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REVIEW

Synergism of fungal and bacterial cellulases and hemicellulases: a novel perspective for enhanced bio-ethanol production

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Abstract The complex structure of lignocellulose requires the involvement of a suite of lignocellulolytic enzymes for bringing about an effective de-polymerization. Cellulases and hemicellulases from both fungi and bacteria have been studied extensively. This review illustrates the mechanism of action of different cellulolytic and hemi-cellulolytic enzymes and their distinctive roles during hydrolysis. It also examines how different approaches can be used to improve the synergistic interaction between fungal and bacterial glycosyl hydrolases with a focus on fungal cellulases and bacterial hemicellulases. The approach entails the role of cellulosomes and their improvement through incorporation of novel enzymes and evaluates the recent break-through in the construction of designer cellulosomes and their extension towards improving fungal and bacterial synergy. The proposed approach also advocates the incorporation and cell surface display of designer cellulosomes on non-cellulolytic solventogenic strains along with the innovative application of combined cross-linked enzyme aggregates (combi-CLEAs) as an economically feasible and versatile tool for improving the synergistic interaction through one-pot cascade reactions.

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Introduction

In view of depleting petroleum reserves and a potential climate change, the need for alternative bioenergy is expected to increase sharply in coming years. In this vein, utilization of lignocellulosic biomass has emerged as the most prominent technology for efficiently producing bioethanol and other value added products.

Research efforts in the last few decades have emphasized replacing first generation biofuels with second generation biofuels as they are made from cheap and abundant lignocellulosic feedstocks. Lignocellulosic biomass obtained from agricultural residues (wheat, straw, sugarcane bagasse, corn stover), herbaceous grasses (alfalfa, clover, switch grass, miscanthus grass) and forest products (hardwoods and softwoods) provide an abundant source of carbohydrate and present substantial renewable substrates for bioethanol production (Vikari et al. 2012).

Most plant cell walls are composed of approx. 15–40 % cellulose, 10–30 % hemicellulose and pectin, and 5–20 % lignin (Prassad et al. 2007). Recalcitrance of lignocellulosic biomass is related to its complex chemical composition (lignin, hemicellulose and acetyl groups) and the physical features (cellulose

crystallinity and degree of polymerization) of the plant cell wall (Van Dyk and Pletschke 2012). The principal framework of cellulose consists of anhydroglucopyranose molecules connected by β-1,4-glycosidic linkages. The complexity of strong intra- and intermolecular hydrogen bonding found in cellulose results in the formation of microfibrillar chains which play an important role in recalcitrance of biomass and make its hydrolysis a cost intensive process (Himmel et al. 2007). Hemicellulose is a heterogeneous group of branched and linear polysaccharides, consisting mainly of D-xylose, and D-mannose, and a number of substituted sugars (Sanchez 2009). Both cellulose and hemicellulose can be hydrolyzed into simple sugars which may be then fermented to ethanol.

Enzymes involved in the degradation of lignocellulose

Many microorganisms, including fungi and bacteria, produce extracellular enzymes which are capable of degrading and utilizing cellulose and hemicellulose as a carbon source. However, a large variety of enzymes is required to degrade all the components of lignocellulose. In nature, a consortium of lignocellulolytic enzymes works synergistically to degrade lignocellulosic biomass.

Cellulases

The degradation of cellulose to glucose involves the cooperative action of at least three enzymes; exo-1,4-β-glucanases (EC 3.2.1.91) and cellobiohydrolase (EC 3.2.1.176), endo-1,4-β-glucanases (EC 3.2.1.4), β-glucosidases (EC 3.2.1.21) (also termed cellobiases). Structural characterization studies of a variety of cellulases have provided detailed information regarding the structure and function of these enzymes. In general, the structures of cellulases are composed of a catalytic domain (CD), which is responsible for the hydrolysis reaction; a cellulose-binding domain (CBD), that mediates the binding of enzyme to the substrate; and the linker (hinge) region (rich in serine, threonine and proline residues) by which the two domains are linked (Carrard et al. 2000; Rabinovich et al. 2002). New types of enzymes, originally classified as carbohydrate-binding module (CBM) 33 (now termed copper-dependent lytic polysaccharide monooxygenase AA10) and GH61 (now termed polysaccharide monooxygenase AA9), that catalyze the oxidative cleavage of polysaccharides have been identified. For an excellent review on this topic please refer to Horn et al. (2012).

Role of CBMs/CBDs

Based on the initial discovery of several modules, cellulose specific CBMs were previously classified as CBDs. Hydrolysis of cellulose occurs at catalytic modules and CBMs assist in effectively recognizing the surface of the crystalline cellulose materials. CBMs contain from 30 to about 200 amino acids and are present as single, double or triple domains in one protein. These are either linked to the *N*- or *C*-terminus of the CD (Shoseyov et al. 2006). The presence of CBMs is shown to increase the binding of the substrate and targets the enzyme towards specific substrates.

