A Mini-review on the Applications of Cellulose-Binding Domains in Lignocellulosic Material Utilizations

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This manuscript provides a mini review on the fundamentals of cellulose binding domains (CBDs) or cellulose binding modules (CBMs) and their applications using lignocellulosic materials. CBDs, the non-productive part of cellulases, have miscellaneous biological functions and have been widely applied in lignocellulose hydrolysis, protein engineering, structural support, metabolism, energy storage, antibiosis, immunological recognition, targeting, attachment, *etc.* due to their specific affinity to various substrates of lignocelluloses. Understanding of the properties and mechanisms of CBDs is of vital significance because it provides the basis for fine manipulation of cellulose-CBM interactions and eventually improves the bioconversion performance of lignocelluloses into fuels and desired chemicals. In this short review, the fundamentals of CBD, the definition of CBM family, and the structures of different CBM families are introduced. Then recent findings in the applications of CBDs are discussed relative to the lignocelllulosic utilizations.

Keywords: Cellulose-binding domain; Cellulase; Cellulose; Lignocellulose; Utilization

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INTRODUCTION

Sustainable and renewable fuels and materials have received increased attention recently due to concerns about the environmental and unsustainable issues associated with fossil fuels and materials. Lignocellulosic biomass is a major alternative and renewable resource for the production of fuels and chemicals through bioconversion (Nimlos *et al.* 2007). Cellulose, the major component of lignocellulosic biomass and the most abundant biopolymer in this biosphere, can be degraded by enzymes, *i.e.* cellulases, into oligosaccharides and finally monosaccharides, which are then subjected to fermentation into fuels and other chemicals (Pauly and Keegstra 2008). The enzymatic hydrolysis of cellulose is a very complex process, which involves the interaction of enzymes, substrates, and enzymatic environment (Yoshida *et al.* 2008; Zhu *et al.* 2008; Hall *et al.* 2010). In order to complete hydrolysis of cellulose, at least three different enzymes act in a synergistic manner (Lynd *et al.* 2002). These cellulases include exo-β-(1,4)-D-glucanases or cellobiohydrolases (CBHs), endo-β-(1,4)-glucanases (EGs), and β-D-glucosidases. CBHs hydrolyze cellulose chains from the ends to produce mainly soluble cellobiose. EGs hydrolyze internal nicks of the cellulosic chains thereby adding new chain ends for CBHs to act on. To complete the conversion, β-D-glucosidases hydrolyze the soluble disaccharide cellobiose to monomeric glucose (Rabinovich *et al.* 2002b).

A typical cellulolytic enzyme consists of a distinct cellulose binding module or cellulose binding domain (CBM and CBD are interchangeable thereafter) and at least one of several distinct catalytic domains (CDs), which are structurally and functionally discrete units of proteins and are connected by an inter-domain linker peptide (Mattinen *et al.* 1997). CBMs play a very important role in the degradation of cellulose crystalline regions since they can concentrate the enzymes on the insoluble cellulosic surface (Boraston *et al.* 2004; Guillen *et al.* 2010; Herve *et al.* 2010). The specific interactions between CBM and cellulose have been taken advantage of extensively in recent years relative to their applications in biology, material, enzyme purification, and other fields. In regard to the advances of fundamental research in CBMs, there are some good review papers available (Linder and Teeri 1997; Levy and Shoseyov 2002; Boraston *et al.* 2004). Therefore, this mini-review will concentrate on the applications of CBDs in the lignocellulosic utilization, which have been achieved in recent years. A brief description of fundamentals of CBDs will be given in the beginning as well.

