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CELLULASES: CHARACTERISTICS, SOURCES, PRODUCTION, AND APPLICATIONS

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8.1 INTRODUCTION

Cellulose is the most abundant renewable biological resource and a low-cost energy source based on energy content ($3–4/GJ) (Lynd et al., 2008; Zhang, 2009). The production of bio-based products and bioenergy from less costly renewable lignocellulosic materials would bring benefits to the local economy, environment, and national energy security (Zhang, 2008).

High costs of cellulases are one of the largest obstacles for commercialization of biomass biorefineries because a large amount of cellulase is consumed for biomass saccharification, for example, ~100 g enzymes per gallon of cellulosic ethanol produced (Zhang et al., 2006b; Zhu et al., 2009). In order to decrease cellulase use, increase volumetric productivity, and reduce capital investment, consolidated bioprocessing (CBP) has been proposed by integrating cellulase production, cellulose hydrolysis, and ethanol fermentation in a single step (Lynd et al., 2002, 2008).

Cellulases are the enzymes that hydrolyze β-1,4 linkages in cellulose chains. They are produced by fungi, bacteria, protozoans, plants, and animals. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures (Henrissat, 1991). Cellulases contain noncatalytic carbohydrate-binding modules (CBMs) and/or other functionally known or unknown modules, which may be located at the N- or C-terminus of a catalytic module. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases: (1) endoglucanases (EC 3.2.1.4), (2) exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and (3) β-glucosidase (BG) (EC 3.2.1.21). To hydrolyze and metabolize insoluble cellulose, the microorganisms must secrete the cellulases (possibly except BG) that are either free or cell-surface-bound. Cellulases are increasingly being used for a large variety of industrial purposes—in the textile industry, pulp and paper industry, and food industry, as well as an additive in detergents and improving digestibility of animal feeds. Now cellulases account for a significant share of the world's industrial enzyme market. The growing concerns about depletion of crude oil and the emissions of greenhouse gases have motivated the production of bioethanol from lignocellulose, especially through enzymatic hydrolysis of lignocelluloses materials—sugar platform (Bayer et al., 2007; Himmel et al., 1999; Zaldivar et al., 2001). However, costs of cellulase for hydrolysis of pretreated lignocellulosic materials need to be reduced, and their catalytic efficiency should be further increased in order to make the process economically feasible (Sheehan and Himmel, 1999).

Engineering cellulolytic enzymes with improved catalytic efficiency and enhanced thermostability is important to commercialize lignocelluloses biorefinery. Individual cellulase can be enhanced by using either rational design or directed evolution. However, improvements in cellulase performance have been incremental, and no drastic activity enhancement has been reported to date. The further improvement on cellulase performance needs the better understanding of cellulose

hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function, and substrate characteristics.

8.2 CELLULASES AND THEIR ROLES IN CELLULOSE HYDROLYSIS

8.2.1 Cellulase Enzyme Systems for Cellulose Hydrolysis

Cellulolytic microorganisms have evolved two strategies for utilizing their cellulases: discrete noncomplexed cellulases and complexed cellulases (Lynd et al., 2002). In general, most aerobic cellulolytic microorganisms degrade cellulose by secreting a set of individual cellulases (Fig. 8.1A), each of which contains a CBM joined by a flexible linker peptide to the catalytic module. CBM is located in either the N-terminus or the C-terminus of the catalytic module, while its location seems not related to its function (Wilson, 2008). Table 8.1 presents an example for the modular architectures of cellulases from different bacteria. By contrast, most anaerobic microorganisms produce large (>1 million molecular mass) multienzyme complexes (Fig. 8.2B), called cellulosomes, which are usually bound to the cell surface of the microorganisms (Bayer et al., 2004). Only a few of the enzymes in cellulosomes contain a CBM, but most of them are attached to the scaffoldin protein that contains a CBM. Some anaerobic bacteria can produce cellulosomes and free cellulases (Berger et al., 2007; Doi and Kosugi, 2004; Gilad et al., 2003). The architecture and function of cellulosomes have been well reviewed by many experts (Bayer et al., 1998a,b, 2004; Doi, 2008; Doi and Kosugi, 2004; Doi and Tamaru, 2001; Doi et al., 1998, 2003).

TABLE 8.1. Modular Architectures of Cellulases from Different Bacteria

Organism	Modular Architecture	GenBank Code
Anaerocellum thermophilum	GH9-(CBM3)$_3$-GH48	ACM60955
A. thermophilum	GH9-(CBM3)$_3$-GH5	ACM60953
Bacillus subtilis	GH5-CBM3	CAA82317
Clostridium phytofermentans	GH48-Ig-CBM3	ABX43721
C. phytofermentans	GH9-CBM3-(Ig)$_2$-CBM3	ABX43720
Clostridium thermocellum	GH48-(Doc)$_2$	AAA23226
C. thermocellum	GH26-GH5-CBM11-(Doc)$_2$	AAA23225
Clostridium cellulolyticum	GH48-Doc	ACL75108
Cellulomonas fimi	GH48-Fn3-CBM2	AAB00822
Thermobifida fusca	CBM2-Fn3-GH48	AAD39947

GH, glycoside hydrolase; CBM, carbohydrate-binding module; Ig, immunoglobulin-like domain; Doc, dockerin; Fn, fibronectin-like domain.
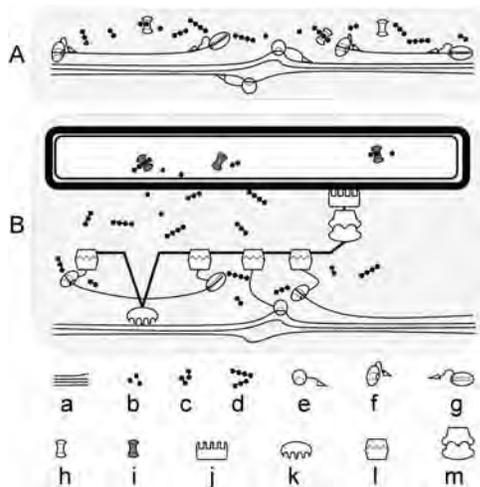

Figure 8.1 Schematic representation of the hydrolysis of cellulose by noncomplexed (A) and complexed (B) cellulase systems. a, cellulose; b, glucose; c, cellobiose; d, oligosaccharides; e, endoglucanase with carbohydrate-binding module (CBM); f, exoglucanase (acting on reducing ends) with CBM; g, exoglucanase (acting on nonreducing ends) with CBM; h, β-glucosidase; i, cellobiose/cellodextrin phosphorylase; j, S-layer homology module; k, CBM; l, type-I dockerin–cohesin pair; m, type-II dockerin–cohesin pair. The figure is not drawn to scale.
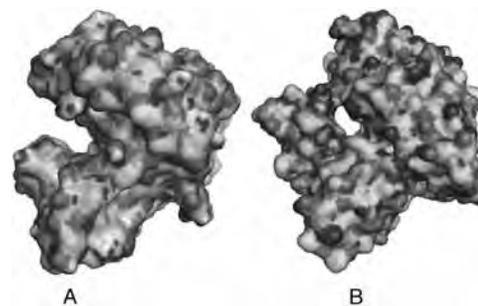

Figure 8.2 Crystal structures of family 6 endoglucanase and exoglucanase. (A) The structure of endoglucanase Cel6A of *Thermobifida fusca* (PDB code: 1TML), which exhibits a deep cleft at the active site. (B) The structure of exoglucanase Cel6A of *Humicola insolens* (PDB code: 1BVW), in which the active site of it bears an extended loop that forms a tunnel. The figure was drawn by using the PyMOL program.

