CONDITIONS OF HYDROLYSIS WITH A SPECIFIC PAIR OF ENDO- AND EXO-CELLULASES

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Abstract

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Enzymatic hydrolysis of cellulose is a technology involved in the production of bioethanol, a potential alternative renewable energy. Many cellulases with endo- and exo-type of activity are known to hydrolyze cellulose synergistically. In this thesis, potential synergy between an endo-cellulase, Cel5B, with and without a carbohydrate- binding module (CBM6), and a new exo-cellulase, CBH1, from *Trichoderma harzianum* FP108 were examined during the hydrolysis of semi- crystalline cellulose (Avicel). Since CBM6 is recognized as having a high affinity for amorphous cellulose, it was hypothesized that this affinity could enhance the synergistic effect between the endo- and exo-cellulases by focusing the action of Cel5B+CBM6 on the amorphous regions of the Avicel substrate. The increased activity of Cel5B+CBM6 over Cel5B alone was confirmed. However, in contrast to our expectations, a synergistic effect was not observed between either endo- and exo-cellulase pairs. From the obtained hydrolysis yield, it was inferred that Cel5B+CBM6 may have exo-type activity that caused a competitive interaction with the exo-cellulase, which resulted in no synergy.

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Chapter 1 - Introduction

With an ever increasing environmental awareness and the inevitable depletion of the world's oil supply (Kerr, 1998), there has been a growing worldwide interest in obtaining an alternative, non-fossil fuel-based, environmentally acceptable energy supply (Sheehan and Himmel, 1999). Under such circumstances, the potential importance of cellulose, the most abundant carbon source on the planet, in the context of conversion of plant biomass into a liquid fuel, bioethanol, has been recognized on a global scale (Mielenz, 2001; Wheals *et al.*, 1999; Lynd *et al.*, 2002).

Current primal use of bioethanol is as a gasoline additive. Due to its high oxygen content, the added ethanol promotes more complete combustion of gasoline, which leads to a reduction of problematic air pollution, such as carbon monoxide (CO) and unburned hydrocarbons (Wyman, 1999; Mielenz, 2001). Besides the advantages in terms of mitigating the emission of pollutants, bioethanol has a great potential to be a gasoline substitute. The renewable nature of biomass makes bioethanol not only a secure source of energy but also an instrument to reduce the net carbon dioxide emission, which is known as the major anthropogenic greenhouse gas responsible for the controversial global warming effect (Wyman, 1999; Sheehan and Himmel, 1999).

Bioethanol can be produced from various plant materials such as cane sugar, starchy materials (e.g. corn and wheat) and lignocellulosic materials (e.g. agricultural residues, trees, and used paper). Although the cost of bioethanol production has been significantly reduced over the last two decades (Wyman, 1999; Wheals *et al.*, 1999), it still requires subsidies from governments to be economically competitive with gasoline (Himmel *et al.*, 1999). One of the key areas where a further cost reduction can be expected is a process of cellulose saccharification into glucose, which is historically carried out by acid hydrolysis techniques

(Wyman, 1999). Alternatively, this conversion can be achieved by using cellulose-hydrolyzing enzymes called cellulases. The acidic hydrolysis is usually a faster reaction than enzymatic hydrolysis (Sheehan and Himmel, 1999), but the latter option has other advantages. For instance, the hydrolysis reaction can be performed in milder conditions, which consumes less energy. Also, the amount of waste chemicals to be disposed is significantly reduced, which lessens not only an economical burden but also environmental concerns (Howard *et al.*, 2003). Furthermore, compared to an already mature technology of acid hydrolysis with more than two centuries of history (Sheehan and Himmel, 1999), enzymatic hydrolysis is based on a rapidly developing biotechnology and holds large potential for further improvements. Therefore, enzymatic hydrolysis of cellulose is a more preferable alternative technology than acid hydrolysis from economical as well as environmental perspectives.

When ethanol is produced through enzymatic hydrolysis, however, the high cost is the price of enzymes, which is a major factor preventing a full diffusion of bioethanol into society (Mielenz, 2001). Thus, the cost resulting from the use of enzymes needs to be reduced for this technology to become a fully feasible means to provide bioethanol. Hence, the general goal of this research was aimed at a reduction of the cost involved in the enzymatic hydrolysis of cellulose by improving the efficiency of the enzymatic reaction.

Chapter 2 - Literature Review

The current technology of enzymatic cellulose hydrolysis is established on a substantial amount of on-going research which started more than six decades ago, and a good understanding of fundamental past discoveries and achievements is an essential step to accomplish a further improvement for this technology. Therefore, this section reviews our current knowledge about characteristics of cellulose and cellulases as well as their interactions.

2.1 Enzymatic Hydrolysis of Cellulose

Cellulose is a linear mono-polymer chain solely comprised of D-glucose units linked to one another via β-1,4-glycosidic bonds. One chain end is termed the reducing end because the hemiacetal of the glucose molecule is able to open and expose the reducing aldehyde. The other chain end is called the non-reducing end because the one carbon in the hemiacetal is involved in the glycosidic bond, which prevents the ring from opening (Mosier *et al.*, 1999). The adjacent glucose molecules are rotated by 180° relative to each other, and thus the repeating unit of a cellulose chain is the cellobiose unit (Figure 1 a). Native cellulose has an average degree of polymerization (DP) ranging from 7000 to 15,000 glucose units. A shorter cellulose chain with glucose units from 3 to 12 is called cello-oligosaccharide, and the chain with DP > 7 is water-insoluble (Zhang and Lynd, 2004). Approximately 30-40 individual cellulose chains are laterally attached together through hydrogen bonds as well as van der Waals forces to form a tight linear bundle called an elementary cellulose fibril (Souza *et al.*, 2002). An important feature of cellulose is that some parts of the chains within the elementary