FUNDAMENTALS OF CELLULOSE BINDING DOMAINS

Typical cellulolytic enzymes contain a CD and one or more CBMs; these are connected by an inter-domain linker peptide (Mattinen *et al.* 1997). Only cellulases originating from a few microorganisms and higher plants have no such domains (Herve *et al.* 2010). According to the amino acid sequence similarity and 3D structure of adsorption module, the different types of cellulases from different sources have been classified into families (Rabinovich *et al.* 2002a). In the latest update of the CAZY database, CBMs are grouped into 71 families, which show notable variations in substrate specificity and show miscellaneous biological functions (http://www.cazy.org/ CBM1_all.html). That database includes all the adsorption modules of carbohydrate binding modules. That is to say, the CBMs listed in the CAZy data not only contain cellulose binding domains, but also include some starch or other polysaccharides binding domains. The relative molecular weight of CBM is between 0.4×10^4 and 2.0×10^4 Daltons (Levy and Shoseyov 2002). Most CBDs have β-sandwich structure and have different number of residues, while some families have β-trefoil, hevein, unique, cysteine knot, β-barrel, and lectin-like folds (Boraston *et al.* 2004; Cantarel *et al.* 2009). Three-dimensional structures of CBM and the protein data bank (PDB) code of main proteins have been summarized in the article by Boraston *et al.* (2004) For example, CBM1, the CBM from family 1, which is found almost exclusively in fungi, consists of 36 amino acid residues, and it is found either at the N-terminal or at the C-terminal extremity of the enzymes (Linder and Teeri 1997). CBMs 2 ~ 6 are mainly found in bacteria.

The structure of CBM1, especially the CBM from CBH I of *Trichoderma reesei*, has been investigated thoroughly. Its NMR structure, as shown in Fig. 1, shows a contiguous independently wedge-shaped structure. One face of the wedge contains three highly conserved aromatic amino acids Y5, Y31, and Y32, which are involved in cellulose recognition and binding. There are two more polar residues (Q7 and N29) that can potentially form hydrogen bonds to cellulose (Beckham *et al.* 2010). This wedge-shaped structure is likely to be shared by all fungal CBDs (Mattinen *et al.* 1997). The specificity of CBMs can be changed through simple mutations (von Schantz *et al.* 2014). It was suggested that tryptophan residues contribute to higher binding affinity than tyrosine residues. Trp→Tyr mutation would decrease the affinity and increase the off-rate

of Cel6A CBM1 on crystalline cellulose (Lehtio *et al.* 2003). However, this effect took place only in the case of one Trp residue mutation. Two Trp residues' mutation was found to yield no further improvement in binding.

Functions of CBMs relative to cellulosic materials embody mainly three aspects (Boraston *et al.* 2004): (i) proximity effects: to increase accessibility of enzyme and insoluble substrates. Proximity effects have been confirmed by multiple mutation studies (Linder *et al.* 1995; Reinikainen *et al.* 1992). (ii) substrate targeting: to target specific carbohydrates in complex structures of plant cell walls, such as CBMs from xylanases or mannanases, which have been shown on large crystals of cellulose with immuno-gold-labeled CBMs (Lehtio *et al.* 2003). (iii) nonhydrolytic cellulose disruption: to disrupt cellulose regular rigid supramolecular structures. Some bacterial CBMs have indirectly confirmed this function (Kataeva *et al.* 2002), such as family 1 CBD and family 2 (Tomme *et al.* 1995).

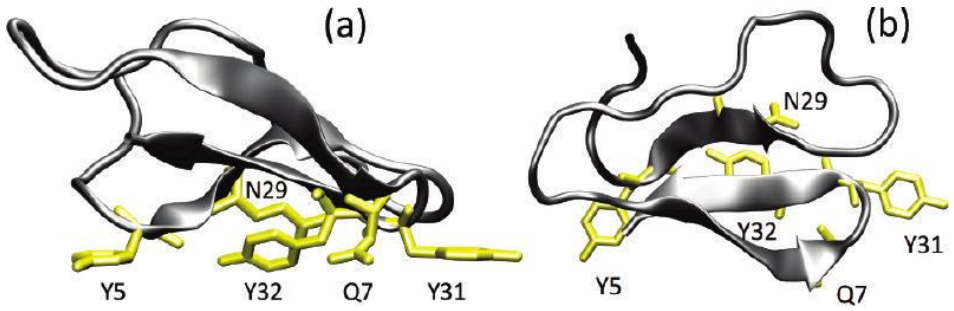

Fig. 1. Carbohydrate-binding module from *T. reesei* Cel7A with Y5, Q7, N29, Y31, and Y32 shown. (a) Side view. (b) Top view. Reprinted from (Beckham *et al.* 2010) with permission from American Chemical Society.

CBM-cellulose binding involves both hydrophobic interactions and polar interaction. But there is not enough evidence to prove that CBM can modify cellulose substrates. The VDW interactions (McGuffee and Elcock 2006; McGuffee and Elcock 2010; Mereghetti and Wade 2011) play a more important role in stabilizing the CBM-fiber binding, whereas CBM and fiber can form a number of hydrogen bonds because of electrostatic interactions (Elcock 2004; Alekozai *et al.* 2013), which may explain why the diffusion of the CBM is hindered on the fiber surfaces.

