

Article

Revisiting the Phenomenon of Cellulase Action: Not All Endo- and Exo-Cellulase Interactions Are Synergistic

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Abstract: The conventional endo–exo synergism model has extensively been supported in literature, which is based on the perception that endoglucanases (EGs) expose or create accessible sites on the cellulose chain to facilitate the action of processive cellobiohydrolases (CBHs). However, there is a lack of information on why some bacterial and fungal CBHs and EGs do not exhibit synergism. Therefore, the present study evaluated and compared the synergistic relationships between cellulases from different microbial sources and provided insights into how different GH families govern synergism. The results showed that CmixA2 (a mixture of TlCel7A and CtCel5A) displayed the highest effect with BaCel5A (degree of synergy for reducing sugars and glucose of 1.47 and 1.41, respectively) in a protein mass ratio of 75–25%. No synergism was detected between CmixB1/B2 (as well as CmixC1/C2) and any of the EGs, and the combinations did not improve the overall cellulose hydrolysis. These findings further support the hypothesis that “not all endo-to exo-cellulase interactions are synergistic”, and that the extent of synergism is dependent on the composition of cellulase systems from various sources and their compatibility in the cellulase cocktail. This method of screening for maximal compatibility between exo- and endo-cellulases constitutes a critical step towards the design of improved synergistic cellulose-degrading cocktails for industrial-scale biomass degradation.

Keywords: cellulose; endo-glucanase; enzymatic hydrolysis; exo-glucanase; glycoside hydrolase; synergy



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1. Introduction

In nature, microorganisms have evolved diverse degradative strategies involving an arsenal of enzymatic machinery to assist them in cellulose de-polymerization [1,2]. Cellulases belong to a large family of glycosyl hydrolases (GH), which display the ability to cleave β -1,4 glycosidic bonds in cellulose chains via an acid hydrolysis mechanism [1,3]. Based on their amino acid sequence and 3D structural similarities, the catalytic machinery of known cellulases has been classified into different glycoside hydrolase (GH) families; 1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 48, 51 and 74 as documented in the Carbohydrate-Active enZymes database (CAZy; <http://www.cazy.org>). Different GH families have been shown to have low sequence similarities with one another; thus, they display a variety of topologies [2,4]. These differences, predominantly in the shape of the active-site pockets, determine the endo- or exo-mode of action of a given enzyme [2,4]. Although cellulases are traditionally viewed as having either exo- or endo-acting activities, recent evidence supports the idea that many enzymes have evolved activities against a broad range of substrates, due to conformational changes in their active sites [5,6].

Traditionally, a system of three main enzymatic activities has been reported to be indispensable for cellulose hydrolysis [7,8]. These include (i) cellobiohydrolases, CBHI/II

(EC 3.2.1.91 and EC 3.2.1.176), which processively cleave cellobiose units from one end (reducing or nonreducing) of the cellulase chain; (ii) endoglucanases, EGs (EC 3.2.1.4), which cleave at random internal regions of the cellulose chain to produce new chain oligosaccharides, and (iii) β -glucosidases, β gl (EC 3.2.1.21), which primarily hydrolyze glycosidic bonds of cellobiose and cellodextrans [8–10]. Efficient enzymatic hydrolysis of cellulose can only be achieved by a complete and balanced composition of cellulases, which co-operate with each other to de-polymerize the substrate [11]. The model generally used to describe this type of collaboration is referred to as enzyme synergism [1,12,13]. Although intensive research on cellulose-degrading enzymes for biomass degradation has mostly focused on the glycoside hydrolases, other carbohydrate-active catalytic domains and proteins (nonhydrolytic), known as lytic polysaccharide mono-oxygenases (LPMOs), swollenins/expansins and CBMs have been shown to contribute to the overall degradation of cellulose by means of increasing cellulose accessibility [14,15]. However, for the purpose of this study, the focus will solely be on the interaction between glycoside hydrolases.

Synergy between cellulolytic enzymes has been documented previously, including, (i) exo–exo synergism, (ii) processive endo–endo synergism, (iii) endo–exo synergism, and (iv) intramolecular synergy between catalytic domains and cellulose binding domains (CBMs) [16–19]. Despite the numerous research efforts and various hypotheses made towards elucidating the synergism between cellulases on crystalline cellulose, the synergistic mechanisms by which cellulases hydrolyze cellulose are not yet fully comprehended [17,20]. Due to the overlapping functions and conflicting natures of endo- and exo-glucanases, the questions as to how these enzymes synergize at a molecular level, or why only a limited number of CBHs and EGs exhibit synergism, remain a mystery [21–23]. Moreover, only a few studies have investigated the synergistic relationships between fungal and bacterial enzymes, thus there is a lack of understanding on how the structural and mechanistic features of various GH families affect the synergistic relationship of a given cellulase cocktail for efficient cellulose hydrolysis. This raises questions, such as how do these enzymes function to depolymerize the diverse regions of the substrate, do all endo- and exo-acting enzymes act synergistically, and what are the factors that govern enzyme synergism? Furthermore, optimizing the composition of cellulase mixtures is an effective method to increase/improve their hydrolytic efficiency and reduce protein loadings during lignocellulose degradation.