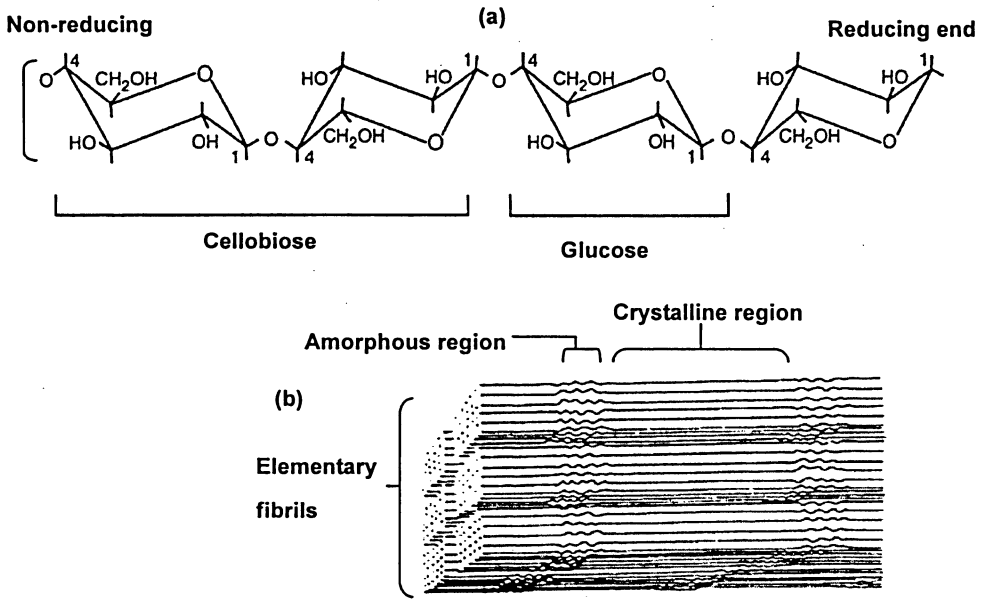


Figure 1. Structure of cellulose: (a) Cellobiose and glucose units, (b) Cellulose elementary fibrils with crystalline and amorphous regions (modified from Bhat and Hazlewood, 2001).

fibril exist in a highly organized crystalline structure due to inter and intra hydrogen bonds while the other parts remain in more loosely arranged amorphous structure (Figure 1 b). The degree of crystallinity, as opposed to amorphousness, varies greatly from 0 to 100% depending on the origin of cellulose materials and their preparation methods (Bhat and Hazlewood, 2001). Most naturally occurring cellulose contains 40 to 90% crystallinity (O'Sullivan, 1997).

Cellulose can be hydrolyzed by many organisms including fungi and microorganisms which produce cellulases. Unlike most other enzymatic reactions, the water-insoluble nature of cellulose necessitates the adsorption of cellulases onto the substrate prior to the hydrolysis reaction taking place (Mansfield *et al.*, 1999). Upon the adsorption, cellulase hydrolyzes cellulose by cleaving the β-1,4-glucosidic bonds between glucose units

in cellulose (Lynd *et al.*, 2002). Despite this seemingly simple interaction between cellulose and cellulase, hydrolysis of cellulose is, in practice, very challenging.

The hydrolysis of cellulose can be observed in terms of the production of soluble reducing sugars such as glucose. The reaction begins with a relatively rapid increase in the yield of reducing sugars, which, however, is typically followed by a distinct slowdown of the rate of sugar production as the reaction proceeds (Mosier *et al.*, 1999). Subsequently, the hydrolysis often stops before the substrate is completely converted into soluble sugars (Mansfield *et al.*, 1999). Over the last few decades, many studies examined factors that limit the hydrolysis (reviews are available: Esteghlalian *et al.*, 2001; Mansfield *et al.*, 1999; Hatfield *et al.*, 1999; Zhang and Lynd, 2004), and the recognized limiting factors include the crystalline structure, the degree of polymerization (DP), available surface area, and size of cellulose substrate. In particular, the crystalline structure is a unique characteristic of cellulose compared to other carbohydrates, such as amylose and xylose. The cellulose chains in a crystalline structure are packed very tightly, which prevents penetration not only of enzymes but also of small molecules such as water (Lynd *et al.*, 2002). Therefore, hydrolysis of crystalline cellulose is considered to be very difficult for cellulases. However, the relative extent to which one factor contributes to the limitation of cellulose hydrolysis compared to others is still poorly understood largely due to tremendous difficulty involved in controlling only one factor without changing the others (Zhang and Lynd, 2004).

2.2 Classification of Cellulases

In order to overcome the recalcitrance of cellulose to hydrolysis, cellulose-degrading organisms produce a mixture of cellulases with different types of activity, and they have been

classified into three major groups, endo-cellulase, exo-cellulase and β-glucosidase, according to their estimated mode of hydrolytic actions, which is summarized in Table 1, and also illustrated in Figure 2. This classification is currently well-accepted and will be used to describe enzymes throughout this paper. However, since the assumed complicated actions, especially those of endo- and exo-cellulases, are based on intensive observations of a respective enzyme's distinct patterns of reducing sugar production from cellulose, it is worthwhile to discuss how cellulases can be designated as endo- and exo-cellulases. Endo-cellulase randomly cleaves bonds in the internal amorphous regions and releases insoluble free chains.