Mechanism of action

Initially, cellulose hydrolysis occurs on the surface of the solid substrates and involves endoglucanases which hydrolyze internal β-1,4-glucosidic linkages randomly in the cellulose chain. Endoglucanases appear to have cleft-shaped open active sites which produce nicks in the cellulose polymer, exposing reducing and non-reducing ends. Exoglucanases have their active sites inside a "tunnel" and progress along the reducing and non-reducing ends of cellulose, liberating cellooligosaccharides and cellobiose units. The products of cellobiohydrolases and endoglucanases are inhibitory to the activities of their enzymes, so finally β-glucosidase (BGL) is required, which acts on cellobiose to liberate glucose (Binod et al. 2011). There is a high degree of coordination between the three enzymes, such as exo/endo, exo/exo and endo/BGL synergy, which is required for the efficient hydrolysis of cellulose crystals.

Fungal and bacterial cellulases

Fungi are key micro-organisms for the degradation of cellulose and the active degraders in this group are divided into two sub-groups:

The first group includes soft rot fungi (e.g. *Trichoderma reesei*), and white rot fungi (e.g. *Phanerochaete chrysosporium*). Cellulase mixtures available

commercially are mainly obtained from the enzyme cocktail produced by *T. reseei* (*Hypocrea jecorina*) and this fungus possesses a total of 200 GH genes (Martinez et al. 2008; Kubicek et al. 2009) (Table 1). Many fungi are known to produce multiple cellulases, for example *T. reesei* produces 2 CBHs (*Cel6A* and *Cel7A*), 8 EGs (*Cel5A*, *Cel5B*, *Cel7B*, *Cel12A*, *Cel45A*, *Cel61A*, *Cel61B* and *Cel74A*) and 7BGLs (*Cel1A*, *Cel1B*, *Cel3A*, *Cel3B*, *Cel3C*, *Cel3D* and *Cel3E*) (Aro et al. 2005). In fungal cellulases, the binding module seems to invariably belong to the CBMI family and targets binding to cellulose surfaces (Costaouec et al. 2013). The CD of exoglucanase *Cel7A* (acts on reducing end) is composed of a β sandwich structure with a long substrate tunnel lined by β-sheets, while four loops cover the tunnel. *Cel7A* of *T. reesei* degrades crystalline cellulose following the phenomenon of processivity, which is the ability of the CBHs to attach to the carbohydrate chain, and decrystallize disaccharide units from the end of the chain without dissociation (Beckham et al. 2010).The lower processivity of *Cel6A* (acts on non-reducing ends) is attributed to a more open and shorter active site tunnel compared to *Cel7A*. Endoglucanase *Cel7B* has an open groove type active site because it lacks four loops of β-sheets that cover the tunnel (Beckham et al. 2010).

The second group comprises of brown rot fungi (e.g. *Postia placenta*) that lacks both CBMs and processive cellulases, however it is found to utilize crystalline cellulose as a sole carbon source, suggesting the involvement of non-enzymatic low molecular weight oxidants through the production of reactive oxygen species (OH-, peroxide- or superoxide-radicals), also known as the Fenton reaction (Dashtban et al. 2009; Wilson 2011).

Many aerobic bacteria secrete various amounts of free cellulases that act synergistically to degrade cellulose. They contain CDs, linker peptides and CBMs which follow a non-processive manner of cellulose degradation. Various aerobic bacteria belonging Table 1 to the genera *Cellulomonas, Pseudomonas, Geobacillus, Erwinia, Streptomyces, Fibrobacter,* as well as *Bacillus* and *Paenibacillus* (Table 1), are known to produce different kinds of cellulases (Table 1) (Maki et al. 2009; Sethi and Scharf 2013).

Anaerobic bacteria have evolved a different type of cellulolytic system that involves complex protein structures supporting enzymes for the hydrolysis of cellulose, known as cellulosomes (Doi and Kosugi 2004). In these bacteria, for example *Clostridium* (Table 1), *Acetivibrio*, *Bacteroides* and *Ruminococcus*, different types of cellulose-degrading enzymes and hemicellulolytic enzymes are assembled on the structural scaffoldin subunits through strong non-covalent protein–protein interactions between the docking modules (dockerin) and complementary modules (cohesins) (Dashtban et al. 2009). Cellulosomes consist of a fibrillar protein scaffoldin. The scaffolding proteins are also called "scaffoldins" and consist of proteins CbpA, Cip A or Cip C (Doi et al. 2003). A few anaerobic cellulolytic thermophilic bacteria, such as *Caldicellulosiruptor* sp., use an intermediate strategy, i.e. they secrete many free cellulases that contain multiple CDs. The *Cald. bescii* CelA comprises of a GH 9 and a GH 48 CD, along with three type III cellulose-binding modules. This CelA drives cellulose hydrolysis, not only through the well-known surface ablation mechanism, but also through excavation into the surface of the substrate, resulting in the formation of extensive cavities (Brunecky et al. 2013).

A different type of cellulolytic degradation mechanism has been found in two Gram negative cellulolytic bacteria, *Fibrobacter succinogenes*, an anaerobic cellulolytic rumen bacterium and *Cytophaga hutchinsonii*, an aerobic soil bacterium. The genome sequences of these bacteria strongly suggest that they do not use free cellulases or cellulosomes, as none of their genes encode for CBMs, scaffoldins or dockerins. Thus these organisms are believed to have evolved a novel cellulolytic mechanism to degrade cellulose fibrils by a complex present on the outer membrane and transport of cellulose molecules through super channels to periplasmic spaces followed by the action of endoglucanase present in the periplasm (Wilson 2011; Sethi and Scharf 2013).