The aim of this study was to provide insights into how the molecular mechanisms of cellulases from different GH families and various microbial sources govern synergism, by means of mapping various CBH to EG interactions. The enzymes used in this study included three CBHs from *Hypocrea jecorina* (HjCel7A), *Clostridium stercoararium* (CsCel48A), and *Trichoderma longibrachiatum* (TlCel7A); two CBHs from a microbial source (Cel6A) and *Clostridium thermocellum* (CtCel5A); three EGs from *Bacillus amyloliquefaciens* (BaCel5A), *Thermotoga maritima* (TmCel5A), and *Trichoderma reesei* (TrCel7B); a cellulase mixture containing three endoglucanases (EglA (Cel9), EglB (Cel12), and EglC (Cel74)) from *Aspergillus niger* (AnEG); and a β -glucosidase from *Aspergillus niger* (Novozyme 188; β gl). This rational approach of screening for maximal compatibility between different GH families could ultimately provide a platform for a better understanding of the specific interactions between the enzymes of interest and how the complex synergistic activities of different enzymes assist one another in degrading cellulose. Consequently, this can shed light on designing synergistic enzyme cocktails, which are tailored to effectively convert biomass into fermentable sugars, thus reducing the overall cellulase loadings required to achieve efficient biomass conversion.

2. Results and Discussion

2.1. Enzyme Characterization and Substrate Specificity Studies

All the cellulases used in this study were characterized based on their physicochemical properties (temperature optima, pH optima, and thermal stability). The pH optima of the enzymes were in the range of 4.0–7.0, with variable temperature optima in the range of

40–60 °C and the enzymes displayed thermal stability between 37 and 50 °C for over 24 h (data not shown). The cellulases were also tested for their specific activities on different cellulosic substrates suspended in sodium citrate buffer (50 mM, pH 5.0) at 37 °C (Table 1).

Table 1. Specific activities of cellulolytic enzymes on various cellulosic substrates.

Enzyme	Specific Activity (U/mg)				
	CMC	Avicel	pNPC	pNPG	Cellopentaol
		<i>Cellobiohydrolase I (CBHI)</i>			
(HjCel7A)	0.35 ± 0.01	0.10 ± 0.00	0.25 ± 0.01	0.06 ± 0.01	Nd
(TlCel7A)	0.70 ± 0.02	0.35 ± 0.01	0.15 ± 0.00	0.03 ± 0.01	Nd
(CsCel48A)	0.70 ± 0.02	0.028 ± 0.00	0.02 ± 0.00	0.09 ± 0.00	Nd
		<i>Cellobiohydrolase II (CBHII)</i>			
(CtCel5A)	4.90 ± 0.20	0.026 ± 0.00	0.017 ± 0.00	0.08 ± 0.00	0.50 ± 0.02
Microbial Cel6A	0.08 ± 0.00	0.05 ± 0.00	0.023 ± 0.00	0.03 ± 0.00	0.48 ± 0.02
		<i>Endo-glucanase (EG)</i>			
(BaCel5A)	21.30 ± 0.80	0.062 ± 0.00	5.59 ± 0.20	0.15 ± 0.00	0.55 ± 0.02
(TmCel5A)	22.01 ± 0.05	Nd	0.85 ± 0.03	0.20 ± 0.01	1.04 ± 0.05
(TrCel7B)	20.16 ± 1.00	0.16 ± 0.00	2.02 ± 0.09	0.05 ± 0.00	1.39 ± 0.06
AnEG (Cel 9, 12, 74)	42.60 ± 1.80	0.41 ± 0.02	0.80 ± 0.03	4.90 ± 0.20	2.05 ± 0.10
		<i>Glucosidase</i>			
βgl	0.30 ± 0.01	Nd	0.30 ± 0.01	53.2 ± 1.30	Nd

One U was defined as the amount of enzyme releasing 1 μmol of product per minute unless stated otherwise. Values are presented as means ± S.D. (n = 3). “Nd” = not detected.

In general, the CBHIs displayed the highest activities on Avicel, CMC, and pNPC, except for CsCel48A which displayed very low activities on Avicel and pNPC. All three CBHIs displayed low activities on pNPG and displayed no cellopentaol activity. Previous studies also reported low or no activity of GH family 48 on crystalline cellulose [24–27]. Interestingly, CsCel48A displayed high CMCase activity (0.70 U/mg), indicating that it exhibited endo-activity. The catalytic domains of *C. thermocellum* (Cel48S) and *C. cellulolyticum* (Cel48F), which have 54–56% amino acid sequence identity to CsCel48A, have previously been solved and found to be structurally different from other processive cellulases [28,29]. It has been proposed that the flexible loops forming the tunnel of Cel48s start as an open tunnel, allowing the enzymes to initiate an endo-cleavage attack on an exposed cellulose chain, followed by the processive hydrolysis of the cellulose chain [25,30]. The minimal activity by CsCel48 towards pNPC agreed with a study by Sánchez and coworkers [27] who reported that the GH48 enzymes show exceptionally low and restricted activity on most common cellulosic substrates.

Among the CBHIIIs, CtCel5A displayed the highest CMCase and cellopentaol activity of 4.90 and 0.5 U/mg, respectively, whereas the microbial Cel6A displayed very low activity on CMC (0.08 U/mg), but higher cellopentaol activity of 0.48 U/mg. Both CBHIIIs displayed exceptionally low activities on Avicel, pNPC, and pNPG [31,32]. Compared to Cel5A, Cel6A displayed minor endoglucanase (CMC) activity, which was probably due to differences in their active site tunnels. Studies have reported that GH5 enzymes may behave as endo-glucanolytic CBHs (multifunctional enzymes), which may be attributable to their typical (α/β)₈-barrel structures, with an open active cleft topology [33–35]. Another noteworthy feature of many GH5 family proteins is that their protein sequences sometimes include additional modules with different functions (particularly CBMs) [36]. Depending on the location (N- or C-terminal), the CBM is appended on the catalytic domain of GH5 enzymes, this may confer the catalytic domains to target different substrates with different chemistry and structures [36].