Table 1. Classification of cellulases based on the estimated mode of action (from Lynd *et al.*, 2002; Irwin *et al.*, 1993; Zhang and Lynd, 2004)

Classification	Estimated mode of action	Other characteristics
Endo-cellulase	Randomly cleaves bonds within the internal amorphous regions and releases shorter free chains with high DC	• Active on amorphous cellulose • Little activity on highly crystalline cellulose • Effective in reducing DP
Exo-cellulase	Acts in a successive manner from the ends of the chains regardless of crystallinity while releasing mainly cellobiose units	• Active on both amorphous and crystalline cellulose • Slow in reducing DP
β-Glucosidase	Hydrolyzes mainly cellobiose units into glucose units	• Not active on insoluble cellulose (DP>7)

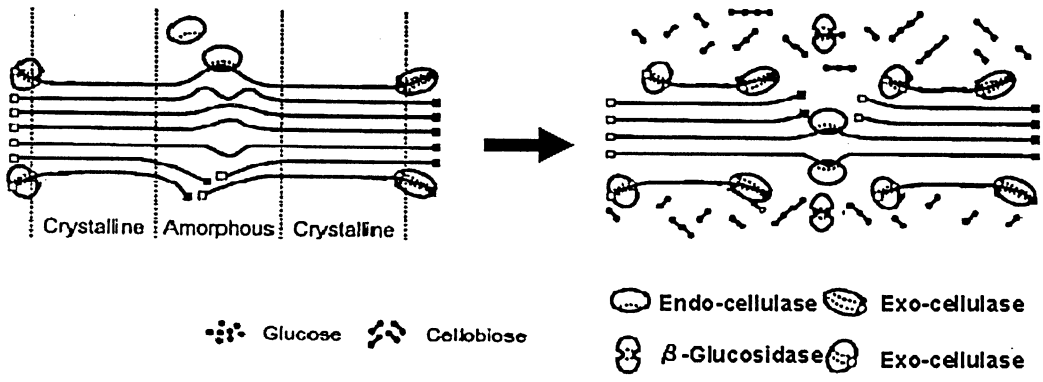


Figure 2. Enzymatic hydrolysis of cellulose (modified from Lynd *et al.*, 2002)

These chains are very difficult parameters to quantify (Moster *et al.*, 1999). Therefore, endo-cellulase is characterized by examining an enzyme's activity in terms of reducing the DP of cellulose, which is usually carried out by using a soluble cellulose derivative, such as carboxymethyl cellulose (CMC; Zhang and Lynd, 2004; Irwin *et al.*, 1993). CMC solutions have a high viscosity, which decreases in proportion to the reduction of the DP of CMC. Thus, an enzyme which quickly reduces the viscosity is considered to have the ability to reduce the DP of cellulose. Therefore, enzymes with a relatively high activity in the reduction of viscosity are classified as endo-cellulases whereas those with little activity are classified as exo-cellulases (Wood and Bhat, 1988; Gilad *et al.*, 2003).

Another way to distinguish the two types of cellulase is based on exo-cellulase-specific characteristics such as successive movements from cellulose chain ends while releasing cellobiose units. However, such characteristics are even more difficult to experimentally prove than those of endo-cellulases (Wood and Bhat, 1988). In fact, despite the wide-spread notion of "successive" and "processive" to describe the mode of exo-cellulase action, no analytical method currently allows a direct observation of such movements. Alternatively, exo-cellulase is often characterized by measuring an enzyme's

activity in terms of producing reducing sugars from a cellulose with a high DC. Accordingly, exo-cellulases are enzymes which have a high activity against mostly crystalline cellulose while endo-cellulases have little activity against it (Wood and Bhat, 1988; Zhang and Lynd, 2004).

An important point to note is that since the classification methods described above essentially rely on the relative activity of an enzyme in comparison to others, no absolute distinction between endo- and exo-cellulase exists (Coutihno and Henrissat, 1999). In other words, there is no clear standard or numerical boundary that distinguishes one type of enzyme from the other because both endo- and exo-cellulase often show a limited but measurable degree of the opposite type of activity. Thus, the ambiguous distinction may lead to confusion when an enzyme shows a relatively high activity in both endo- and exo-cellulase-specific parameters (Henrissat and Davies, 1997). In fact, enzymes with recognized dual activity have been reported (Table 2), and such enzymes are conveniently described as a cellulase with both endo- and exo- activity (Tomme *et al.*, 1996a), endo-resembling exo-cellulase (Kim and Kim, 1995), a processive endoglucanase (Gilad *et al.*, 2003), and successive exo-cellulase with endo-type activity (Boisset *et al.*, 2000). Therefore, it is important to bear in mind that the classification of endo- and exo-cellulases is very useful in terms of characterizing enzymes and understanding mechanisms of enzymatic hydrolysis of cellulose but the distinction between endo- and exo- cellulases may not be as clear-cut as it seemed to be.

Table 2. Cellulases with recognized dual endo- and exo- types of activity

Organism	Primary type	Characteristics	References
Cellulomonas fimi	Endo-	higer activity on BMCC than an exo-cellulase from the same organism, possibly successive from reducing side	Tomme *et al.*, 1996a
Clostridium stercorarium	Endo-	Highly active on CMC and Avicel	Riedel *et al.*, 1997
Bacillus circulans	Exo-	Active in hydrolying CMC and Avicel but slow in reducing CMC viscosity	Kim and Kim, 1995
Clostridium thermocellum	Endo-	Active in hydrolyzing CMC and Avicel, random active on CMC and successive movement on filter paper	Gilad *et al.*, 2003
Humicola insolens	Exo-	Active on BMCC, successive action and cut internal bonds of BMCC	Boisset *et al.*, 2000
Thermobifida fusca	Endo-	Highly active on filter paper and BMCC, active on CMC, and processsive movement	Wilson, 2004

Compared to the endo- and exo- division, characteristics of β-glucosidase are more explicit, and it mainly hydrolyzes cellobiose although some β-glucosidase can hydrolyze soluble cello-oligosaccharides (Lynd *et al.*, 2002). Since β-glucosidase does not hydrolyze insoluble cellulose, solubilization of cellulose by reducing the DP has to be carried out by endo-cellulase and/or exo-cellulase. An important feature about the solubilization by endo- and exo-cellulases is that due to the different preferences between the two types of cellulases on which part of the substrate they tend to act on, their hydrolytic actions are often complementary to each other, which results in a faster hydrolysis reaction and a higher yield of reducing sugars as compared to when these cellulases are working separately. This beneficial phenomenon is known as synergism.

