Research Article

A minimal set of bacterial cellulases for consolidated bioprocessing of lignocellulose

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Cost-effective release of fermentable sugars from non-food biomass through biomass pretreatment/enzymatic hydrolysis is still the largest obstacle to second-generation biorefineries. Therefore, the hydrolysis performance of 21 bacterial cellulase mixtures containing the glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase (BsCel5), family 9 *Clostridium phytofermentans* processive endoglucanase (CpCel9), and family 48 *C. phytofermentans* cellobiohydrolase (CpCel48) was studied on partially ordered low-accessibility microcrystalline cellulose (Avicel) and disordered high-accessibility regenerated amorphous cellulose (RAC). Faster hydrolysis rates and higher digestibilities were obtained on RAC than on Avicel. The optimal ratios for maximum cellulose digestibility were dynamic for Avicel but nearly fixed for RAC. Processive endoglucanase CpCel9 was the most important for high cellulose digestibility regardless of substrate type. This study provides important information for the construction of a minimal set of bacterial cellulases for the consolidated bioprocessing bacteria, such as *Bacillus subtilis*, for converting lignocellulose to biocommodities in a single step.

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1 Introduction

Cellulosic material is the most abundant renewable bioresource. Cellulose biodegradation mediated by cellulases and/or cellulolytic microorganisms represents one of the largest flows in the global carbon cycle [1]. The utilization of a small fraction of cellulosic materials (e.g., 5–10%) for the production of biofuels and value-added chemicals would greatly decrease reliance on crude oil, promote rural economy, decrease net greenhouse emissions, and increase national energy security [1–3]. Cost-effective release of fermentable sugars from non-food biomass through biomass pretreatment/enzymatic hydrolysis is still the largest obstacle to second generation biorefineries [1, 4, 5].

Although cellulose $(C_6H_{10}O_5)_n$ is a linear polysaccharide of several hundred or over ten thousand β-1,4-glucosidic bond-linked anhydroglucose units, its enzymatic hydrolysis requires three cellulase components with different action modes – endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase – to work together [2, 6, 7]. In general, endoglucanases (EC 3.2.1.4) randomly cut accessible β-glucosidic-1,4-bonds of cellulose chains, generating new ends for cellobiohydrolases. Two different types of cellobiohydrolases (EC 3.2.1.91) processively act on reducing and non-reducing ends, releasing cellobiose (mainly) from solid cellulose to the aqueous phase. β-Glucosidase (EC 3.2.1.21) is responsible for cleaving cellobiose to glucose so to eliminate cellobiose inhibition to endoglucanase and cellobiohydrolase.

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Abbreviation: RAC, regenerated amorphous cellulose

Table 1. A comparison of key characteristics of different cellulosic materials

Substrates	CrI	DP	CAC (m^2/g)	References
Avicel	0.5–0.6	150–500	2.38 ± 0.10	[23, 39, 42]
Regenerated amorphous cellulose	~0	30–200	41.9 ± 2.2	[23, 39, 42]
Cotton	0.8~0.95	1000–3000	N.A.	[39]
Pretreated lignocellulosic substrates	0.1–0.7	400–1000	5–30	[4, 23, 27, 39, 45]
Bacterial microcrystalline cellulose	0.8~0.95	600–2000	33.5 ± 1.5	[39, 42]

N.A. – not applicable

High cellulase cost remains as one of the cost-limiting factors for biomass biorefineries, which are estimated to consume nearly 100–200 g cellulase per gallon of ethanol produced [8, 9]. To decrease cellulase consumption per gallon of cellulosic ethanol production, intensive cellulase engineering efforts have been made by enhancing individual components using rational design or directed evolution [10–12] and reconstitution of different action-mode cellulase components (cocktail) on cellulosic substrates [13–17]. Novozymes and Genencor have worked on fungal enzyme cocktails involving more than ten cellulase components for improved mass-specific activity on diluted acid pretreated biomass. Recently, Walton and his coworkers reconstituted the fungal cellulase cocktail containing core hydrolases (including cellobiohydrolase, endoglucanase, β-glucosidase, endoxylanase, and β-glucosidase), along with accessory enzymes (including esterases, proteases, nonhydrolytic proteins, and glycosyl hydrolases), on AFEX-pretreated biomass, which contains a large amount of hemicellulose [18]. On the other hand, a study of a minimal fungal cellulase mixture suggested that some pretreated lignocellulosic biomass was hydrolyzed well by a careful combination of several enzymes [16]. However, the studies on minimal sets of bacterial cellulases are relatively sparse, and the bacterial cellulase cocktail with the performance on Avicel close to that of an integrated Clostridial cellulosome [19, 20] has not been determined yet in spite of the attempts at constructing an artificial aggregated system [21]. For example, two noncellulosomal cellulases of *Clostridium thermocellum*, Cel9I and Cel48Y, hydrolyze crystalline cellulose with a 2.1-times synergism [22].

