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REVIEW

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Lignocellulases: a review of emerging and developing enzymes, systems, and practices

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Abstract

The highly acclaimed prospect of renewable lignocellulosic biocommodities as obvious replacement of their fossil-based counterparts is burgeoning within the last few years. However, the use of the abundant lignocellulosic biomass provided by nature to produce value-added products, especially bioethanol, still faces significant challenges. One of the crucial challenging factors is in association with the expression levels, stability, and cost-effectiveness of the cellulose-degrading enzymes (cellulases). Interestingly, several recommendable endeavors in the bid to curb these challenges are in pursuance. However, the existing body of literature has not well provided the updated roadmap of the advancement and key players spearheading the current success. Moreover, the description of enzyme systems and emerging paradigms with high prospects, for example, the cell-surface display system has been ill-captured in the literature. This review focuses on the lignocellulosic biocommodity pathway, with emphasis on cellulase and hemicellulase systems. The paradigm shift towards cell-surface display system and its emerging recommendable developments have also been discussed. The attempts in supplementing cellulase with other enzymes, accessory proteins, and chemical additives have also been discussed. Moreover, some of the prominent and influential discoveries in the cellulase fraternity have been discussed.

Keywords: Cellulases, Lytic polysaccharide mono-oxygenases, Cellulase systems, Cell-surface display systems, Autodisplay systems, Cellulosomes

Background

The demand for cellulosic biocommodities as an alternative to fossil-based chemicals has surged within the last few decades. This burgeoning exploration could partly be attributed to the prevailing economic and environmental concerns of fossil-based chemicals. Lignocellulosic biomass is one of the abundant, low-cost, and renewable/sustainable feedstock for the production of biochemicals (including biofuels) due to its rich cellulose content (Roedl 2010; Doherty et al. 2011; Gallezot 2012). Unfortunately, the production of cellulosic biocommodities has been technically challenging owing to the recalcitrance of lignocellulose, which comprises hemicellulose (20–30%), cellulose (30–40%), and lignin (20–30%) (Chang et al. 2011; Park et al. 2011). This recalcitrance has been identified as a major hindrance toward lignocellulose depolymerization. Technically, the resistance to enzymatic hydrolysis is ascribed to morphological and physicochemical factors such as lignin content (Hendriks and Zeeman 2009), degree of crystallinity (Park et al. 2010), degree of polymerization (Kim et al. 2015b), hemicellulose sheathing (Mosier et al. 2005), accessibility of inner microfibrils and porosity (Sharrock 1988), and moisture content and particle size of substrate (Chandra et al. 2007).

Also, the enzymatic hydrolysis of the cellulose and hemicellulose content of lignocellulosic biomass to their constituent monomeric sugars capable of use in the production of biocommodities (e.g., bioethanol and other

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value-added biochemicals) has been hindered in so many ways. The hydrolysis mostly requires multiple enzymes with different specificities to deconstruct the complex lignocellulosic structure (Boyce and Walsh 2015). Specifically, a synergetic action of lignocellulases—cellulases, hemicellulases, lignases (ligninolytic enzymes) and, most recently, lytic polysaccharide mono-oxygenases (LPMO)—is required for an effective deconstruction activity. Remarkably, many efforts toward finding sustainable means of producing significant quantities of cellulosic biochemicals are in pursuance.

Consequently, various reviews focusing on lignocellulose-degrading enzymes, structure, and mode of actions have been remarkably reported (Rabinovich et al. 2002; Haki 2003; Ulrich et al. 2008; Wilson 2009; Juturu and Wu 2014; Bornscheuer et al. 2014). There are also reviews on cellulase engineering and other in vitro strategies towards improving the functionality of cellulases (Bayer et al. 2008; Himmel et al. 2010; Schoffelen and van Hest 2012). However, the fraternity still faces challenges in terms of robustness, hydrolysis efficiency, and cost of these crucial enzymes. Some exemplary accounts on cellulase improvement strategies have been reported (van den Burg 2003; Percival Zhang et al. 2006; Beckham et al. 2012; Elleuche et al. 2014). Nevertheless, these pronounced reviews individually could not provide an updated framework of the advancements and key players spearheading the current success. Moreover, the paradigmatic shift from cell-free systems to robust surface display systems has been ill-captured in the literature. Thus, the recommendable achievements have been uncoupled with the roadmap of cellulose-degrading enzymes.

This review provides an overview of lignocellulases and discusses the roadmap of enzymes and enzyme systems in ensuring that high levels of reduced sugars are obtained from the lignocellulosic biomasses for industrial use. The attempts in supplementing cellulase with other enzymes, accessory proteins, and chemical additives have also been discussed. Herein, the sterling progress in the surface display of enzymes has been emphasized. Moreover, some of the prominent and influential discoveries in the cellulase fraternity have been discussed.

Cellulases and their functional properties

Cellulases are glycoside hydrolases (GHs) that decompose cellulose—a hydrophilic, water-insoluble polymer composed of repeated units of D-glucose interlinked by β-1,4-glycosidic bonds—into shorter chain polysaccharides such as cellodextrin, cellobiose, and glucose. They commonly have a catalytic domain (CD) that cleaves the glycosidic bond; carbohydrate-binding module (CBM) that targets the CD to the polysaccharide substrate; and, in many cases, additional types of ancillary modules such as FN3-like modules (Moraïs et al. 2012; Garvey et al. 2013). Cellulases are distinctly categorized into three (i.e., endoglucanases, exoglucanases or cellobiohydrolases, and β-glucosidases or cellobiases) as per their structure and function, but work collaboratively to enforce the hydrolysis of the complex cellulose microfibrils of the plant cell wall.

The endo- and exoglucanases functionally perform the same task—the hydrolysis of glycosidic bonds—but they differ structurally in terms of the site (loop) for cellulose binding (Juturu and Wu 2014). For instance, endoglucanases (E.C.3.2.1.4) are characterized by short loops, defining open active site clefts that can bind to any accessible site (especially the amorphous sites) along cellulose chains to yield long-chain oligomers (Juturu and Wu 2014; Wilson 2015). They exhibit rapid dissociation compared with other cellulases, and their action on cellulose has been identified as the enzyme activity with greatest liquefaction ability that results in a decrease in the chain length and viscosity (Boyce and Walsh 2015).

For exoglucanases, they have long loops and affinity for the crystalline sites along cellulose chains and yield primarily cellodextrin (Segato et al. 2014). Most often, the loops form a tunnel around the catalytic residues; therefore, substrates usually are directed from the end of the tunnel to encounter the active site of the enzyme (Juturu and Wu 2014). Exoglucanases are in two forms—the reducing end (E.C.3.2.1.176) and non-reducing end (E.C. 3.2.1.91) cellobiohydrolases—but act uni-directionally on the long-chain oligomers (Juturu and Wu 2014). These classifications are based on the portion of the oligosaccharide chain each enzyme favorably attacks; however, they work "processively" to ensure the breakdown of the polysaccharide. For example, *Trichoderma reesei* cellobiohydrolases (Cel7A and Cel6A) progressively hydrolyze cellodextrin from the reducing and non-reducing chain ends, respectively (Wahlström et al. 2014). On the other hand, β-glucosidases possess a rigid structure with active site residing in a large cavity, called the active site pocket, which favors the entry of disaccharides (Nam et al. 2010); even though β-glucosidases are also capable of hydrolyzing soluble cellodextrins with degree of polymerization ≤6 (González-Candelas et al. 1989; Zhang and Lynd 2004). The active site pocket is encased in four hydrophobic loops with different conformations to enhance substrate binding (Czjzek et al. 2000; Nam et al. 2010). Like exoglucanase, β-glucosidases are classified into two sub-families, namely: sub-family A and sub-family B. Sub-family A includes plant and non-rumen prokaryotic cellobiases, and sub-family B includes fungal cellobiases (e.g., *Trichoderma reesei, Aspergillus niger*, and *A. aculeatus*) and rumen bacteria cellobiases, for example, from the anaerobic bovine symbiotic *Butyrivibrio fibrisolvens* (Park et al. 2011).

The complementary functions of these cellulases are crucial for efficient cellulose deconstruction. The classical hydrolysis theory explains that endoglucanases catalyze random deconstruction of cellulose chains along the amorphous regions through the cycles of adsorption and desorption, producing mainly cellodextrin; cellobiohydrolases processively hydrolyze the crystalline cellulose regions either from the reducing or non-reducing end, liberating cellobiose as their main product; and β-glucosidases finally hydrolyze the released soluble cello-oligomers to glucose (Wahlström et al. 2014). The cascading depolymerization activity is governed by (1) synergism, (2) processivity, and (3) substrate-channeling ability of the enzyme, and the catalytic mechanism (Fig. 1) follows the classical acid-catalyst hydrolysis model (Garvey et al. 2013). Two critical amino acid residues—one as a proton donor and the other as a nucleophile—facilitate the enzymatic cleavage of glycosidic bonds by the stereochemical modification (i.e., retention or inversion) of the anomeric carbon configuration (Koshland 1953; Garvey et al. 2013). It is worth noting that the products of both endo- and exoglucanases can inhibit the respective enzyme in a process, called feedback inhibition. For this reason, exoglucanases and β-glucosidases are essentially required to relieve endo- and exoglucanases, respectively, from feedback inhibition. Similarly,