8.2.2   Sequence Families of Cellulases and Their Three-Dimensional Structures

The Carbohydrate-Active Enzymes database (CAZy; http://www.cazy.org) provides continuously updated information of the glycoside hydrolase (EC 3.2.1.-) families (Cantarel et al., 2009). Glycoside hydrolases, including cellulase, have been classified into 115 families based on amino acid sequence similarities and crystal structures. A large number of cellulase genes have now been cloned and characterized. They are found in 13 families (1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 51, and 48), and cellulase-like activities have been also proposed for families 61 and 74 (Schulein, 2000).

Based on the data in CAZy database, the three-dimensional structures of more than 50 cellulases except the family 3 cellulases are available now. All of cellulases cleave β-1,4-glucosidic bonds, but they display a variety of topologies ranging from all β-sheet proteins to β/α-barrels to all α-helical proteins.

8.2.3   Catalytic Mechanisms of Cellulases

Glycoside hydrolases cleave glucosidic bonds by using acid–base catalysis. The hydrolysis is performed by two catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base (Davies and Henrissat, 1995). Depending on the spatial position of these catalytic residues, hydrolysis occurs *via* retention or inversion of the anomeric configuration. For "retaining" cellulases, the anomeric C bearing the target glucosidic bond retains the same (substituent) configuration after a double-displacement hydrolysis with two key glycosylation/deglycosylation steps. By contrast, for "inverting" cellulases, the anomeric C invert its (substituent) configuration after a single nucleophilic displacement hydrolysis (Vocadlo and Davies, 2008). The detailed catalytic mechanisms have been well characterized and reviewed (Davies and Henrissat, 1995; Vocadlo and Davies, 2008; Zechel and Withers, 2000).

8.2.4   Endoglucanase

Endoglucanase, or CMCase, randomly cut β-1,4-bonds of cellulose chains, generating new ends. Different endoglucanases are produced by archaea, bacteria, fungi, plants, and animals with different catalytic modules belonging to families 5–9, 12, 44, 45, 48, 51, and 74. Fungal endoglucanases in general possess a catalytic module with or without a CBM, while bacterial endoglucanases may possess multiple catalytic modules, CBMs, and other modules with unknown function (Table 8.1).

The catalytic modules of most endoglucanases have a cleft/grove-shaped active site (Fig. 8.2A), which allows the endoglucanases to bind and cleave the cellulose chain to generate glucose, soluble cellodextrins or insoluble cellulose fragment. However, some endoglucanases can act "processively," based on their ability to hydrolyze crystalline cellulose and generate the major products as cellobiose or longer cellodextrins (Cohen et al., 2005; Li and Wilson, 2008; Mejia-Castillo et al., 2008; Parsiegla et al., 2008; Yoon et al., 2008; Zverlov et al., 2005).

8.2.5   Exoglucanase

Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either cellobiose or glucose as major products. Exoglucanases can effectively work on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri, 1997).

CBH is the most-studied exoglucanase. Different CBHs are produced by many bacteria and fungi, with catalytic modules belonging to families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Aerobic fungal CBHs are in families 6 and 7 only; aerobic bacterial CBHs are in families 6 and 48; anaerobic fungal CBHs are in family 48; and anaerobic bacterial CBHs are in family 9 as well as 48. In other words, family 7 CBHs only originate from fungi, and family 48 CBHs mostly originate from bacteria.

The most significant topological feature of CBHs' catalytic module is the tunnel structure which is formed by two surface loops (Fig. 8.2B). The tunnel may cover the entirety (e.g., family 7 CBH) or part of the active site (e.g., family 48 CBH). Figure 8.2 shows the crystal structures of family 6 endoglucanase and exoglucanase. Although these two enzymes share the similar folding, the active site of endoglucanase is the deep cleft structure while it is a tunnel for exoglucanase. The tunnel-shape active site of exoglucanase enables the enzyme to hydrolyze cellulose in a unique "processive" manner (Koivula et al., 2002; Vocadlo and Davies, 2008).

The glycoside hydrolase family 48 exoglucanases are widely believed to play important roles in crystalline cellulose hydrolysis mediated by bacterial cellulase systems. Their role is believed to be somewhat similar to that of the *Trichoderma* CBHI (Cel7A) (Teeri, 1997; Zhang et al., 2006b). Family 48 exoglucanases is dominant catalytic components of cellulosomes, such as *Clostridium cellulolyticum* CelF (Reverbel-Leroy et al., 1997), *Clostridium cellulovorans* ExgS (Liu and Doi, 1998), *Clostridium jusui* CelD (Kakiuchi et al., 1998), and *Clostridium thermocellum* CelS (Kruus et al., 1995; Wang et al., 1994), or as key noncomplexed cellulase components, such as *Cellulomonas fimi* CbhB (Shen et al., 1995), *Clostridium stercorarium* Avicelase II

(Bronnenmeier et al., 1991), *Thermobifida fusca* Cel48 (Irwin et al., 2000), *Paenibacillus barcinonensis* BP-23 Cel48C (Sánchez et al., 2003), and *Ruminococcus albus* 8 Cel48A (Devillard et al., 2004). For example, *C. thermocellum* CelS accounts for ~30% of the weight of the cellulosome isolated from the Avicel-grown culture but its level decreases to ~10% of the weight of that isolated from cellobiose-grown culture, implying that CelS plays a key role for crystalline cellulose hydrolysis (Bayer et al., 1985; Zhang and Lynd, 2005).

8.2.6 β-Glucosidase

β-Glucosidases (BGs) that do not contain a CBM hydrolyze soluble cellodextrins and cellobiose to glucose. The activity of BG on insoluble cellulose is negligible. BGs degrade cellobiose, which is a known inhibitor of CBH and endoglucanase. Different BGs are produced by various archaea, bacteria, fungi, plants, and animals, with different catalytic modules belonging to families 1, 3, and 9. Based on either experimental data or structural homology analysis, the stereochemistry of families 1 and 3 BGs is of the retaining type, while the stereochemistry of family 9 BG is of the inverting type. Most of the time, aerobic fungi produce extracellular BGs, and anaerobic bacteria keep their BGs in cytoplasm. BGs have a pocket-shaped active site, which allows them to bind the nonreducing glucose unit and clip glucose off from cellobiose or cellodextrin.