Hemicellulases

Hemicellulose, and xylan in particular, represents 20–35 % of lignocellulosic biomass. The conversion of the hemicellulosic fraction (either in the monomeric form or in the oligomeric form) is vital for increasing the overall yield of bioethanol. Hemicellulose has a more varied structure than cellulose and thus it requires a large suite of enzymes for effective hydrolysis (Van

Table 1 Cellulolytic and hemicellulolytic enzymes found in industrially important fungal and bacterial strains

Microorganisms	Enzymes	Glycoside hydrolase family	References
Aerobic fungi	*Free enzymes*		
Trichoderma reesei	Endoglucanase (Cel74A)	GH 74	Adapted from Adav et al. (2012)
	β-1,6-Glucanase	GH 5	
	Exo-1,3-β-D-glucanase	GH 55	
	Glucan 1,3-β-glucosidase	GH 17	
	Glucan 1,4-β glucosidase	GH 15	
	β-Glucosidase	GH 31	
	Glucan endo-1,3-β-glucosidase	GH 17	
	β-1,4-Glucosidase	GH 3	
	Glucan endo-1,3-β-glucosidase (Agn1)	GH 71	
	β-glucosidase (TrBg12)	GH 1	Lee et al. (2012)
	β-xylosidase	GH 3	Rojas et al. (2005)
Aspergillus niger	Endoglucanase (Egl C)	GH 74	Hasper et al. (2002)
	Endo β-1-4 xylanse (Xln A)	GH 10	Do et al. (2013)
	Endoglucanase (EglA)	GH 12	Pham et al. (2011)
	Xyloglucanase (AnXEGl2A)	GH 12	Master et al. (2008)
	α-L rhamnosidase	GH 13	Liu et al. (2012)
Trichoderma harzianum	β-mannosidase	GH 2	Nascimento et al. (2014)
	(1–3) α-glucanases	GH 71	Grun et al. (2006)
	Xylanases	GH 11	Shrivastava et al. (2007)
Humicola insolens (thermophilic)	Cellobiohydrolase (Cel6A)	GH 6	Adapted from Li et al. (2011)
	Endoglucanases (Cel6B)	GH 6	
	Endoglucanase (EGI)	GH 7	
	Cellobiohydrolase (Cel7B)	GH 7	
	Endoglucanase (EGV)	GH45	
Anaerobic bacteria	*Cellulosomes*		
Clostridium acetobutylicum	Endoglucanase (CelA)	GH5	Adapted from Doi and Kosugi (2004)
	Endoglucanase (CelE)	GH9	
	Exoglucanase (CelF)	GH48	
	Endoglucanase (CelG)	GH9	
	Exoglucanase (CelH)	GH9	
	Endoglucanase (CelL)	GH9	
	Endoglucanase (EngA)	GH44	
	Mannanase (ManA)	GH5	
	Sialidase (CAC0919)	GH74	
	Endoglucanase (CAC3469)	GH5	
Clostridium thermocellum	Xyloglucanase (Xgh74A)	GH 10	Zverlov et al. (2005)
	Endoxylanase (Xyn 10D)	GH 74	
	Cellulase (Cel 48S, Cel 48Y)	GH 48	Olson et al. (2010)
	Lichenase (LicB)	GH16	Adapted from Doi and Kosugi (2004)
	Mannanase (ManA)	GH26	
	Xylanase/acetyl xylan esterase (XynA/XynU)	GH11	

Table 1 continued

Microorganisms	Enzymes	Glycoside hydrolase family	References
	Xylanase(XynB/XynV)	GH11	
	Xylanase/feruloyl esterase (XynY)	GH10	
Anaerobic fungi	*Cellulosome*		
Orpinomyces PC 2	Cellulases (CelA, CelC, CelD, CelF, CelH and CelI)	GH 6	Adapted from Ljungdahl (2008)
	Cellulases (CelB, CelE, CelG, and CelJ)	GH 5	
	β-glucosidase (BglA)	GH 1	
	Xylanase (XynA)	GH11	
	Lichenase	GH16	
	β-glucanase	GH16,	
	Mannanase (ManA)	GH5	
	Acetyl xylan esterase (AxeA)	CE 6	
	Feruloyl esterase (FaeA)	CE 1	
Aerobic bacteria	*Free enzymes*		
Bacillus halodurans	α-amylase G-6 (BH0413)	GH13	Lombard et al. (2014) (CAZy database, http://www.cazy.org/)
	α-galactosidase (MelA)	GH4	
	β-1-3-glucanase/laminarinase (BH0236)	GH81	
	β-glucanase (CelB)	GH5	
	β-glucosidase (BglA)	GH1	
	β-xylosidase (BHXyl39)	GH39	
	β-xylosidase (XynB)	GH43	
	Acetyl xylan esterase (Rgae)	CE12	
	Endo- β-1-3(4)-glucanase (BH2115)	GH16	
	Endo-β-*N*-acetylglucosaminidase	GH85	
	Xylanase (BH0899)	GH11	
	Xylanase A (Xyn A)	GH10	
Bacillus cellulolyticus	endo β-1-4-glucanase A (Cel A, Cel B, Cel C)	GH5	Lombard et al. (2014) (CAZy database, http://www.cazy.org/)
Paenibacillus mucilaginosus	β-glucosidase (BglPm)	GH1	Lombard et al. (2014) (CAZy database, http://www.cazy.org/)
	β-glucosidase (Bglb)	GH3	
	Xylanase A (Xyn A)	GH52	
	β-glucosidase (BglC, BglA)	GH1	
	Xylanase A (XynD, Xynb2)	GH43	
Bacillus licheniformis	Cellulases (EglA, Cel A, CelB, CelD)	GH9, GH48, GH5, GH5	Lombard et al. (2014) (CAZy database, http://www.cazy.org/)
	Arabinofuranosidases AbfA	GH51	
	β-glucosidase (BglC, BglS)	GH1, GH16	
	Xylanase (Xynb2, XynC, XynD)	GH43, GH30, GH43	

