenomics	Xylanase, β-glucanase, CMCase	51
ISHI-3	nd	nd	nd	Avicelase, CMCase, xylanase	58
WS-1M, SG-M, CS-M	GH3, 43, 13, 10, 29, 28, 16, 4, 92	AA2, 6, 10	Metagenomics	Arabinanase, arabinoxylanase, β-glucanase, xylanase, galactomannanse, rhammogalactomannanse	63

nd, not determined/not reported.

more thermotolerant and more stable in the presence of 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), an ionic liquid (IL) used for biomass pretreatment and applied for saccharifying [C2mim][OAc]-pretreated switchgrass at elevated temperatures (up to 80°C). In a later study, the IL-tolerant enzyme mixture from the consortium was found to comprise six glycoside hydrolase proteins mostly of *Paenibacillus* origin, including 3 EGs (GH5 and 9) and one CBH (GH48), in addition to a GH10 xylanase and an arabinofuranosidase (62). An IL-tolerant cellulase cocktail, called JTherm was formulated by combining consortium enzymes with recombinant CBH (CBM3-GH5 of CelB from *Caldicellulosiruptor saccharolyticus*) and β-GLU from *Thermotoga petrophila*. The cocktail saccharified IL-pretreated biomass at a higher temperature and higher IL concentration compared with a commercial cocktail; moreover, it is compatible with single-pot bioprocessing involving simultaneous IL pretreatment and saccharification (62).

Natural lignocellulosic substrates are composed of high and low complexity polysaccharides, in which the latter are degraded by microbes more readily than the former. Consortia with greater capacity to digest high complexity plant polysaccharides as well as lignin can be obtained by a two-step enrichment process. In this method, consortia are initially isolated by culturing with substrate in the conventional manner. The partially-degraded substrate from the culture is recovered and used as substrate in the second enrichment step with the consortia microbes from the first enrichment. Using this method, *Enterobacteriales*, *Pseudomonadales*, *Flavobacteriales*, *Bacillales* and *Burkholderiales* microbes were found to be relatively more abundant in the second enrichment from partially degraded wheat straw, switchgrass and corn stover compared with the first enrichment step (63). Analyses of the CAZymes in the meta-secretomes of consortia from the second-step enrichment showed a variety of glycosyl hydrolases and auxiliary enzymes (AA6, AA10 and AA2), highlighting the potential of the two-step enrichment method for obtaining consortia with enhanced lignocellulose degrading activity. Potential of the meta-secretome on production of oligosaccharides directly from agricultural wastes was demonstrated.

Single origin enzyme with synergistic partners: *Trichoderma reesei* system Fungal cellulase blends generally contain cellulase preparations from a core fungal strain and side hemicellulase activities as a base (64). CBHs are generally the major component in commercial cellulase blends for crystalline cellulose breakdown (65). Supplementation of cellulase preparations with additional accessory or auxiliary enzymes as crude preparations or isolated enzyme components are generally required to achieve maximal hydrolysis efficiency. *T. reesei* cellulase is the most widely used cellulase in bio-industry; however, this enzyme has several drawbacks, e.g., low β-GLU activity. Cellulases from alternative microbial sources with higher specific activity and less susceptible to inhibition by cellobiose and lignin-derived compounds have been assessed for their potential as alternative candidates for industrial application (65). The comparative GH profiles of *T. reesei*, *Talaromyces cellulolyticus* and *Penicillium funiculosum*, the selected potential cellulase producers are shown in Fig. 5. All three species shared Endo-/Exo-glucanase in GH 5, 6, and 7 with GH12 in *T. reesei* and *T. cellulolyticus*. Their xylanolytic enzymes are mostly in GH11 with debranching enzymes in different families. Their cellulolytic enzyme systems are described in detail below.

At present, most commercially available cellulases used for biomass degradation (e.g., Celluclast 1.5 L, Cellic CTec2 and HTec2 from Novozymes, Cellulase TRL from Solvay Enzyme, and Accellerase1500 from Genencor) are produced from derivatives of the RUT-30 strain, a cellulase hyper-producing mutant of *T. reesei* (*Hyporia jacorina*) obtained by random mutagenesis (66). Genome sequencing of the RUT-30 strain revealed more than 200 GH genes (67), and the important genetic changes that underpin the hyper-cellulolytic phenotype of this strain have been identified (68). A variety of different molecular tools have been employed to engineer the expression and properties of cellulolytic enzymes in *T. reesei* including enzyme engineering, enzyme profile modification, gene expression, and epigenetics (68,69). Bio-processes for cellulase production from hyper-producing *T. reesei* strains have been thoroughly explored using model cellulosic substrates or agricultural wastes with the highest cellulase titer of up to 90.3 FPU/ml and the productivity of 627.1 FPU/l/h using a fed-batch process with a mixture of glucose and β-disaccharides as the substrate (70).