Among the EGs assessed, AnEG displayed the highest CMCase, Avicelase, glucosidase, and cellopentaol cleaving activities. BaCel5A displayed the highest activity on pNPC of 5.59 U/mg, whereas TmCel5A, TrCel7B, and AnEG exhibited 0.85, 2.02, and 0.80 U/mg activity on pNPC, respectively. BaCel5A, TmCel5A, and TrCel7B displayed similar CMCase activity at 21.3, 22.01, and 20.16 U/mg, respectively, and displayed little to no activity on Avicel. BaCel5A, TmCel5A, and TrCel7B displayed 0.15, 0.20, and 0.05 U/mg glucosidase

activity, respectively, and displayed 0.55, 1.04, and 1.39 U/mg cellopentaol cleaving activity, respectively. The catalytic sites of EGs exhibit extended open substrate-binding clefts, offering an easier entry to the cellulose chain [37]. Thus, CMC is the preferred substrate for determining EG activity, since CMC contains carboxymethyl substituents that can only enter enzyme active sites with an open conformation [3,38,39]. These enzymes displayed higher Avicelase activity compared to the CBHI enzymes assessed (excluding *TiCel7A*), as well as all the CBHII enzymes assessed in this study, thus, suggesting that these EGs exhibit exo-glucanase activity on micro-crystalline cellulose [8]. In addition, Kim [40] reported that endoglucanases from a *Bacillus* sp. and various fungi, may exhibit Avicelase activity in addition to CMCCase activity with the help of an exo-activity resident in the same molecule. Studies have revealed that EGs may exhibit processive behavior due to a CBM attached to the catalytic domain and have performed studies to support the critical role of CBMs for EG processivity [36,41]. Although the mode of processivity seems to be more common in CBHIs, studies have reported that EGs can change their character to facilitate a processive mode of action on recalcitrant substrates [36,41]. Their ability to cleave internal sites of the cellulose chain and to release soluble oligosaccharides prior to separating from the cellulose chain has resulted in the introduction of a subclass of cellulases, referred to as processive endo-cellulases [23,25,30].

Finally, β gl exhibited high glucosidase activity of 53.2 U/mg and minimal activity on CMC and *p*NPC. No detectable enzyme activity was observed on Avicel or cellopentaol. The enzyme was, therefore, assumed to have exhibited true β -glucosidase-like catalytic properties.

2.2. Exo–Exo Synergy between CBHI (*TiCel7A*) and CBHII (*Microbial Cel6A/CtCel5A*)

Exo–exo synergy between CBHI (*TiCel7A*) and CBHII (microbial *Cel6A*) was investigated to determine the optimal CBH mixture (CBHI + CBHII) cocktail required for cellulose hydrolysis (Figure 1a). *TiCel7A* was selected because it was the most active enzyme on Avicel (as seen in Table 1). A confirmatory study of the CBHI to CBHII synergistic ratio was performed by swapping CBHII (microbial *Cel6A*) with a CBHII from a different GH family and source (*CtCel5A*) (Figure 1b).

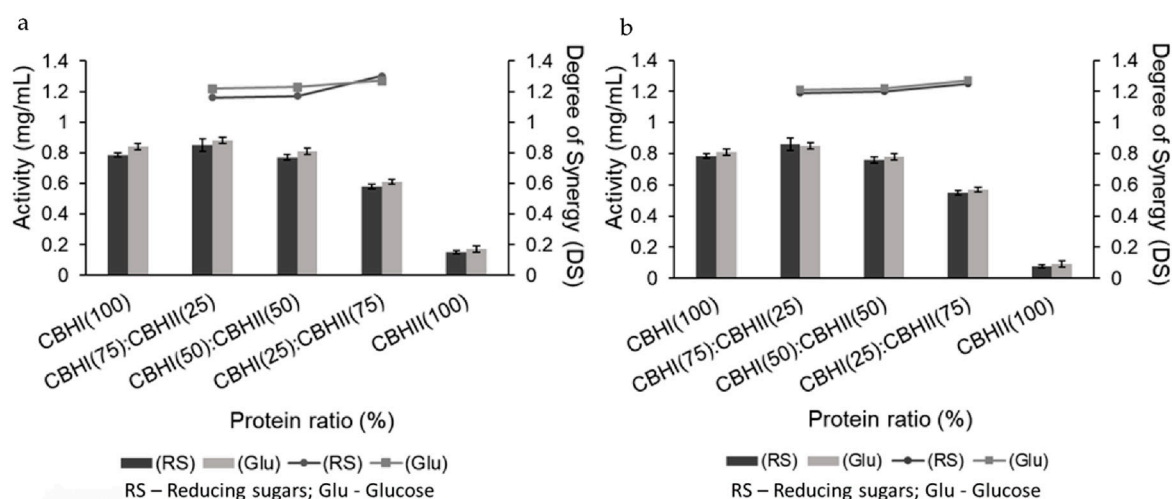


Figure 1. Synergistic degradation of Avicel by various ratios of *TiCel7A* (CBHI) and microbial *Cel6A* (CBHII) (a) and *TiCel7A* (CBHI) and *CtCel5A* (CBHII) (b). Assays were performed in sodium citrate buffer (0.05 M; pH 5.0) and incubated at 37 °C for 24 h. Values represented as mean values \pm S.D. ($n = 3$).