Microcrystalline cellulose (Avicel) and regenerated amorphous cellulose (RAC) are two model cellulosic materials [6]. Avicel is made from wood pulp by acid hydrolysis, which can remove most amorphous cellulose and all hemicellulose, but Avicel still contains a significant fraction of amorphous cellulose [23]. RAC is prepared from Avicel through a series of steps: cellulose slurrying in water, cellulose dissolution in concentrated phosphoric acid, and regeneration in water [24]. As a result, RAC, a completely disordered insoluble substrate, has much larger cellulose accessibility than that of Avicel [25] but has the same degree of polymerization when ice-cold concentrated phosphoric acid is used [26]. Different hydrolysis patterns have been observed on these two substrates by using fungal cellulase [24]. Since most types of pretreated lignocellulosic biomass containing amorphous and crystalline cellulose have substrate accessibility ranges falling between the ranges of Avicel and RAC (Table 1) [27], the information pertaining to minimal bacterial cellulases sets on Avicel and RAC would be useful for developing better bacterial enzyme mixtures expressed by recombinant cellulolytic consolidated bioprocessing bacteria, such as *Bacillus subtilis*, which would efficiently hydrolyze pretreated cellulosic materials.

In this study, we investigated the synergistic action of a ternary bacterial cellulase component mixture – glycoside hydrolase family 5 *Bacillus subtilis* endoglucanase (BsCel5) [10], family 9 *Clostridium phytofermentans* processive endoglucanase (CpCel9) [28], and family 48 *C. phytofermentans* cellobiohydrolase (CpCel48) [29] on Avicel and RAC. CpCel9 is validated to be a critical component for microbial cellulose hydrolysis [30]; CpCel48 is among the most active family 48 enzymes, possibly due to its low processivity [29]. As was demonstrated elsewhere, the two noncellulosomal cellulases of *C. thermocellum*, of the families Cel9 and Cel48 have provided efficient synergistic hydrolysis of crystalline cellulose [22, 31]. The BsCel5, CpCel9, and CpCel48 were thought to have respective functions of fungal *Trichoderma reesei* EG, CBH II, and CBH I (Table 2), where CBH I (TrCel7A) and CBH II (TrCel6A) hydrolyze glucosidic bonds close to reducing and non-reducing ends, respectively. This study of minimal bacterial cellulase cocktails on crystalline cellulose and amorphous cellulose could help develop better enzyme mixtures for pretreated cellulosic materials and create recombinant cellulolytic consolidated bioprocessing microorganisms that can express different cellulase components with proper ratios [2, 32].

Table 2. Comparison of key cellulase components in fungal and bacterial cellulase systems

	EC #	Modular structure	Function	Molecular mass (kDa)	Optimum pH	Optimum Temp. (°C)	Reference
Fungal cellulase							
T. reesei cellobiohydrolase I (TrCel7A)	3.2.1.91	GH7-CBM1	Reducing-end acting CBH	54.1	4	60	[46]
T. reesei cellobiohydrolase II (TrCel6A)	3.2.1.91	CBM1-GH6	Non-reducing end acting CBH	49.6	5	44	[47]
T. reesei endoglucanase I (TrCel7B)	3.2.1.4	GH7-CBM1	Random β-bond cleaving EG	48.2	5	60	[48]
Bacterial cellulase components							
C. phytofermentans Cel48 (CpCel48S)	3.2.1.91	GH48-Ig-CBM3-His6	Reducing-end acting CBH	98.5	5–6	60	[29]
C. phytofermentanas Cel9 (CpCel9)	3.2.1.4	GH9-CBM3	Non-reducing end acting processive endoglucanase	104.8	6.5	65	[28]
B. subtilis Cel5 (BsCel5)	3.2.1.4	GH5-CBM3	Random β-bond cleaving EG	52.4	6	60	[35]

2 Methods and materials

2.1 Chemicals

All chemicals were reagent grade or higher, purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Avicel PH105, microcrystalline cellulose, was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was made from Avicel as described elsewhere [24]. The *Trichoderma* cellulase (Novozyme® 50013, 84 filter paper units/mL) and β-glucosidase (Novozyme® 50010, 270 U/mL) were gifts from Novozymes North American (Franklinton, NC). Cellulase and β-glucosidase were measured on filter paper and cellobiose at pH 4.8 and 50°C, respectively, as described elsewhere [33]. Cellodextrins were prepared by hydrolysis of a mixture of concentrated HCl/H_2SO_4 and separated by chromatography [34]. A broad range protein marker (2–212 kDa) in SDS-PAGE was purchased from New England Biolabs (Ipswich, MA).