Secretome analysis revealed a variety of cellulases and hemicellulases enzymes in its crude enzyme preparation, involving a number of glycosyl hydrolases distributed in different GH families (71). *T. reesei* secretomes are dominated by a Cel7A CBH, which has a processive mode of catalysis with endo-initiation activity. This enzyme is responsible for the majority of the hydrolysis turnover, together with another CBH, Cel6A, several EGs (GH5, 7, and 12) and GH3 β-GLU (68). However, *T. reesei* is suboptimal for cellulolysis owing to the relatively low activities of β-GLU, hemicellulose and LPMO. *T. reesei* also lacks the downstream β-xylosidase activity necessary for complete hydrolysis of the generated oligosaccharides to sugar monomers (72). Together with the need for acidophilic culture conditions and poor thermal stability, these drawbacks in biochemical properties remain major obstacles for using *T. reesei* native cellulases for efficient hydrolysis of lignocellulose substrates.

Synergism of *T. reesei* cellulase enzymes with various CAZymes has been extensively studied, leading to the development of intra-species or inter-species enzyme blends with increased catalytic efficiency. The first generation cellulase from Novozymes, Celluclast 1.5 L, is usually supplemented with a β-GLU, particularly that

CAZY family	*Trichoderma reesei*	*Talaromyces cellulolyticus*	*Penicillium funiculosum*
GH2	3	0	2
GH3	2	0	11
GH5	3	12	6
GH6	1	1	11
GH7	2	2	12
GH10	2	1	0
GH11	4	7	22
GH12	1	4	0
GH13	0	5	0
GH15	1	3	1
GH16	3	0	0
GH17	3	0	0
GH18	2	0	0
GH20	1	0	0
GH26	0	1	0
GH27	0	0	5
GH28	2	16	10
GH31	1	0	1
GH35	0	0	10
GH38	1	0	0
GH43	1	22	0
GH45	0	2	0
GH47	2	0	1
GH49	0	0	1
GH53	0	1	2
GH54	0	1	0
GH55	1	0	1
GH57	1	0	0
GH61	2	1	0
GH62	0	1	8
GH67	2	2	1
GH71	4	0	0
GH72	1	0	0
GH74	2	1	0
GH78	0	12	1
GH92	2	0	0

FIG. 5. Comparative CAZyme profiles of selected cellulolytic fungi used as sources for commercial cellulases. *Trichoderma reesei* (secretome) (71); *Talaromyces cellulolyticus* (genome) (99); *P. funiculosum* (secretome) (122).

from *Aspergillus niger* (73). The second-generation cellulase, Cellic CTec2, was further improved for increased β-GLU activity less susceptible to glucose inhibition with additional hemicellulases and LPMO. Further formulation of Cellic CTec2 with endoxylanase preparation HTec2 is recommended to boost cellulose hydrolysis for substrates with high hemicellulose contents (74). A number of studies on synergism of *T. reesei* cellulase, either as in-house or commercial preparations with various accessory hemicellulases and auxiliary components from various microbial origins have been conducted. The conditions tested and sugar yields obtained from each mixture are summarized in Table 2.

Screening programs have revealed varying degrees of synergism of *T. reesei* cellulase with crude enzymes comprising downstream and hemicellulolytic activities from different fungi, e.g., *P. funicusolum* (75), *Humicola insolens* (76), and *Chaetomium globosum* (77). The degree of synergism of hemicellulase correlates with the xylan content of the substrates, and is also dependent on the pretreatment method and the hydrolysis time (78). Notable examples demonstrating synergism of *T. reesei* cellulase with protein partners from different fungi include the following: an extracellular enzyme preparation from wood decaying fungi *Laetiporus sulphureus* and *Pleurotus ostreatus* with rich activities of GH5- and GH45-EGs, GH3-β-GLU, and GH10-xylanases (79); an *Aspergillus awamori* mixture with high β-GLU, xylanase, and ferulic acid esterase activities (80); a crude enzyme from *Aspergillus oryzae* with ferulic acid esterase and xylanase activities (81); *A. niger* secretome containing feruloyl esterases, xylanases, and other auxiliary hemicellulolytic enzymes (71); a combination of crude enzyme from *Aspergillus aculeatus* and a bacterial expansin from *Bacillus subtilis* (82) and commercial pectinases of fungal origins (83).

Mono-component enzymes can also be combined with *T. reesei* cellulase for increasing the efficiency of hydrolysis for different cellulosic substrates. Mono-component enzymes including endoglucanase, hemicellulase (main-chain acting endo-xylanase), downstream enzymes (e.g., β-xylosidase), debranching enzymes (e.g., feruloyl esterase), α-arabinofuranosidase, CE8 pectin esterase and α-glucuronidase derived from various fungi (e.g., *Aspergillus*), bacteria (e.g., *Clostridium*, *Streptomyces*, *Bacillus*, *Geobacillus*, and *Dictyoglomus*) (84–86) and metagenome (87) have been shown to enhance hydrolysis when combined with *T. reesei* cellulase. In some cases, the synergistic effects are highly substrate specific, e.g., synergism of GH10 xylanase for hydrolysis of steam pretreated agricultural residues and hardwood and GH5 xyloglucanase for softwood substrates (88).

In addition to Cx factors, several C1 factors also synergize with *T. reesei* cellulase and thus could be important for development of new commercial lignocellulolytic systems. LPMOs from different

TABLE 2. Cellulase mixtures based on *Trichoderma reesei* enzyme.