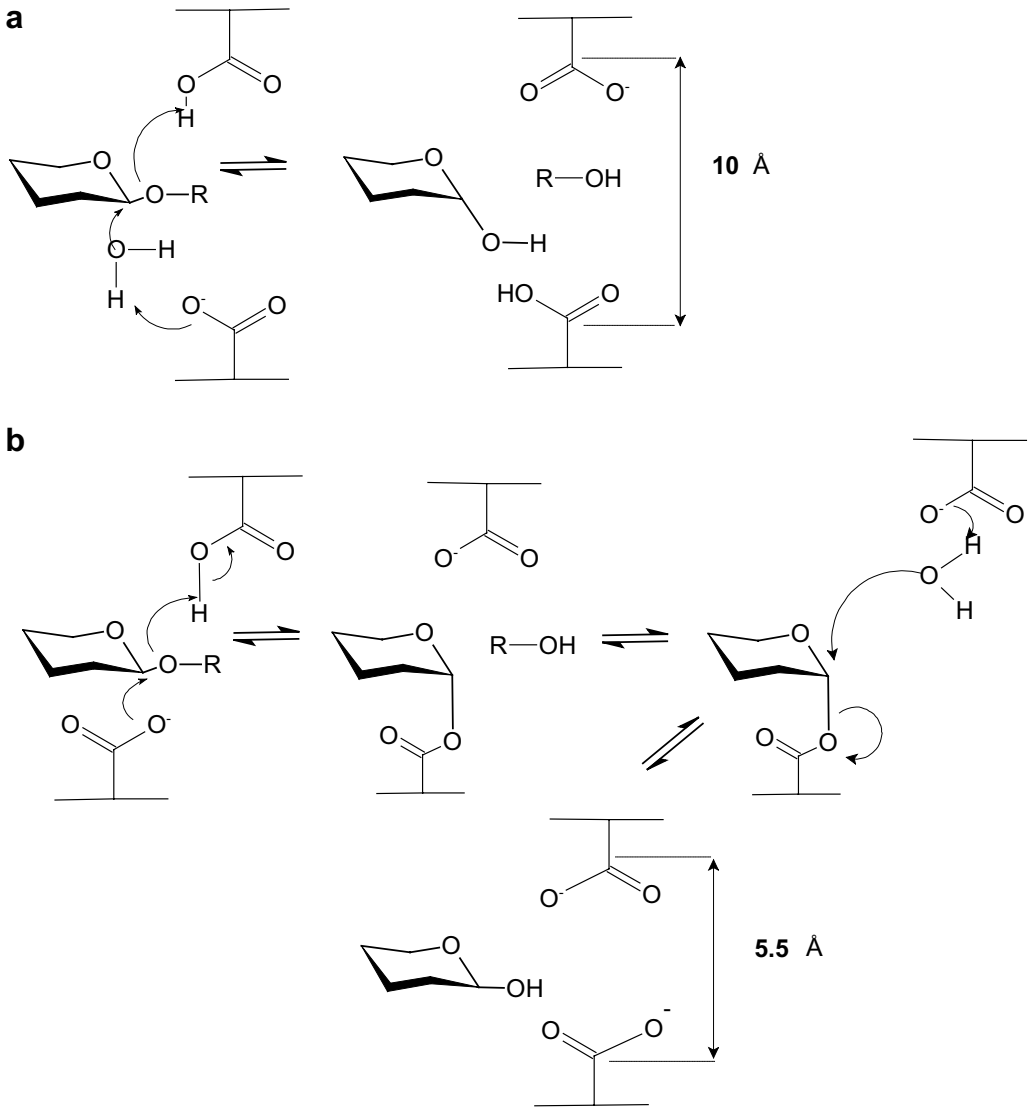

Fig. 1 The two major catalytic mechanisms of GHs, namely: the inversion (**a**) and retention (**b**) mechanisms. These two mechanisms lead to the effective hydrolysis of cellulosic substrates. The reader is referred to Zechel and Withers (2000) and Koshland (1953) for a detailed review

β-glucosidases also face glucose inhibition and, thus, the search for glucose-tolerant β-glucosidases is developing.

Recent insights have revealed oxidative enzymes, lytic polysaccharide mono-oxygenases (LPMOs), as key players in biomass decomposition. According to reports, LPMOs complement the functionality of the canonical cellulases by improving substrate accessibility and introducing chain breaks in the cellulose strand by oxidative means (Vaaje-Kolstad et al. 2010; Horn et al. 2012). The emergence of these auxiliary enzymes has critically disputed the classical concept of carbohydrate polymer saccharification and, thus, has provided additional insight into how saprophytes effectively attack cellulosic substrate (Hemsworth et al. 2013a). LPMOs have been further discussed in "Lytic polysaccharide mono-oxygenases (LPMOs)." Figure 2 describes the contemporary understanding of cellulose degradation.

Common and developing sources of cellulases

Cellulases have been commonly sourced from different organism, mainly fungi, bacteria, and protozoans, although plant and animal cellulases are known (Kim and Kim 2012). Among the organisms, fungi and bacteria express functionally diverse multiple isoforms of cell wall degrading enzymes as a result of genetic redundancy, differential mRNA processing, or post-translational modification (Badhan et al. 2007). Therefore, fungi and bacteria have become the focus of the recent cellulase industry. Table 1 displays some cellulolytic fungi and bacteria with their sources.

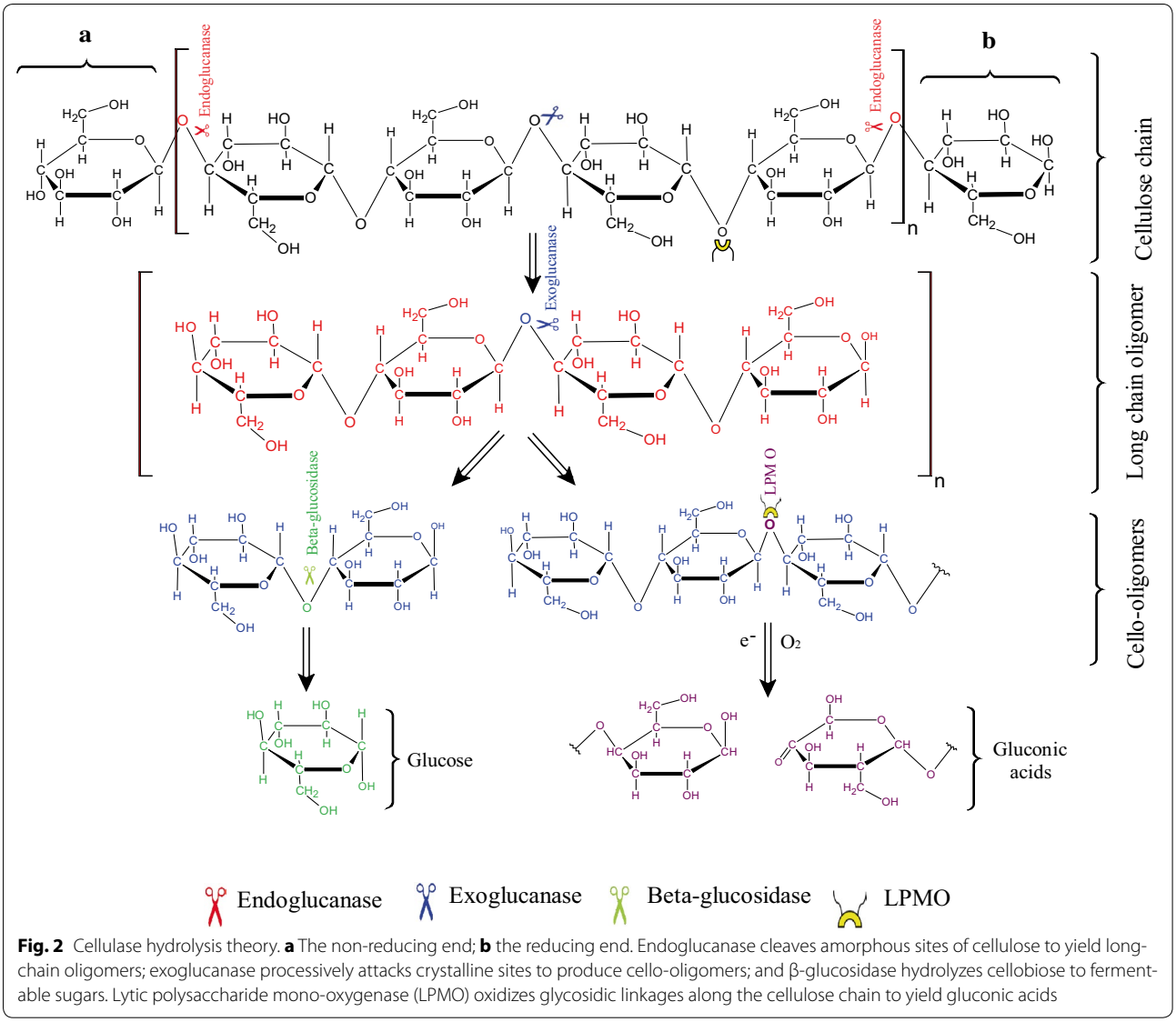

Fig. 2 Cellulase hydrolysis theory. **a** The non-reducing end; **b** the reducing end. Endoglucanase cleaves amorphous sites of cellulose to yield long-chain oligomers; exoglucanase processively attacks crystalline sites to produce cello-oligomers; and β-glucosidase hydrolyzes cellobiose to fermentable sugars. Lytic polysaccharide mono-oxygenase (LPMO) oxidizes glycosidic linkages along the cellulose chain to yield gluconic acids

Table 1 Some cellulolytic microbes and their sources. Modified from: Himmel et al. (2010)