2.3 Synergism between cellulases

Synergism between endo- and exo-cellulases is considered to be a result of sequential interaction between them while hydrolyzing cellulose (Bhat and Hazlewood, 2001). As shown in Figure 2, endo-cellulases cleave a long cellulose chain on the internal amorphous regions, which results in providing more substrate (new ends of chains with crystalline cellulose in-between) for exo-cellulases to work on. This mechanism is strongly supported by three kinds of experimental evidence. Firstly, a single type of cellulase can not achieve the same extent of hydrolysis that is achieved by a mixture of endo- and exo-cellulase when the total amount of the enzymes is equal (Irwin *et al.*, 1993). Secondly, synergism between end- and exo-cellulases only takes place on a cellulose substrate with crystalline structure but not on a substrate in a completely amorphous structure (Irwin *et al.*, 1993; Bhat and Hazlewood; 2001). Finally, when cellulose is first treated with endo-cellulase alone, the subsequent hydrolysis of the treated cellulose by an exo-cellulase produces a higher yield than an untreated cellulose would, but not vice versa (Valjamae *et al.*, 1999; Nidetzky *et al.*, 1994a). However, it should be noted that not all endo- and exo-cellulase pairs can complement each other's actions to exhibit a synergistic effect, and the reason is not yet well-known (Bhat and Hazlewood; 2001; Klyosov, 1990). The endo-exo synergism further leads to providing more substrates, mainly cellobiose, for β-glucosidase rather than when endo- and exo-cellulases are working separately. Since accumulation of cellobiose often causes feedback inhibition against endo- and exo-cellulases, it becomes important to supply enough β-glucosidase to prevent such inhibition. Consequently, the simultaneous presence of these three types of enzymes increases the hydrolysis yield compared to these enzymes working separately. A quantitative representation of the extent of synergism is usually expressed in terms of a degree of synergy (DS), which is the ratio of activity exhibited by a

mixture of cellulases divided by the sum of the activity of the individual cellulases evaluated separately (Zhong and Lynd *et al.*, 2004).

The above-described endo-exo synergism is the predominant synergistic interaction found in literature. However, synergism was also reported to exist between two distinct exo-cellulases (Table 3). That is, the simultaneous presence of two exo-cellulases can produce a higher hydrolysis yield than the sum of the yields individually produced by the two cellulases working separately (Nidetzky *et al.*, 1993). This type of synergism was originally reported only between fungal exo-cellulases (Woodward, 1991), but now it appears to be wide-spread for bacterial cellulases as well (Hoshino *et al.*, 1997; Baker *et al.*, 1995; Wilson, 2004; Zhang *et al.*, 1995). However, it is very difficult to explain how two exo-cellulases can interact complementarily to each other so as to develop a synergistic effect because two

Table 3. Synergistic effect between two exo-cellulases

Organism for exo-and exo cellulases	DS	Substrate	Reference
Trichoderma reesei & T. reesei	1.97	BMCC[1]	Nidetzky *et al.*, 1993
T. reesei & T. reesei	2.1	Filter paper	Irwin *et al.*, 1993
T. reesei & T. reesei	3.1	Cotton	Hoshino *et al.*, 1997
Irpex lacteus & T..reesei	2.7	Cotton	Hoshino *et al.*, 1997
Humicola insolens & H. insolens	4.3	Avicel	Boisset *et al.*, 2000
Thermomonospora fusca & Trichoderma reesei	0.9	Sigmacel	Baker *et al.*, 1995

[1]Bacterial microcrystalline cellulose

exo-cellulases are expected to act on the similar spots within a cellulose chain (the chain ends), unlike endo-exo combinations. Until recently, all exo-cellulases were believed to act only from the non-reducing ends of cellulose chains, but improved techniques to label substrates revealed that some exo-cellulases prefer hydrolyzing from the reducing ends (Barr *et al.*, 1996). This finding further verified that exo-exo synergy takes place only between exo-cellulases with the opposite chain end preferences (Teeri, 1997). For instance, in Table 3 on the lowest row, exo-cellulases from *Thermomonospra fusca* and *Trichoderma ressei* tend to act from reducing ends of the chains (Teeri, 1997). Thus, actions of these cellulases do not complement to each other but rather interfere with each other for substrate which results in a DS of less than 1. The other combinations presented are assumed to contain exo-cellulases with preferences for opposite chain ends though such conditions were not always confirmed (Hoshino *et al.*, 1997). Still, the existence of two kinds of exo-cellulases with opposite chain ends preference is by no means fully accountable for the mechanisms of exo-exo synergism because they can merely act on different spots on cellulose, but do not seem to enhance or facilitate each other's action by providing more substrate for each other.

Furthermore, there are also some reports suggesting the presence of synergism between distinct endo-cellulase pairs (Table 4). However, as in the case of exo-exo synergism, given their similar mode of action, it is hard to picture that the action of one endo-cellulase could facilitate that of another endo-cellulase. Usually, when cellulose is hydrolyzed by a pair of two different endo-cellulases, the value of DS rises only slightly above 1, as indicated by the results of Baker *et al.* (1995) and Irwin *et al.* (1993) in Table 4. A DS value of less than 1 indicates a competitive interaction between the cellulases for substrate, instead of a complementary interaction (Baker *et al.*, 1995). Thus, a reasonable explanation is not available for the mechanism for endo-endo synergism. However, since endo-cellulases with a

relatively high exo-type activity have been reported (Table 2), a possibility exists that one or both of the endo-cellulases which showed a synergistic effect with another endo-cellulase had an exo-type activity (Baker *et al.*, 1995; Gilad *et al.*, 2003).