Core enzyme	Supplemental components	Substrate	Sugar yield	Hydrolysis condition	Reference
Trichoderma reesei cellulase	Commercial enzymes 1. Cellulases from *Trichoderma reesei* 2. β-GLU from *A. niger* 3. EG from *Talaromyces emersonii* Crude enzymes produced from fungal strains 1. *Laetiporus sulphureus* 2. *Pleurotus ostreatus*	Alkaline-sulfite pretreated sugarcane bagasse	87% glucan and 94% xylan conversion from mixture of *T. reseei* cellulase and *L. sulfureus* crude enzyme (5% and 6%, respectively, increase compared to *Trichoderma reesei* enzyme alone)	45°C, pH 5.0, 72 h, 500 FPU/g bagasse enzyme loading	79
Trichoderma reesei cellulase	Crude enzyme from *Aspergillus awamori*	Steam-pretreated sugarcane bagasse	Glucose yield 3.9 g/L (41% cellulose hydrolysis) equivalent to 200% increase compared to (*Trichoderma reesei* enzyme alone)	50°C, pH 4.8, 72 h, 20 FPU/g bagasse enzyme loading	80
Trichoderma reesei cellulase	Crude enzyme from *Humicola insolens*	NH_4OH-treated rice straw	79.8% hydrolysis efficiency was obtained from 75%:25% (v/v) mixture of *Trichoderma reesei* and *H. insolens* (10% higher than *Trichoderma reesei* enzyme alone)	50°C, pH 5.0, 3 h	76
Trichoderma reesei cellulase	Crude enzyme from *Penicillium funiculosum*	Paper and microcrystalline cellulose	Glucose yield from mixture of crude enzymes from *T. reessei* and *P. funiculosum* (ratio 1:1) equivalent to 72, 82, 63, 65 and 50% increase compared to *Trichoderma reesei* enzyme alone on foolscap paper, filter paper, microcrystalline cellulose, newspaper and office paper hydrolysis, respectively	45°C, 3 h	75
Celluclast 1.5 L	1. Commercial α-L-arabinofuranosidase 2. Commercial acetyl xylan esterase 3. Commercial β-GLU C6105 4. Recombinant EG 5. Recombinant xylanase 6. Recombinant expansin	Hydrothermally pretreated sugar cane bagasse	Glucose yield is 24.23 g/L equivalent to 72% conversion of cellulose presence in the biomass (Celluclast 1.5 L alone = 49.11%)	50°C, pH 4.8, 72 h, 10 FPU per gram of dried bagasse enzyme loading	85
Celluclast 1.5 L	1. Crude enzyme from *Aspergillus aculeatus* BCC199 2. Recombinant expansin	Alkaline-pretreated rice straw	Total reducing sugar is 769 mg/g biomass (108% increase compared to *Trichoderma reesei* enzyme alone)	50°C, pH 5.0, 48 h, 2.82 FPU/g biomass enzyme loading	82
Celluclast 1.5 L	1. Recombinant EG (Cel9) (GH9) from bagasse metagenome 2. Recombinant xylanase (Xyn11) (GH11) from bagasse metagenome	Alkaline-pretreated rice straw	Total reducing sugar is 104 mg/g biomass (658% increase compared to *Trichoderma reesei* enzyme alone)	50°C, pH 6.0, 24 h, equivalent to Celluclast dosage of 0.01 FPU/g substrate	87
Celluclast 1.5 L	Crude enzyme from *Aspergillus oryzae* P21C3	Hydrothermally pretreated bagasse	Glucose and xylose conversion yields were 51.2% and 78.1%, respectively (36% increase compared to *Trichoderma reesei* enzyme alone)	50°C, pH 4.8, 24 h, 10 FPU/g dried bagasse enzyme loading	81
Accellerase 1500	1 Endo-1,4-xylanases (GH11) from *P. funiculosum* (XynC 11/CAC15487) 2. Feruloyl esterase (CE1) from *C. thermocellum* (CtFAE/ ATCC27405)	Phosphoric acid pretreated sugarcane bagasse	83.79 % conversion from mixture of Accellerase, XynC11 and CtFAE while 60.01% was obtained by Acellerase alone.	50°C, pH 5.0, 48 h, 7.5 mg/g biomass enzyme loading	84
Accellerase XY	1. Crude enzyme from *C. globosum* BCC5776 2. Novozyme 188	Alkaline-pretreated rice straw	Glucose yield is 256.4 mg/FPU (2.9 folds compared to *C. globosum* enzyme alone)	50°C, pH 5.5, 48 h, 1.49 FPU/g biomass enzyme loading	77
Accellerase 1500	1. Recombinant α-L-arabinofuranosidase (GH62) 2. Recombinant pectin esterase (CE8) 3. Recombinant xylanase (GH10)	Alkaline-pretreated rice straw	Reducing sugar yield 340 mg/g biomass (47.3% increase compared to *Trichoderma reesei* enzyme alone)	50°C, pH 5.0, 48 h, 0.5 mg/g biomass enzyme loading	86
Trichoderma reesei cellulase	GH61 gene from *Thermoascus aurantiacus* incorporated to *Trichoderma reesei* SMA135	Pretreated corn stover	91% of cellulose hydrolyzed from mixture with GH61 (1.9-folds enzyme reduction compare to enzyme without GH 61)	50°C, pH 5.0, 144 h, 4.2 mg/g substrate enzyme loading of enzyme with GH61	89