2.2 Strains and media

Escherichia coli DH5a was used as a host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as the host for recombinant protein expression. The Luria-Bertani (LB) medium was used for *E. coli* cell growth and recombinant protein expression. Ampicillin (100 μg/mL) was added in the *E. coli* media.

2.3 Purification of cellulase components

The cellulase cocktails were composed of BsCel5, CpCel9, and CpCel48. The strains *E. coli* BL21 (pET20b-Bscel5), *E. coli* BL21(pET20b-Cpcel9), and *E. coli* BL21(pET20b-Cpcel48) were cultivated in the LB media supplemented with 1.2% glycerol at 37°C for expression. 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures when A_{600} reached ~2.0, and the temperature of the cultures was decreased to 18°C for 16 h. The recombinant BsCel5 and CpCel9 were purified through a simple affinity adsorption on RAC followed by desorption by ethylene glycol [25]. The recombinant CpCel48 was purified using a nickel-resin column and a PD-10 desalting column as described elsewhere [29]. The individual cellulase specific activity measurement is based on the methods described elsewhere [28, 29, 35].

2.4 Hydrolysis of cellulose

Enzymatic hydrolysis of cellulose was carried out in 50 mM 2-*N*-morpholino-ethanesulfonic acid

(MES) buffer (pH 7.5) containing 1 mM $CaCl_2$ and 5 g/L RAC or Avicel PH105 at 37°C. One milliliter of the hydrolysis solution was prepared by mixing 500 µL 10 g/L RAC or Avicel slurry, concentrated MES buffer, and enzyme solutions. Initial reaction rates for individual enzymes at 10 µg/mL were measured on 1% substrate (Avicel and RAC) after 20 min hydrolysis. The reactions were terminated by boiling for 5 min. After centrifugation, aliquots of the supernatants were assayed for the release of reducing sugars by the modified 2,2′-bicinchoninate (BCA) method [26]. For the cocktail experiments, total enzyme loadings were 50 mg cellulase mixture/L (i.e., at cellulose/cellulase of 100) for both bacterial and fungal cellulases. The specific activity of the fungal enzyme was approximately 1 filter paper unit/mg protein. Excess β-glucosidase was supplemented for eliminating cellobiose inhibition. The final concentration of β-glucosidase added to the reaction was 30 U/g glucan, which was sufficient to convert all the soluble oligomers into glucose, according to our preliminary test by HPLC for the hydrolysis products [36]. Enzymatic hydrolysis experiments were conducted in a miniaturized digestion apparatus holding 2.0-mL Wheaton glass sample vials. Each cellulase composition was tested in duplicate. The vials were placed in a rack and the rack was then fixed with screws on a rotary shaker. The reactions were then continuously mixed at 250 rpm at 37°C. At 2, 12, and 72 h, 150-µL aliquots were withdrawn. The samples were boiled for 5 min to stop the reactions. After centrifugation, the soluble sugar concentration in the supernatants was measured by the phenol-sulfuric acid method, which accurately measures primary hydrolysis products regardless of sugar chain lengths [37]. All hydrolysis experiments were conducted in triplicate. Contour plots based on average experimental data with an SD of less than 5% were generated by using Origin Pro 8.1 (Northampton, MA) with a ternary contour option. The contour lines shown in the figures represent iso-surfaces fitted to the experimental results.

2.5 Other assays

Protein mass concentration was measured by the Bradford assay [38]. The purity of protein samples was examined by 10% SDS-PAGE. The soluble cellodextrins released from cellulosic materials were measured using a Beckman HPLC equipped with a Bio-Rad HPX-42A column (Richmond, CA) at a flow rate of 0.4 mL/min water [34].

3 Results and discussion

3.1 Production and purification of bacterial cellulase components

The recombinant bacterial enzymes (BsCel5, CpCel9, and CpCel48) were produced in *E. coli* BL21 (DE3) harboring the respective plasmid. *E. coli* BL21 (DE3) (pET20b-Bscel5) produced ~310 mg soluble BsCel5/L cell culture and approximately 100 mg BsCel5 was purified with a purification yield of ~ 36%. Approximately 61 mg CpCel9 and 19 mg CpCel48 were purified per liter of *E. coli* BL21 (DE3) (pET20b-Cpcel9) and BL21 (DE3) (pET20b-Cpcel48) cell cultures, respectively, with similar purification yields of 32%. The purified bacterial cellulases appeared to be homogeneous when examined by SDS-PAGE (Fig. 1A). BsCel5, CpCel9 and CpCel48 had apparent molecular masses of 50, 102 and 96 kDa, consistent with their estimated molecular mass (52.4, 104.8, and 98.5 kDa) based on their deduced amino acid sequences. The specific activities of BsCel5, CpCel9 and CpCel48 were 42.9 ± 0.1, 36.3 ± 1.3, and 4.6 ± 0.1 U/µmol enzyme on Avicel, respectively. Clearly, all the enzymes exhibited higher activities on RAC than on Avicel (Table 3). On RAC, primary