8.2.7 Substrate, Synergy, and Model

Natural cellulosic materials are heterogeneous although its chemical structure is very simple—a linear polymer made of anhydroglucose units linked by β-1,4-glucosidic bonds. Cellodextrins, oligosaccharides, decrease their solubility in water greatly as their degree of polymerization (DP) increases. Cellodextrins with DP from 2–6 are soluble in water; cellulosic materials become weakly soluble when their DPs are more than 6; cellulosic materials with DP of more than 30 are regarded as insoluble cellulose (Zhang and Lynd, 2003, 2004a). Low solubility of cellulosic materials is mainly attributed to strong hydrogen bonds among inter-sugar chains. For crystalline cellulose, highly orderly hydrogen bonds among the adjacent cellulose chains and van der Waal's forces result in low accessibility of cellulose (Zhang and Lynd, 2004a). Therefore, depolymerization rates of crystalline cellulose mediated by enzymes or chemical catalysts are much slower as compared to those of amorphous cellulose with much larger surface area (Zhang et al., 2006a) or those of soluble starch (Zhang and Lynd, 2004a). Therefore, an increase of cellulose accessibility

to cellulase (CAC) results in faster hydrolysis rates (Hong et al., 2007; Zhang et al., 2006a).

Synergy among exoglucanase (CBH) and endoglucanase is vital for complete hydrolysis of cellulosic materials. A number of studies have been conducted to investigate the optimal ratio between exoglucanase and endoglucanase for a maximum synergy degree, and so on. But it is difficult to draw any clear conclusion due to great variations in enzyme components, substrates, concentrations of enzymes and substrates, product assays (soluble-reducing ends or soluble glucose equivalent), reaction time, and so on. In order to seek for clarity, the functionally based kinetic model for enzymatic hydrolysis of pure cellulose by individual exoglucanase/endoglucanase has been developed (Zhang and Lynd, 2006). This model includes the different action mode CBHI, CBHII, and endoglucanase I, and incorporates two measurable and physically interpretable substrate parameters: the DP and the fraction of beta-glucosidic bonds accessible to cellulase, F_a.

This model has been used to calculate the degree of synergy (DS) between endoglucanase and exoglucanase ("endo–exo synergy") as a function of substrate properties and experimental conditions. The DS predicted by the model increases significantly with increasing DP (Fig. 8.3A). As summarized elsewhere (Zhang and Lynd, 2004b), DS values in the range of 4–10 have been reported for bacterial cellulose and cotton (DP ≥ 2000), whereas DS values for Avicel (DP ~300) are 1.5–2.5, and for PASC (DP ~60) are about 1.0. This model predicts increasing DS as the fraction of accessible bonds, F_a, increases (Fig. 8.3B). In addition, the maximum DS is predicted to occur at a somewhat higher exo/endo ratio as F_a increases. Comparison of Figure 8.3A,B suggests that DP is a much more important substrate property than F_a in influencing the DS.

Predicted DS values increase sharply with increasing total enzyme loading (Fig. 8.3C), consistent with reported results (Nidetzky et al., 1994; Woodward et al., 1988). Predicted DS also increases with increasing reaction time (Fig. 8.3D), consistent with experimental data in the absence of product inhibition (Fierobe et al., 2001, 2002b). Also, the optimal ratio of CBHI/EG obtained for the maximum DS increases with increasing enzyme loading and prolonging reaction time before the limited accessibility of the solid substrate is saturated (Fig. 8.3C,D).

This cellulase kinetic model involving a single set of kinetic parameters is successfully applied to a variety of cellulosic substrates, and it describes more than one behavior associated with enzymatic hydrolysis (Table 8.2). This model has potential utility for data accommodation and design of industrial processes, structuring, testing, and extending understanding of cellulase
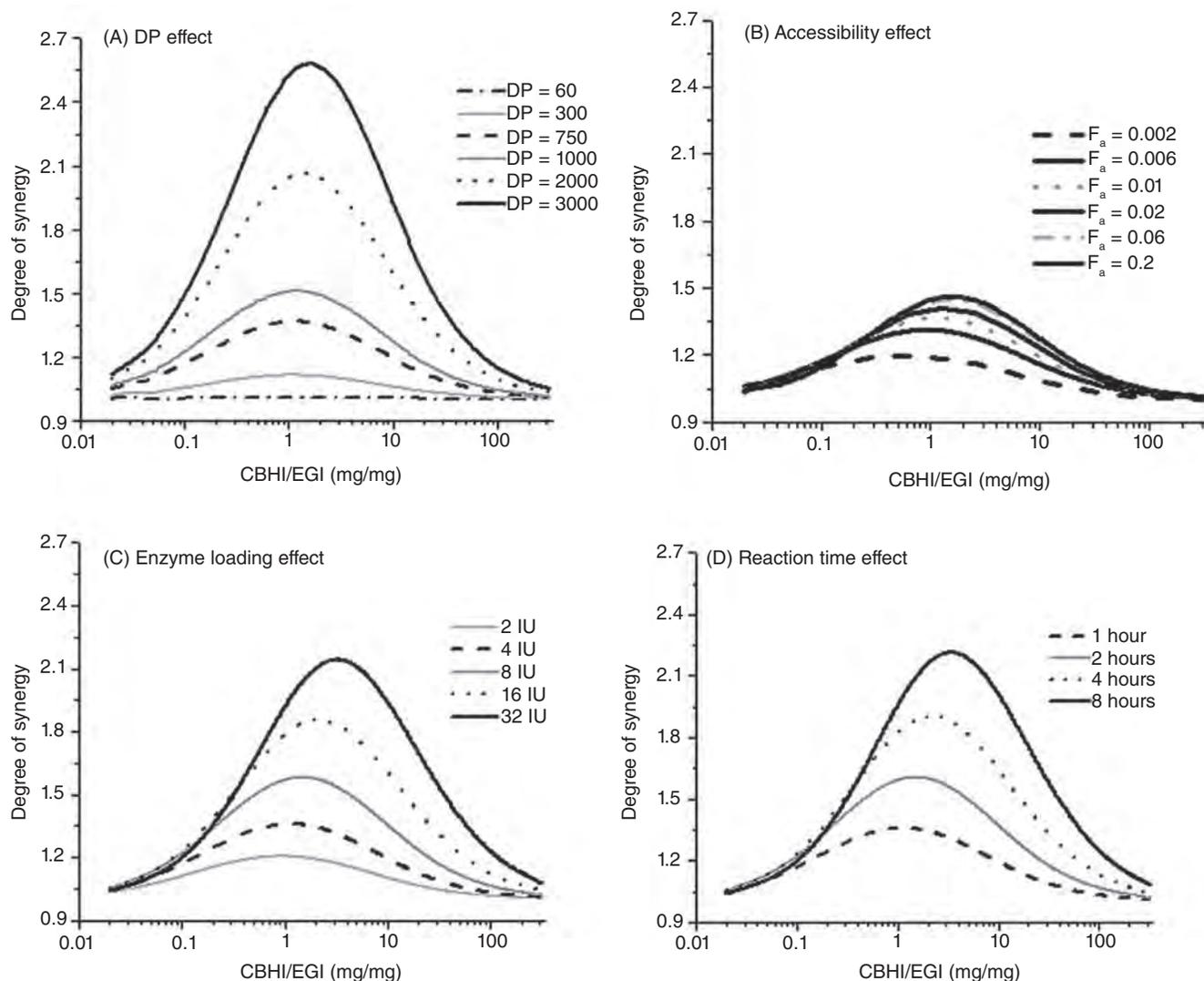
Figure 8.3 The simulation of the synergy between endoglucanase and exoglucanases in terms of substrate characteristics (degree of polymerization, A; and accessibility, B) and experimental conditions (enzyme loading, C; and reaction time, D). Modified from Zhang and Lynd (2006). Biotechnol. Bioeng. 94:888–898.