Dyk and Pletschke 2012). Enzymes that degrade hemicellulose can be divided into two groups; firstly, a group of de-polymerizing enzymes which cleave the backbone called core enzymes, and a second group called as de-branching enzymes that remove substituents which may pose steric hindrances to de-polymerizing enzymes and subsequently increase the overall yield of reducing sugars during hydrolysis. This second group of enzymes is also known as ancillary and/or auxiliary enzymes. The core enzymes required to degrade the hemicellulases include endo-β-1,4 xylanases (EC 3.2.1.8), xylan 1,4-β-xylosidases, (EC 3.2.1.37) endo-1,4-β mannanases (EC 3.2.1.78) and β-1-4 mannosidases (EC 3.2.1.25). The ancillary enzymes include α-L-arabinofuranosidase (EC 3.2.1.55), β-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73) and p-coumaric acid esterase (EC 3.1.1-) (Lairson et al. 2008).

Role of CBMs

Hemicellulases are modular proteins and consists of CBMs and dockerins, in addition to CDs. CBMs are known to localize the soluble enzyme to its target substrate. Most of the CBMs are composed of a β-jelly roll structure of two β-sheets and one planar hydrophobic surface that helps in binding of crystalline cellulose or a deep cleft that allows the binding of a single polysaccharide molecule (Rabinovich et al. 2002).

Mechanism of enzyme action

Xylanases (EC 3.2.1.8) are the group of enzymes responsible for the hydrolysis of β-1-4 bonds in the backbone of xylan. The main enzymes involved in this group are endo 1,4- β xylanases and β-xylosidases. Most of the xylanases belong to GH family 10 and 11, and are also distributed between families 5, 8 and 43 (Sapag et al. 2002). Xylanases belonging to the GH 10 family possess a cellulose binding domain and a CD connected by a linker. Xylanases belonging to this family have a (β/α)fold(TIM barrel), whereas GH family 11 has low molecular weight enzymes which have a β jelly roll structure (Paes et al. 2012). β-xylosidases are grouped into five families—GH families 3, 39, 43, 52 and 54, while most of the fungal β-xylosidases belong to families 3 and 43 (Van den Brink and De Vries 2011). β-mannanase hydrolyzes the mannan based hemicellulose and liberates short β-1-4 manno-oligomers which can be further hydrolyzed to mannose by β-mannosidase. β-mannanase sequence comparison studies permit assignment of these enzymes to either glycoside hydrolase family 5 or 26. Most mannanases often display a modular organization and usually consist of two-domain proteins. β-mannanases in both families belong to the 3-D structure group (β/α)eightfold catalytic module characteristic of a clan A glycoside hydrolase (Stoll et al. 2000).

Several accessory enzymes are also required, such as α-D-glucuronidase, which cleaves the α-1-2-glycosidic bond of the 4-*O*-methyl-D-glucuronic acid side chain of xylan. α-L-Arabinofuranosidases and arabinases hydrolyze the arabinofuranosyl containing hemicellulose and are distributed within GH families 3,43,51,54 and 62. Acetylxylan esterases hydrolyze the *O*-acetyl groups from positions 2 and 3 on the β-D xylopyranosyl residues of acetylxylan. Feruloyl esterases hydrolyze the hydroxyl cinnamoyl ester bonds at O_2 or O_5 of the arabinoside chain and glucuronyl esterases hydrolyze the methyl esters bonds at the O_6 position of methylated glucuronic acid (Van Dyk and Pletschke 2012).