Bacteria		Fungi	
Species	**Source**	**Species**	**Source**
Aerobes (free, non-complexed cellulases)			
Mesophilic bacteria		Mesophilic fungi	
Bacillus brevis[a]	Termite gut	*Aspergillus nidulans, A. niger, A. oryzae*	Soil, wood rot
B. thuringiensis[a]	Caterpillar gut		
Bacillus cereus[a], *B. subtilis*[a]	Soil, rumen	*Agaricus bisporus*	Compost
Cellulomonas fimi[a]	Soil	*Coprinus truncorum*	Soil, compost
Cellvibrio japonicas	Soil	*Geotrichum candidum*	Soil, compost
Cytophaga hutchinsonii	Soil, compost	*Penicillium chrysogenum*	Soil, wood rot
Paenibacillus polymyxa	Compost	*Phanerochaete chrysosporium*	Compost
Pseudomonas fluorescens	Soil, sludge	*Rhizopus oryzae*	Soil, dead organic matter
Pseudomonas putida	Soil, sludge	*Trichocladium canadense*	Soil
Saccharophagus degradans	Rotting marsh grass	*Trichoderma reesei*	Soil, rotting canvas
Sorangium cellulosum	Soil	*Trichoderma longibrachiatum*	Soil
Thermophilic bacteria		Thermophilic fungi	
Acidothermus cellulolyticus	Hot spring	*Chaetomium thermophilum*	Soil
Thermobifida fusca	Compost	*Corynascus thermophilus*	Mush compost
		Paecilomyces thermophile	Soil, compost
		Thielavia terrestris	Soil
Anaerobes (complexed or free, non-complexed cellulases)			
Mesophilic bacteria		Mesophilic fungi	
Acetivibrio cellulolyticus	Sewage	*Neocallimastix patriciarum*	Rumen
Bacteroides cellulosolvens	Sewage	*Orpinomyces joyonii*	Rumen
Clostridium cellulolyticum	Compost	*Orpinomyces PC-2*	Rumen
Clostridium cellulovorans	Wood fermenter	*Piromyces equi*	Rumen
Clostridium josui	Compost	*Piromyces E2*	Feces
Clostridium papyrosolvens	Mud (freshwater)		
Clostridium phytofermentans	Soil		
Fibrobacter succinogenes	Rumen		
Prevotella ruminicola	Rumen		
Ruminococcus albus	Rumen		
Ruminococcus flavefaciens	Rumen		
Thermophilic bacteria			
Anaerocellum thermophilum	Hot spring		
Caldicellulosiruptor Saccharolyticus	Hot spring		
Clostridium thermocellum	Sewage, soil, manure		
Clostridium stercorarium	Compost		
Thermotoga maritima	Mud (marine)		
Rhodothermus marinus	Hot spring		

[a] Most *Cellulomonas* and *Bacillus* strains are facultative anaerobes that can also grow anaerobically

Fungi sources

Currently, fungi are the most studied group of cellulose-degrading microorganisms, owing to their high protein secretion capabilities and multi-component, synergetic, cellulolytic, enzyme activity (Ulrich et al. 2008; Juturu and Wu 2014). The most extensively studied cellulolytic enzymes are *T. reesei* cellulases because of their application in commercial cellulase preparations (Wahlström et al. 2014). The cellulase mixtures of *T. reesei* (the 'gold standard') consist predominantly of exoglucanases, which contribute up to 80% of the total protein; endoglucanases (up to 15% of the total protein); and lesser amounts of enzymes with other hydrolytic activities (Garvey et al. 2013). According to Parisutham et al.

(2014), *T. reesei* also possesses intracellular β-glucosidase to avoid effects of cellobiose feedback inhibition during cellulose hydrolysis. However, the levels of β-glucosidases are mostly low and, thus, require supplementation from other sources such as Aspergilli.

The emergence of filamentous fungi of the genus *Aspergillus* as one of the key cellulase-producing organisms has made an outstanding impact in bioprocessing. For example, *Aspergillus oryzae* (Chandel et al. 2011; Begum and Alimon 2011), *A. unguis* (Rajasree et al. 2013), *A. tubingensis* (Decker et al. 2001), *A. fumigatus* (Watanabe et al. 1992; Anthony et al. 2003; Soni et al. 2010; Sherief et al. 2010; Liu et al. 2011; Das et al. 2013), and the most pronounced *A. niger* (Kang et al. 2004; Hanif et al. 2004; Varzakas et al. 2006; Sohail et al. 2009; Sakthi et al. 2011; Bansal et al. 2012; Oberoi et al. 2014) have been studied for their cellulolytic benefits. The *Aspergillus* species produce different isoforms of enzymes such as cellulases, xylanases, laccases, and other accessory proteins necessary for biomass depolymerization. The multiplicity is due to the presence of diverse protein encoding genes, differential glycosylation of common polypeptide chains, and post-translational modification disparities (Willick and Seligy 1985; Decker et al. 2001). Moreover, physical and nutritional factors may also account for reported differences in enzyme expression and expression levels. Enzymes from Aspergilli are mostly reported to exhibit low total cellulase activity (Falkoski et al. 2013); however, their high β-glucosidase expressing levels have made them relevant game changers for industrial applications. One remarkable property of the species in the genus is their tolerance against osmotic gradients. For example, the high glucose-tolerance of β-glucosidases from *Aspergillus* sp. has been reported (Riou et al. 1998; Günata and Vallier 1999; Rajasree et al. 2013; Das et al. 2015), and this revelation has been vital in the roadmap to the 'green' future.

Yeast has also had its use in cellulolytic investigations. Foreseeably, yeast found its application as a common expression platform for enzyme systems because of its robustness. Interestingly, a recombinant yeast has been able to express three copies each of endoglucanase and exoglucanase, and one copy of β-glucosidase for cellulose depolymerization (Matano et al. 2013; Parisutham et al. 2014). According to Juturu and Wu (2014), yeast provides numerous advantages when used as a host for recombinant protein expression. The benefits include: (1) the ability to perform eukaryotic post-translational modifications; (2) the ability to secrete recombinant proteins; (3) the ability to grow to very high cell densities; (4) the wide availability of yeast strains for recombinant protein expression; and (5) the relatively toxin-free nature of yeast cells in comparison with endotoxin-associated bacterial strains, whose products may require purification (if ingestible or injectable). The unending stream of science has more to uncover regarding fungal cellulases, owing to their capabilities of producing copious amounts of enzymes.

Bacteria sources

Although much of the cellulases used for lignocellulosic biomass hydrolysis are derived from fungi, yet the isolation and characterization of novel carbohydrate-degrading enzymes from bacteria are now widely exploited. This is because of the efficient heterologous production, high specific activity, and less stringent pH requirement of bacterial systems. The most effective natural cellulolytic system known is produced by bacteria (Stern et al. 2015). Well-known genera for bacteria-based cellulolytic enzymes are mostly *Bacillus, Cellulomonas, Streptomyces, Cytophaga, Cellvibrio*, and *Pseudomonas*. Although many types of proteins have been produced by *Escherichia coli*, there is no report on natural cellulolytic *E. coli* in the past several years (Yamada et al. 2013). However, through metabolic engineering *E. coli* are made tractable such that they can be endowed with an efficient cellulolytic system capable of producing high-value compounds from lignocellulosic biomass.

In bacteria, cellulases are mostly present as extracellular aggregated structures attached to the cells (Juturu and Wu 2014). However, the expression of highly active cellulases of fungal origin in bacterial expression platforms has been a persisting challenge, with many resulting in diverse expression inefficiencies (Garvey et al. 2013). *E. coli* remains the most commonly used system for recombinant cellulase protein production, particularly for the expression and characterization of novel cellulolytic proteins, including those from extreme habitats or animal guts (Garvey et al. 2013). The high protein secretion capacity of *Bacillus subtilis*, with its high-activity endoglucanase, has also been used to engineer recombinant cellulase strains that thrive on cellulose as a sole carbon source without any other organic nutrient (Zhang 2011).

Remarkably, the future of cellulolytic enzyme sources is gradually shifting toward bacterial sources. The discovery of the exceptional cellulolytic properties of bacteria from the genera *Clostridium* and *Thermotoga* has contributed to the gradual shift from the dominant fungi sources to that of bacteria. The nature of cellulases from these species are thermostable and optimally active at elevated temperatures between 60 and 125 °C (Vieille and Zeikus 2001; Schiraldi and De Rosa 2002; Haki 2003); thus, making them essential candidates for improving the techno-economics of biomass saccharification (Parisutham et al. 2014). Notably, running enzymatic hydrolysis at higher temperatures has the penchant to (1) promote biomass

disorganization; (2) increase substrate solubility; (3) improve rheological properties (e.g., viscosity); and (4) reduce the risk of microbial contamination (Vieille and Zeikus 2001; Boyce and Walsh 2015). Bacteria from the genera *Clostridium* and *Thermotoga* also produce self-assembled scaffolded multimodular enzyme systems, termed cellulosomes, to efficiently hydrolyze the complex and rigid structure of cellulose (Brunecky et al. 2012). The extreme stabilities (e.g., pH and thermal) and multifunctional nature of enzymes produced by these cellulosome-expressing bacteria have revolved the attention of scientist on to understanding the structure and function of their genetic makeup in order to mimic the innate abilities. On account of the obvious benefits reported in the literature, scientists have consistently investigated the gene (Yagüe et al. 1990; Zverlov et al. 2003; Koeck et al. 2013), fusion/modification of enzymes (Ciolacu et al. 2010; Lee et al. 2010; Ye et al. 2010; Lee et al. 2011; Nakashima et al. 2014), and the optimal growth (Islam et al. 2013; Reed et al. 2014) of these useful microbes to harness their inherent benefits. For example, the biochemical and biophysical characteristics of multimodular enzymes from *Clostridium thermocellum* (Zverlov et al. 2005; Tachaapaikoon et al. 2012; Brunecky et al. 2012; Hirano et al. 2013; Yuan et al. 2015) and *Thermotoga maritima* (Chhabra et al. 2002; Carvalho et al. 2004; Pereira et al. 2010; Wu et al. 2011) have been reported.