enzyme systems when experimental data are available, and providing guidance for functional design of cellulase systems at a molecular scale.

8.2.8 Cellulase Activity Assays

A number of cellulase activity assays have been summarized (Ghose, 1987; Wood and Bhat, 1988; Zhang et al., 2009). Cellulase activity assays can be classified into initial rate assays and end-point assays (Zhang et al., 2009). The former is the same as regular enzyme assays on soluble substrates. The latter is specifically designed for cellulase. Taking filter paper activity as an example, it requires the release of a fixed amount of soluble sugars (i.e., 4 mg glucose, measured by the DNS assay with glucose as reference) within a fixed time (i.e., 1 hour) from 50 mg of Whatman No. 1 filter paper. The enzyme solution needs a series of dilution for this assay (Zhang et al., 2009).

Also, cellulase samples can be individual samples or their mixture; the substrates can be soluble or insoluble; the substrates can be soluble cellulose derivatives (e.g., carboxymethylcellulose [CMC]), or pure cellulose (microcrystalline, regenerated amorphous cellulose, bacterial microcrystalline cellulose, filter paper), or pretreated biomass. So many variations in experimental conditions from the enzymes to substrates, the complicated relationship between physical heterogeneity of

TABLE 8.2. Questions Addressed by the Functionally Based Model for Enzymatic Cellulose Hydrolysis

Questions before This Model	New Insights or Explanations
What key cellulose characteristics influence hydrolysis rate?	Accessibility and DP are used; many phenomena can be explained.
What substrate characteristics affect the degree of endo–exo synergy?	DP is primary, accessibility is modest, in agreement with literature data.
Do other factors affect the degree of synergy?	Cellulase loading, reaction time can, in agreement with literature data.
Is there a constant optimal ratio of endo/exo for maximum synergy degree?	No. It is a dynamic ratio, depending on experimental conditions and substrate characteristics.
Why do relative hydrolysis rates on various substrates change with enzyme loading?	A clear mathematical explanation is provided.
What is the top priority for pretreatment?	Increasing cellulose accessibility.
Which enzyme parameter is the most important for overall cellulase activity?	Depending on the substrates selected (e.g., the exchange of cellulose-binding module for EGI could be powerful for low F_a substrates but not for high F_a ones).

the cellulosic materials, and the complexity of cellulase enzyme systems (synergy and/or competition) result in great challenges in cellulase activity assays (Ghose, 1987; Wood and Bhat, 1988; Zhang and Lynd, 2006; Zhang et al., 2009).

8.3 CELLULASE IMPROVEMENT EFFORTS

Cellulase engineering contains three major directions: (1) directed evolution for each cellulase, (2) rational design for each cellulase, and (3) the reconstitution of designer cellulosome or cellulase mixtures (cocktails) active on insoluble cellulosic substrates, yielding an improved hydrolysis rate or better cellulose digestibility (Fig. 8.4).

8.3.1 Directed Evolution

Directed evolution is a robust protein-engineering tool independent of knowledge of the protein structure and of the interaction between enzymes and the substrate (Arnold et al., 2001). The greatest challenge of this method is developing tools to correctly evaluate the performance of the enzyme mutants generated (Zhang et al., 2006b). The success of a directed-evolution experiment greatly depends on the method chosen for finding the best mutant enzyme, often stated as "you get what you screen for."
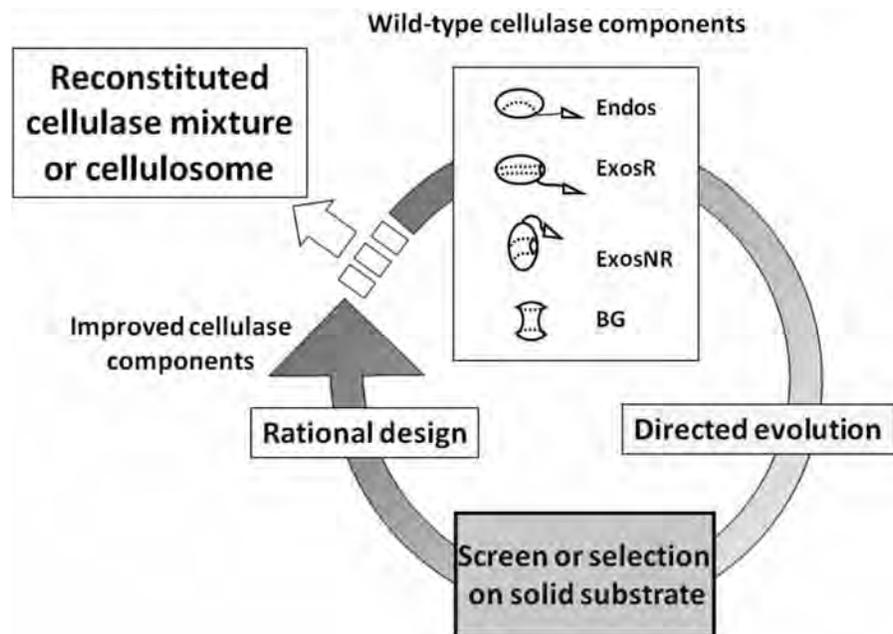

Figure 8.4 Scheme of cellulase engineering for noncomplexed cellulases. Endos, endoglucanases; exosR, exoglucanases acting on reducing ends; exosNR, exoglucanases acting on nonreducing ends; BG, β-glucosidase.