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Table 2 (*continued*)

Core enzyme	Supplemental components	Substrate	Sugar yield	Hydrolysis condition	Reference
Celluclast 1.5 L	Recombinant AA9 LPMO and GH10 xylanase from *Gloeophyllum trabeum*	Alkaline pretreated wheat straw	Supplementation of LPMO and Xyn to Celluclast increased total reducing sugar production by 54%, with 40% and 57% increases in glucose and xylose recovery	50°C, pH 5.0, 48 h, 1.5 mg/g biomass of Celluclast supplemented with 0.5 mg/g biomass of LPMO and GH10	90
Celluclast 1.5 L	AA9 LPMO from *Chaetomium globosum*	Avicel	1.7-fold synergism with Celluclast in the hydrolysis of Avicel	50°C, pH 5.0, 48 h, 0.9 FPU/g cellulose of Celluclast and 0.9 mg/g cellulose of LPMO	91
Crude cellulase from *T. reesei*	Recombinant swollenin from *T. reesei*	Native cock's-foot grass	1.5-fold boosting of the reducing sugar release	40°C, pH 5.0, 164 h, 2 mg/g substrate supplemented with 0.02 μM of SWO	92
Cellulclast 1.5 L	Novozyme 188 and Swollenin from *Trichoderma harzianum*	Pretreated sugarcane bagasse	Supplementation of swollenin led to 2-folds increase in reducing sugars compared to commercial enzyme	18 h	93
Celluclast 1.5 L	Recombinant expansins BpEX from *Bacillus pumilus* CmEX from *Clavibacter michiganensis*	1. Avicel PH101 2. Phosphoric acid-swollen cellulose 3. Arabinoxylan	BpEX and CmEX showed 2.5- and 1.9-folds enhancement on hydrolysis of Avicel. The greatest enhancement was achieved on arabinoxylan with 11.4- and 12.2-fold greater reducing sugar yield than the reaction with Celluclast alone.	50°C, pH 5.0, 48 h, 0.06 FPU/g substrate of Celluclase and 100 μg/g substrate of the bacterial expansin	35

organisms synergize with *T. reesei* cellulase, and the first report of LPMO synergy for the AA9 (formerly GH61) proteins TaGH61A from *Thermoascus auranticus* and TtGH61E from *Thielavia terrestris* showed 1.1–1.3 fold synergistic activities at industrially high conversion yield (89). The enhancing effect was also observed by co-expression of recombinant TaGH61A in *T. reesei*, which enabled 90% cellulose conversion for saccharification of acid-pretreated corn stover with half the enzyme dosage. Synergy of *T. reesei* enzyme with LPMOs derived from different microbial sources, e.g., *Gloeophyllum trabeum* and *C. globosum* was demonstrated to enhance saccharification efficiency of model cellulosic substrates and agricultural wastes (90,91). Fungal swollenin (92,93), plant expansin (94), and bacterial expansin (35) proteins enhance the hydrolysis of model cellulosic and hemicellulosic substrates and native biomass; however, these effects have only been shown under certain conditions different from those relevant to biorefinery such as low cellulase dosage. The degrees of synergism of expansins depend on the crystallinity state and chemical structure of the substrates as well as intrinsic properties of the expansins of different origins.

Single origin enzyme with synergistic partners: *Talaromyces cellulolyticus* system *T. cellulolyticus* (formerly known as *Acremonium cellulolyticus* (95)) is another promising fungus for cellulase production for lignocellulose hydrolysis. The strain was firstly isolated in 1982 from soil in Japan (96) and had been continually modified to increase its total cellulase activity by random mutagenesis to obtain the cellulase hyper-producing strains TN and C-1 (97). The most efficient hyper-producing strain CF-2612 was isolated with a high protein secretion level of 18 g/L (97). The cellulase mixture produced by this fungus is commercially produced by Meiji Seika Pharma Co. (Tokyo, Japan) with the name *Acremonium* cellulase.

T. cellulolyticus secretes a complex cellulase and hemicellulase system with higher β-GLU activity than that of *T. reesei*. The cellulase derived from *T. cellulolyticus* is superior to *T. reesei* for hydrolysis of various lignocellulosic materials including eucalyptus, Douglas fir and rice straw (98) with an additional advantage of strong mannan-degrading activity applicable on hydrolysis of mannan-rich substrate (98). The *T. cellulolyticus* strain Y-94 genome of size 36.4 Mbp contains a number of genes encoding various enzymes including cellulases, hemicellulases, pectinases, and amylases that cooperatively degrade lignocellulosic biomass (99). At least 249 ORFs are annotated as GH family proteins, including 133 potentially secreted proteins based on the presence of signaling sequences. The number of GH family genes of *T. cellulolyticus* is greater than that of *T. reesei* (67). According to the CAZy database, 22 cellulases (12 GH5s [including hemicellulases such as mannanase], 1 GH6, 2 GH7s, 4 GH12s, 1 GH61, and 2 GH45s), 37 hemicellulases (22 GH43s, 1 GH10, 7 GH11s, 1 GH74, 1 GH62, 1 GH53, 1 GH54, 2 GH67s, and 1 GH26) and 38 pectinases (16 GH28s, 12 GH78s, 4 PL1s, 2 PL4s, 2 CE8s, and 2 CE12s) are represented in the genome (99). These results support the ability of *T. cellulolyticus* on efficient degradation of various types of biomass (98,100,101).