Table 4. Synergistic effect between two endo-cellulases

Organism for endo- and endo-cellulases	DS	Substrate	Reference
Clostridium thermocellum	1.22	Avicel	Tuka *et al.*, 1992
Gleophyllum sepiarium & *G. trabeum*	1.12	Pulp	Mansfield *et al.*, 1998
Tricoderma reesei & *Acidothermus cellulolyticus*	0.7	Sigmacel	Baker *et al.*, 1995
Thermomonospora fusca & *Trichoderma reesei*	0.9	Sigmacel	Baker *et al.*, 1995
Thermomonospora fusca & *Acidothermus cellulolyticus*	0.68	Sigmacel	Baker *et al.*, 1995
Thermomonospora fusca & *Thermomonospora fusca*	0.5	Filter Paper	Irwin *et al.*, 1993

2.4 Parameters Affecting the Degree of Synergism

Although our current knowledge can not comprehensively explain the mechanisms of synergism, especially that of exo-exo and endo-endo synergism, it is well-recognized that synergism plays an important role in cellulose hydrolysis. An improved rate and yield of hydrolysis will allow reducing the amount of enzymes required for hydrolysis since a higher synergistic effect can achieve better hydrolysis with fewer enzymes (Kim *et al.*, 1998). As already indicated in Table 3 and 4, the DS is not constant but varies depending on such

parameters as the degree of polymerization of cellulose and enzyme ratios. Since a higher DS is more desirable to achieve the hydrolysis more efficiently, the following section discusses major DS influential factors and their potential mechanisms with a focus on endo-exo synergism.

Degree of polymerization

Considering the complementary interaction between endo- and exo-cellulases for synergism, the degree of polymerization (DP) of cellulose is theoretically expected to have a direct influence on the DS. Assuming that other characteristics of cellulose such as the degree of crystallinity are negligible, a cellulose with a high DP has a fewer chain ends than a cellulose with a low DP at an equivalent total length or weight. For instance, in Figure 3, a high DP cellulose (H) has only 2 chain ends, while a cellulose with a low DP (L) has 6 of them. Thus, at this state, independently working exo-cellulases have 2 and 6 spots

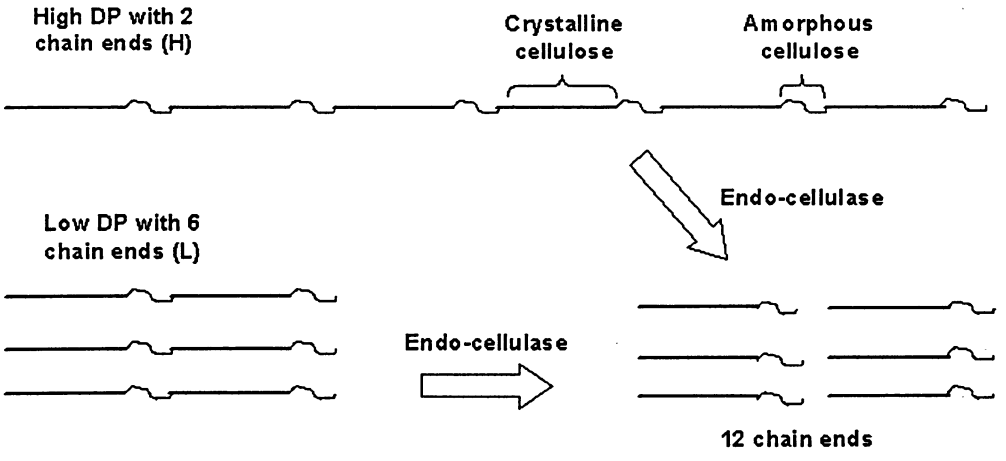


Figure 3. Potential action of endo-cellulases on cellulose of degree of polymerization

to work on for cellulose H and L, respectively. However, when endo-cellulases cut all internal amorphous regions available on both cellulose H and L, the total chain ends become 12 in either case. That is, compared to the initial state, the action of endo-cellulase can provide 6 times more substrate for exo-cellulases to work on for cellulose H whereas exo-cellulases receive only twice the ends for cellulose L. Consequently, when endo- and exo-cellulases work together to hydrolyze these two celluloses, the DS for the cellulose H is expected to be higher than that for the cellulose L. This theoretically expected relationship between the DS and the DP is supported by experimental data reported by other researchers. Table 5 contains ranges of reported DS between endo- and exo-cellulases for several different substrates along with their estimated DP and the degree of crystallinity, which is expressed in terms of crystallinity index (Crl). The values of both DS and DP are increasing from the top

Table 5. Degree of synergy from endo- and exo-cellulase pairs on cellulose substrates

Substrate	Crl[4]	DP	DS[5]	References
PASC[1]	0 – 0.04	100	1.0 – 1.8	Nidetzky *et al.*, 1993 Zhang and Lynd, 2004
Avicel[2]	0.63 - 0.82	300	1.4 – 4.9	Hoshino *et al.*, 1997 Zhang and Lynd, 2004
Filter Paper	0.35 - 0.45	750	2.9 – 7.8	Irwin *et al.*, 1993 Zhang and Lynd, 2004
Cotton	0.81 - 0.95	1000 – 3000	3.9 – 7.6	Hoshino *et al.*, 1997 Zhang and Lynd, 2004
BMCC[3]	0.76 - 0.95	2000	5.0 – 10	Wilson, 2004 Zhang and Lynd, 2004

[1]Phosphoric acid swollen cellulose, [2]microcrystalline cellulose derived from pulp, [3]bacterial microcrystalline cellulose, [4]crystallinity index (the ratio of crystalline cellulose to amorphous cellulose), [5]Reported degree of synergism from endo- and exo-cellulase pairs.

row to the bottom although the correlation is not as clear as it is expected in theory. This is because, unlike the simplified cellulose H and L in Figure 3, in practice, the DC as well as the distribution pattern of amorphous regions formed in cellulose is not identical from one type of substrate to another (O'Sullivan, 1997); these parameters also influence the DS.