Selection and Screening. Selection is always preferred over screening because it has several orders of magnitude higher efficiencies than does screening (Zhang et al., 2006b). Selection requires a phenotypic link between the target gene and its encoding product that confers selective advantage to its producer. Selection on solid media in Petri dishes is commonly used because a number of mutants can be identified conveniently by visual inspection of growth. But selection power may be weak for secretory enzymes because of cross-feeding of the soluble products (Rouvinen et al., 1990; Zhang et al., 2006b).

Screening can be divided into two categories: (1) facilitated screening, which distinguishes mutants on the basis of distinct phenotypes, such as chromosphere release or halo-forming and (2) random screening, which picks mutants randomly (Taylor et al., 2001). Nearly all directed-evolution examples for endoglucanases have been reported by using facilitated screening on solid plates containing CMC followed by Congo Red staining (Catcheside et al., 2003; Kim et al., 2000; Lin et al., 2009; Murashima et al., 2002; Wang et al., 2005).

Random screening is the last choice if facilitated screening is not available. Random screening is often implemented using 96-well microtiter plates or toward 384-well and higher density plate formats (Sundberg, 2000). Filter paper activity assay, the most popular cellulase assay (Ghose, 1987; Zhang et al., 2009), has been miniaturized into 96-well microplates for high-throughput cellulase assays on solid cellulosic substrates (Decker et al., 2003; Xiao et al., 2004), but no better cellulase has been reported based on these methods until now.

β-Glucosidase Improvement. Identification of desired mutants from a large mutant library remains challenging for cellulase engineering. We have demonstrated a novel combinatorial selection/screening strategy for finding thermostable BG on its natural substrate—cellobiose, as shown in Figure 8.5 (Liu et al., 2009). First, selection has been conducted through complementation of BG for non-cellobiose-utilizing *Escherichia coli* so that only the cells expressing active BG can grow on the M9 synthetic medium with cellobiose as the sole carbon
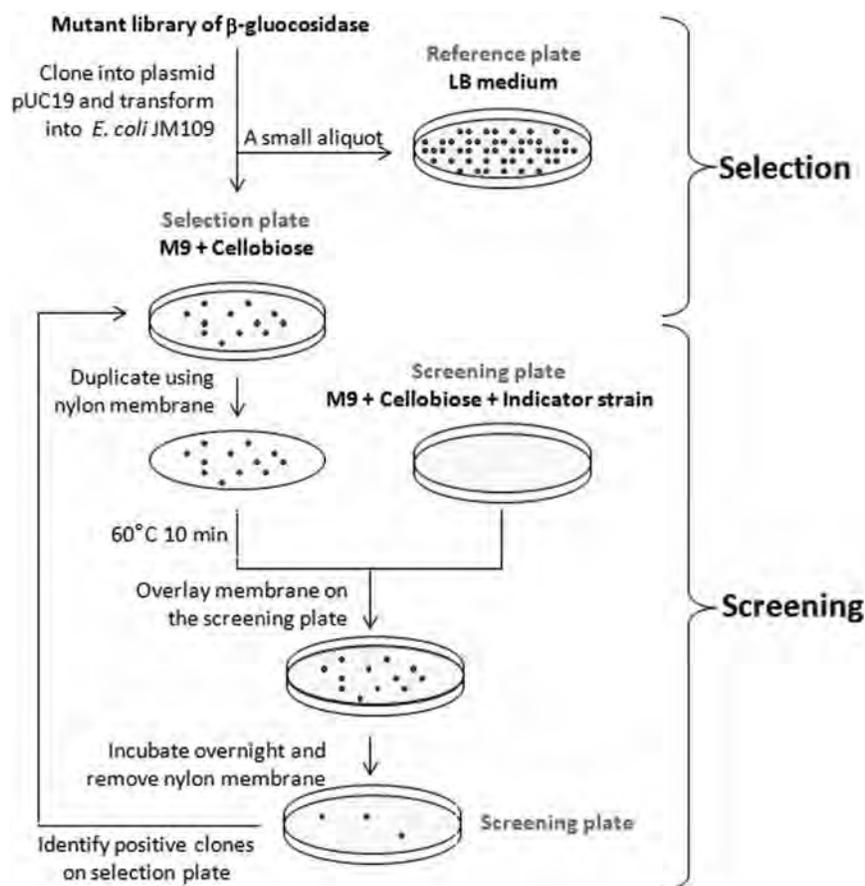

Figure 8.5 Scheme of the selection/screening approach for fast identification of thermostable β-glucosidase mutants active on cellobiose.

source (selection plate). Second, the clones on the selection plates are duplicated by using nylon membranes. After heat treatment, the nylon membranes are overlaid on M9/cellobiose screening plates so that the remaining activities of thermostable BG mutants hydrolyze cellobiose on the screening plates to glucose. Third, the growth of an indicator *E. coli* strain that can utilize glucose but not cellobiose on the screening plates helps detect the thermostable BG mutants on the selection plates. Wild-type *E. coli* cannot utilize cellobiose as a carbon source to support its growth but has a cellobiose transport system. Several thermostable mutants have been identified from a random mutant library of the *Paenibacillus polymyxa* BG. The most thermostable mutant A17S has an 11-fold increase in the half-life of thermo-inactivation at 50°C (Liu et al., 2009).

Endoglucanase Improvement. The efficient screening strategy for hydrolytic enzymes requires the target enzymes to be secreted or be displayed on the surface of the outer membrane of the gram-negative bacterium *E. coli* (Fig. 8.6). This screening strategy involves (1) extracellular hydrolytic enzyme can access polymeric substrates, (2) the enzyme hydrolyzes cellulosic materials to soluble sugars, and (3) the microbe assimilates soluble sugars and utilize the sugar source for its growth.

After testing several different cell-surface display technologies, we have found that the fusion of the target protein with an ice-nucleation protein (INP) (Kawahara, 2002) is very efficient for expression of large-size enzymes on the surface of *E. coli*.