Development of media and bioprocesses for *T. cellulolyticus* have increased its cellulase productivity and application. Tests on different polysaccharides, disaccharides, monosaccharides and their mixtures as carbon sources revealed that Solka Floc and soluble soybean polysaccharide enhanced production of cellulase by the fungus. Furthermore, lactose induces cellulase synthesis when used as a sole carbon source, and it can synergize with Solka Floc for induction of cellulase (102). Besides lactose, other inexpensive carbon sources can induce cellulase production in *T. cellulolyticus*, including wet milled rice straw (103) and waste milk packaging pretreated with cellulase (104). The crude secreted *T. cellulolyticus* enzyme has been tested with different synergistic partners on various substrates, as shown in Table 3. The synergistic partners include enzymes with complementary activities that are lacking in the *T. cellulolyticus* system, such as β-mannanase and β-mannosidase (105) as well as a commercial hemicellulase preparation Optimash BG and crude enzyme from *Trichoderma asperellum* (106). The *T. cellulolyticus* enzyme system can be applied for bioethanol production, as shown by simultaneous saccharification and fermentation of paper sludge organic materials (107) and co-culture with *Saccharomyces cerevisiae* in a one-pot bioethanol production (108).

The cellulolytic capability of *T. cellulolyticus* has the potential to be developed further owing to the variety of genetic tools available for this organism. Genetic modification of *T. cellulolyticus* is facilitated by the use of an uracil auxotrophic strain, in which genes can

TABLE 3. Cellulase mixtures based on *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) enzyme.

Core enzyme	Supplemented components	Substrate	Sugar yield	Hydrolysis condition	Reference
Crude enzyme from *Talaromyces cellulolyticus*	–	Hot-compressed water pretreated rice straw	Glucose, xylose, and arabinose yields were 70%, 35%, and 50%, respectively.	45°C, pH 5.0, 72 h, 20 mg/g substrate of enzyme loading	103
Crude enzyme from *Talaromyces cellulolyticus*	–	Ball-milling pretreated eucalyptus, Douglas fir, and rice straw	Glucose yield obtained from eucalyptus, Douglas fir and rice straw hydrolysis are 65%, 62% and 65%, respectively.	50°C, pH 5.0, 72 h, 90 mg/g substrate of enzyme loading	98
Crude enzyme from *Talaromyces cellulolyticus*	1. Commercial enzyme GM5 from *Aspergillus niger* 2. Purified β-mannanase and β-mannosidase from GM5 3. Crude enzyme from *Talaromyces cellulolyticus* cultured with mannan.	Ball-milling pretreated Douglas fir	Mannose yield (4.5%) from *Talaromyces cellulolyticus* crude enzyme alone were improved to 79.7%, 73.5%, and 56.8% by adding component 1, 2, and 3, respectively.	45°C, pH 5.0, 72 h, 13.2 mg/g substrate of *Talaromyces cellulolyticus* enzyme and 1 U/g substrate β-mannosidase loading	105
Crude enzyme from *Talaromyces cellulolyticus*	1. Commercial enzyme Optimash BG from *Trichoderma reesei* 2. Crude enzymes from *Trichoderma asperellum*	Ball-milling pretreated rice straw	Supplementation of component 1 (1.69) and 2 (0.48 mg/g-substrate) led to 4-folds decrease in the protein loading of *Talaromyces cellulolyticus* crude enzyme with 67% theoretical xylose yields	45°C, pH 5.0, 72 h, 8.93 mg/g substrate of *Talaromyces cellulolyticus* enzyme loading	106

be modified using a uracil prototrophy marker and a marker recycling system (109,110). Transgene expression can be regulated via a starch-inducible homologous promoter (111). Improvement of the cellulolytic capability of *T. cellulolyticus* can be achieved by mutation of endogenous genes, or expression of transgenes with enzymatic functions that are absent in the wild-type fungus. Examples of mutating endogenous genes include a xylanase regulator gene (xlnR), which modulates cellulase activity (112), and disruption of catabolite repressor CreA protein, which enhances enzyme production (113). Reports of expressing transgenes with complementing activities include a recombinant CBHI (Cel7A) and xylanases from *T. cellulolyticus* (111,114,115), and expression of a homologous β-xylosidase under the control of the cellobiohydrolase I promoter, which resulted in conversion of the accumulated xylooligosccharides to xylose (116).

Single origin enzyme with synergistic partners: *P. funiculosum* system *Penicillium* fungi are increasingly recognized as a potential source of cellulase for biorefinery. Several *Penicillium* species produce crude enzymes with a better FPase to β-GLU activity ratio compared with *T. reesei* (117). A few *Penicillium*-derived cellulases are now available as commercial products, e.g., *Penicillium oxalicum* cellulase (117). *P. funiculosum* produces an enzyme system which contains a complicated set of glycosyl hydrolases and auxiliary components relevant to complete hydrolysis of lignocelluloses with a well-balanced amount of the main enzymatic activities (118,119), particularly a higher activity of β-GLU, when compared with commercial *T. reesei* cellulase preparations. Among *P. funiculosum* strains, NCIM1228 is a potent hypercellulolytic strain which saccharifies biomass comparable to *T. reesei* RUT C-30 (120,121). The majority of its secreted proteins (58%) under cellulase inducing conditions constitute CAZymes (120). More than 50 proteins including various cellulolytic, hemicellulolytic and proteolytic enzymes were identified in Rovabiot Excel (Adisseo, Anthony, France), a cellulase-based feed additive prepared from protein secreted by this fungus (122). Among prominent CAZymes secreted by this fungus, GH7 cellobiohydrolase (CBH1) is the major protein in the secretome of strain NCIM1228 (121) in addition to a GH6 CBH and several EG (GH5, 7, and 12) and GH 3 β-GLU similar to *T. reesei* (120).