This exo–exo synergism is a phenomenon that was first confirmed by Fägerstam and Pettersson in 1980 [42] and has since then been confirmed by other researchers [9,16,43,44]. According to the exo–exo synergy model, it has been hypothesized that CBHI and CBHII prefer to hydrolyze cello-oligosaccharides from opposing sides of the cellulose chain

(CBHI—reducing and CBHII—nonreducing end). Consequently, this renders a strong synergistic relationship between the two different CBHs [28]. Based on these results, the ratio of 75% CBHI (*Ti*Cel7A):25% CBHII (microbial Cel6A or *Ct*Cel5A) was selected as the benchmark (optimal ratio) CBH mix (Cmix) for subsequent experiments. This ratio agrees with the CBHI (70%):CBHII (30%) ratio reported by previous researchers [9,44].

2.3. Synergism between Exo- and Endo-Glucanases

Cellulose hydrolysis and the synergistic interactions between various Cmix combinations (CmixA1, CmixA2, CmixB1, CmixB2, CmixC1, and CmixC2) and endo-glucanases were investigated (Table 2), whereby CmixA1 is (*Ti*Cel7A + microbial Cel6A), CmixA2 is (*Ti*Cel7A + *Ct*Cel5A), CmixB1 is (*Hj*Cel7A + microbial Cel6A), CmixB2 is (*Hj*Cel7A + *Ct*Cel5A), CmixC1 is (*Cs*Cel48A + microbial Cel6A), and CmixC2 is (*Cs*Cel48A + *Ct*Cel5A).

2.3.1. Synergy between CmixA1 (*Ti*Cel7A and Microbial Cel6A) or CmixA2 (*Ti*Cel7A and *Ct*Cel5A) and Various Endoglucanases

CmixA1 and *Ba*Cel5A displayed the highest degree of synergy (DS) at the ratio of 75%:25% followed by a ratio of 50%:50%. The total reducing sugars and glucose released from Avicel by this enzyme combination (75%:25%) were 1.13 mg/mL and 1.07 mg/mL, respectively. The highest DS around 1.40 also demonstrated that CmixA1 and *Ba*Cel5A were co-operating during hydrolysis. This observation agreed with previous studies [9,16,45] who determined that the optimum synergistic effect is achieved when a higher ratio of CBH to EG is used in the reaction. However, this is dependent on the nature and characteristics of the EG and the substrate used [46]. In contrast, there was no co-operation between CmixA1 and *Tm*Cel5A. CmixA1 and *Tm*Cel5A had a DS of 1.0, additionally, the total reducing sugars and glucose released were not higher than that of the CmixA1 (100%). The DS around 1.0 suggests that only CmixA1 was hydrolyzing the Avicel to produce the total reducing sugars. When combining CmixA1 with *An*EG, the highest amount of total reducing sugars were produced by a 25% CmixA1:75% *An*EG combination. This combination was unique, because the *An*EG enzyme (at 100%) in this case displayed a higher activity compared to the optimized CmixA1 (at 100%). In addition, at 50% CmixA1:50% *An*EG, the synergy displayed significantly high amounts of total reducing sugars, suggesting that both the CmixA1 and *An*EG enzymes were cooperating in hydrolyzing Avicel. For CmixA1 and *Tr*Cel7B, there was no synergy, and this was supported by the DS values, which were below 1.0.

Generally, due to the high synergistic effect exhibited by this enzyme combination (CmixA1 and EGs) and the significant increase in soluble sugars released, we propose that CmixA1 (CBHI and CBHII) was the main contributor to Avicel hydrolysis and that the presence of EG induced the synergistic efficiency by shearing the β -1,4 glycosidic bonds from internal filaments, releasing smaller fragments of cellulose to provide new sites for CBH binding [16,17,46]. An alternative explanation for endo–exo synergism has been proposed by Våljamäe and coworkers [18], known as the surface erosion model. This model is based on the hypothesis that CBHI modifies the substrate structure, thereby generating obstacles that results in slowing dissociation as well as stalling itself, leading to an overall loss in catalytic efficiency. However, the eroded substrate is more accessible for EG activity, which then cleaves the substrate into smaller fragments to release the trapped CBHs [17,22]. As a result, the synergistic interaction of endo- and exo-cellulases results in the acceleration of cellulose hydrolysis [18,44].

Table 2. Summary of the reducing sugars (RS) and glucose (Glu) released after 24 h hydrolysis of Avicel by the synergistic action of cellulases from different GH families and microbial sources.