Fungal and bacterial hemicellulases

Fungi produce a suite of hemi-cellulolytic enzymes that can degrade hemicellulose into mono- or disaccharides. The most studied fungal hemicellulases are from *A. niger,* which include two endo-xylanases, one β-xylosidase, one endomannanase, one β-mannosidase, two α-galactosidases, one β-galactosidases, one α-glucuronidases, one acetyl xylan esterases and two feruloyl esterases(Van den Brink and De Vries 2011). Similarly, aerobic fungi *T. reesei* are also known to secrete various hemicellulases (Table 1) (Shallom and Shoham 2003). Most of the fungal endo-xylanases belonging to families 10 (formerly known as F) and 11 (formerly known as G) have been reported in literature. Multiple β-xylosidases have been reported in the filamentous fungi *Asp. niger* and *Penicillium wortmanii* (Juturu and Wu, 2012). β-xylosidases from *Asp. niger* and *Asp. awamori* are included in glycosyl hydrolase families 3 and 54 and these enzymes follow a double displacement mechanism with retention of the anomeric center configuration. Similarly, β-

xylosidase of the filamentous fungi *Cochliobacillus carbonum* follows the mechanism of inversion of the anomeric center. The most mannanolytic group among fungi belongs to genera *Aspergillus, Agaricus, Trichoderma* and *Sclerotium* (Dhawan and Kaur 2007). With the exception of a few anaerobic fungi, most of the fungal mannanases belong to the family 5 glycosyl hydrolases. Among the fungal mannanases, the *T. reesei man1* gene was the first fungal mannanase gene to be characterized (Stalbrand et al. 1995). Tang et al. (2001) demonstrated that the CD of CEL4 from *Agaricus bisporus* displayed the highest amino acid sequence similarity with Ascomycete mannanase from *Asp. aculeatus* and *T. reesei* (43 and 42 %, respectively), which belong to glycosyl hydrolase family 5.

Bacterial hemicellulases have been reported from both Gram positive and Gram negative strains. Most of the bacterial xylanases are categorized under GH family 10 and GH family 11. Two endo-xylanases and one β-xylosidase from *T. maritima* have been functionally characterized (Zhengqiang et al. 2001). Multifunctional xylanases with a feruloyl esterase module and an acetyl xylan esterase module have been characterized from *Clostridium thermocellum* (Prates et al. 2001) and *C. cellulovorans* (Kosugi et al. 2002), respectively. A multi-enzyme complex with xylanase, mannanase, arabinofuranosidase and xylosidase activity was identified and characterized by Van Dyk et al. (2010). The hemicellulolytic arsenal of *Thermobacillus xylanilyticus* exhibiting xylanase, arabinofuranosidase, xylosidase, feruloyl esterase and acetyl esterase activity was revealed by Rakotoarivonina et al. (2012). Bacterial mannanases have been annotated to both glycosyl hydrolases families 5 and 26. Multifunctional enzymes that contain CDs belonging to different GH families, like *Paenibacillus polymyxa cel*44C-*man*26A, have been detected. The GH44 domain possesses cellulase, xylanase and lichenase activities, and the enzyme domain GH 26 exhibits mannanase activity (Han et al. 2006). Multiple β-mannanases in *Cellvibrio japonicus* have been classified in both families 5 and 26 (Hogg et al. 2003). Besides this, β-mannanases from different *Bacillus* spp are also found in both families (Hatada et al. 2005). Mannanases can be produced from various bacterial sources such as *Bacillus* spp., *Streptomyces* spp., *Caldibacillus cellulovorans*, *Caldicellulosiruptor Rt8b*, and *Caldocellum saccharolyticum* (Zhang et al. 2006).

Synergy between fungal and bacterial lignocellulolytic enzymes

Most of the current commercial lignocellulolytic mixtures are based on fungal cellulases and hemicellulases (Hu et al. 2011). The question arises as to what more can be done to improve the fungal enzymes or if there could there be a way to design novel cocktails based on fungal and bacterial glycosyl hydrolases. Studies have conducted a parallel comparison of fungal and bacterial enzymes but these could not provide any conclusive results (Johnson et al. 1982; Irwin et al. 1993). It is well known that fungi produce copious amounts of cellulases (Sweeney and Xu 2012); cellobiohydrolases account for nearly 70 % (w/w) of secreted proteins and enzymes in cellulolytic fungi, followed by endoglucanases (∼20 % w/w), while hemicellulases account for only less than <1 % of total weight of the secreted proteins (Sweeney and Xu 2012). Many studies have documented the presence of bacterial strains (Mohanram et al. 2013) that produce cellulases with high specific activities but these have low titre values (Lynd et al. 2002). The hemicellulolytic machinery of bacterial hemicellulases has been well studied and reviewed (Maki et al. 2009). The ability of bacterial strains to inhabit extreme environmental conditions and industrial niches provides us with a unique gene pool of cellulases and hemicellulases which (in combination with modern molecular tools for engineering which impart improved properties) can be added to existing commercial cellulase and hemicellulase mixtures dominated by fungal enzymes. Only a few studies have reported on exo/exo and exo/endo synergy between fungal and bacterial enzymes (Baker et al. 1998, 1995). Gao et al. (2010) reported the use of a hybrid mixture of fungal cellulases and bacterial hemicellulases, which effectively maximized the saccharification of AFEX-treated corn stover, resulting in 95 % glucan and 65 % xylan conversion at pH 4.5 and 50 °C. The synergistic action of a diverse set of accessory hemicellulases from different bacterial sources and core fungal cellulases resulted in high glucose (80 %) and xylose (70 %) yields with moderate enzyme loadings (∼20 mg protein/g glucan) compared to commercial enzymes (Gao et al. 2011).