Degree of crystallinity

Influences of the degree of crystallinity (DC) on the DS seem to be somewhat more difficult to predict than that of the DP because, as indicated in the last section, the distribution patterns of amorphous regions in a cellulose chain can be different even among celluloses with the same DC, which is also expected to have influence on the DS between endo- and exo-cellulases. For example, in Figure 4, cellulose chains A and B have the same DC, which is lower than the DC of cellulose C, but A has a higher frequency of the amorphous cellulose distribution than B does. When endo-cellulase cuts all internal amorphous regions of each cellulose chain, the resulting available cellulose chains from cellulose A will be 12 whereas those for cellulose B and C will be 6. As compared to the initially available chain ends, which is 2 for all celluloses, the action of endo-cellulases produces 6 times more chain ends for cellulose A while it releases 3 times more chain ends for cellulose B and C. That is, when endo- and exo-cellulases work together to hydrolyze these three types of cellulose, the DS for A, would be higher than that for B and C. Therefore, even when two cellulose chains have the same DC, as in the case of A and B, if the distribution pattern of amorphous regions is different, the DS can be different. On the other hand, a change in the DC alone, as in the case of B and C, does not have a direct influence on the DS. This is probably a reason why there is

no correlation seen between the DS and the Crl in Table 5.

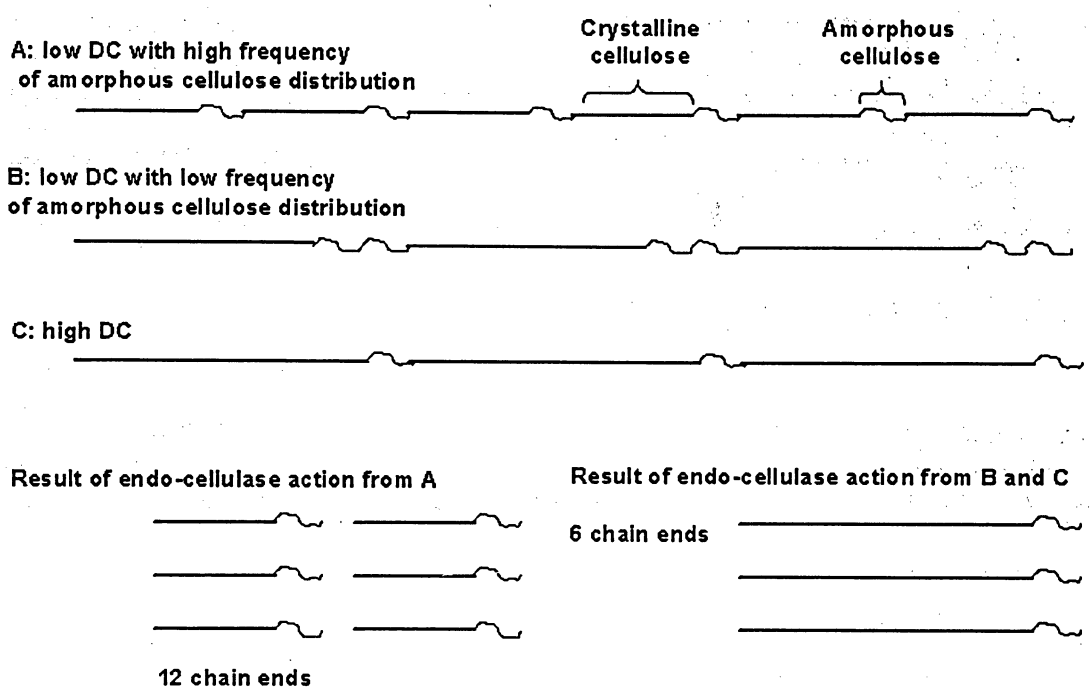


Figure 4. Potential action of endo-cellulase on cellulose of different degree of crystallinity

Endo-/exo-cellulase ratio

The distribution pattern of amorphous cellulose is also expected to influence the required endo-/exo- cellulase ratio to achieve a high DS. Since endo-cellulases cleave bonds in amorphous regions, the number of endo-cellulases required for hydrolysis depends on the amount of amorphous regions available in the substrate, which is determined by the distribution pattern or frequency of amorphous regions in cellulose (see Figure 4). On the other hand, the amount of exo-cellulase needed to hydrolyze cellulose depends on the number of chain ends, which will be available after the amorphous regions in the substrate are cleaved by the action of endo-cellulase. Thus, the required amount of exo-cellulase also

depends on the distribution pattern of the amorphous regions (see Figure 4). Accordingly, the endo-/ exo-cellulase ratio required to achieve a high DS is expected to vary depending on the distribution pattern of the amorphous regions in cellulose. Therefore, an estimation of the required endo-/exo-cellulase ratio seems to be possible in theory with accurate information of the distribution patterns of amorphous regions in the substrate. However, to the best knowledge of the author, such an attempt has not been made to decide an endo-/exo-cellulase ratio for the hydrolysis, which is probably because the distribution of amorphous regions in cellulose is not always homogeneous in cellulose (O'Sullivan, 1997), making its measurement a very challenging task (Zhong and Lynd, 2004). Meanwhile, the ratio of endo-/exo-cellulase to maximize the DS has been examined solely experimentally (Table 6). Typically, a small ratio of endo-cellulase (10 – 25 %) in the endo- and exo- cellulase mixture appears to be necessary to maximize the DS when Avicel, which has DC of 63-82%, (Zhang and Lynd, 2004) is used as a substrate.

Substrate/enzyme ratio

In addition to the endo-/exo-cellulase ratio, the ratio of the total amount of endo- and exo-cellulase to the amount of substrate also influences the DS (Walker *et al.*, 1993; Watson *et al.*, 2002; Woodward *et al.*, 1988; Tuka *et al.*, 1992). Ideally, the adsorption of endo-cellulase onto cellulose, which is required prior to the cleavage of bonds, needs to be only within the amorphous regions because endo-cellulases do not hydrolyze other parts of cellulose. Similarly, the adsorption of exo-cellulase is only needed onto the chain ends. In practice, although there seems to be a slight distinction between endo- and exo-cellulase's binding sites on cellulose (Jeoh *et al.*, 2002; Medve *et al.*, 1998), endo-cellulases can also