The biochemical properties of *P. funiculosum* enzymes have been characterized, including the GH11 enzymes (XynC) (123,124) and a GH10 xylanase (XynD) with potential application for xylo-oligosaccharide production (125). A CBH1 cellobiohydrolase from this fungus showed approximately 18-fold higher turnover rate (k_{cat}) and a six-fold higher catalytic efficiency than its homolog in

TABLE 4. Cellulase mixture based on *Penicillium funiculosum*.

Core enzyme	Supplemented components	Substrate	Sugar yield	Hydrolysis condition	Reference
Crude enzyme from *P. funiculosum*	–	Diluted acid and alkaline pretreated sugarcane bagasse	Glucose yield after pre-hydrolysis step is 64 g/L equivalent to 81.9% conversion	Pre-hydrolysis was performed with 100 g of bagasse at 50°C and pH 5.0 for 12 h, total enzyme load of 12.5 FPU/g of total solid	127
P. funiculosum ATCC 11797	Crude enzymes produced from fungal strains 1. *Trichoderma harzianum* IOC 3844 2. *Aspergillus niger* ATCC 1004	Pretreated sugarcane bagasse	Glucose yield was 94.1 g/L corresponding to 64% glucan conversion yield (70.59% increase from *P. funiculosum* enzyme alone)	50°C, pH 5.0, 48 h, 24 mg/g cellulose enzyme loading	126
Crude enzyme from *P. funiculosum*	Crude enzyme from *Trichoderma harzianum*	Diluted acid pretreated sugarcane bagasse	97% hydrolysis was obtained from mixture of crude enzyme from *P. funiculosum* and *Trichoderma harzianum*	50°C, pH 5, 18 h, 18 FPU/g biomass enzyme loading	119

T. reesei (TrCBH1) (121). The cellulolytic enzymes of *P. funiculosum* can also be combined with enzymes from other organisms with complementary activities to make more efficient enzyme cocktails. In one study, experimental mixture design was performed to develop an enzyme mixture with optimal activity from crude enzyme preparations of *P. funiculosum*, *Trichoderma harzianum* and *A. niger* (126). Studies on formulation of *P. funiculosum* enzymes are summarized in Table 4.

P. funiculosum can be cultured in bioreactors for large-scale production of cellulase and is suitable for the simultaneous saccharification and fermentation process using synthetic and agricultural substrates. In one study, a cellulase productivity of approximately 900 IU/L was achieved for Avicelase and FPase activities after 60 h of fermentation using sugarcane bagasse pretreated with acid and alkali as the substrate (127). Optimization of media and conditions for production of cellulase from *P. funiculosum* in a stirred tank bioreactor was also studied using statistical design experimental strategy, resulting in 508 U/L for FPase, 9204 U/L for EG, and 2395 U/L for β-GLU (128).

The dynamic changes in the secretome of a non-model hypercellulolytic *P. funiculosum* in response to several model and composite cellulase inducers was studied (129). The levels of cellulolytic enzyme were shown to be substrate dependent and suggested that the fungus secretes cellulolytic enzymes in waves which initially act to hydrolyze the composite substrates in the culture environment before a second wave of proteins which tend to be more tailored to the specific substrate.

Improvement of *P. funiculosum* by genetic modification has been studied through overexpression of key cellulolytic enzyme components and deregulating the expression of key proteins for enhancing its capability on cellulase production for industrial use (130). However, overexpression of the key rate limiting enzymes CBHI and CBHII in the secretome, previously identified as activities absent in *P. funicosolum* (120), did not lead to the desired results as it disturbed the expression of other accessory enzymes. In contrast, modification of the global regulatory mechanisms of cellulase induction by disruption of the catabolite repressor Mig1 homolog led to alleviation of carbon catabolite repression (CCR) (131). This resulted in prolonged cellulase induction in the production medium resulting in two-fold higher cellulase activity than the parental strain with the maximum secreted protein titer of >14 g/L. The CCR-disrupted strain showed better growth with enhanced performance on carbon source utilization. Therefore, the CC4-disruptant and other genetically modified strains could be used for enhanced production of cellulase.