Models for improving synergy between fungal and bacterial glycosyl hydrolases

The quest for optimizing new enzyme cocktails for enhanced saccharification and fermentation of lignocellulosic biomass has to continuously evolve and, due to the structural complexity of raw substrates, different types of pre-treatment strategies and the economic constraints associated with commercialization, novel strategies have to be implemented to supplement and improve the existing commercial cellulase-hemicellulase cocktails. In this review we briefly focus on some models that could be used to exploit and implement synergistic interactions between fungal and bacterial enzymes (Fig. 1).

Designer cellulosomes

Cellulosomes are high molecular weight extracellular complexes secreted by anaerobic microorganisms consisting of scaffoldins and cellulosomal enzymes that are capable of degrading plant cell walls (Doi and Kosugi 2004). We have discussed cellulosomes in our previous section and will therefore focus on designer cellulosomes. The concept of designer cellulosomes involves the preparation of chimeric scaffoldins with specific dockerin-binding capacities by using cohesins from various scaffoldins (Doi and Kosugi 2004). The construction of divergent cohesins and matching dockerin bearing enzymes enable specific recognition and binding of cohesins and dockerins, allowing for greater control over the spatial distribution and rearrangement of the desired enzymes in designer cellulosomes (Bayer et al. 2007). Fierobe et al. (2002, 2005) developed a comprehensive set of bi-functional and tri-functional chimera cellulosomes using divergent cohesin and dockerin tagged cellulases from *C. cellulolyticum* and *C. thermocellum*. Arfia et al. (2014) demonstrated the promotion of cellulose degradation through the integration of bacterial lytic polysaccharide monooxygenases into designer cellulosomes bearing *Thermomonospora fusca* cellulases. The cohesion and dockerin moieties were derived from *A. cellulolyticus*, *C. thermocellum* and *B. cellulovens*.

In the first study of its kind, Mingardon et al. (2007) demonstrated the successful engineering of a non-

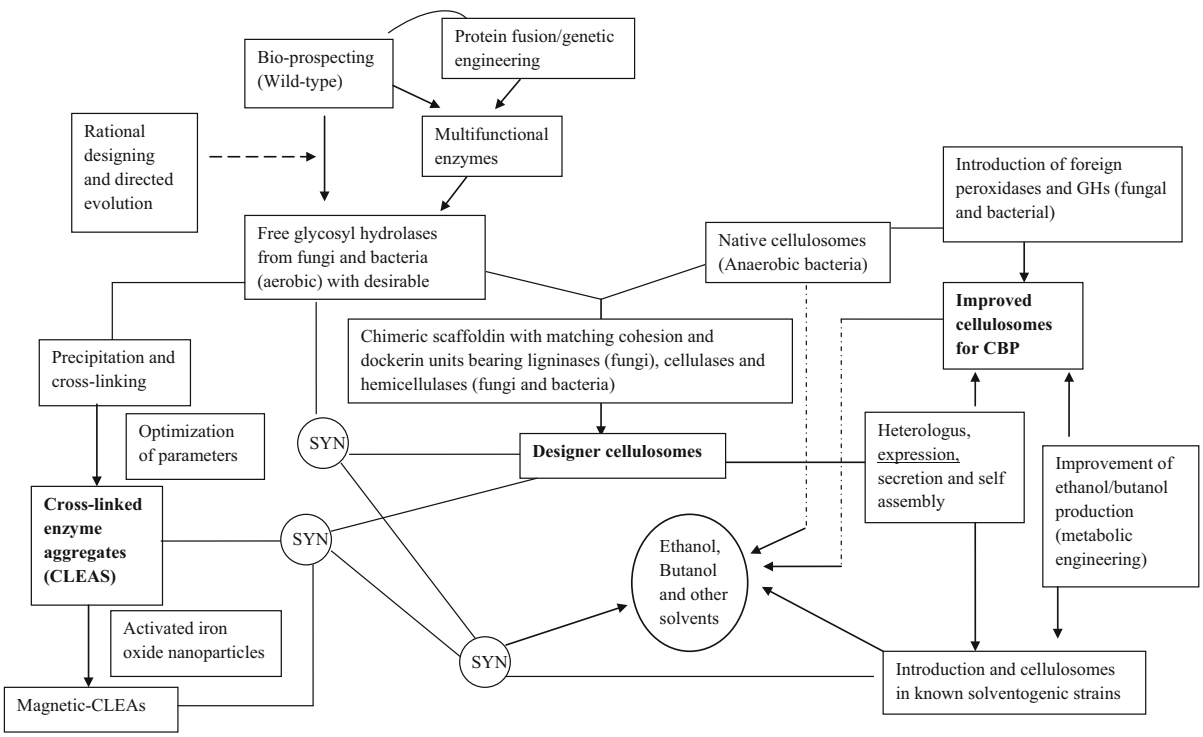

Fig. 1 A synergistic model involving fugal and bacterial glycosyl hydrolases for enhanced saccharification and fermentation of lignocellulosic biomass