Table 6. Endo-/exo-cellulase ratio for maximum degree of synergy

Organism for endo- and exo-cellulases	% of endo[1]	DS	Substrate	Reference
Thermomonospora fusca	21	1.8	Avicel	Walker *et al.*, 1993
Trichoderma reesei	20	2.1	Avicel	Woodward *et al.*, 1988
T. fusca and *T. reesei*	13	4.8	Avicel	Boisset *et al.*, 2001
Humicola insolens	25	3.8	Avicel	Boisset *et al.*, 2001
Thermobifida fusca	10	1.7	Avicel	Watson *et al.*, 2002

[1]Percentage of endo-cellulase in cellulase mixture for the maximum DS

adsorb onto crystalline cellulose while exo-celluloses can adsorb onto internal cellulose chains as well (Zhang and Lynd *et al.*, 2004). Therefore, binding of either endo- or exo-cellulases to cellulose can result in blocking the adsorption of the other type of cellulase. This unproductive competitive binding is pronounced when the ratio of enzyme to a substrate is high as opposed to the substrate (Jeoh *et al.*, 2002; Medve *et al.*, 1998). Consequently, the DS is expected to be smaller as the cellulase/substrate ratio is increased, which is experimentally demonstrated by several researchers (Walker *et al.*, 1993; Watson *et al.*, 2002; Woodward *et al.*, 1988). On the other hand, when the amounts of either endo- or exo-cellulases are insufficient to cleave all available bonds in amorphous regions or to cleave from all available chain ends, these hydrolysable spots will be left untouched, which results in a slower hydrolysis reaction. That is, a low enzyme/substrate also reduces the DS.

Thus far, major influential factors on the DS between endo- and exo- cellulases were described. However, even when these parameters are experimentally controlled as consistent

to the greatest extent possible, a DS of a pair of endo- and exo-cellulases is higher than that of other pairs. This is because every cellulase has a different relative activity against cellulose (Klyosov, 1990). Such difference between cellulases at an individual level can be elucidated to some extent with more recent findings about structural differences in the enzyme proteins and their associated functions. Therefore, the following section will review our current knowledge about structures and functions of cellulase protein.

2.5 Domain Structure of Cellulase and Functions

Most cellulases are constructed with two functionally distinct domains (also called modules): a catalytic domain (CD) and a non-catalytic carbohydrate-binding module (CBM). CD is directly responsible for the catalytic activity of the enzyme and comprises a larger portion of the overall protein (Bhat and Hazlewood, 2001). CBMs, whose size ranges from 40 to 200 amino acids, are mostly located at one end of the amino acid sequence of the enzyme protein (Tomme *et al.*, 1998). CD and CBM are connected via a highly glycosylated linker peptide. CDs as well as CBMs are classified into structurally homologous individual families based on their DNA sequence similarities (Henrissat *et al.*, 1998).

Catalytic domain structure

At present, CDs of cellulases are divided into more than 15 families (Hilden and Johansson, 2004). An important achievement made over the last decade is the determination of the three dimensional (3-D) structures of CDs through such techniques as X-ray crystallography. Figure 5 shows 3-D structures of CDs from families 6 and 7. Their overall protein structures are significantly different among those in different families. For example,

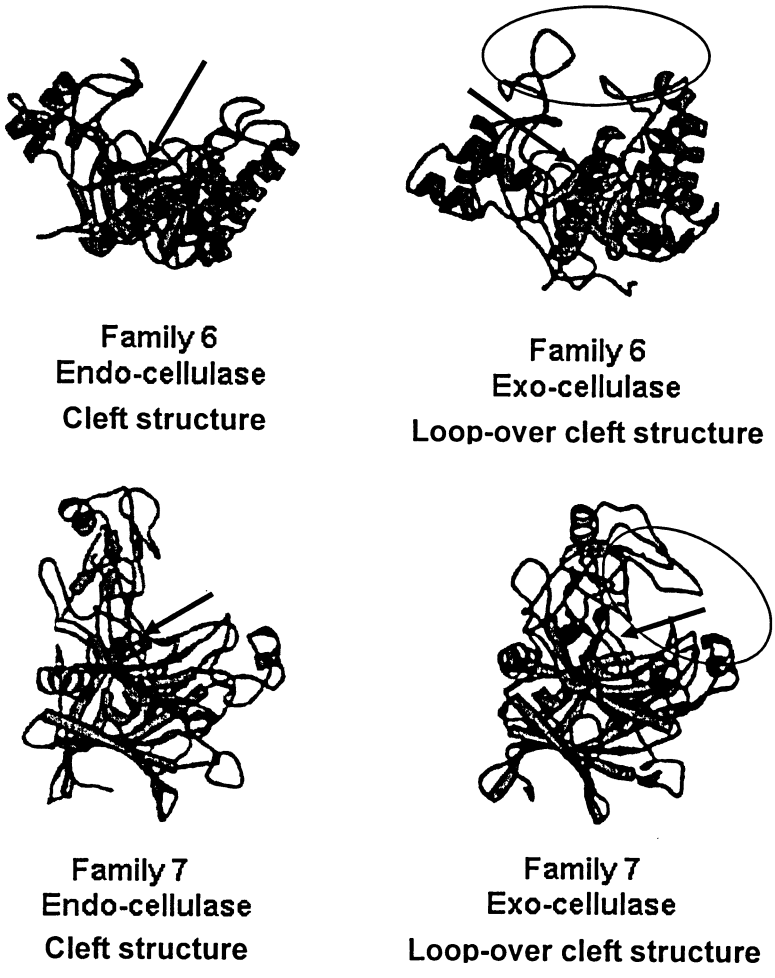


Figure 5. Three dimensional structures of family 6 and 7 catalytic domains of cellulases (modified from Davies and Henrissat, 1995)

family 6 CDs exhibit boat-like shapes whereas those in family 7 are more elongated vertically. Arrows indicate the proximate locations of the catalytic center, which typically exist inside of the cleft-shape structure and near the center of the protein (Davies and Henrissat, 1995). The active catalytic center typically contains aspartic acid and glutamate acid residues, which are