Open reading frame Cphy1163 encoding a family 5 glycoside hydrolase (Cel5A) from an anaerobic cellulolytic bacterium *Clostridium phytofermentans* ISDg was cloned and expressed in *E. coli*. Cel5A has been displayed on the surface of *E. coli* surface by using *Pseudomonas syringae* INP as an anchoring motif for identification of Cel5A mutants with improved thermostability (Fig. 8.6). Several identified more stable mutants displayed on the cell surface are not stable when they are expressed in free form. The half-life times of thermo-inactivation of the wild-type and mutant Cel5A fused to a CBM3 have been significantly prolonged when the fusion proteins are prebound before thermo-inactivation to the substrates, especially to the insoluble substrates. The addition of either a CBM3 on the N-terminus or a CBM2 on the C-terminus does not influence the enzyme activities on the soluble substrate CMC but drastically decreases the activities on the insoluble substrates—regenerated amorphous cellulose (RAC) and microcrystalline cellulose (Avicel). The resulting Cel5 mutant has similar activity to the wild-type
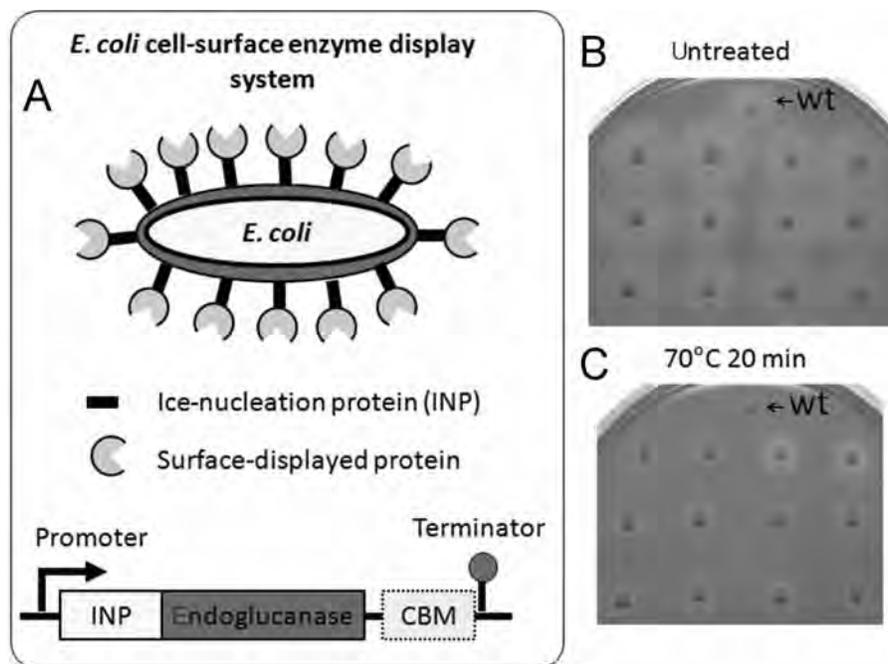

Figure 8.6   Improvement of the thermostability of endoglucanase Cel5A by directed evolution and surface display technologies. (A) Schematic representation of the protein expression cassette and the surface display strategy. (B and C) The detection of endoglucanase activity of wild-type Cel5A and its mutants without (B) or with (C) the heat treatment. The position for strain expressing wild-type Cel5A is indicated.

but increases half-life time of thermo-inactivation on CMC, RAC, and Avicel (1.92-, 1.36-, and 1.46-fold, respectively). All of the above results suggested high complexity of cellulase engineering (Liu et al., 2010).

8.3.2   Rational Design

Rational design requires detailed knowledge of protein structure, of the structural causes of biological catalysis or structural-based molecular modeling, and of the ideal structure–function relationship. The modification of amino acid sequence can be achieved through site-directed mutagenesis, exchange of elements of secondary structure, and even exchange of whole domains and/or generation of fusion proteins. The faith in the power of rational design relies on the belief that our current scientific knowledge is sufficient to predict function from structure. Even if the structure and catalysis mechanism of the target enzyme are well characterized, the molecular mutation basis for the desired function may not be achieved (Arnold, 2001; Zhang et al., 2006b).

Heterogeneous cellulose hydrolysis is a very complicated process (Lynd et al., 2002; Zhang et al., 2006b), which makes rational design of cellulase difficult. Site-directed mutagenesis has been applied to investigation of cellulase mechanisms and enhancement in enzyme properties, which has been reviewed elsewhere (Schulein, 2000; Wilson, 2004; Wither, 2001). For example, Baker and coworkers have increased the activity of endoglucanase Cel5A from *Acidothermus cellulolyticus* on microcrystalline cellulose by decreasing product inhibition (Baker et al., 2005). By the integration of computer modeling and site-directed mutagenesis, Escovar-Kousen et al. have improved the activity of the *T. fusca* endocellulase/exocellulase Cel9A on soluble and amorphous cellulose by 40% (Escovar-Kousen et al., 2004). Rignall et al. have reported that a single mutation in active-site cleft significantly changes the mixture of products released from phosphoric acid-swollen cellulose (Rignall et al., 2002). Beside site-directed mutagenesis, the insertion or domain deletion has been adopted to alter the cellulase performance (Lim et al., 2005; Park et al., 2002). The recent reports have showed that the CBM engineering can successfully improve hydrolytic activity of both endoglucanase and exoglucanase on crystalline cellulose (Mahadevan et al., 2008; Voutilainen et al., 2009). Using a SCHEMA structure-guided recombination method, more thermostable CBH hybrids have been obtained (Heinzelman et al., 2009a,b).

Despite continuing efforts to enhance noncomplexed cellulase performances, there is no general rule for improving cellulase activity on solid cellulase substrates.

Therefore, rational design for cellulase remains on a trial-and-test stage.

8.3.3   Designer Cellulosome

Cellulosomes with proximity synergy among cellulase components exhibit increased activity on crystalline cellulose. A designer cellulosome combines multiple enzymes and forms a single macromolecular complex, which is useful for understanding cellulosome action and for biotechnological applications (Bayer and Lamed, 1992; Bayer et al., 1994, 2007; Nordon et al., 2009). Designer cellulosomes include the construction of chimeric scaffoldins that contain divergent cohesins and matching dockerin-bearing enzymes. This arrangement allows researchers to control the composition and spatial arrangement of the resultant designer cellulosomes (Bayer et al., 2007).

In vitro small-size artificial cellulosomes have been constructed by Fierobe and his coworkers for the first time (Fierobe et al., 2001). The controlled incorporation of components finds out CBM's targeting function on the substrate surface and the proximity of enzyme components in the complexes (Fierobe et al., 2002a).

In order to get more efficient designer cellulosomes, more free glucoside hydrolases have been incorporated into cellulosomes (Caspi et al., 2008, 2009). Bayer et al. have cellulosomized two exoglucanases produced by *T. fusca*: Cel6B and Cel48A (Caspi et al., 2008). The recombinant fusion proteins tagged with dockerins have shown to bind efficiently and specifically to their matching cohesins. However, the lack of a cellulose-binding module in Cel6B has a deleterious effect on its activity on crystalline substrates. By contrast, the dockerin-bearing family 48 exoglucanase shows increased levels of hydrolytic activity on CMC and on two crystalline substrates, compared to the wild-type free enzyme. More recently, *T. fusca* Cel5A has been also integrated into designer cellulosome complexes (Caspi et al., 2009). The combination of the converted Cel5A endoglucanase with the converted Cel48A exoglucanase also exhibits a measurable proximity effect on Avicel. The length of the linker between the catalytic module and the dockerin has little effect on the activity. It is also found that positioning of the dockerin on the C-terminus of the enzyme, consistent with the usual position of dockerins on most cellulosomal enzymes, results in an enhanced synergistic response.