CBMs have a high specificity and functional diversity. They come from different sources, and their functions are diverse from each other. It was established that the binding sites of families 1, 2, and 3 CBDs are adapted to bind to a surface, while family 4 CBD binds to a single molecule (Shoseyov *et al.* 2006). For instance, CBM2, similar to CBM1 from fungi, can bind with the bacterial microcrystalline cellulose and partially crystalline phosphate expanding fibers (PASC) (Rabinovich *et al.* 2002b); whereas CBM4, with an obvious coupling groove structure as shown in Fig. 2, cannot bind bacterial microcrystalline cellulose, but it can combine with the amorphous region of PASC (Jervis *et al.* 1997; McLean *et al.* 2002). In addition, CBM adsorption and desorption involving cotton cellulose allomorphs (I, II, and III) has been investigated (Ciolacu *et al.* 2014). The results indicate that CBM1 has the highest adsorption capacity on cellulose I. There are some CBDs that have two or more multi-specific CBMs with similar sequences. For example, Xylanase 10A from *Rhodothermus marinus* has two CBMs, *i.e.* CBM4-1 and CBM4-2 (Abou-Hachem *et al.* 2003), though the cited article does not specify whether they have the same functions. Another enzyme, Cel9B from

Paenibacillus barcinonensis, is composed of the two cellulose-binding domains CBM3b and CBM3c. Chiriac *et al.* (2010) demonstrated that CBM3b and CBM3c have different functions; CBM3b shows the ability to bind to cellulose while CBM3c does not.

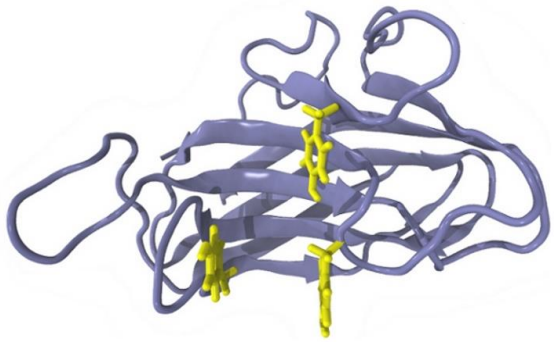

Fig. 2. A cleft structure of the hydrophobic residues (yellow) of carbohydrate-binding module family 4. Reproduced with permission from (Prates *et al.* 2013).

APPLICATIONS OF CBDs IN LIGNOCELLULOSIC UTILIZATIONS

CBDs' natural function is involved in some biological processes such as structural support, metabolism, energy storage, antibiosis, immunological recognition, targeting, and attachment, *etc.* (Guillen *et al.* 2010).

CBDs also can play an important role in other applications, such us the fusion of CBM and other proteins to produce complexes that offer the possibility of developing paper-based diagnostic tools (Kong and Hu 2012) for infectious diseases (Rosa *et al.* 2014). Such structures can target immobilization of antibodies (Cao *et al.* 2007; Hussack *et al.* 2009), proteins (Ofir *et al.* 2005), bacteriophages (Tolba *et al.* 2008), and bacteria (Wang *et al.* 2002) onto celluloses. It is possible even to detect multiple targets.

CBD can also be employed in the utilization of lignocellulosic biomass. During the application of lignocelluloses, enzymes are usually used to break down or modify a cellulose surface for improved properties. In order to lower the cost of enzymes, it is necessary to modify the enzymes for improved performance. Due to the specific binding between CBD and cellulose, it is rational to modify the CBDs of enzymes. By this means one can fine-tune the interactions between CBD and substrate cellulose for better performance. In this review, these applications are grouped according to the methodology by which they are used and the type of CBMs (CBM family) they employ.

Applications of CBDs Solely

Even though there are a number of applications of CBDs for bioconversion in the literature, there are only a few reports on the applications of CBDs solely, *i.e.* using only the CBD portion of enzyme and without the CD segment involved. Hall *et al.* (2011a) utilized the CBDs of cellulases from *T. reesei* to pretreat biomass prior to enzymatic hydrolysis. CBDs of cellulases from *T. reesei* had a noticeable impact on cellulose structure upon incubation. The crystallinity of different types of cellulose (microcrystalline and fibrous) was reduced after pretreatment with CBDs, rendering the substrates less recalcitrant to enzymatic hydrolysis, and an increase up to 25% in glucose concentration during hydrolysis was obtained. In addition, the bleached chemical pulp properties, viscosity and strength after PFI refining, can be equally affected by the presence of CBD in intact cellulases (Suurnäkki *et al.* 2000).