Minimal enzyme systems based on mono-component enzymes Designing an enzyme system based on combinations of selected mono-component enzymes is considered an efficient approach for customizing a highly effective enzyme mixture for a target cellulosic substrate i.e., a specified cellulosic material pretreated by a specific method. This tailor-made enzyme is made up of the core cellulases (EG, CBH, and β-GLU) and further complemented with selected hemicellulases or auxiliary components. Their stoichiometric ratios are usually optimized using systematic statistical experimental design. By identifying all enzyme activities (major and minor) in mono-component enzymes, it is possible to design combinations of the minimal number of required enzyme activities for optimized biomass processing of different agricultural residues that vary in their physical and chemical properties, and for different pretreatment methods (132). So far, a number of synergistic enzyme mixtures based on isolated enzyme components, either using purified endogenous or recombinant enzymes for hydrolysis of several cellulosic substrates have been reported. This strategy is considered advantageous to crude enzyme-based mixtures as enzyme dosage is potentially lower (101). Moreover, integrated enzyme sets can be expressed in well-established heterologous hosts for consolidated bioprocessing (133).

Minimal enzyme systems previously reported for hydrolysis of different biomass are summarized in Table 5. The earliest reports of minimal systems described mixtures of recombinant cellulases from *H. insolens* optimized for hydrolysis of crystalline cellulose represented by bacterial cellulose ribbons (134). A later study of synergistic enzyme combinations for digesting ammonia fiber expansion-pretreated corn stover reported an optimized mixture of four cellulases together with two hemicellulases obtained by heterologous expression in *Pichia pastoris* (135). *P. pastoris* heterologous expression of an CBH derived from *T. cellulolyticus*, a EG from *T. terrestris* and a β-GLU and an endo-β1,4-xylanase (XYN) from *A. aculeatus* led to a development of a blend optimized for hydrolysis of sugarcane bagasse (136). Heterologous expression in *Escherichia coli* was employed to study CAZymes from the anaerobic fungus *Orpinomyces*, in which minimal systems for corn stover and switchgrass degradation were obtained (137). In addition to recombinant enzymes, endogenous enzymes purified from crude multi-enzyme cocktails have been explored, e.g., highly purified enzymes from the crude multi-enzyme secreted by *Trichoderma viride* (138) and *T. cellulolyticus* (101) were tested in different ratios for optimization of biomass degradation. In both reports, the optimized mixture of purified enzymes showed greater efficiency than the crude multi-enzyme. Minimal enzyme systems composed of commercially available enzymes optimized for different types of biomass can also be developed, e.g., formulation of synergistic enzyme mixtures made from cellulolytic and xylanolytic cocktails of different commercially available enzymes (139). The optimal ratios of each cocktail varied according to the biomass pretreatment method employed. The rationally design enzyme formulations showed comparable if not better efficiency compared with commercial cellulases for hydrolysis of hardwood substrates tested. The development of tailor-made enzyme cocktail was also reported using enzyme components including core cellulases, accessory hemicellulases and a lytic polysaccharide monoxygenase from *Myceliophthora thermophile* where different combinations of core cellulases are needed for hydrolysis of phosphoric acid swollen cellulose and birchwood (140). Different ratios of a cellulolytic core-set (CelMix) and a xylanolytic core-set (XynMix) were required for formulation of the HoloMix enzyme system for hydrolysis of the biomass pretreated by different methods (139).

Monosaccharide yields from enzymatic biomass degradation can be substantially enhanced by the addition of accessory and auxiliary enzymes to minimal enzyme systems. The addition of four different bacterial hemicellulases, an α-arabinofuranosidase and an α-glucuronidase to a blend of fungal cellulases led to markedly increased xylose and glucose yields for saccharification of corn stover pretreated by ammonia fiber expansion compared with a commercial enzyme blend (141). Heterologously-expressed *Sporotrichum thermophile* GH61 (*St*Cel61a), an LPMO AA9 enzyme, synergized with a mixture of mono-component cellulases for hydrolysis of substrates with varying lignin content (142). Accessory enzymes can increase hydrolysis efficiency, even for minimal enzyme systems with ratios of core enzymes optimized for different substrates. Addition of an endo-polygalacturonase (EPG) from *A. aculeatus* was shown to synergize with an optimized (hemi)cellulolytic enzyme mixture on direct saccharification of cassava pulp with no need for the high-temperature gelatinizing step (143).

In addition to optimization of component enzyme ratios in minimal enzyme systems, protein engineering can be employed to improve the biochemical properties of the components.

TABLE 5. Minimal synergistic enzyme systems.