Cmix	Cmix/Endo (%)	BaCel5A				TmCel7A				AnEG				TrCel7B			
		RS (mg/mL)	Glu (mg/mL)	DS _{RS}	DS _{Glu}	RS (mg/mL)	Glu (mg/mL)	DS _{RS}	DS _{Glu}	RS (mg/mL)	Glu (mg/mL)	DS _{RS}	DS _{Glu}	RS (mg/mL)	Glu (mg/mL)	DS _{RS}	DS _{Glu}
A1	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.04	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.71 ± 0.03	0.70 ± 0.04	1.19	1.26	0.47 ± 0.01	0.49 ± 0.02	1.12	1.16	1.29 * ± 0.06	1.18 * ± 0.05	1.08	1.10	0.69 ± 0.02	0.65 ± 0.02	0.90	0.88
	50/50	0.95 * ± 0.03	0.90 * ± 0.04	1.30	1.28	0.61 ± 0.02	0.64 ± 0.02	1.09	1.09	1.27 * ± 0.05	1.13 ± 0.03	1.15	1.31	0.83 ± 0.03	0.82 ± 0.03	0.95	0.99
	75/25	1.13 * ± 0.05	1.07 * ± 0.04	1.38	1.40	0.80 ± 0.03	0.78 ± 0.04	1.11	1.14	1.26 * ± 0.04	1.10 ± 0.05	1.15	1.11	0.86 ± 0.03	0.83 ± 0.03	0.96	1.00
	100/0	0.84 ± 0.03	0.85 ± 0.04	–	–	0.84 ± 0.03	0.85 ± 0.04	–	–	0.84 ± 0.03	0.85 ± 0.04	–	–	0.84 ± 0.03	0.85 ± 0.04	–	–
A2	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.04	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.80 ± 0.03	0.75 ± 0.02	1.35	1.37	0.50 ± 0.02	0.50 ± 0.02	1.26	1.2	1.43 * ± 0.07	1.35 * ± 0.06	1.22	1.29	0.70 ± 0.02	0.69 ± 0.02	0.92	0.95
	50/50	1.06 * ± 0.05	0.95 * ± 0.04	1.38	1.37	0.73 ± 0.02	0.72 ± 0.03	1.22	1.17	1.40 * ± 0.07	1.32 * ± 0.06	1.20	1.20	0.80 ± 0.03	0.78 ± 0.04	0.93	0.90
	75/25	1.21 * ± 0.06	1.10 * ± 0.05	1.47	1.41	0.89 ± 0.03	0.82 ± 0.03	1.23	1.22	1.32 * ± 0.05	1.31 * ± 0.05	1.30	1.27	0.85 ± 0.03	0.82 ± 0.04	0.92	0.94
	100/0	0.89 ± 0.04	0.83 ± 0.03	–	–	0.89 ± 0.04	0.83 ± 0.03	–	–	0.89 ± 0.04	0.83 ± 0.03	–	–	0.8 ± 0.04	0.83 ± 0.03	–	–
B1	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.04	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.25 ± 0.01	0.29 ± 0.01	0.63	0.64	0.21 ± 0.00	0.26 ± 0.00	0.98	0.80	0.98 ± 0.4	0.94 ± 0.03	0.98	1.00	0.45 ± 0.02	0.45 ± 0.02	0.77	0.69
	50/50	0.31 ± 0.01	0.35 ± 0.01	0.64	0.64	0.30 ± 0.01	0.33 ± 0.00	0.94	0.80	0.88 ± 0.04	0.84 ± 0.03	1.00	0.99	0.43 ± 0.01	0.45 ± 0.02	0.72	0.67
	75/25	0.37 ± 0.01	0.40 ± 0.02	0.77	0.72	0.38 ± 0.02	0.41 ± 0.02	0.99	0.91	0.76 ± 0.03	0.75 ± 0.02	1.00	0.96	0.43 ± 0.02	0.43 ± 0.01	0.74	0.68
	100/0	0.40 ± 0.02	0.42 ± 0.02	–	–	0.40 ± 0.02	0.42 ± 0.02	–	–	0.40 ± 0.02	0.42 ± 0.02	–	–	0.40 ± 0.02	0.42 ± 0.02	–	–
B2	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.04	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.28 ± 0.00	0.34 ± 0.00	0.72	0.72	0.23 ± 0.01	0.28 ± 0.01	0.98	0.87	1.04 ± 0.05	1.00 ± 0.05	1.03	0.95	0.56 ± 0.02	0.53 ± 0.01	0.98	0.82
	50/50	0.34 ± 0.01	0.38 ± 0.00	0.76	0.72	0.29 ± 0.01	0.37 ± 0.02	0.97	0.84	0.85 ± 0.04	0.80 ± 0.03	1.02	0.98	0.54 ± 0.02	0.54 ± 0.00	0.94	0.83
	75/25	0.37 ± 0.01	0.44 ± 0.01	0.88	0.82	0.34 ± 0.01	0.38 ± 0.01	0.99	0.88	0.69 ± 0.04	0.66 ± 0.03	0.97	0.86	0.51 ± 0.02	0.52 ± 0.02	0.94	0.85
	100/0	0.40 ± 0.02	0.44 ± 0.02	–	–	0.40 ± 0.02	0.44 ± 0.02	–	–	0.40 ± 0.02	0.44 ± 0.02	–	–	0.40 ± 0.02	0.44 ± 0.02	–	–
C1	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.04	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.15 ± 0.00	0.23 ± 0.01	0.73	0.95	0.00 ± 0.00	0.09 ± 0.00	0	0.98	0.75 ± 0.03	0.69 ± 0.02	0.94	0.99	0.39 ± 0.02	0.41 ± 0.02	1.16	1.00
	50/50	0.14 ± 0.00	0.20 ± 0.00	0.76	0.93	0.00 ± 0.00	0.13 ± 0.00	0	1.23	0.45 ± 0.02	0.40 ± 0.01	0.65	0.78	0.36 ± 0.01	0.38 ± 0.01	1.18	1.05
	75/25	0.12 ± 0.00	0.19 ± 0.00	0.88	0.86	0.09 ± 0.00	0.15 ± 0.00	1.28	1.24	0.16 ± 0.00	0.21 ± 0.01	0.38	0.46	0.29 ± 0.01	0.33 ± 0.01	1.18	1.04
	100/0	0.10 ± 0.00	0.15 ± 0.00	–	–	0.10 ± 0.00	0.15 ± 0.00	–	–	0.10 ± 0.00	0.15 ± 0.00	–	–	0.10 ± 0.00	0.15 ± 0.00	–	–
C2	0/100	0.18 ± 0.00	0.23 ± 0.01	–	–	0.00 ± 0.00	0.00 ± 0.00	–	–	1.16 ± 0.06	1.10 ± 0.05	–	–	0.45 ± 0.01	0.47 ± 0.02	–	–
	25/75	0.14 ± 0.00	0.19 ± 0.00	0.70	0.85	0.00 ± 0.00	0.06 ± 0.00	0.00	0.78	0.79 ± 0.04	0.71 ± 0.03	1.00	1.00	0.41 ± 0.02	0.41 ± 0.02	1.09	0.99
	50/50	0.13 ± 0.00	0.18 ± 0.00	0.72	0.85	0.00 ± 0.00	0.08 ± 0.00	0.00	0.86	0.53 ± 0.02	0.51 ± 0.02	0.96	1.00	0.36 ± 0.01	0.39 ± 0.01	1.18	1.04
	75/25	0.10 ± 0.00	0.16 ± 0.00	0.83	0.80	0.07 ± 0.00	0.13 ± 0.00	1.20	1.23	0.25 ± 0.00	0.30 ± 0.01	0.58	0.66	0.29 ± 0.01	0.31 ± 0.01	1.17	1.06
	100/0	0.09 ± 0.00	0.14 ± 0.00	–	–	0.09 ± 0.00	0.14 ± 0.00	–	–	0.09 ± 0.00	0.14 ± 0.00	–	–	0.09 ± 0.00	0.14 ± 0.00	–	–