Mello and Polikarpov (2014) cloned, expressed, and purified the CBM from CBH I from *Trichoderma harzianum*. Then it was used, in combination with either a commercial cellulase preparation, *T. reesei* CBH I, or its separate catalytic domain, to hydrolyze filter paper. In all cases the amount of glucose released was increased, reaching up to 30% gain when the carbohydrate-binding module was added to the reaction.

In another report (Kitaoka and Tanaka 2001), CBDs was added into the pulp with anionic polyacrylamides (A-PAM). Wet strength and dry tensile strengths of the paper sheets were significantly improved. Yokota *et al.* (2009) went further and grafted CBMs derived from *T. viride* and *T. reesei*, and *Thermomyces lanuginosus*, respectively, into anionic polyacrylamide *via* a peptide condensation reaction. The prepared CBM-conjugated A-PAMs displayed different retention behaviors, depending on the kind of pulp substrates, *i.e.* hardwood and softwood fibers.

Applications of Proteins with Fused CBDs

Fusion of one or more CBDs to a neutral enzyme

A neutral enzyme, which has low activity in its original state, can be enhanced significantly in its performance by fusion one or more CBDs into the neutral enzyme. For instance, Cel5A, an endoglucanase (EG), contains a single catalytic domain but no distinct cellulose-binding domain. It has been reconstructed by attaching one or two CBDs for enhanced performance by Tang *et al.* (2014). They reconstructed it by fusion of a CBM containing a linker from *Corticium rolfsii*. Results showed that the degradation of cellulose by redesigned EG was about double that by original enzyme; while the optimal pH and temperature, pH stability, and heat stability of redesigned EG were similar to the original one. Telke *et al.* (2013) fused a family 6 CBM from *Saccharophagus degradans* to the C-terminus of a mutant of Cel5A, which resulted in a 7-fold increase in thermostability than the native Cel5A. The final fusion protein showed much higher affinity and 7-fold higher hydrolytic activity than the native Cel5A on Avicel and filter paper.

Due to the specific adsorption of CBD to cellulose substrates, an efficient purification process has been developed for some proteins by tagging a CBD. This scalable method can purify the CBM-tag protein by using a simple unit operation, involving centrifugation or filtration. For example, the cathelicidin-derived human peptide LL37, which has a broad spectrum of antimicrobial and immunomodulatory activities, was cloned to the N- and C-termini of a CBM3 fused to the linker sequence (LK-CBM3) from *Clostridium thermocellum* (Ramos *et al.* 2010). In the course of processing, the purification was achieved using cellulose CF11 fibers, taking advantage of the CBM3's specific affinity for cellulose. To improve the performance of cellulose substrate in the CBM-tagged protein purification process, Hong *et al.* (2008) developed an ultra-high capacity cellulosic adsorbent regenerated amorphous cellulose (RAC), with a binding capacity of up to 365 mg protein per gram of RAC, enabling it to efficiently capture (dilute) proteins.

Fusion of one more CBD to an enzyme for improved activity

Due to the specific affinity between CBD and cellulose, one or more CBDs can be fused to an enzyme that has its own CBDs for improved affinity and other properties.

The family 1 cellulose-binding module (CBM1) from fungi is the most extensively investigated CBM. It has been fused with glucose-tolerant β-glucosidase

(BGL) (Zhao *et al.* 2013), CSCMCase (Thongekkaew *et al.* 2013), endo-1,4-mannanases (Tuan Anh *et al.* 2010), and laccase (Ravalason *et al.* 2009), for improved properties. Using the BGL-CBD as the catalyst, the yield of glucose reached a maximum of 90% from 100 g/L cellobiose, and the BGL-CBD could retain over 85% activity after five batches with the yield of glucose all above 70%. The performance of the BGL-CBD on microcrystalline cellulose was also studied; the yield of the glucose was increased from 47% to 58% by adding the BGL-CBD to the cellulase, instead of adding the Novozyme 188. Compared with CSCMCase, the recombinant fusion enzymes had acquired an increased binding affinity to insoluble cellulose and the cellulolytic activity toward insoluble cellulosic substrates (SIGMACELL® and Avicel) was higher than that of the native enzyme. The fused mannanase–CBM enzyme was more thermostable, and showed that mannanase–CBM improved the glucose yield compared to wild-type and recombinant mannanases for softwood hydrolysis when it was used in association with a *T. reesei* enzymatic cocktail. Laccase-CBM was able to bind to a cellulosic substrate and, to a greater extent, to softwood kraft pulp. Laccase-CBM was further investigated for its softwood kraft pulp bio-bleaching potential and compared with the *P. cinnabarinus* laccase. Addition of a CBM was shown to greatly improve the delignification capabilities of the laccase in the presence of 1-hydroxybenzotriazole (HBT). In addition, ClO_2 reduction using 5U of chimeric enzyme per gram of pulp was almost double than that observed using 20U of *P. cinnabarinus* laccase per gram of pulp.