By using a protease-deficient *Bacillus subtilis* as the host for the heterologous expression, Doi and colleagues have constructed *in vitro* minicellulosomes that use recombinant cellulosomal enzymes and truncated scaffoldin components from *Clostridium cellulovorans* (Murashima et al., 2002). The reconstituted

cellulosomes exhibited synergistic activity on cellulosic substrates. The ability of two *B. subtilis* strains to cooperate in the synthesis of an enzyme complex (an *in vivo* minicellulosome) has been demonstrated, and the minicellulosomes formed by "intercellular complementation" show their respective enzymatic activities (Arai et al., 2007).

Recently, Chen et al. have demonstrated the display of a functional minicellulosome on the yeast cell surface (Tsai et al., 2009). The recombinant yeast cells with the surface displayed mini-scaffoldin is mixed with the *E. coli* lysates containing dockerin-containing cellulases. Later, an assembly of a functional minicellulosome on the yeast surface has been obtained. The displayed minicellulosome exhibits the synergistic effect between endoglucanase and exoglucanase. When a BG is incorporated, the cells displaying the minicellulosome exhibit significantly enhanced glucose liberation and produce ethanol directly from phosphoric acid-swollen cellulose. Designer cellulosomes are still less active than natural cellulosomes. It is believed that some unknown factors are critical to cellulolytic activity of cellolosome.

8.4 THE APPLICATIONS AND PRODUCTIONS OF CELLULASE

8.4.1 Industrial Applications of Cellulases

Cellulases are used in the textile industry, in detergents, pulp and paper industry, improving digestibility of animal feeds, and food industry. They account for a significant fraction of industrial enzyme markets. Cellulase market will grow rapidly. For example, if the goal of the Department of Energy that the production of 45 billion gallons of cellulosic ethanol in 2030 comes true with an assumption of cellulase cost ($0.20 dollar per gallon), the projected yearly cellulase market will be as large as 9 billion dollars. Obviously, cellulase will be the largest industrial enzyme as compared to the current market size for all industrial enzymes ($~2 billion).

8.4.2 Cellulase Production

Cellulase can be produced by either solid or submerged fermentations. But nearly all companies have chosen submerged fed-batch fermentation for producing low-cost cellulase because they can produce more than 100 g of crude cellulase (weight) per liter of broth. It is estimated that cellulase production costs based on dry protein weight may be as low as $10–40 per kilogram of dry protein weight. Most enzyme companies (e.g., Novozymes, Genencor, Iogen, etc.) produce commercial cellulases based on *Trichoderma* and *Aspergillus* or their derivative strains except Dyadic's *Chrysosporium luc-*

knowense. During the past years, Genencor and Novozymes claimed a 20- to 30-fold reduction in cellulase production costs to 20–30 cents per gallon of cellulosic ethanol (Himmel et al., 2007). This achievement has been implemented mainly by (1) reduction of sugar costs from lactose to glucose plus a small amount of sophorose, (2) enzyme cocktail for higher specific activity, and (3) better thermostability. But this achievement seems overclaimed. It is estimated that current cellulase costs may range from 1.00 to 1.50 U.S. dollars per gallon of cellulosic ethanol.

8.5 CONSOLIDATED BIOPROCESSING

Consolidated bioprocessing (CBP) integrates cellulase production, cellulose hydrolysis, and pentose and hexose fermentations in a single step (Lynd et al., 1999, 2002, 2005). CBP offers the potential for lower production costs, lower capital investment, and higher conversion efficiency as compared to the processes featuring dedicated cellulase production. In addition, CBP could realize higher hydrolysis rates, and hence reduce reactor volume and capital investment as a result of enzyme–microbe synergy, as well as the use of thermophiles or other organisms with highly active cellulases. Moreover, cellulose-adherent cellulolytic microorganisms may successfully compete for products of cellulose hydrolysis with nonadhered microbes, including contaminants, which could increase the stability of industrial processes based on microbial cellulose utilization.

Not a CBP-enabling microorganism is suitable for industrial applications yet. CBP microorganism development can proceed *via* a native cellulose utilization strategy, a recombinant cellulose utilization strategy, or a combination of recombinant cellulose utilization and product-producing ability (Fig. 8.7). The native cellulolytic strategy involves engineering product metabolism to produce desired products based on naturally cellulolytic microorganisms (e.g., *C. thermocellum, Trichoderma reesei*; Xu et al., 2009). The recombinant cellulolytic strategy involves introducing heterologous cellulase genes into an organism, whose product yield and tolerance credentials are well-established (e.g., *Saccharomyces cerevisiae*). The recombinant cellulolytic and ethanologenic combination can be conducted in some model microorganisms (e.g., *E. coli*) or industrially safe microorganisms (e.g., *B. subtilis*).

The recombinant cellulolytic strategy involves engineering noncellulolytic organisms that exhibit high product yields by producing a heterologous cellulase system enabling cellulose utilization. The early research advances have been reviewed previously (Lynd et al., 2002). The yeast *S. cerevisiae* is an attractive host
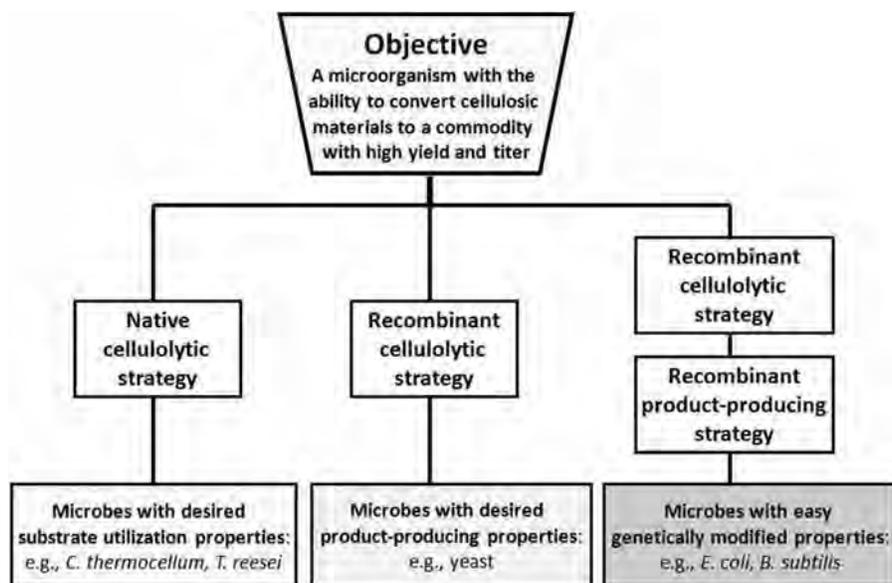
Figure 8.7   Three different consolidated bioprocessing development strategies to obtain organisms useful in processing cellulosic feedstocks.