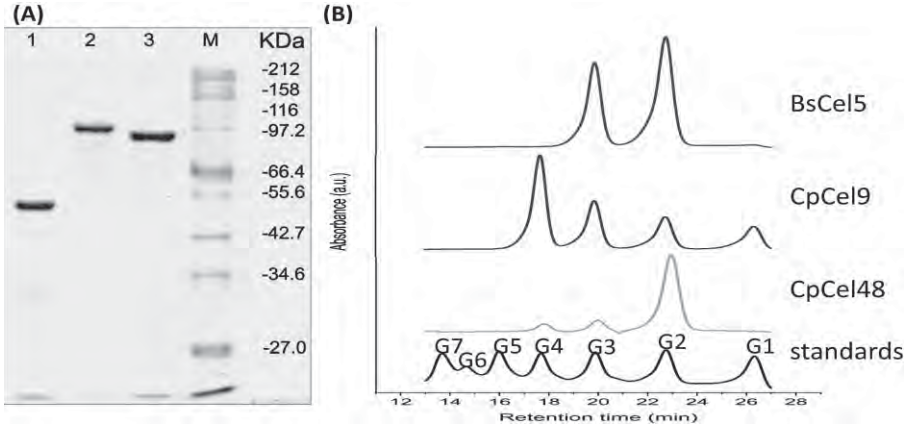

Figure 1. (**A**) SDS-PAGE analysis of purified bacterial enzymes. Lane 1, BsCel5; lane 2, CpCel9; lane 3, CpCel48; and lane M, protein makers. (**B**) HPLC spectra of the hydrolysis products by BsCel5, CpCel9 and CpCel48. HPLC standards G1–G7 are glucose and water soluble cellodextrins with a degree of polymerization from 2 to 7.

Table 3. Specific activity of purified bacterial cellulases on Avicel PH105 and RAC at pH 7.5 and T 37°C without addition of β-glucosidase

Enzyme	Substrate	Specific activity[a] (U/μmol)
BsCel5	Avicel PH105	42.9 ± 0.1
	RAC	105 ± 2.5
CpCel9	Avicel PH105	36.3 ± 1.3
	RAC	95.7 ± 4.3
CpCel48	Avicel PH105	4.6 ± 0.1
	RAC	10.6 ± 0.6

a) One unit of enzyme was defined as one μmol of reducing sugars generated per min. Reducing sugars were measured by the modified BCA method [26]

hydrolysis products of BsCel5, CpCel9 and CpCel48 were examined by HPLC (Fig. 1B). Endoglucanase BsCel5 produced cellobiose and cellotriose as the two sole major products. Processive endoglucanase CpCel9 was able to produce glucose, cellobiose, cellotriose, and cellotetraose, where cellotetraose was the most dominant product. Cellobiohydrolase CpCel48 produced cellobiose as a major product plus small amounts of cellotriose and cellotetraose. Since primary hydrolysis mediated by endoglucanases and cellobiohydrolases is a rate-limiting step [39], excess β-glucosidase was added in the following experiments to eliminate the possible influences due to cellobiose and long cellodextrin inhibition on the cellulase mixtures.

3.2 Hydrolysis with ternary bacterial cellulase mixtures

Ternary mixtures of three cellulases (i.e., CpCel48, CpCel9 and BsCel5) were reconstituted into 21 different compositions. Each corner of the triangle represented the purified cellulase only (100%) without the other two cellulase components; each side represented a mixture of the binary cellulases with a step length of 20% (i.e., six compositions were tested for each binary mixture at weight ratios of 100:0; 80:20; 60:40, 40:60, 20:80; 0:100, and the points inside contained a ternary cellulase mixture, where the composition was defined by its triangle coordinates. Of the 21 compositions, 3 were individual cellulase only (at the triangle corners), 12 were binary mixtures (on the three sides of the triangle), and 6 were ternary mixtures (inside the triangle). The total cellulase concentration was 50 mg/L, i.e., substrate/enzyme = 100:1, which is close to future practical enzyme loadings in cellulosic ethanol biorefineries [8, 9] (Note: a current typical substrate/enzyme ratio is ~50:1).