Mixture components	Substrate	Sugar yield	Hydrolysis condition	Reference
Purified enzymes from *Trichoderma viride* mutant strain 1. CBH: Cel7A, Cel6A, Cel6B (GH7A, GH6A, GH6B) 2. EG: Cel7B, Cel12A, Cel61A (GH7B, GH12A, GH61A) 3. β-GLU (GH1)	Steam-exploded corn stover	Glucose yield 15.5 mg/ml	50°C, pH 4.8, 72 h, total enzyme dosage 1.1 mg/ml	138
Purified enzymes from *Talaromyces cellulolyticus* 1. EG (Cel5A) (GH5A) 2. CBH (Cel6A) (GH6A) 3. CBH (Cel7A) (GH7A) 4. Xylanase (Xyn10A) (GH10) 5. β-GLU (Bgl3A) (GH3)	Dilute acid-pretreated corn stover	41.6% of glucan conversion	45°C, pH 5.0, 48 h, 2.55 mg/g glucan enzyme dosage	101
Recombinant enzymes from *Myceliophthora thermophila* 1. EG (GH5) 2. EG (GH7) 3. β-GLU (GH1) 4. Feruloyl-esterase 5. LPMO 6. CBH (CBH6) 7. CBH (CBH7) 8. Mannanase 9. Xylanase	Hydrothermally pretreated wheat straw	Reducing sugar yield 27%	50°C, 48 h, 20 mg/g substrate enzyme loading	140
Recombinant and purified enzymes 1. EG (GH7B) from *Thielavia terrestris* 2. β-GLU (GH1) from *A. aculeatus* 3. Xylanase (GH10) from *A. aculeatus* 4. CBH (GH7A) from *Talaromyces cellulolyticus*	Alkaline catalyzed-steam exploded pretreated sugarcane bagasse	Glucose yield 543 mg/g biomass equivalent to 92.4% glucose recovery.	50°C, pH 5.0, 72 h, 20 enzyme loading	136
Recombinant and purified enzymes 1. EG (GH12) from *A. aculeatus* 2. β-GLU (GH1) from *A. aculeatus* 3. Xylanase (GH10) from *A. aculeatus* 4. Endopolygalacturonase (GH28) from *A. aculreatus* 5. CBH (GH7A) from *Talaromyces cellulolyticus* 6. Commercial raw starch degrading amylase (Stargen)	De-starched cassava pulp and raw cassava pulp	Glucose yield 600 mg/g biomass equivalent to 91.3% glucose recovery.	50°C, pH 5.0, 72 h, 5 mg/g fiber degrading enzyme loading and 0.5 mg/g Stargen	143
Purified commercial enzyme mixtures 1. CelMix - EG (Egl) from *A. niger* - CBH (Cel7A) from *Trichoderma longibrachiatum* - β-GLU (Novozyme 188, Bgl1) from *A. niger* 2. XynMix - Xylanase (Xyn2A) from *Trichoderma viride* - Xylanase (XT6) from *Geobacillus stearothermophilus* - α-Glucuronidase (AguA) from *Geobacillus stearothermophilus* - Xylosidase (SXA) from *Selenomonas ruminantium* 3. Laccase (AbLac) from *Agaricus bisporus*	Steam pretreated Acacia and Poplar wood	Sugar yield = 70–100%	50°C, pH 5.0, 24 h, 27.5 mg protein/g biomass of CelMix and XynMix enzyme loading	139
Purified enzymes from commercial preparations and recombinant enzymes: 1. EG I (GH7B) from Speczyme CP 2. CBH I (GH7A) from Speczyme CP 3. CBH II (GH6A) from Speczyme CP 4. β-GLU (GH3) from Novozyme 188 5. Endo-xylanase (EX, GH11) from Multifect xylanase 6. β-Xylosidase (βX, GH3) from *A. nidulans*	Ammonia fiber expansion (AFEX) treated corn stover	80% glucose and 56% xylose recovery	50°C, pH 5.0, 24 h, 33 mg/g substrate enzyme loading	135

(*continued on next page*)

Table 5 (*continued*)

Mixture components	Substrate	Sugar yield	Hydrolysis condition	Reference
Recombinant enzymes from *P. verruculosum* 1. EG II (N194A) 2. CBH I (N45A) 3. CBH II (N219A)	Aviel and Aspen wood	+	40°C, pH 4.5, 72 h, 10 mg/g substrate enzyme loading	144
Recombinant enzymes from *Orpinomyces* strain C1A Cel6A (GH6) + EG(CH5) + β-xylosidase Bgxg1 (GH39) + xylanase (GH11) supplemented with swollenin (Swo1)	Pretreated corn stover and switchgrass	Total conversion 66.4%–73.8% obtained from AGF4 mixture. Supplementation of Swo1 increased glucan and xylan conversion by up to 6.8% and 5.6%, respectively.	50°C, pH 6.0, 72 h, 0.4 mg/g substrate enzyme loading	137

Dotsenko et al. (144) reported the design of a binary and a ternary enzyme mixture comprising mutant forms of EG II, CBH I and CBH II from *P. verruculosum* with enhanced cellulase activities obtained by engineering of the enzyme's N-glycosylation sites. The optimized mixture of mutant enzymes showed 11–40% greater glucose release compared with the corresponding wild-type mixtures at the same loading on hydrolysis of Avicel and milled aspen wood.

FUTURE PROSPECTS

Development of more efficient cellulose blends for biomass industry will be driven by screening of new cellulolytic enzymes and novel auxiliary components from microbial resources using more advanced high-throughput genomic and relevant multi-omic techniques from unexplored sources, e.g., thermophilic fungi (145) and actinomyces (146). Together with more understanding of ligninolytic enzymes, this will lead to the development of more efficient cellulase producers and an extended pool of enzymes for synergistic formulations comprising components of fungal and bacterial origins. The search on effective enzymes will be greatly complemented by advances in enzyme engineering and heterologous hosts for high-level production of cellulases (147). Enzyme efficiency can be further improved by chemical additives, e.g., surfactants in the cellulase blends (148). In addition, enzymes can be recycled via the incorporation of binding domains and immobilization to matrices (149), and bioreactor designs could be improved by separation of product from the reaction and running at lower substrate loading (150). Altogether, these strategies will reduce the cost of enzyme in the biorefinery biomass conversion process.

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