organism for this strategy because of its high ethanol productivity with high yields, high osmo and ethanol tolerance, natural robustness in industrial processes, ease of genetic manipulation, and its generally regarded safe status. Cellulases from bacterial and fungal sources have been transferred to *S. cerevisiae*, enabling the hydrolysis of cellulosic derivatives (Lynd et al., 2002), or growth on cellobiose (McBride et al., 2005; van Rooyen et al., 2005). Three recombinant enzymes—*T. reesei* endoglucanase II, *T. reesei* CBHII, as well as *Aspergillus aculeatus* BG—have been coexpressed in *S. cerevisiae* via individual fusion proteins with the C-terminal-half region of α-agglutinin (Fujita et al., 2004). But this strain cannot grow on cellulose as self-catalyzing cellulolytic microorganisms, possibly because of poor active cellulase expression.

To achieve the self-supporting growth based on recombinant cellulases, it is appropriate to estimate minimum cellulase expression levels. On the basis of the sufficiency of expression of growth-enabling heterologous enzymes, the level of enzyme expression required to achieve a specified growth rate can be calculated as a function of enzyme specific activity (McBride et al., 2005). For growth enabled by cellulase with specific activities in the range available, required expression levels are well within the range reported (1–10% of cellular protein) (Park et al., 2000; Romanos et al., 1991). Protein expression at this level has been reported in both *S. cerevisiae* (Park et al. 2000) and *E. coli* (Hasenwinkle et al., 1997), although not for active cellulases.

Although a large number of cellulose-utilization microorganisms are discovered from diverse environments, advances in recreating recombinant cellulolytic microorganisms are limited because recombinant cellulolytic microorganisms must be capable of (1) overexpressing high-level recombinant active cellulase, (2) secreting or displaying most of active cellulases outside the (outer) membrane so that cellulase can hydrolyze insoluble cellulose, (3) producing several cellulase components, (4) regulating expression of cellulase components for a maximal synergy for hydrolysis, (5) producing sufficient ATP for cellulase synthesis, and (6) having right sugar transport systems for cellulolytic products (cellodextrins and oligoxylosaccharides). van Zyl and his coworker (Den Haan et al., 2007) have constructed a recombinant *S. cerevisiae* with the *T. reesei* endoglucanase (EGI) and the *Saccharomyces fibuligera* BG that can grow on regenerated amorphous cellulose in the presence of 0.5% yeast extract and 1% tryptone.

For native cellulolytic strategy, a number of efforts have been made to increase ethanol yield. For example, metabolic engineering has been applied to a mesophilic cellulolytic *C. cellulolyticum* for enhancing ethanol yields by heterologous expression of *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase (Guedon et al., 2002). *T. saccharolyticum*, a thermophilic anaerobic bacterium that ferments xylan and biomass-derived sugars has been modified to produce ethanol at high yields (Shaw et al., 2008).

Combined recombinant cellulolytic and product-producing strategy is initiated by Prof. Ingram. Prof.

TABLE 8.3. Comparison of Potential CBP Microorganisms for Production of Industrial Biocommodities

Key Feature	Saccharomyces cerevisiae	Escherichia coli	Clostridium thermocellum	Bacillus subtilis
C5 sugar utilization	–/+	+++	–	+++
Oligosaccharide utilization	–	–/+	+++	+++
Protein secretion capacity	+	–/+	+++	+++
Easiness of genetic modifications	+++	+++	–/+	++
Medium cost benefits	++	–	++	+++
Resistant to product inhibition	+++	–	–/+	+++
Resistant to salt/toxic inhibition	+	–	–	+++
Value of cell residues	++	–	–	+++
Growth rate	++	+++	+	+++
Anaerobic fermentation	+++	++	+++	+
Culture temperature	~30°C	~37°C	~60°C	30~45°C

Ingram and his coworkers have introduced heterologous cellulases and ethanol production pathway into *E. coli* and *Klebsiella oxytoca* for higher ethanol yields and lower cellulase use (Wood and Ingram, 1992; Zhou and Ingram, 2001; Zhou et al., 2001). They demonstrated that the recombinant *K. oxytoca* M5A1 containing endoglucanase genes from *Erwinia chrysanthemi* (*celY* and *celZ*) grows on amorphous cellulose with supplementary 0.5% yeast extract and 1% tryptone. Recently, our laboratory has developed the first recombinant cellulolytic *B. subtilis* that can grow on cellulose as the sole carbon source in defined M9 media by producing sufficient cellulase (Zhang et al., submitted). Table 8.3 presents the advantages of *B. subtilis* as compared with other potential CBP microorganisms.

Costly rich media for commodity production is economically prohibited (Lawford and Rousseau, 1996; Wood et al., 2005). The supplementary yeast extract or other organic nutrients (e.g., amino acids) in media works as the carbon source and nitrogen source at the same time. Their additions drastically decrease synthesis burden of amino acids. One of the milestones for CBP development will be the construction of a recombinant microorganism that can grow on cellulose without the addition of costly organic nutrition to produce value-added biocommodities.

In a word, industrially safe cellulolytic microorganisms that can produce desired product in one step are not available now. It is expected that such CBP microorganisms will be obtained soon.

8.6   PERSPECTIVES

Biomass saccharification is the largest technical and economic obstacle to biorefinery and biofuels production. To address this challenge, there are three different approaches or their combinations: (1) biomass pretreatment/fractionation and enzymatic hydrolysis, (2) cellulase engineering, and (3) CBP. Biomass pretreatment/fractionation must be further enhanced for better overall cost efficiency; for example, increasing substrate reactivity so as to decrease cellulase use (Sathitsuksanoh et al., 2009, 2010), co-utilization of ligno-cellulose components (e.g., lignin, hemicellulose) for high-value products (Sathisuksanoh et al., 2009a,b; Zhang, 2008), recycling costly cellulase by readsorption of free enzymes or desorbed enzymes (Zhu et al., 2009). Cellulase engineering must be focused on (1) increasing cellulase specific activity on pretreated biomass through enzyme cocktail or rational design or directed evolution, (2) increasing cellulase stability for cellulase recycling, and (3) decreasing enzyme production costs ($ per kilogram of dry protein). CBP microorganisms or consortium would simplify the whole process and increase productivity. In practice, the above three approaches would be integrated together for maximizing the returns from biorefinery and biofuels.

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