Most currently, the main interest of the biobased industries has been on the application of extremozymes (Demirjian et al. 2001; Egorova and Antranikian 2005). These enzymes derived from extremophilic microorganisms (acidophiles, alkaliphiles, halophiles, thermophiles, psychrophiles, and piezophiles) are rich sources of natural tailored enzymes, which are functionally more superior over their mesophilic counterparts for applications at extreme/harsh conditions that were long thought to be destructive to proteins (van den Burg 2003; Elleuche et al. 2014). Extremozymes are capable of catalyzing their respective reactions in non-aqueous environments, water/solvent mixtures, at extremely high pressures, acidic and alkaline pH, at temperatures up to 140 °C, or near the freezing point of water (Schiraldi and De Rosa 2002; Elleuche et al. 2014). The outstanding prospects of these enzymes have created a surge in their investigation for use in biotechnological and industrial applications. In conformity with industrial demands, the cellulolytic prospects of the anaerobic extremophile, *Caldicellulosiruptor bescii* (formerly *Anaerocellum thermophilum*, isolated from a geothermally heated pool), have been exemplified in literature (Yang et al. 2009; Kanafusa-Shinkai et al. 2013). The *C. bescii* and some of its relatives in the same genus secrete free (non-cellulosomal) biomass-degrading enzymes rich in CBMs (specifically CBM3 family) that target the enzymes to crystalline cellulose, but show high degree of multi-modularity (Harris et al. 2014). This Gram-positive, non-spore-forming, neutrophilic, cellulolytic/hemicellulolytic bacterium grows in a temperature range of 40–90 °C, with an optimum temperature of 72–80 °C, and efficiently degrades crystalline cellulose, xylan, and non-pretreated plant biomass such as Napier grass, switch grass, and hardwood poplar (Yang et al. 2009; Kanafusa-Shinkai et al. 2013). For example, the *C. bescii* CelA (comprising a GH family 9 and a family 48 CD, as well as three type-III CBMs) and its fragments can depolymerize lignocellulosic biomass to glucose, cellobiose, and xylose via a combined surface ablation and cavity-forming mechanism without the help of accessory proteins (Brunecky et al. 2012). These abilities of *C. bescii* make it a potential candidate for thorough investigation and implementation. Table 2 shows a list of some industrially relevant thermostable cellulases that have been isolated and characterized.

Developing practices for improving the production and performance of cellulases

Some cellulase improvement techniques

Recently, there have been several attempts to acquire highly efficient cellulases with improved cellulolytic activity and stability (di Lauro et al. 2006; Mesas et al. 2012; Jagtap et al. 2013). Various improvement methods including rational design and directed evolution in complementation with techniques like DNA family shuffling and error-prone polymerase chain reaction (PCR) have been prominent. For example, Wang et al. (2014) have reported the application of random mutagenesis followed by genome shuffling to improve the cellulase production of *Trichoderma koningii* D-64. Also, structure-based protein design has been successfully used to increase thermal resistance and modify substrate specificity of glucosidases (Lee et al. 2012). The uses of random insertion domain strategies to allosterically modify enzymes have also been reported (Ribeiro et al. 2015). These allosteric enzymes present spatially distinct locations for regulation and catalysis and offer oligomeric states where tertiary and quaternary structural changes are transmitted across protein–protein interfaces to facilitate the communication between effector binding and modulation of catalytic activity (Ribeiro et al. 2015). The random insertion strategy has been relevant for curbing the hindrance of inhibition. However, the very large and costly nature of random insertion libraries, and associated bias towards certain insertion points have been challenging; therefore, the design of smaller high-quality libraries using a semi-rational approach is developing. Convincingly, the application of metagenomic techniques to exploit the functional genes in uncultured natural

Table 2 Some thermostable cellulases of industrial significance

Cellulase	Source/organism	Maximum/optimum activity	Stability ($T_{1/2}$)	References
Cellulase	*Desulfurococcus fermentans* (Hyperthermophilic archaea)	80–82 °C (pH 6)	85 °C, >3 days	(Perevalova 2005)
Endoglucanase (GH5)	Hyperthermophilic archaea	109 °C (pH 6.8)	100 °C, 5 h	(Graham et al. 2011)
Endoglucanase (GH5)	*Dictyoglomus thermophilum*	50–85 °C (pH 5)	70 °C, 336 h	(Shi et al. 2013)
β-Glucosidase (GH1)	*Thermotoga thermarum* DSM 5069T	90 °C (pH 4.8)	90 °C, 2 h	(Zhao et al. 2013)
β-Glucosidase (GH1)	Hydrothermal spring metagenome	90 °C (pH 6.5)	90 °C, >1.5 h	(Schröder et al. 2014)
β-Glucosidase (GH1)	*Alicyclobacillus acidocaldarius*	65 °C (pH 5.5)	65 °C, >3 h	(di Lauro et al. 2006)
β-Glucosidase (GH3)	*Thermofilum pendens*	90 °C (pH 3.5)	90 °C, >2 h	(Li et al. 2013)
β-Glucosidase (GH3)	*Pholiota adiposa* SKU0714	65 °C (pH 5)	65 °C, 23 h	(Jagtap et al. 2013)
β-Glucosidase	*Pyrococcus furiosus*	102–05 °C (pH 5)	100 °C, 85 h	(Kengen et al. 1993)
β-Glucosidase	*Oenococcus oeni* ST81	40 °C (pH 5)	40 °C, 50 days	(Mesas et al. 2012)
β-Glucosidase	*Sphingopyxis alaskensis*	50 °C (pH 5.5)	NA	(Shin and Oh 2014)
CMcellulase	*Bacillus pumilus* S124A	50 °C (pH 6.0)	NA	(Balasubramanian and Simões 2014)
Multi-domain (Hemi)cellulase	*Caldicellulosiruptor bescii*	72–80 °C (pH 5–6)	NA	(Kanafusa-Shinkai et al. 2013)

NA not analyzed

microorganisms could help in overcoming the limit of pure cultivation methods (Chang et al. 2011). Moreover, harnessing glycosylation—a form of post-translational modification—to improve cellulase activity looks promising (Easton 2011; Beckham et al. 2012).

Cellulase supplementations

The supplementation of cellulases with additives (biological and non-biological) for lignocellulose saccharification has been witnessed. This developing practice is based on the understanding that the effective degradation of the complex structure of lignocellulose requires not only cellulases, but also supplementary enzyme blends of ligninases (laccases), hemicellulases, and accessory proteins, depending on the morphological characteristics of the lignocellulosic biomass. Chemical additives have also been used to improve the functionality of cellulases.

Biological additives

Laccases Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are multi-copper oxidases, capable of catalyzing one-electron oxidation of various substrates such as phenolic and non-phenolic subunits of lignin (Lahtinen et al. 2009; Dwivedi et al. 2011; Chandel et al. 2013). Laccases have four copper atoms present in their active sites which are distributed at three different copper centers, namely: Type-1 (blue copper center), Type-2 (normal copper), and Type-3 (coupled binuclear copper centers) (Dwivedi et al. 2011). These copper atoms serve as a catalytic metal and reducing agent for the oxidation of various carbons (C-1, C-4, and C-6) in the polymeric structure (Segato et al. 2014). Ascorbate oxidase, ferroxidases, ceruloplasmin, and bilirubin oxidases are examples of members in the multi-copper protein family.

The primary substrate of laccases is lignin, an amorphous, complex cross-linked polymer consisting of phenylpropane units (Claus 2004; Moilanen et al. 2011). In general, laccases break down lignin into less harmful products, using electron transfer and hydrogen atom transfer mediators. Laccases are widely distributed in plants, fungi, and bacteria and exhibit diverse functions and stability, depending on their source organism and physiology. Molecular structure elucidations and the electrochemical assessment of laccases have resulted in three classifications, namely: high, medium, and low redox potential laccases (Mot and Silaghi-Dumitrescu 2012; Mate and Alcalde 2015). Plant and bacterial laccases belong to the low redox potential category, whereas fungal laccases are categorized as either high or medium redox potential laccases. The magnitude of the redox potential correlates with the substrate range and oxidation capacity of the enzyme (Mate and Alcalde 2015). As a result, fungal laccases exhibit high wood depolymerization activity and are widely distributed in ascomycetes, deuteromycetes, and basidiomycetes; the most efficient species known is the white-rot fungus (Dwivedi et al. 2011; Pandiyan et al. 2014). Bacterial laccases are also active lignin degraders, but with high thermal and pH stability compared with fungal laccases, and hence more compatible with almost all industrial processes when immobilized (Dwivedi et al. 2011). Some examples of bacterial laccase sources are *Azospirillum lipoferum*,

Bacillus subtilis, *Anabaena azollae*, *Streptomyces cyaneus,* and *Streptomyces lavendulae*. Contrary to fungal and bacterial laccases which accelerate lignin degradation and aid in bioremediation, plant laccases typically facilitate the biosynthesis of lignin in the plant cell wall (Lahtinen et al. 2009). Some sources of plant laccases are *Rhus vernicifera*, *Rhus succedanea*, *Populus euramericana*, *Nicotiana tobacco*, and *Zea mays*.

Remarkably, the complex plant cell wall lignin depolymerization property of laccases (fungi and bacteria) has been vital in the deconstruction of residual lignin that may be present after pretreatment. For instance, the presence of lignin oxidases (laccases) in cellulose hydrolysis boosts cellulase activity by liberating cellulases from unproductive binding sites on lignocellulosic substrates to increase the effective concentration of free cellulases in solution (Berlin 2013). Also, laccases could possibly address issues regarding phenolic compound inhibition of cellulases. For example, Hyeon et al. (2014) achieved 2.6-fold increase in the yield of reduced sugar from pretreated barley straw using cellulase–laccase blends. Moilanen et al. (2011) employed blend of commercial cellulases and laccases on pretreated spruce and obtained 12% increase in hydrolysis yield. Furtado et al. (2013) and Ribeiro et al. (2011) have also demonstrated the improvement in synergy and catalytic performance of fused laccases–(hemi)cellulase complex for biomass hydrolysis.

Hemicellulases Hemicellulases commonly share similar activities with cellulases because of the common β-1,4-glycosidic bonds in the backbone of the hemicellulose component of plant biomass (Chang et al. 2011). The hemicellulose substrate is a complex carbohydrate structure consisting of different easy hydrolysable polymers such as pentoses (e.g., xylose and arabinose), hexoses (e.g., mannose, glucose, and galactose), and sugar acids (Hendriks and Zeeman 2009). Pretreated lignocellulosic biomass hydrolysis is strongly affected by the presence of hemicellulose—the most thermo-chemically sensitive among cellulose, hemicellulose, and lignin—which connects lignin to cellulose fibers and gives the whole cellulose–hemicellulose–lignin network more rigidity (Hendriks and Zeeman 2009). For instance, xylans—the dominant component of hemicellulose from hardwood and agricultural plant—and xylooligomers putatively have a direct inhibitory effect on cellulases (Hendriks and Zeeman 2009; Harris et al. 2014); hence, the need for its depolymerization to reduce the burden on cellulases and improve sugar yields.