The hydrolysis performance of the cellulase cocktails was examined on Avicel at 2, 12 and 72 h (Fig. 2). For this partially ordered low-accessibility microcrystalline cellulose, the contour line patterns changed greatly over time, suggesting that optimal enzyme ratios for maximum cellulose digestibility were dynamic but not fixed, depending on several aspects. Regardless of reaction time, the highest di-

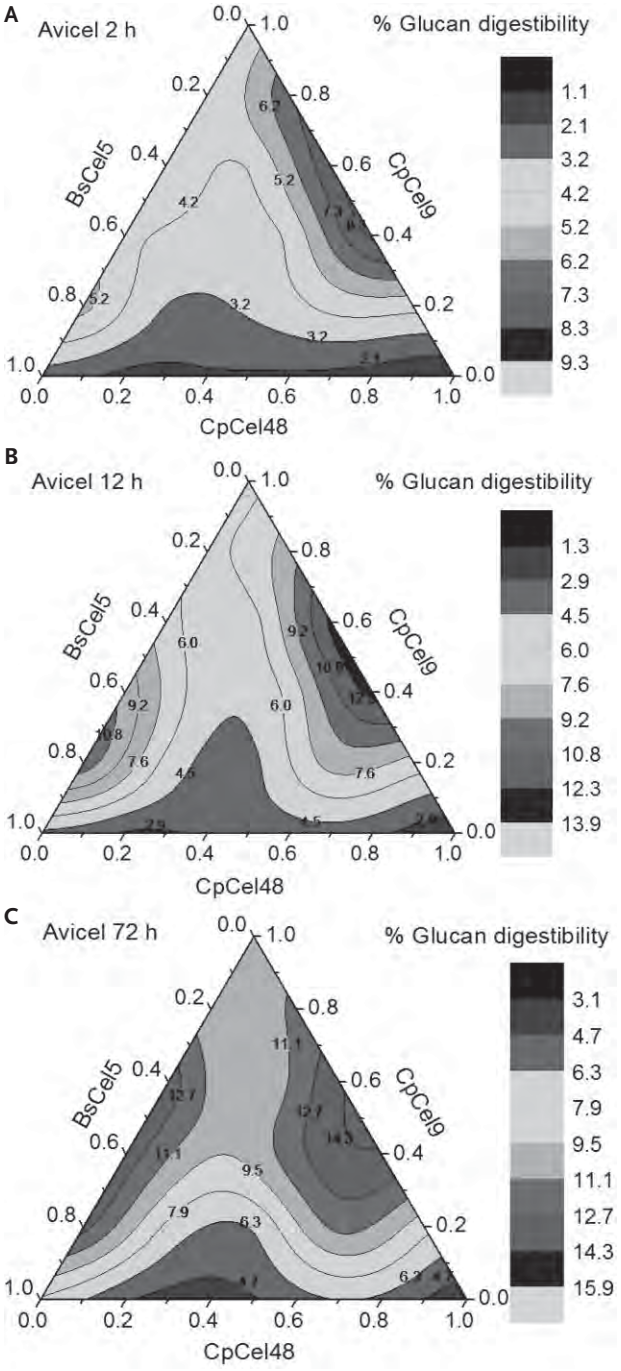

Figure 2. Contour plots of enzymatic hydrolysis by ternary bacterial cellulase mixtures (BsCel5, CpCel9 and CpCel48) on Avicel at different times (**A**, 2 h; **B**, 12 h; and **C**, 72 h). Enzymatic hydrolysis was carried out in 50 mM MES buffer (pH 7.5) containing 1 mM $CaCl_2$ and 5 g/L Avicel at 37°C. The total mass concentration of cellulases was 50 mg cellulase mixture/L.

gestibility contour lines were observed along the CpCel9 line (binary mixtures between CpCel9 and CpCel48) and the lowest digestibilities were obtained along the CpCel48 side (binary mixtures between BsCel5 and CpCel48). Both results suggested that the family 9 processive endoglucanase was the most important in hydrolysis of microcrystalline cellulose, in good agreement with previous in vitro and in vitro experimental reports [30]. The second most important cellulase component was initially the family 48 cellobiohydrolase CpCel48. Not surprisingly, it was long thought that family 48 exoglucanase would play a central role in crystalline cellulose hydrolysis because these enzymes are the dominant components in microbial cellulase systems [29], and their expression levels were enhanced when crystalline cellulose was a growth substrate as compared to soluble cellobiose [40]. The least important cellulase component was BsCel5, although it had the highest specific activity on Avicel at short reaction times (e.g., 20 min) (Table 3). At 72 h, a second highest digestibility zone was observed along the BsCel5 side (Fig. 2C), suggesting that the key role of CpCel48 might be replaced with BsCel5. This relatively unimportant role of the family 48 cellobiohydrolase in hydrolysis of microcrystalline cellulose was partially supported by an in vivo Cel48S knockout experiment for *C. thermocellum* [41].