The activity values (RS and Glu) are presented as means ± S.D. ($n = 3$). RS—reducing sugars, Glu—glucose, DS_{RS}—degree of synergy for reducing sugars, DS_{Glu}—degree of synergy for glucose. CmixA1 is (TmCel7A + microbial Cel6A), CmixA2 is (TmCel7A + CtCel5A), CmixB1 is (HjCel7A + microbial Cel6A), CmixB2 is (HjCel7A + CtCel5A), CmixC1 is (CsCel48A + microbial Cel6A), and CmixC2 is (CsCel48A + CtCel5A). * represents the product release values that are significantly higher than those generated by the most active single enzyme.

The results obtained with CmixA2 were comparable to the synergistic relations established with CmixA1. The results showed that CmixA2 displayed the highest synergistic effect with *BaCel5A* (DS for reducing sugars: 1.47 and DS for glucose: 1.41) in a protein ratio of 75–25%. This enzyme combination (CmixA2 75%:*BaEngCel5A* 25%) also produced significantly more soluble sugars than the total amount produced by these enzymes individually at 100% protein loading. The highest quantity of sugars (reducing sugars: 1.43 mg/mL and glucose: 1.35 mg/mL) was also liberated by the combined activities of *AnEG* and CmixA2 at a protein ratio of 75–25%, respectively. No synergy between CmixA2 and the other two EGs (*TmCel5A* and *TrCel7B*) was observed, which was supported by the low DS values (<1.0). We, therefore, propose that *Trichoderma* endoglucanases do not synergize with CmixA (both CmixA1 and CmixA2).

This further confirms the observations and hypotheses of previous researchers who stated that the free chain ends produced by the endo-glucanase, *TrCel7B*, are not always accessible to CBHI [47] or that overlapping functions or competition for the same binding sites on the substrate result in antisynergy [28,48,49]. When the activity of one enzyme does not facilitate the activity of another enzyme then, antisynergy is observed (DS < or = 1.0), whereas this phenomenon may also occur when competitive behavior between the enzymes is observed (enzymes competing for the same binding sites on the substrate; DS < 1.0) [48–50].

2.3.2. Synergy between CmixB1 (*HjCel7A* and Microbial *Cel6A*) or CmixB2 (*HjCel7A* and *CtCel5A*) and Various Endoglucanases

No synergism was detected between CmixB1 and any of the EGs, and the combinations did not improve the overall cellulose hydrolysis. At 100% protein loading, CmixB1 produced 0.40 mg/mL of reducing sugars and 0.42 mg/mL of glucose, which was higher than the sugars liberated by all the tested combinations. The results also showed that as the *AnEG* amount (%) in the enzyme combination decreased, the total amount of reducing sugars and glucose also decreased. This was a clear indication that there was no cooperation between CmixB1 and *AnEG* during the hydrolysis of Avicel. For synergy between CmixB1 and *BaCel5A/TmCel5A*, a similar trend was observed in the opposite direction, whereby CmixB1 resulted in the increased sugar release from the Avicel, indicating that there was still no interaction/cooperation observed between the enzymes.

The results for CmixB2 were similar to the findings between CmixB1 and EGs. No synergism was detected between CmixB2 and any of the EGs, and the overall cellulose hydrolysis was not improved. When CmixB2 was used individually (100% protein loading), it liberated 0.40 mg/mL of reducing sugars and 0.44 mg/mL of glucose. Based on these results, we propose that CmixB2 does not interact with any of the endo-glucanases (bacterial and fungal), and therefore, it cannot be a suitable component of the enzyme cocktail for effective biomass hydrolysis.

These results provide clear evidence that not all endo- and exo-cellulases act synergistically. One may ask why pronounced synergism was established between various combinations of EGs and *TiCel7A*, but not with *HjCel7A*? What differentiates these CBHs from the same glycoside hydrolase family (7) from each other? Various studies have reported that observed synergism could be related to their adsorption capacities. If both enzymes are weakly bound to the substrate, synergism will be shown to a much lesser degree [51]. Furthermore, we assume that these discrepancies in our results are likely due to differences in these enzymes' specific activities on the cellulose substrate. As mentioned previously, *TiCel7A* had 2-to-3-fold higher activity on Avicel and CMC (Table 1) compared to *HjCel7A*. Here, we propose that the low Avicelase activity of *HjCel7A* cannot effectively access the individual chains in the cellulose provided by EGs and is, therefore, considered rate-limiting in effective cellulose hydrolysis. This makes *HjCel7A* an unsuitable candidate in designing an optimal cellulase cocktail.