Hemicellulases are mostly modular proteins possessing CDs, CMBs, and other functional modules to facilitate the cleavage of either glycosidic or esterified acid side groups (Shallom and Shoham 2003; Decker et al. 2008). For instance, α-glucuronidases, α-arabinofuranosidases, α-D-galactosidases, and mannanases attack glycosidic bonds whereas acetyl or feruloyl esterases hydrolyze ester bonds of acetate or ferulic acid side groups in the plant cell wall structure. In most cases, hemicellulases are employed in concert with cellulases in the depolymerization of lignocellulosic biomass to fermentable sugars. Relative to the theoretical sugar content, Gao et al. (2011) reported recommendable quantities of reduced sugars from corn stover pretreated by ammonium fiber expansion (99% glucose and 55% xylose), dilute acid (97% glucose and 68% xylose), and ionic liquid (88% glucose and 53% xylose) using cellulase–hemicellulase cocktail. Since hemicellulose presents a rich source of carbon, its successful hydrolysis improves the yield of fermentable sugars.

Lytic polysaccharide mono-oxygenases (LPMOs) LPMOs are copper-dependent enzymes mostly found in saprophytic fungi (e.g., *Thermoascus aurantiacus, Gloeophyllum trabeum, Lentinus similis, Pichia pastoris, Neurospora crassa*) and bacteria (e.g., *Bacillus amyloliquefaciens, Enterococcus faecalis*) (Quinlan et al. 2011; Phillips et al. 2011; Beeson et al. 2012). They were previously grouped among GHs because of their weak endocellulase activities (Karlsson et al. 2001; Karkehabadi et al. 2008). However, modern understanding of their characteristics has resulted in their reclassification as auxiliary activity (AA) family enzymes. Based on mainly structural differences, bacterial (AA10; formerly CBM33) and fungal (AA9; formerly GH61) LPMOs have been studied and classified. Moreover, a supportive classification based on Peptide Pattern Recognition sequencing has recently been reported (Busk and Lange 2015). Nevertheless, their functional distinctions and associated mechanisms are yet to be fully elucidated to help exploit their maximum benefits. Accordingly, studies focusing on the structure (Harris et al. 2010; Aachmann et al. 2012; Hemsworth et al. 2013b; Borisova et al. 2015; Frandsen et al. 2016) and interactions (Isaksen et al. 2014; Eibinger et al. 2014; Courtade et al. 2016; Kracher et al. 2016) of LPMOs are surfacing.

According to structural discussions, the active sites of LPMOs are held in the center of an extended flat face structure—unlike the tunnel-shaped structures housing the active sites of canonical hydrolases (i.e., endo- and exoglucanase)—for an efficient interaction with substrates such as cellulose (including cello-oligosaccharides) and chitin (Hemsworth et al. 2013a; Isaksen et al. 2014). Technically, the active site is said to possess a monomeric type II copper ion (Cu^{2+}) aligned by an N-methylated N-terminal histidine in a network, termed histidine brace, to help the enzyme interact with substrates (Quinlan et al. 2011; Hemsworth et al. 2013b).

The LPMO substrate catalysis is a consequence of the binding of active oxygen molecule from the atmosphere to the monomeric Cu^{2+}, which culminates in the interaction of the active site with available chains within the polysaccharide matrix (substrate). LPMOs assist in the biomass decomposition process by oxidatively attacking the most accessible and most reactive C–H bonds (i.e., C-1 and C-4) along the cellulose strand using molecular oxygen, an external electron donor and, putatively, CBM (Hemsworth et al. 2013a; Walton and Davies 2016). In other words, the enzymes promote the abstraction of hydrogen atoms and assist in the scission of β-1,4-glycosidic linkages between C-1 and C-4 of the cellulose chain.

The role of LPMOs is dependent on substrate dynamics and process conditions. Practically, the overall saccharification yield increases when LPMOs are combined with the three common cellulases, especially in the processing of dry matter with relevant remnants of lignin (Cannella and Jørgensen 2014). Jung et al. (2015) investigated LPMO from *Gloeophyllum trabeum* in concert with cellulases and xylanase. Though no significant individual LPMO activity was observed, the work reported an accelerated synergistic degradation of pretreated kenaf and oak (Jung et al. 2015). Also, Müller et al. (2015) studied the activity of LPMOs with Celluclast® on lignocellulosic biomass of high dry matter concentration and reported an improved product generation. However, their work revealed the need to reconsider process conditions to favor the oxygen and free electron demands of LPMOs (Müller et al. 2015). Nevertheless, Westereng et al. (2015) showed that the lignin component of lignocellulosic substrates provides a reserve of electrons capable of promoting the activity of LPMOs. The effects of divalent cations on LPMO effectiveness was previously stressed by Harris et al. (2010). Also, Cannella et al. (2012) have unveiled the possible inhibition of β-glucosidase activity by the LPMO products (e.g., cellobionic and gluconic acids).

'Non-hydrolytic' accessory proteins The common non-hydrolytic proteins known are expansins and swollenins. Expansins are phytoproteins capable of loosening the plant cell wall and disrupting the cellulose crystal structure, whereas swollenins are expansin derivatives from fungi (e.g., *T. reesei*, *Aspergillus fumigatus*, etc) and bacteria (e.g., *Bacillus subtilis*). Swollenin also exhibits crystal-disruption activity on cellulosic materials (Nakashima et al. 2014). There are proofs that these non-hydrolytic accessory proteins can enhance cellulase activity through their ability to disrupt hydrogen bonds to reduce cellulose crystallinity while increasing cellulase accessibility to enzymes (Harris et al. 2014).

In response to the known benefits, researchers are investigating the enhancing effects of these non-hydrolytic accessory proteins on cellulose degradation, especially in a reaction mixture. Nakatani et al. (2013) demonstrated, for the first time, the synergetic effect of co-displayed cellulase and expansin-like protein on a *Saccharomyces cerevisiae* cell surface, and they reported 2.9-fold higher degradation activity on phosphoric acid-swollen cellulose (PASC) compared with the activity of cellulase-expressing strain only. Nakashima et al. (2014) also studied fused *Bacillus subtilis* expansin and *Clostridium thermocellum* endoglucanase for the degradation of highly crystalline cellulose and reported about 35% digestibility by the fused proteins. The use of these accessory enzymes in cellulase blends for industrial applications is liable to improve the level of reduced sugar obtainable from lignocellulosic substrates, thus, requires more investigation.

Chemical additives

Chemical additives have been used with cellulases to provide enzymatic process enhancement in the form of metal cofactors or activators. These activators come in the form of metal ions and chelating agents, yielding significant effects on enzymatic activities by assisting in the biochemical transformations. Some of these additives (e.g., surfactants) are effective for lignocellulose depolymerization, in that they putatively prevent enzyme denaturation and inactivation by reducing the unproductive adsorption of enzymes onto the substrate via hydrophobic interactions with lignin (Eriksson et al. 2002). For a quick example, Fontes and Gilbert (2010) explained that calcium is pivotal for dockerin (a facet of most enzyme structures) stability and function, and in the presence of ethylenediaminetetraacetic acid (EDTA, a chelating agent), dockerins are unable to interact with cohesins (another facet of most enzyme structures).

Boyce and Walsh (2015) studied the effect of various additives, such as $CaCl_2$, EDTA, $MgCl_2$, Tween 20, and Triton X-100, on *Alicyclobacillus vulcanalis* endoglucanase activity by adding specified concentration of these additives to the enzyme sample and immediately measuring their influence on the enzyme activity. Relative to the control (enzyme without additives), they reported that $CaCl_2$ (10 mM) and EDTA (2 mM) yielded, respectively, 97 and 98% activities; whereas $MgCl_2$ (10 mM) yielded 86%, but exhibited a slight inhibitory effect on the activity of the endoglucanase. They further reported that the inclusion of 0.1% Tween 20 or 0.5% Triton X-100 in the enzyme solution improved the enzyme thermal stability while enhancing the enzyme activity with 124 and 126%, respectively. They attributed the significant beneficial effect of Tween 20 and Triton X-100 to (1) reduced

unproductive adsorption of enzymes to lignin; (2) changes in the enzyme reaction milieu, and (3) reduced enzyme denaturation as a result of the surfactant binding on enzyme secondary and tertiary structures.

Also, Kim et al. (2015a) analyzed the effects of metal ions and a chelating agent on the activity of xylanase–cellulase fusion protein (Xyl10g GS Cel5B) and reported that the endoglucanase and xylanase activities increased by 39 and 15%, respectively, in the presence of 1 mM $CoCl_2$. They, however, reported a complete inhibition of activity of the fused protein by $HgCl_2$.

Moreover, in an experiment to characterize a β-glycosidase (Aab-gly) from the thermoacidophilic bacterium (*Alicyclobacillus acidocaldarius*), Lauro et al. (2006) reported that divalent cations, namely: Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} (each at 5 mM, 65 °C, and on 2 mM 2NP-β-Glc) had significant activation effect on Aab-gly. However, Zn^{2+} and Co^{2+} inhibited the enzyme by 33 and 96%, respectively. Mesas et al. (2012) examined the effects of chloride salts ($MgCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$, $CoCl_2$, $CaCl_2$, and $CuCl_2$) on the activity of β-glucosidase from *Oenococcus oeni* ST81 and reported that only Mn^{2+} seemed to slightly increase the enzyme activity; whereas Cu^{2+}, Fe^{2+}, Zn^{2+}, and Co^{2+} clearly reduced the catalytic activity of the enzyme from 8 to 54%, depending on the identity and concentration of the metal ion. There are more other interesting additive-effect observations reported in literature (Kengen et al. 1993; Schülein 2000; Li et al. 2013; Zhao et al. 2013; Jagtap et al. 2013; Balasubramanian and Simões 2014).

The major concern of additive experiments has been the ill-explained discrepancies of the data obtained. The discrepancies in enzyme-additive reportage reinforce the phenomenon of enzyme selectivity in the use of cofactors. Interestingly, even cations of the same valency have yielded different results. The discrepancy could be associated with the charge density of the additive and the size of the active site pocket of the enzyme, but this point should be proved experimentally. It is rational to conclude that the effects of these metal cofactors are enzyme and/or organism depended, and hence thorough studies should be focused on this to consolidate existing understanding.