A novel gene encoding a swollenin-like protein, POSWOI, was isolated from the filamentous fungus *Penicillium oxalicum* by Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Kang *et al.* 2013). It consisted of a CBM1 followed by a linker connected to a family 45 endoglucanase-like domain. Using the CBH I promoter, recombinant POSWOI was efficiently produced in *T. reesei* with a yield of 105 mg/L, and it showed significant disruptive activity on crystalline cellulose. Simultaneous reaction with both POSWOI and cellulases enhanced the hydrolysis of crystalline cellulose Avicel by approximately 50%. Using a POSWOI-pretreatment procedure, cellulases can produce nearly twice as many reducing sugars as without pretreatment.

A two-CBM1 strategy was also used to construct recombinants for improved performance. Linder *et al.* (1996) constructed a recombinant by fusing the CBDs of two *T. reesei* cellobiohydrolases via a linker peptide similar to the natural cellulase linkers. The double CBDs exhibited much higher affinity on cellulose than either of the single CBD, indicating an interplay between the two components. In another report, Gundllapalli *et al.* (2007) claimed that they linked the β-glucosidase enzyme (BGL) from *Saccharomycopsis fibuligera* to the CBM1 of CBH II in *T. reesei* with single and double copies of CBD fused at the N-terminus of BGL. The results indicated that the recombinant enzymes of BGL-CBD displayed a 2- to 4-fold increase in their hydrolytic activity toward cellulosic substrates such as Avicel, amorphous cellulose, bacterial microcrystalline cellulose, and carboxymethyl cellulose in comparison with the native enzyme.

CBMs from other families are also employed to construct new proteins. The family 2 cellulose-binding module (CBM2) of xylanase CflXyn11A from *Cellulomonas flavigena* was fused to cellulase Cel7B from *T. reesei*, which has a CBM1 (Pavon-Orozco *et al.* 2012). The results showed that there is a high degree of synergy (6.3) between CflXyn11A and TrCel7B in hydrolysis of sugar cane bagasse after 12 h in the equimolar mixture.

The two CBDs of family 3 (CBM3) from *Clostridium cellulovorans* were fused together by Levy *et al.* (2002) to produce a cellulose cross-linked protein (CCP), which

was found to improve the filter performance of paper. A very interesting aspect is that the single CBD protein can also improve the mechanical properties of paper, but the protein with double CBM3 yielded very poor results. This may be explained by the effect of linker length. Maybe in this CCP, the distance between two CBDs is too short and the two CBDs cannot fully develop their binding ability. Cel9B from *Paenibacillus barcinonensis* is a modular endoglucanase with a novel molecular architecture among family 9 enzymes that comprises a catalytic domain (GH9), a family 3c cellulose-binding domain (CBM3c), a fibronectin III-like domain repeat (Fn31,2), and a C-terminal family 3b cellulose-binding domain (CBM3b). Chiriac *et al*. (2010) demonstrated that CBM3b and CMB3c have different functions. Deletion of CBM3c produced a notable reduction in hydrolytic activity, while it did not affect the cellulose-binding properties, as CBM3c did not show the ability to bind to cellulose. On the contrary, CBM3b exhibited binding to cellulose. Cadena *et al.* (2010) used this Cel9B as a highly efficient biocatalyst in pulp refining to reduce the associated energy. In their work, they found that the truncated form containing the catalytic domain (GH9-CBD3c) has a strong effect on fiber morphology. Comparing its effect with that of the whole cellulase (Cel9B), the truncated enzyme contributed to increasing paper strength through improved tensile strength, burst strength, and tear resistance. Therefore, the catalytic domain of Cel9B has biorefining action on pulp. Although cellulose binding domains (CBDs) are less efficient toward pulp refining, evidence obtained in their work suggested that CBD3b alters fiber surfaces and influences paper properties as a result.