cellulosomal *Neocallimastix patriciarum* (fungi) cellulase (Cel6A) using a bacterial dockerin and its subsequent incorporation into a mini-cellulosome with bacterial family 9 endoglucanases from *C. cellulolyticum* exhibiting enhanced activity on crystalline cellulose. Based on this study, elaborate designer cellulosomes containing family 6 and family 7 cellobiohydrolases from *T. reseei*, family 9 endoglucanase (CelF) from *C. thermocellum,* xylanase/acetyl xylan esterase (Xyn A) from *T. fusca* and Man A from *C. thermocellum*/*C. cellulolyticum* can be engineered using chimeric scaffoldin bearing specific cohesion-dockerin modules. Cellulolytic enzymes from the thermophilic fungi, *Thermoascus aurantiacus* and *Penicillium citrinum*, cellulases and hemicellulases from bacterial and archeal species across different genera, such as *Bacillus, Thermobacillus, Clostridium, Fervidobacterium, Rhodothermus, Thermoplasma, Thermotoga, Pyrococcus*, *Sulfolobus*, *Thermococcus* and *Desulfurococcus,* provide a vast pool of extremophilic cellulases and hemicellulases that can be engineered into designer cellulosomes.

Improving the saccharolytic machinery of cellulosomal bacteria

The cellulosomes of cellulolytic bacteria can be redesigned to improve their efficiency for consolidated bioprocessing (CBP). *C. thermocellum* with its anaerobic nature and tolerance to high temperature has been proposed as a suitable candidate for CBP. The synergistic degradation achieved by the cellulosome of *C. thermocellum* (due to the presence of the different enzymes) results in the formation of large concentrations of the major soluble disaccharide end product cellobiose.

Like other bacterial and fungal cellulase systems, the multienzyme cellulosome system of *Clostridium thermocellum* is strongly inhibited by the major end product cellobiose. The inhibition of *C. thermocellum* cellulosomal cellulases in the presence of cellobiose, and the inhibition of cell growth and metabolism by toxic end products such as lactic acid and acetic acid could be solved by heterogeneous expression or redesign of the cellulosome with β-glucosidases from *Aspergillus niger* and gene knockouts of lactic and acetic acid encoding genes, respectively (Maki et al. 2009). In a recent study, a chimeric cohesin-fused β-glucosidase (BglA-CohII) was successfully merged to an unoccupied dockerin on the scaffoldin subunit of the *Clostridium thermocellum* cellulosome (Gefen et al. 2012). The fusion of a cellulosome and BglA-CohII resulted in an increased hydrolysis of microcrystalline cellulose and pre-treated switch grass compared to the native cellulosome alone or in combination with wild-type BglA in solution.

Similarly, chimeric cohesins fused with fungal cellobiohydrolases like GH7 CBH-I and GH6 CBH-II can be merged to an unoccupied dockerin on the scaffoldin subunit. The opposing specificities render GH7 and 6 with a high degree of synergistic action on crystalline cellulose that could enhance the hydrolytic efficiency of the C. *thermocellum* cellulosome. The degradation of lignin is imperative for the commercial success of biomass conversion, because it not only reduces the cellulase/hemicellulase adsorption on lignin, but also increases the accessibility of cellulolytic enzymes for cellulose. White rot and brown rot fungi secrete significant levels of oxidoreductase enzymes, whose co-presence with hydrolytic enzymes could move the bioconversion of lignocellulose biomass to a new level. The incorporation of peroxidases and laccases on the unoccupied dockerin modules could result in a highly versatile *C. thermocellum* with the ability to produce ethanol from different lignocellulosic substrates (Fig. 1).

Expression of cellulolytic ability in fermentative microbes

The current cost of biomass conversion technologies can be significantly reduced by the use of organisms that hydrolyze the cellulose and hemicellulose in biomass and produce a valuable product such as ethanol at a high rate and titer. The engineering of non-cellulolytic organisms with high product yields so that they express a heterologous cellulase system able to utilize cellulose is identified as a recombinant cellulolytic strategy. A number of bacterial (*E. coli*, *Zymomonas abilis*, *Klebsiella oxytoca*) and yeast (*Sacch. cerevisiae*, *Pichia pastoris, P. stipis*) strains have been employed for this purpose and have been well reviewed (Elkins et al. 2010; La Grange et al. 2010). Our focus is on fungal and bacterial synergy models. The *cel5* E endoglucanase gene and *bgl3A* β-glucosidase gene from *C. thermocellum* and *Saccharomycopsis fibuligera,* respectively, were expressed in a *Sacch. cerevisiae* strain, resulting in higher

endoglucanase activity with improved conversion of PASC to ethanol (Jeon et al. 2009). Wen et al. (2010) used the *Sacch. cerevisiae* α-agglutinin anchor to tether the chimeric scaffoldin containing three *C. thermocellum* cohesins, as well as the *C. thermocellum* CBD to the yeast cell surface. *C. thermocellum* dockerins were added to *T. reesei* Cel5A (EGII) and Cel6A (CBHII) and a β-glucosidase from *Asp. aculeatus*. Proximity as well as enzyme–enzyme synergy was observed between the fungal and bacterial enzymes. The genomic sequence of *P. stipitis* shows numerous lignocellulolytic genes, including xylanase, endo-1,4-β-glucanase, exo-1, 3-β-glucosidase, β-mannosidase, and α-glucosidase. The ability of *P. stipitis* was enhanced by the addition of *C. thermocellum* endoglucanase-encoding gene *celD* (Piotek et al. 1998) and co-expression of fungal xylanase and xylosidase-encoding genes (Den Haan and Van Zyl 2003).