The chronology of cellulolytic GH systems

Lignocellulosic substrates require several enzymatic strategies, even after pretreatment, to ensure significant generation of fermentable sugars and subsequent production of biochemicals. These strategies may be conducted separately or in combination, and they involve the following dominant microbial paradigms: cell-free enzyme systems, multi-enzyme (cellulosome) complexes, and multifunctional enzyme systems. These underlined systems have their associated pros and cons, and hence require continual studies and improvement. Figure 3 shows the common configurations of microbial cellulase systems.

GH cell-free systems

The concept of cell-free enzymes was presented by Buchner in 1897, where he claimed that biological processes could be carried out without living cells (Khattak et al. 2014b). Typically, cell-free systems are used for cofactor-independent reactions, and often exhibit reaction-rate-limited kinetics, resulting from the direct access to substrate in solution (Smith et al. 2015). Cell-free enzymes have been exploited both in single (Kengen et al. 1993; Kim et al. 2010; Böhmer et al. 2012) and multiple domain systems (Kanafusa-Shinkai et al. 2013). Several immobilization practices have been reported (Kazenwadel et al. 2015). The general concept of immobilization has been highlighted in a subsequent section.

Among the numerous microorganisms known for their cellulolytic potentials, few has been identified to produce significant quantities and a complete set of cell-free lignocellulases in vitro (Patagundi et al. 2014). According to Khattak et al. (2014a), GH cell-free systems are considered as possible solution for surmounting all complexities and shortcomings associated with conventional enzyme hydrolysis by providing the following advantages: (1) well-regulated, continuous, and prolonged processing of substrate conversion; (2) easy evaluation of the effect of additional cofactors; and (3) no consumption of reduced sugar for cell energy requirement. Cell-free system dramatically reduces the time and effort needed to obtain proteins since it does not require gene transfection and extensive purification procedures (Kim et al. 2010). Moreover, it provides flexible reaction conditions for the introduction of several additives (such as chaperones, detergent, and affinity tags) into the reaction mixture as compared to in vivo systems (Kim et al. 2010).

Interestingly, the cell-free system has grabbed a tremendous interest in the production of various biocommodities, not only reduced sugars but also recombinant proteins, proteinaceous antibiotics, vaccines, hormones, and dihydrofolic acid reductase, etc. (Rollin et al. 2013; Khattak et al. 2014b). However, numerous limitations have confronted cell-free systems, especially when a mixture of enzymes constituting cascade of reactions is employed to produce bioproducts. Some of these shortcomings are the subjects of instability, reusability, and inactivation during biochemical processes (Khattak et al. 2014b). Problems of overall cellulase viability in the presence of high substrate and product concentration are also possible (Khattak et al. 2012, 2014b). The development of synthetic cell-free enzyme systems, with reprogrammed

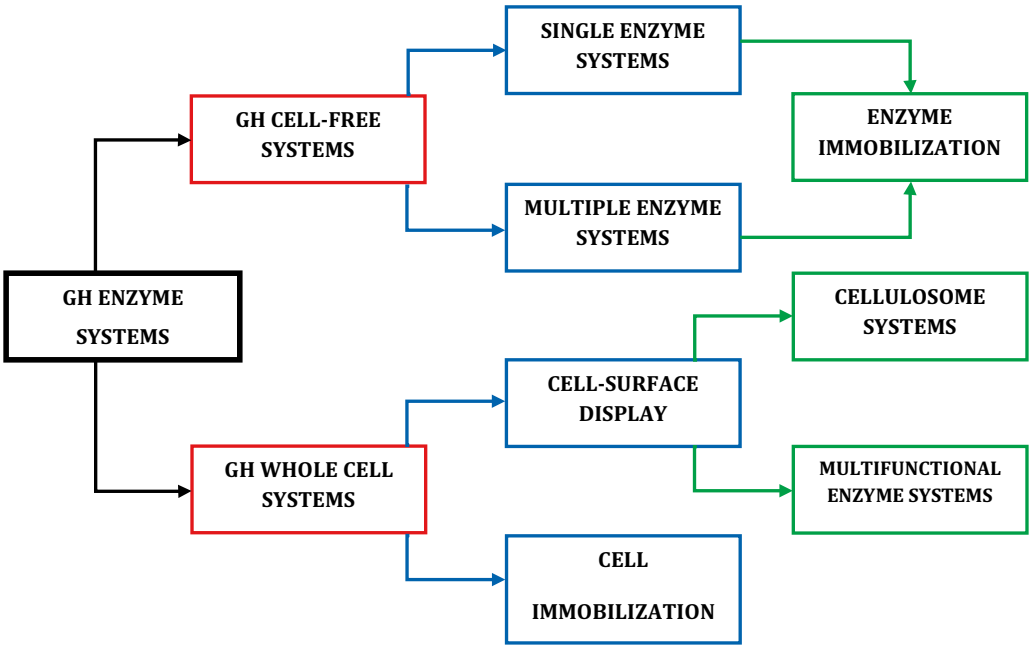

Fig. 3 Developments in GH systems. Pioneer systems in *red*; systems under continual improvement in *blue*; and emerging systems in *green*. The immobilization of both cell-free systems and whole-cell biocatalyst is trending

or newly constructed metabolic pathways to produce high-volume reduced sugars, are believed to be much more efficient due to reasons including the absence of external barriers (Percival Zhang 2010). Well-established approaches for the development of synthetic cell-free enzyme pathways include micro-compartmentalization, ionic channeling, co-polymerization, and protein fusion. Notably, the synthetic cell-free enzyme systems favor maximum enzyme–substrate interaction, product-oriented substrate utilization, and a higher concentration of biocatalyst (Khattak et al. 2014b). However, factors such as cofactor balance, thermodynamics, reaction equilibrium, and product separation and purification still need to be addressed (Zhang 2011).

GH whole-cell systems

The whole-cell biocatalyst system was developed to overcome the cost and complexities associated with enzyme purification via intracellular and extracellular localization of enzymes. In the former, the microorganism provides the most favorable working environment for the enzymes by (1) availing all necessary cofactors and regeneration networks; and (2) providing sufficient protection of enzymes from effects such as destabilization and degradation, while allowing both the substrate and product to cross the membrane barrier (Jose et al. 2012). On the other hand, the extracellular localization of enzymes involves the display of enzymes on the surface of the microorganism (thus, the designation "cell-surface display") to avoid possible substrate–product transport complexities across the cell membrane (Schüürmann et al. 2014).

The advent of whole-cell systems has helped to overcome some of the challenges faced by cell-free systems. The whole-cell systems convey several advantages such as stability, resistance, lower cost, reusability, and reduced labor, while providing products with high purity (Brault et al. 2014; Kim et al. 2014; Khattak et al. 2014b). The reduced proneness to cell injury; improved resistance to physiological and environmental factors, such as variation in pH, elevated temperature, and system inhibition; high metabolic productivity; and reduced incubation time make the whole-cell system more promising for biotechnological implementation.

Currently, the introduction of new knowledge and techniques, including genetic engineering, peptide engineering, and metabolic engineering, with specializations such as system and synthetic biology, has successfully improved the whole-cell system in various ways (Turner 2003; de Carvalho 2011; Pearsall et al. 2015). For example, the whole-cell biocatalyst system has been enhanced to immobilize the enzymes and improve substrate–enzyme contact, while increasing the catalytic potential of the enzymes by extending their overall lifetime (Kisukuri and Andrade 2015).

Immobilized and co-immobilized systems

Conventional enzyme immobilization is the practice of restraining the movement of enzymes, for example, by direct cross-linking, covalent coupling, entrapment, micro-encapsulation, and tethering onto a solid support to improve technical performance, usability, and industrial process economy. The technical performance includes enzyme stability, substrate specificity, enantioselectivity, and reactivity (Mateo et al. 2007; Schoffelen and van Hest 2012). The target of most immobilization practices is mainly to achieve fewer side reactions, high tolerance of structural variation of the substrates, high productivity and space–time yield, and high durability of the biocatalyst (Cao et al. 2003).

Immobilization is widely practiced in both cell-free and whole-cell systems. However, concerns regarding thermal instability at elevated temperatures, ineffective substrate utilization, by-product formation, and downstream industrial processing cost of end-product make the conventional immobilized system an ineffective approach for process industrialization (Khattak et al. 2014b). These consequences possibly result from intrinsic alterations in the catalytic activity, the overall stability, and the morphological structure of the individual enzymes in the new microenvironment. However, the use of efficient catalyst base; the use of hydrophilic and inert spacer arms; and the careful selection of the enzyme residues involved in the immobilization are some of the strategies toward curbing the steric obstacles. For example, the use of affinity tags (e.g., histidine tag) to selectively immobilize enzymes onto surfaces like cells, DNA scaffolds, and chelating supports is microbiologically practicable.

Cell-surface display systems

The cell-surface display system is the practice whereby whole cells are empowered to extracellularly degrade substrates and, sometimes, internalize resulting products to produce value-added end-products. Regardless of the host organism, surface display systems often have three core features in common. These are: (1) a signal peptide to direct the protein of interest toward the secretory pathway; (2) an endogenous surface protein pliable to recombination (i.e., insertion, deletion, and fusion) to facilitate a stable surface anchorage of the target protein; and (3) an epitope tag to facilitate the detection of successful surface display (Smith et al. 2015). In the surface display system, the amount of cellulase displayed is strictly dependent on the cell surface area, unlike cell-free systems, where there are no such limits (Yamada et al. 2013).

The cell-surface display system serves as an inherent biological platform for immobilizing enzymes, and thus offers three main advantages: (1) no protein diffusion into surrounding media; (2) enhanced biomass hydrolysis stemming from the close proximity, and induced synergy of enzymes present; and (3) easy recoverability and reusability by simple sedimentation or centrifugation. According to Yamada et al. (2013), the low diffusion rate of cell-surface displayed enzymes, owing to its insolubility in the substrate, is however a disadvantage. Arguably, when these surface displayed enzymes are aligned cooperatively to work synergistically, there would be a more efficient hydrolysis via substrate channeling, resulting in high enzymatic activity with high monomer yields. Common surface display systems that have been explored are cellulosomal (multi-enzyme) systems and multifunctional enzyme systems. The most recent subset is the autotransporter display (autodisplay) system, which is described in subsequent section.