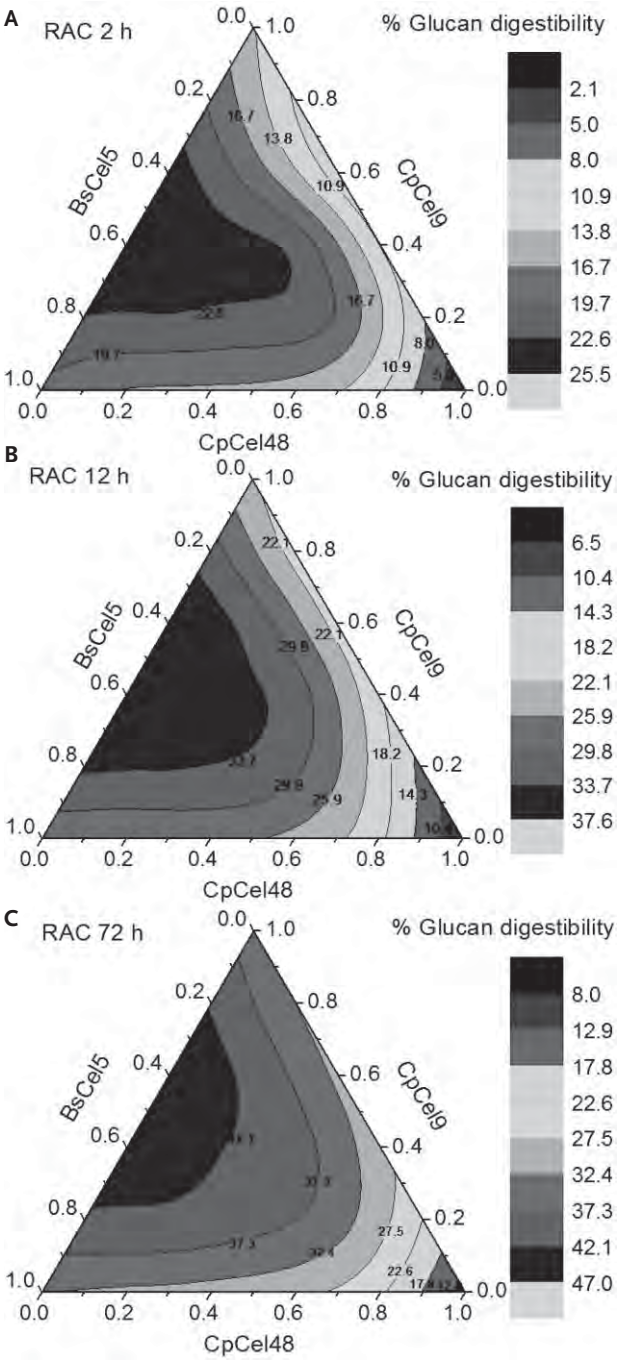

Figure 3. Contour plots of enzymatic hydrolysis by ternary bacterial cellulase mixtures (BsCel5, CpCel9 and CpCel48) on RAC at different times (**A**, 2 h; **B**, 12 h; and **C**, 72 h). Enzymatic hydrolysis was carried out in 50 mM MES buffer (pH 7.5) containing 1 mM $CaCl_2$ and 5 g/L RAC at 37°C. The total mass concentration of cellulases was 50 mg cellulase mixture/L.

For high-accessibility RAC, the hydrolysis contour plots of the cellulase cocktails are shown in Fig. 3. The contour lines for RAC did not change their patterns significantly, which was completely different from those of Avicel (Fig. 2). The highest digestibility zones were obtained along the BsCel5 side and inside area along this side, suggesting that the best enzyme cocktails contained both BsCel5 and CpCel9 plus an optional CpCel48. The lowest digestibility zones appeared at the CpCel48 corner, suggesting the relatively unimportant role of Cel48 in amorphous cellulose hydrolysis. This result seemed consistent with the fact that Cel48 expression of *C. thermocellum* was repressed when amorphous cellulose was used as the carbon source compared to Avicel [40]. The regions of highest digestibility on RAC were more than three times larger than those on Avicel PH105, suggesting that it was easier to determine the nearly constant optimized enzyme cocktails for amorphous cellulose than for Avicel.