The family 6 carbohydrate-binding module (CBM6) was used to fuse with xylanase (Qiao *et al.* 2014) to increase the ability to digest hemicelluloses. Bae *et al*. (2003) went further, fusing endoglucanase Cel5 of *Ruminococcus albus* at the C-terminus with the single-cellulose binding domain (CBM6) of *Clostridium stercorarium* xylanase A. The recombinant Cel5-CBM6 improved the affinity of enzyme to the insoluble substrate cellulose.

The family 22 of carbohydrate binding module (CBM22) binds xylan but not to cellulose. Xylanase A of *Thermotoga neapolitana* contains binding domains both at the N- and C-terminal ends of the catalytic domain. In the N-terminal position it contains two CBMs that belong to family 22. Mamo *et al.* (2007) took advantage of this type CBM and fused to an alkaline active GH10 xylanase from *Bacillus halodurans* S7 and expressed in *Escherichia coli*. The fusion of the CBM structures enhanced the hydrolytic efficiency of the xylanase against insoluble xylan, but it decreased the stability of the enzyme. Meanwhile, the optimum temperature and pH for the activity of the xylanase did not change.

Carbohydrate binding modules (CBMs) with specific affinities for crystalline (CBM2a) or amorphous (CBM44) cellulose were used to track specific changes in the surface morphology of cotton fibers during amorphogenesis (Gourlay *et al.* 2012). The extents of phosphoric acid-induced and swollenin-induced changes to cellulose accessibility were successfully quantified using this technique. The adsorption of substructure-specific CBMs can be used to accurately quantify the extent of changes to cellulose accessibility induced by non-hydrolytic disruptive proteins. The technique provided a quick and quantitative measure of the accessibility of cellulosic substrates.

CBD not only exhibits specific adsorption to cellulose substrate, but also shows evidence of acting as a thermostabilizing domain for enzymes. The CBD of cellobiohydrolase Cel7A from *T. reesei* melted at 65 to 66 ^{o}C, the catalytic domain obtained via papain-catalyzed proteolysis was shown to denature at 51 ^{o}C, while the

whole enzyme displayed a melting point of 59 ^{o}C (Hall *et al.* 2011b). Its thermostability has also been demonstrated in xylanase 2 from *T. reesei* (He *et al.* 2009) .

OUTLOOK

Lignocellulose, which has been the main raw material for many commodities closely related to people's life for centuries, is one of the richest sources for renewable fuels and chemicals for future. How to utilize it efficiently and effectively is an issue of great importance. Enzymes can catalyze the reactions associated with lignocellulose and its components of cellulose, hemicellulose, and lignin efficiently and effectively. Due to the specific affinity between CBD and cellulose, as well as the matrix structure of lignocellulose, CBDs adsorbed on cellulose surface concentrate the enzymes on the insoluble materials and therefore can improve the performance of not only cellulases, but also mananases, xylanase, laccases, and other enzymes. CBD can be fused to a neutral enzyme without a CBD or to a typical enzyme, which has its own CBD for improved affinity and activity. It has been fused to cellulases, mananases, xylanase, laccases, and other enzymes to promote the degradation of the components of lignocellulose for biofuels and biochemicals. Therefore, understanding of CBMs properties and the mechanisms in ligand binding is vital to the comprehensive utilization of lignocellulose.

Due to the versatile functions of CBMs, there is a large pool of CBMs available for potential utilization. This offers numerous choices to improve the performance of enzymes by fusion of one or more CBMs to an existing enzyme to fine-tune its affinity to the substrate and therefore to manipulate its performance as desired. As a consequence of the activity improvement, the consumption and the cost of enzymes can be reduced dramatically. So far, the most extensively studied CBM is the CBM from family 1, and some scientific reports and commercial applications are related to family 3 CBM, but only a few applications have involved the CBMs from other families. The interactions between CBMs and cellulose is not fully understood yet, especially the CBMs from other families.

Better understanding of CBMs properties and the mechanisms in ligand binding will promote development of new cellulose-recognition technologies and provide the basis for fine manipulation of the CBM-cellulose interactions. Such efforts will benefit the utilization of lignocellulose materials eventually.

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