2.3.3. Synergy between CmixC1 (CsCel48A and Microbial Cel6A) or CmixC2 (CsCel48A and microbial Cel6A) and Various Endoglucanases

It was observed that CmixC1 (CsCel48A + microbial Cel6A) or EG alone at a 100% protein mass loading released the same quantity or even more sugars than their combinations. Therefore, no synergistic relationships were established between CmixC1 and any of the EGs assessed in this study. Similar results to that obtained between CmixC1 and EGs were observed when assessing the synergistic relationships between CmixC2 (CsCel48A + CtCel5A) and the EGs. It was observed that CmixC2 (CsCel48A + CtCel5A) or EG alone at a 100% protein mass loading released the same quantity or even more sugars than any of the combinations tested. Again, no synergistic relationships were established between CmixC2 and any of the EGs.

We suggest that although the endo- and exo-cellulases behaved synergistically, the overall hydrolysis was not enhanced due to the insufficient exo-cellulase activity exhibited by CsCel48A, thereby restricting the activity of the EGs to completely hydrolyze the substrate. Similar findings were reported by Irwin and coworkers [52]; however, their study assessed the synergistic relationship between fungal exo-glucanases and bacterial endo-glucanases on filter paper. The highest synergistic association (DS 1.28 and 1.24 for reducing sugars and glucose) was established when CmixC1/CmixC2 and *TmCel5A* were combined in a protein ratio of 75–25%. We postulate that the ability of *TrCel7B* to synergistically interact with CsCel48A and not with *HjCel7A*, is due to the low specific activity exhibited by CsCel48A on Avicel, which agreed with a study conducted by Sánchez and coworkers [27]. They suggested that the low activity of Cel48A makes it incompatible with the conventional role of exo-cellulases, as it cannot effectively gain access in the individual chains of cellulose during cellulose hydrolysis. Thus, it is not clear whether the mechanism by which Cel7A and Cel48A synergize with other enzymes is similar. Furthermore, they found that Cel48A from *T. fusca* synergized well with Cel9A from *T. fusca* and that these two enzymes play similar roles to that of *TrCel7B* and *TrCel7A*, respectively. Thus, we assume that *TrCel7B*, a processive endo-cellulase, with relatively high activity on Avicel played the role of CsCel48A, this leads to noticeable synergy, but not enhanced cellulose hydrolysis. These results further support the evidence that synergy and high product formation do not always go hand-in-hand [13,53].

In contrast to the synergistic behavior observed between CmixA1/CmixA2 and *BaCel5A*, as well as with *AnEG*, CmixC1/CmixC2 displayed antis synergistic behavior. Although we postulate that this is attributed to the insufficient degree of cellulose hydrolysis displayed by CsCel48A, it remains unclear as to why a synergistic relationship would be established when CsCel48A was combined with some processive enzymes, *TrCel7B* or *TmCel5A*, but not with the other processive enzymes, *AnEG* and *BaCel5A*. These results support the evidence that the relationships between enzymes are overly complex and are highly dependent on the type of enzymes and the degree to which their activities complement each other. Therefore, there is no “guaranteed” optimal ratio of endo-cellulase/exo-cellulase for achieving the maximum degree of synergy, since the phenomenon that causes the synergistic effect depends not only on the particular enzyme classes involved but also on the nature and catalytic properties of the enzyme [43].

3. Materials and Methods

3.1. Materials

Avicel pH-101; carboxymethyl-cellulose (CMC); *para*-nitrophenyl substrates (*p*-nitrophenyl- β -D-cellobioside and -glucopyranoside); cellobiohydrolase I from *Hypocrea jecorina* (Cel7A); and a cellulase containing three endoglucanases (EglA (Cel9), EglB (Cel12), and EglC (Cel74)) from *Aspergillus niger* were purchased from Sigma Aldrich (St Louis, MO, USA). The 1,4- β -D-cellopentaol, CBHI from *Trichoderma longibrachiatum* (Cel7A); CBHII from a microbial source (Cel6A); and three endoglucanases (EG) *Bacillus amyloliquefaciens* (Cel5A), *Thermotoga maritima* (Cel5A), and *Trichoderma reesei* (Cel7B) were purchased from Megazyme™ (Bray, CW, Ireland). CBHI from *Clostridium stercorarium* (Cel48A) and CBHII

from *Clostridium thermocellum* (Cel5A) were purchased from NZYTech Ltd. (Paço do Lumiar, Lisboa, Portugal), while β -glucosidase (Novozyme 188) was purchased from Novozymes (Bagsvaerd, Hovedstaden, Denmark). The enzymes used in this study were chosen according to their GH family affiliations, the source of organism (fungal or bacterial) and their commercial availability.

3.2. Protein Determination

The protein concentrations of the cellulases were determined by the Bradford method with bovine serum albumin (BSA) was used as a suitable protein standard [54].