3.3 Comparison of bacterial cellulase mixtures and a commercial fungal cellulase

Nearly all commercial cellulases are produced by fungi because aerobic fungal fermentation can produce secretory protein levels of more than 100 g/L and their protein production costs may be as low as ~2.4 US dollar/kg dry protein weight [6]. These cellulase expenditures could range from 20 to

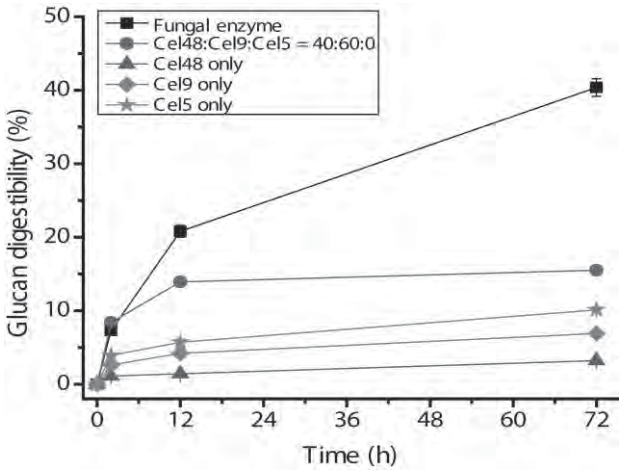

Figure 4. Hydrolysis profiles of an optimal bacterial cellulase mixture (CpCel48:CpCel9:BsCel5 = 40:60:0), three individual cellulases, and a commercial fungal mixture on Avicel. The experimental conditions were the same as that of Fig. 2. All substrate/cellulase weight ratios were 100:1. Error bars represent SD from triplicate samples.

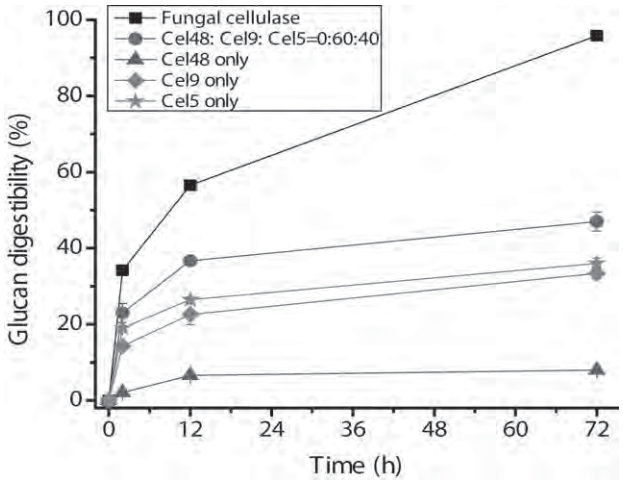

Figure 5. Hydrolysis profiles of an optimal bacterial cellulase mixture (CpCel48:CpCel9:BsCel5 = 0:60:40), three individual cellulases, and a commercial fungal mixture on RAC. All substrate/cellulase weight ratios were 100:1. Error bars represent SD from triplicate samples.

100 cents /gallon ethanol produced [36]. However, the enzyme expenditures in starch ethanol industry are as low as 2–5 cents/gallon ethanol (i.e., substrate/enzyme = ~500:1), where starch-hydrolyzing enzymes are produced by bacterial *Bacillus* spp. This large difference has raised a question – is it possible to hydrolyze pretreated cellulosic biomass using bacterial cellulase cocktails? Therefore, we compared the hydrolysis performance of the best ratio bacterial enzyme mixture (Cel48:Cel9 = 40:60) (Table 4) and a commercial fungal cellulase mixture on Avicel (Fig. 4). At the same protein mass concentration, a Cel48 and Cel9 mixture exhibited faster hydrolysis rates and better digestibilities than any individual cellulase component, indicating the well-known endo/exo synergic effect. At 2 h, the bacterial enzyme mixture reached a digestibility of 8.4%, slightly higher than that of the fungal cellulase (7.3%). The bacterial cellulase reached a digestibility of 13.9% at 12 h and leveled off later. In contrast, the fungal cellulase retained most of its hydrolysis ability, and the digestibility was 40.4% at 72 h. The lower digestibility for the bacterial cellulase mixture was not attributed to their denaturation (data not shown).