There are a number of strains such as *E. coli*, *K. oxytoca C. acetobutylicum*, *K. marxianus*, *H. polymorpha* and many others with fermentative ability. *C. acetobutylicum* has generated special interest as strains belonging to *C. acetobutylicum* have been used for the large scale production of acetone and butanol; it expresses multi-complex structures on its surface but lacks hydrolytic activity towards crystalline cellulose. Glycoside hydrolases (GHs, Cel8A, Cel9B, Cel48S and Cel9K) and a range of synthetic genes encoding *C. thermocellum* cellulosomal scaffoldin proteins (CipA variants), as well as synthetic cellulosomal operons that direct the synthesis of Cel8A, Cel9B and a truncated form of CipA, were integrated into the *C. acetobutylicum* genome using recently developed Allele-Coupled Exchange (ACE technology) (Kovacs et al. 2013). The successful expression, secretion and self-assembly of the heterologous cellulosome by *C. acetobutylicum* provides a fantastic opportunity to introduce CBH-I, CBH-II and β-glucosidase from *T. reesei* and *A. niger,* thereby enhancing the cellulolytic efficiency of this strain (Fig. 1).

Cross-linked enzyme aggregates

Cellulosomes have a distinct advantage over free enzymes due to their close proximity, high processivity and enhanced synergy resulting from efficient substrate channeling. However, they suffer from certain limitations that arise because of the technical challenges that accompany the successful engineering of chimeric scaffoldins and/or fusion of new enzymes into existing cellulosomes. The large size of cellulosomes may limit their movement on un-treated raw substrates as they may not be able to access the cellulose microfibrils due to the presence of lignin and non-specific adsorption on lignin. Such inactivation may have a greater impact on cellulosomes compared to free enzymes, as their high molecular weight implies that for equal loadings on mass basis, cellulosomes would be present in much lower molar concentrations, resulting in substantial loss of activity.

Considering the fact that economic feasibility of the process is imperative for commercialization of biological conversion of biomass, a model approach that includes the advantages of cellulosomes but reduces their inadvertent disadvantages can be achieved using free enzymes through the application of cross-linked enzyme aggregates (CLEAs) (Fig. 1). The preparation of combi-CLEAs involves the physical aggregation of different combinations of enzymes using precipitants followed by chemical cross-linking (cross-linking agents) (Dalal et al. 2007; Bhattacharya et al. 2013). Considering a model combi-CLEAs preparation containing a thermo-stable and processive GH 6 and 48 cellobiohydrolase from fungi and bacteria, respectively—a thermostable endoglucanase and β-glucosidase from fungi along with highly stable hemicellulase (xylanase–xylosidase, mannanase–mannosidase and de-branching enzymes) cocktail from thermophilic bacterial strains, could include all the desirable traits and allow the preparation of defined substrate based cocktails with high economic viability. The process control and the operation stability can be improved even further by the use of magnetic-CLEAs (Talekar et al. 2013; Bhattacharya and Pletschke 2014). Combi-CLEAs therefore provide an excellent option of harnessing the synergistic advantages of multi-enzyme complexes and circumventing the technical and steric hindrances associated with cellulosomes.

Future prospects

The effective de-polymerization of lignocellulosic biomass holds the key towards a greener future. In order to overcome the recalcitrance of the biomass, novel approaches have to be pursued. Due to the complexity of the substrates and the prevalence of

different pre-treatment strategies, commercialization of lignocellulolytic enzymes is possible if effective sugar release is achieved under low protein loadings and with very high fermentative yields. Therefore, equal importance has to be given towards the strategic improvement of both saccharification and fermentation. Enzyme cocktails prepared based on the synergistic interaction of fungal and bacterial glycosyl hydrolases could benefit from the huge gene pool of cellulases and hemicellulases—not only on the basis of the enormous diversity that this would offer but also in bringing together the cutting edge research and development strategies in each of these separate fields. Advances in molecular techniques like directed evolution, site-directed mutagenesis, rational designing, gene fusion, DNA shuffling and other techniques have to be extended for improving the properties of the free enzymes. The application of multiple enzyme complexes and designer cellulosomes in conjunction with free enzymes has to be encouraged to create novel substrate-based defined enzyme cocktails. Improving the fermentative efficiency of cellulosomal strains and/or expression, assembly and secretion of cellulosomes in non-cellulolytic solventogenic strains will hold the key towards the validation of a successful CBP approach (Fig. 1). However, Nature may still hold the key for delivering novel enzymes. Exploring new habitats, genome analysis, data mining and proteomic analysis of secretomes (fungal and bacterial) could open the gates for novel enzymes and unravel the mysteries of enzyme interaction and synergism. It is important for us to understand that the best hydrolysis of lignocellulose occurs under natural conditions and that our aim should be to replicate those conditions which will maximize our goal towards the economically feasible production of biofuels.

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