3.3. Substrate Specificity Determination

Avicelase and endo-glucanase activities were determined using the polymeric substrates, Avicel and CMC, as described by [55]. The reactions were conducted at 37 °C for 24 and 1 h for Avicelase and CMCCase assays, respectively, whereas nonreducing end cellobiosidase activity was determined using cellopentaol, a modified oligosaccharide [55]. The 3,5-dinitrosalicylic acid (DNS) method was used for measuring the amount of reducing sugars liberated, whereby glucose was used as suitable standard [56]. Glucosidase and reducing end cellobiosidase activities were assayed using 2 mM *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) and *p*-nitrophenyl- β -D-cellobioside (*p*NPC), respectively [55]. In all studies, one unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of product per minute under standard assay conditions.

3.4. Synergy Studies

The synergistic relationships between the enzymes were determined by setting up various reactions containing different protein mass ratios of exo/endo- and exo-cellulases. The total protein loading for all enzyme cocktail experiments was kept at 1.375 mg protein/g of Avicel, which was supplemented with Novozyme 188 (10% mass ratio of the total protein loading) to ensure complete hydrolysis of cellobiose. The assays were carried out in 400 μ L reaction volumes, containing 2% (*w/v*) substrate suspended in sodium citrate buffer (50 mM, pH 5.0). The reactions were conducted at 37 °C with mixing at 25 rpm for 24 h.

The hydrolysis was terminated by heating the enzymes at 100 °C for 5 min followed by cooling down at 4 °C. Hydrolysis controls included substrate (containing only the substrate without the enzyme) and enzyme controls (containing only the enzyme without the substrate). Analysis for reducing sugars and glucose released was conducted according to the methods described previously [56]. All enzyme hydrolysis assays were performed in triplicate.

3.4.1. Determination of the Optimal CBHI: CBHII Ratio (Exo–Exo Synergy) and the Effect of CBHII Swapping on Synergy

We investigated exo–exo synergy between CBHI (*Ti*Cel7A) and CBHII (microbial Cel6A) to determine the optimal CBH (CBHI + CBHII) cocktail required for cellulose hydrolysis. We later swapped the microbial Cel6A (CBHII) with a CBHII from a different GH family and enzyme source (*Ct*Cel5A) to compare their hydrolysis and synergistic profile with CBHI. The ratio of CBHI:CBHII combination that resulted in increased cellulose hydrolysis was chosen as the benchmark, denoted *C*_{mix}, for subsequent experiments.

3.4.2. Synergistic Interactions between Endo- and Exo-Acting Cellulases from Different GH Families

The benchmark *C*_{mix} (CBHI (*Ti*Cel7A):CBHII (microbial Cel6A or *Ct*Cel5A)) at 75%:25% protein loading was mapped with the activities of four different EGs (*Ba*Cel5A, *Tm*Cel5A, *Tr*Cel7B, and *An*Cel9, 12, 74) at different protein ratios, and assessed for their capacity to liberate reducing sugars and glucose (individually and in combination) from Avicel.

3.4.3. CBHI Swapping

The CBHI (*TiCel7A*) in the benchmark mixture was then swapped with a CBHI from a different GH family and/or microbial source (*HjCel7A* or *CsCel48A*) to assess whether this approach of CBHI swapping had any influence on the synergism established between endo- and exo-cellulases. The ratio of 75%:25% protein loading between CBHI: CBHII was kept consistent throughout all the experiments.

3.4.4. Calculation of the Degree of Synergy (DS)

The degree of synergy was calculated as the ratio between the observed activity produced by the combination of enzymes performing in synergy to the sum of the activities produced by the individual enzymes [23,46].

$$DS = (\text{observed activity of combined enzyme 1 \& 2}) / (\text{activity of enzyme 1}) + (\text{activity of enzyme 2})$$

The three outcomes for DS are: (i) $DS > 1.0$, enzymes act synergistically to degrade a substrate; (ii) $DS = 1.0$, no synergy, and (iii) $DS < 1.0$, enzymes do not interact synergistically, which could be a result of competitive behavior [13,49].

3.5. Data Analysis

One-way analysis of variance (ANOVA) was used for the evaluation of significant increase in activity exhibited by enzyme combinations (considered with respect to reducing sugars and glucose released) compared to the theoretical sum of the individual enzyme controls for each combination. All pairwise comparison procedures were based at 95% and 99% confidence level ($p < 0.01$ and 0.05) and were conducted using the Data Analysis feature in Microsoft Excel 2016 (Redmond, WA, USA).

4. Conclusions

In this investigation, cross synergism was explored by swapping various endo- and exo-cellulases from different GH families and mapping their activity at different protein ratios. The results showed that *CmixA2* displayed the highest synergistic effect with *BaCel5A* (DS for reducing sugars: 1.47; DS for glucose: 1.41) in a protein ratio of 75–25%. The different family GHs were successfully characterized with respect to their biochemical characteristics. It was evident that although the enzymes are substrate specific (based on their 3D structure and sequence similarity), it is apparent that some endo-glucanases may have dual functions due to their processive nature, thus, they display both endo- and exo-glucanase activity. Synergy studies showed that swapping CBHII had minor effects on the synergistic and hydrolysis patterns observed between cellobiohydrolases, suggesting that CBHII enzymes might share similar catalytic mechanisms. On the other hand, CBHI swapping exhibited drastic changes even for same GH family CBHs. It was also shown that synergy between endo- and exo-glucanases is not always achieved; it seemed the synergistic effect depends not only on the enzyme class but also on the nature of the enzymes involved. We, therefore, postulate that due to the different substrate specificities exhibited by cellulases from different GH family affiliations and microbial sources, enzymes are not always compatible with each other for efficient cellulose degradation. It appears that only by examining the specific activities of an enzyme (individually and in combination), we can substantiate whether an enzyme/enzyme cocktail will offer any advantages towards enhancing cellulose hydrolysis.

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