The best bacterial cellulase mixture (Cel9:Cel5 = 60:40, Table 5) was then compared with the fungal cellulase for RAC hydrolysis (Fig. 5). Similarly to the Avicel case, the fungal cellulase exhibited faster hydrolysis rates and higher digestibilities than the bacterial cellulase mixture. The highest digestibility was 95.8% for the fungal cellulase at 72 h, about two times of that of bacterial cellulase (47%). The superiority of the ratio of the fungal to bacterial cellulase on Avicel (2.6) was larger than that on RAC, suggesting that cellulase performance evalu-

Table 4. Different cellulase compositions for obtaining the top three highest Avicel digestibilities at 2, 12 and 72 h

Time (h)	Ternary cellulase system (Cel48: Cel9: Cel5)	Glucan digestibility (%)
2	60:40:0	9.3 ± 0.2
	40:60:0	8.4 ± 0.1
	20:80:0	7.5 ± 0.1
12	40:60:0	13.9 ± 0.3
	60:40:0	13.7 ± 0.1
	0:20:80	13.6 ± 0.6
72	0:60:40	15.9 ± 0.6
	0:20:80	15.7 ± 0.1
	40:60:0	15.5 ± 0.5

Table 5. Different cellulase compositions for obtaining the top three highest RAC digestibilities at 2, 12 and 72 h

Time (h)	Ternary cellulase system (Cel48: Cel9: Cel5)	Glucan digestibility (%)
2	40:40:20	25.5 ± 0.1
	20:20:60	24.7 ± 1.4
	0:40:60	23.7 ± 0.4
12	20:20:60	38.3 ± 0.8
	0:60:40	36.7 ± 0.2
	0:20:80	35.7 ± 0.7
72	0:60:40	47.0 ± 2.5
	0:20:80	44.6 ± 3.2
	0:80:20	43.1 ± 3.6

ation was strongly associated with the substrates tested.

These reconstitution experiments of the minimal bacterial cellulases (Figs. 2 and 3) clearly suggested that the optimal ratios of cellulase mixtures really depend on the substrates and reaction times. Regardless of substrate type, family 9 processive endoglucanase was the most important because this enzyme was a bifunctional enzyme with endo- and exo-glucanase activities [28]. On partially ordered microcrystalline cellulose, family 48 cellulase was important initially, but its importance decreased with an increase in conversion. For example, two noncellulosomal cellulases of *C. thermocellum*, Cel9I and Cel48Y, hydrolyze crystalline cellulose synergistically and a maximum synergism of 2.1 was determined at a Cel48/Cel9 ratio of ~95:5, suggesting an important role for Cel48 at the beginning of hydrolysis [22]. The changed role of family 48 cellulase in the enzyme cocktails may be explained by a great change in substrate reactivity of Avicel over conversion [42]. For the high-accessibility amorphous cellulose, family 48 was the least important.

4 Concluding remarks

Developing an optimal enzyme mixture depends greatly on substrate characteristics, reaction time, and chosen substrate/enzyme ratio. Several commonly used biomass pretreatments, such as dilute acid and steam explosion, aim to remove hemicellulose to break down the linkage among cellulose, hemicellulose and lignin; ammonia-based pretreatments attempt to remove lignin and hemicellulose to achieve a similar disruption [4, 27, 43]. Although they can increase substrate accessibility to cellulase, post-pretreatment fiber structures and relatively high crystallinity index suggested that common pretreatments cannot break highly ordered hydrogen bonds in cellulose fibers, resulting in relatively low substrate accessibilities (Table 1) [23, 43]. The high heterogeneity of pretreated biomass resulted in huge challenges in identifying the best ratio of cellulase mixtures. In contrast, cellulose solvent-based biomass pretreatment is a recently developed technology aimed at dissolving cellulose fibers in cellulose solvents so that the regenerated amorphous cellulose have much higher substrate accessibilities than substrates handled by common pretreatments [4, 18, 27]. Pretreated high-accessibility biomass has higher digestibilities and faster hydrolysis rates [4, 27], consumes less enzyme [36], and may simplify enzyme cocktail formulation (Fig. 3).

Comparison of the hydrolysis ability of fungal cellulase with that of bacterial cellulase (Figs. 4 and 5) clearly indicated that fungal cellulase exhibited much faster hydrolysis rates than bacterial cellulase at the same temperature and protein mass concentration over a long period. However, this observation seemed to conflict with another observation that cellulolytic bacteria can hydrolyze crystalline cellulose faster than fungi [2]. Such conflicting results may be explained by the followings: (i) some bacterial cellulase components can form complexed cellulase (cellulosome), which increases mass-specific cellulase activity by several fold [19–21]; (ii) bacterial cellulase displayed on the surface of microorganisms can exhibited several-fold higher activity compared to non-cell-bound enzymes [44]; and (iii) some bacterial cellulases can work at much higher temperatures than commercial fungal cellulase [2]. To increase mass-specific bacterial cellulase activity, future research directions would be to engineer individual cellulase performance by rational design or directed evolution as well as displaying the linked cellulase complex on the surface of microorganisms [2, 32]. A combination of cellulase engineering and metabolic engineering would help develop industrially important cellulolytic microorganisms that can produce cellulase, hydrolyze cellulose and ferment sugars to the desired product in a single step [2, 32, 35].

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