Cellulosomal (multi-enzyme) systems Cellulosomes can be described as one of nature's most elegant and elaborate nanomachines (Fontes and Gilbert 2010). They are organized multi-enzyme complexes consisting of carbohydrate-binding modules (CBMs), catalytic domains (CDs), and scaffoldin subunits, which selectively integrate different CDs of enzymes in close proximity onto their individual unified complexes through a cohesin–dockerin interaction. The embedded enzymes work cooperatively and synergistically to ensure efficient depolymerization of the cellulose material.

The cellulosome phenomenon is a mimicry of interesting in vivo activities involving co-localization of enzymes for cascading reactions. Many crucial cellular functions such as biosynthesis (e.g., Krebs TCA cycle) and cellular signaling are controlled in living organisms by multistep simultaneous enzymatic reactions with excellent efficiency and specificity. A key characteristic of these highly efficient enzyme pathways is the cooperative and spatial organization of enzymes to ensure the sequential conversion of substrates (Fontes and Gilbert 2010; Park et al. 2014). The effect of this systematic organization of enzymes is very distinct, in that it enhances the overall efficiency of molecular activities by increasing the local enzyme–substrate concentrations and channeling intermediates between consecutive enzymes to avoid competition with other reactions present in the cell (Park et al. 2014).

The genesis of cellulosomal enzymes in microbes is linked to the discovery of *Clostridium thermocellum* and its potentials, which initiated the call to investigate the cellulosome genomics and metagenomics: cellulosomics (Bayer et al. 2008). The cellulosome architecture (Fig. 4) is dictated by a primary scaffoldin subunit, consisting of repeating units of cohesin (type I) modules that engage in high specificity and or affinity protein–protein

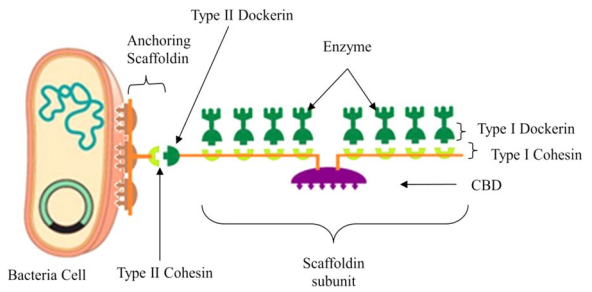

Fig. 4 Schematic architecture of bacterial cellulosome. The desired enzymes are assembled onto a suitable peptide with the help of cohesin–dockerin arrangements. The anchoring scaffoldin ensures the surface attachment of the cellulosome onto the bacteria surface [The figure was modified from Juturu and Wu (2014)]

interaction ($K_D < 10^{-9}$ M) with type I dockerin-containing enzyme, allowing the assembly of multiple enzymes in a spatially defined manner (Park et al. 2014; Stern et al. 2015). Most scaffoldins contain 6–9 different cohesins, which can bind up to 26 different cellulosomal enzymes (Juturu and Wu 2014). The primary scaffoldin interacts by means of type II cohesin–dockerin interaction with an anchoring scaffoldin to enforce cellulosome attachment to the cell surface via an S-layer homology (SLH) module (Stern et al. 2015). However, the intermodular cohesin–dockerin interaction dictates the assembly of the cellulosome complex; hence, granting the possibility of expressing different cellulosomes within a single organism, depending on the enzyme subunit compositions (Moraïs et al. 2012; Juturu and Wu 2014).

The CBM has multiple roles in the hydrolysis of cellulose: (1) to increase the concentration of cellulase close to the substrate; (2) to target the CD of the enzyme to specific sites on the substrate; and (3) to disrupt the crystalline structure of the substrate all through hydrophobic interaction between the three hydrophobic amino acid residues on the flat face of the CBM (Wahlström et al. 2014). There is also the possibility that CBMs assist in the improvement of the overall structure of the multimeric enzyme, leading to an increase in the hydrolysis yield (Fan et al. 2009). However, the number and position of CBMs in the multi-enzyme complex may cause effects (such as product inhibition) on the enzymes during cellulose degradation (Moraïs et al. 2012); thus, it requires investigation.

The CBM modules are classified into three, namely type A, type B, and type C, to define CBMs in terms of their binding specificity. Type A CBMs bind the surface of complex polysaccharides, type B CBMs (with specificity for amorphous regions) recognize internal glycan chains (endo type), and type C CBMs (with specificity for crystalline regions) bind the termini of glycans (exo type), according to Bornscheuer et al. (2014) and Fontes and Gilbert (2010).

The practice of the use of cellulosomes is interestingly surging in cellulose degradation activities. In this case, the GH enzyme assembly is attached onto the surface of the organism (mostly fungi and bacteria) for an effective saccharification process. The repeating scaffoldin-cohesins are docked individually with different dockerin-bearing GHs to enforce efficient cascade reaction, leading to high yields of fermentable sugars.

According to Zhang (2011), the most investigated in vitro multi-enzyme complex—even in the conversion of cellulose into fermentable sugars—are cellulosomes. This stems from the highly active-synergistic hydrolytic effect of the enzymes. To effectively evaluate the proposed benefits of cellulosomal enzymes over free enzymes, it is imperative to compare the optimized-state characteristics of each system on the same substrate (Moraïs et al. 2010). Park et al. (2014) reported 23-fold glucose production enhancement over that of free enzymes after their investigation of the effects of localization, surface accessibility, and functionality of synergetic enzymes on Scaf3-decorated bacteria outer membrane vesicles (OMVs) using phosphoric acid-swollen cellulose (PASC) as substrate. Yuan et al. (2015), in an investigation to biochemically characterize and structurally analyze cellulase/xylanase from *Clostridium thermocellum*, also revealed equally insightful results. Advancement in cellulosome investigation has led to the advent of its artificial counterpart, called designer cellulosomes, described below.

Designer cellulosomes (Chimeras) Designer cellulosomes (also known as chimeras)—unlike native cellulosomes—are artificial constructs, composed of chimeric scaffoldin and enzymes with cohesins and dockerins of divergent specificities, thus providing interdomain flexibility in the enzyme complex while maintaining (to some extent) the original wild-type functionality (Fierobe et al. 2002; Stern et al. 2015). Although synthetic cellulosomes present faster hydrolysis rates than non-composite cellulase mixtures, Zhang (2011) remarked that there is a limitation in the understanding of why synthetic cellulosomes constructed to date have been much less active than their natural counterparts. This may be due to factors such as changes in the microenvironments of the active sites, possible unproductive competition between functionally similar enzymes, difficulties in component arrangement as well as the nature of the peptide linker.

Cota et al. (2013) investigated and assembled a complex xylanase–lichenase (XylLich) chimera—both enzymes from *Bacillus subtilis*—through all-atom molecular

dynamics simulations. Contrary to the remark by Zhang (2011), Cota et al. (2013) reported based on comparison between the recombinant protein yield and the hydrolytic activity achieved that the production of chimeric enzymes is more efficient (in terms of cost and catalytic efficiency) than wild-type proteins and could be more profitable in streamlining biomass conversion strategies than separate production of single enzyme. Cota et al. (2013) further reported that the mode of operation of their chimera was exactly similar to that of the parental enzymes. Moreover, Moraïs et al. (2012) reported that their designer cellulosome system from *Thermobifida fusca* exhibited "equal or superior" activity to that of the free system. This presumably reflects the combined proximity effect of the enzymes and high flexibility of the designer cellulosome components to enable efficient enzymatic activity of the catalytic modules.

The higher flexibility and structural conformations of the fused CDs of designer cellulosomes explicate their more efficient enzymatic abilities (Moraïs et al. 2012). Stern et al. (2015), based on extensive combinatorial analysis, devised and developed a designer cellulosome concept consisting of chimeric scaffoldins for controlled incorporation of recombinant polysaccharide-degrading enzymes. Their results supported the argument that for a given set of cellulosomal enzymes, the relative position of enzymes within a scaffoldin can be critical for optimal degradation of microcrystalline cellulosic substrates. Liang et al. (2014) also constructed a penta-functional minicellulosome by co-expressing lytic polysaccharide mono-oxygenases (LPMOs) and cellobiose dehydrogenases (CDH) with cellobiohydrolases, endoglucanases, and β-glucosidases in *Saccharomyces cerevisiae* for simultaneous saccharification and ethanol fermentation of PASC. The synergetic activity of this penta-enzyme complex increased the ethanol titer from 1.8 to 2.7 g/l.

Engineering multi-domain enzymes that are capable of catalyzing two or more reactions is a potential strategy to reduce enzyme costs in bio-industrial processes, as multiple catalytic properties on a single polypeptide conceivably simplify production and purification operations of biochemicals (Ribeiro et al. 2011). Similarly, the cost-effective optimization of chimeras to prevent unproductive competition between functionally similar enzymes by testing the importance of both the positions of enzymes and CBMs for an efficient use in bioprocessing industries is necessary, though demanding a vigorous investigation. However, the successful expression of the essential cellulolytic enzymes (*i.e.*, endoglucanases, exoglucanases, and β-glucosidases) on a single peptide chain, in a processive order, such that their proportionate quantities favor maximum hydrolysis efficiency has been highly challenging (Tozakidis et al. 2016). Also, the determination of simple and reliable structural organization of the chimeric domains has been a significant drawback in the construction of a protein chimera, but the advent of small-angle X-ray scattering (SAXS) with flexible analytical models (e.g., molecular dynamics (MD) and Monte Carlo simulations) has provided not only successful computational data validation approaches, but also accurate fitting of the scattering profile due to their potential to explore the protein conformation in space (Cota et al. 2013).

A critical factor for success in the creation of enzyme chimeras is the compatibility and cooperativity among the involved CBMs and CDs, with respect to their physicochemical requirements such as solubility, optimum pH, and temperature (Kim et al. 2010; Ribeiro et al. 2011). Howbeit, Stern et al. (2015) suggested that the optimal order for the positioning of enzymes as per their investigation is processive endoglucanase, exoglucanase, and non-processive endoglucanase; and for overall higher enzymatic activity the CBM should not be placed in the middle of the scaffoldin.

In parallel with the designer cellulosome approach, another interesting attempt to increase enzyme synergism, in the form of multifunctional enzyme conjugates, has been reported recently, and it is believed that this strategy may provide a component cost-reducing advantage over designer cellulosomes in future industrial applications (Moraïs et al. 2010). However, the multifunctional enzyme strategy is limited to small numbers of enzymes and restricted to suboptimal equimolar ratios of enzymes. This paradigm permits the expression of single enzymes on the surface of a suitable microorganism such that blending complexities could be overcome.

Multifunctional enzyme systems Multifunctional enzymes—comprising the direct surface display of multiple enzymes in a non-complex form—are very high-molecular weight proteins of one or several CBMs and two or more CDs for effective substrate targeting and efficient degradation of plant cell walls, respectively (Moraïs et al. 2012; Smith et al. 2015). The several catalytic modules on the same polypeptide chain are assembled such that their enforced proximity account for an enhanced concerted action on substrates (Moraïs et al. 2012). The enzyme assemblies in multifunctional enzyme systems enable metabolic control and prevent metabolic crosstalk between competing pathways (Conrado et al. 2008). Multifunctional enzymes are formed by linking the CD of desired enzymes, using flexible peptide linkers or linkers containing CBMs, with that of the parent enzyme (Fan et al. 2009). The resulting enzyme may retain similar properties (example, pH and temperature profiles, kinetics, etc.) as the parent enzyme and exhibit synergetic effects

in the hydrolysis of the target substrate. Though the multifunctional enzyme system is under thorough investigation, a broader understanding of (1) how the structure of an enzyme relates to its function and (2) what changes can be tolerated within a multifunctional enzyme framework are needed to promote industrial applications.

Moraïs et al. (2012) employed a synthetic biology approach to convert two different cellulases from the free enzyme system of *Thermobifida fusca* into bifunctional enzymes with different modular architectures and examined their performance compared to those of the combined parental free enzyme and equivalent designer cellulosome systems. They reported that the different architectures of the bifunctional enzymes displayed "somewhat inferior" cellulolytic activity to that of the wild-type free enzyme system. However, Ribeiro et al. (2011) created two bifunctional enzymes with xylanase–laccase activity using rational design methods and reported catalytic properties similar to the parental enzymes. Moreover, Chang et al. (2011) reported an excellent performance of a bifunctional xylanase/endoglucanase (RuCelA), which distinguishes it as an ideal candidate for industrial applications. Cho et al. (2008) reported a multifunctional enzyme Cel44C-Man26A (secreted by *Paenibacillus polymyxa* GS01) with cellulase, xylanase, lichenase, and mannanase activities. The construction of multifunctional enzymes is putatively dependent on the nature and anatomy of source organism as well as the design technique, and thus more insights are required for this justification.

The autodisplay systems The autodisplay system is an induced superior advancement of the whole-cell biocatalyst strategy. It mostly involves the recombinant surface display of proteins or peptides by means of autotransporter proteins in Gram-negative bacteria (Jose et al. 2012). The autotransporter proteins—the peptide chains that 'link' or hold the passenger protein onto the outer membrane of the organism—are common secretion proteins of most Gram-negative bacteria, and are synthesized as precursor protein containing all domains needed to transport the passenger (e.g., cellulases, proteases, lipases, esterases etc.) to the cell surface (Jose 2006; Jose et al. 2012). This provides the possibility to transport protein (recombinant or natural passenger) to the outer membrane so long as its coding region lies between a typical signal peptide and a C-terminal "β-barrel" domain (Schumacher et al. 2012). Tozakidis et al. (2016) has published a proof of concept of cellulose hydrolysis using autodisplay cellulases.

The hypothetical model of the autodisplay secretion mechanism (Fig. 5) has been described by Himmel et al. (2010). It typically involves the transport of a polyprotein precursor across the inner membrane (IM) of the cell into the periplasm, with the help of the sec signal peptide (SP). A typical precursor protein comprises a signal peptide (SP) followed by the autotransporter protein, composed of an N-terminal passenger domain (α-domain) and a C-terminal translocator domain (β-domain). The β-domain, as its name signifies, involves β-barrel and linker. Inside the periplasm, the C-terminal part of the precursor forms a porin-like structure (β-barrel) within the outer membrane (OM). Subsequently, the passenger proteins translocate to the cell surface through the pores, with anchorage from the free mobile β-barrel, unlike in other display systems where they are covalently attached to the cell envelope. Complementarily, the peptide linker ensures the full surface exposure and functionality of the passenger protein.

The recombinant expression principle of autotransporter proteins has several advantages. The flexible transfer of these proteins from one Gram-negative bacterium to the other needs little or no additional machinery for its propagation. Moreover, a large number (more than 10^5) of recombinant proteins or peptide molecules can be displayed on the surface of a single microorganism, without reducing cell viability or integrity (Jose and Meyer 2007). In addition, the relatively simple modular structure of autodisplay systems allows the easy interaction of passenger proteins on the bacterial cell surface, thus displaying desired heterologous enzymes. The autodisplayed proteins normally expressed as monomers are capable of forming multimers upon membrane interaction after expression (Schumacher et al. 2012; Smith et al. 2015). Furthermore, the autodisplay secretion method makes subsequent, often costly, purification steps to recover the enzyme of interest unnecessary (Kranen et al. 2014).

Conclusions

The bioprocess industry is constantly seeking to obtain useful products from the highly abundant lignocellulosic feedstock. Thus, lignocellulases have been vital in the production of reduced sugars for the manufacturing of biocommodities. The industrial pursuit of obtaining high level of fermentable sugars from lignocellulosic biomass depends substantially on the successful expression and blend of cellulases, hemicellulases, lignases, and other accessory proteins in a non-competing, progressive, and synergetic order, in one complex. However, the challenge has been the successful assembly of an entire suite of these enzymes that could function optimally at the same time and under different conditions to completely digest lignocellulosic biomass to simple sugars. Many cellulase improvement practices and enzyme systems (i.e., cell-free or whole-cell) have surfaced and presently the fraternity is witnessing a gradual shift towards the cell-surface display system. However, the challenge has been

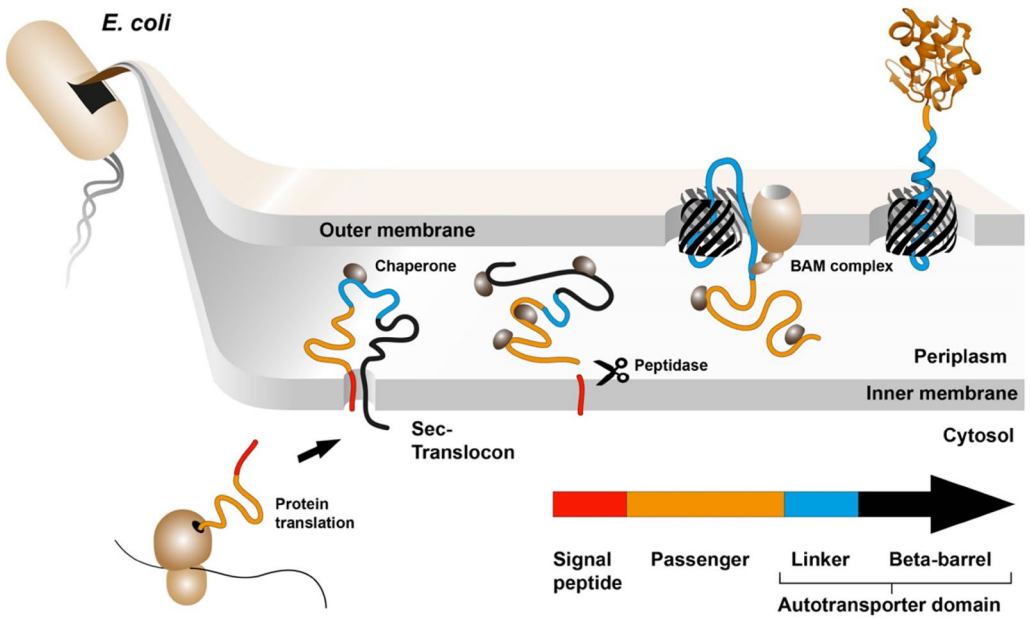

Fig. 5 The hypothetical model of the autodisplay secretion mechanism. The transport protein (e.g., enzyme) translates from the cytosol through the inner membrane, where the signal peptide is cleaved off by specific peptidases. The autotransporter and the passenger enzyme move across the outer membrane for outward exposure. The β-barrel helps in anchoring the passenger onto the surface of the bacteria whereas the linker enforces the outward exposure of the passenger [The figure was adapted from Tozakidis et al. (2015)]

the achievement of high-level expressions necessary for industrial use. Techniques such as directed evolution and rational design have been used in improving cellulases. The practice of harnessing glycosylation to improve cellulase activity looks promising. A success in these ventures would be influential to the proposed 'green' future.

Abbreviations

LPMOs: lytic polysaccharide mono-oxygenases; GHs: glycoside hydrolases; CBM: carbohydrate-binding module; CD: catalytic domain; PCR: polymerase chain reaction; EDTA: ethylenediaminetetraacetic acid; SLH: S-layer homology; IN: inner membrane; OM: outer membrane; OMV: outer membrane vesicle; PASC: phosphoric acid-swollen cellulose; CDH: cellobiose dehydrogenase; SAXS: small-angle X-ray scattering; MD: molecular dynamics; SP: signal peptide.

Authors' contributions

EMO and SNNA drafted the manuscript. CMO, CB, RM, and JJ revised and approved the content of the manuscript. All authors read and approved the final manuscript.

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Competing interests

RM and JJ are the founding members of Autodisplay Biotech GmbH (Germany); CMO is the project lead consultant; CB is the project associate consultant; EMO and SNNA are the postgraduate students at Universiti Malaysia Sabah.

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