believed to be responsible for the catalytic reaction of the enzyme (Withers, 2001). An important feature which distinguishes exo-cellulases from endo-cellulases is the structure highlighted by circles, where long loop-like peptides cover part of the clefts. This loop-over cleft structure, also known as a tunnel structure, is assumed to restrict the hydrolytic action of exo-cellulases to the chain ends by narrowing the gap between loops so that a cellulose chain needs to be inserted into the cleft from the side. In addition, the limited space allows exo-cellulases to hold an inserted cellulose chain under the loop so that the enzyme can move successively along the chain while releasing cellobiose units (Davies and Henrissat, 1995; Meinke *et al.*, 1995). In contrast, the cleft structures of CDs originating from endo-cellulases are wide-open and are estimated to allow endo-cellulases to act randomly on the cellulose surface because a cellulose chain can be laterally inserted into the cleft (Davies and Henrissat, 1995; Schulein, 2000). This structural distinction between endo- and exo-cellulases, originally proposed by Davies and Henrissat (1995), now appears to be shared by most CDs from other families as well (Mandelman *et al.*, 2003; Varrot and Davies, 2003; Khademi *et al.*, 2002; Stahlberg *et al.*, 2001; Guimaraes *et al.*, 2002; Varrot et al., 2003, Sandgren *et al.*, 2001; Sulezenbacher *et al.*, 1997). This distinction allows one to speculate that an exo-cellulase could obtain endo-type activity by shortening the loops covering the cleft (Warren, 1996), and, in fact, this speculation was experimentally proved (Meinke *et al.*, 1995). When the loop-peptides of an exo-cellulase-originated CD from *Cellulomonas fumi* was shortened by site-directed mutagenesis, the resulting enzyme showed an enhanced endo-type activity in terms of reduction in the viscosity of soluble cellulose (CMC) solution. Additionally, removal of the loop structure from an exo-cellulase from *T. reesei* also showed an enhanced endo-type activity (Ossowski *et al.*, 2003). These studies not only prove the speculation but also strongly suggest that the distinction between endo- and exo-cellulases is

not absolute, but there could be an enzyme with a dual type of activity, possibly by having an intermediate type of structure or a flexible one.

Carbohydrate-binding module

Cellulose-recognizable CBMs, which include those originating from other carbohydrate-hydrolyzing enzymes, such as xylanases, can adsorb onto cellulose but do not have the ability to hydrolyze it. However, removal or site-directed mutagenesis of CBM often reduces the affinity of the remaining CD or of the modified enzyme for insoluble cellulose, which results in a decline of the hydrolysis rate (Nidetzky *et al.*, 1993; Nidetzky *et al.*, 1994a; Nidetzky *et al.*, 1994b; Lemos *et al.*, 2003; Wang *et al.*, 2003). Moreover, addition of a foreign CBM can improve the catalytic activity of the fused CD (Karita *et al.*, 1996; Garrard *et al.*, 2000; Gill *et al.*, 1999). Therefore, the primal role of CBM is generally considered to be facilitating the hydrolytic activity of CDs by increasing the overall affinity for cellulose (Tomme *et al.*, 1995; Linder and Teeri, 1997; Hilden and Johansson, 2004). The affinity of CBM for cellulose is mainly attributed to the presence of aromatic amino acid residues, such as tryptophan and tyrosine, and polar residues, including glutamic acid and aspartic acid, which are often located in close proximity to the aromatic residues. The aromatic residues develop hydrophobic stacking interactions with glucose-rings of cellulose chains while the polar residues seem to establish hydrogen bonds with cellulose chains (Linder and Teeri, 1997; Boraston *et al.*, 1999).

At present, carbohydrate-binding modules, including those that bind to other carbohydrates (e.g. chitin and mannnose), are classified into 42 different families according to the DNA sequence similarity, and cellulose-recognizable CBMs belong to 15 families of these (Table 7). A notable feature that emerged with the classification and advanced study of

Table 7. Classification of cellulose-recognizable CBMs

Family	No. of entries	Approx. size (a.a.)	3-D structure	cellulose for adsorption
1	193	40	Yes	Cellulose, Chitin
2	151	100	Yes	Cellulose, Chitin, Xylan
3	85	150	Yes	Cellulose, Chitin
4	21	150	Yes	Amorphous cellulose, xylan
5	104	60	Yes	Cellulose, Chitin,
6	65	120	Yes	Amorphous cellulose, xylan
8	1	150	Yes	Cellulose
9	23	170	Yes	Amorphous cellulose
10	16	50	Yes	Cellulose
11	5	180 -200	Yes	Cellulose
12	52	40 - 60	Yes	Cellulose, Chitin
15	2	40	Yes	Amorphous cellulose
16	18	160	no	Amorphous cellulose
17	12	200	Yes	Amorphous cellulose
28	13	200	yes	Amorphous cellulose

(data are from CAZy server at http://afmb.cnrs-mrs.fr/CAZY/acc.html)

the adsorption behavior of CBMs is that CBMs in certain families (4, 6, 9, 15, 16, 17, and 28) have specific affinity for amorphous cellulose but have little or no affinity for crystalline cellulose (Coutinho and Henrissat, 1999; Tomme *et al.*, 1996b). Furthermore, family 2 CBMs are further divided into two groups (family 2a and family 2b) according to the structural similarity of their binding sites. CBMs in family 2a were demonstrated to have a specific affinity for crystalline cellulose but not for amorphous cellulose (Simpson *et al.*, 2000; McLean *et al.*, 2002) whereas those in family 2b bind only to xylan (McLean *et al.*, 2000;

Boraston *et al.*, 2002). The specific affinity of CBMs for either amorphous or crystalline cellulose is most probably attributed to the structural distinction between the binding site of CBMs (Boraston *et al.*, 1999), but such a distinction is still under speculation (Jamal *et al.*, 2004).

2.5 Research Objectives

Based on the above-mentioned recent discovery of CBMs with a specific affinity for amorphous cellulose, the author came up with a completely new approach for enhancing the synergistic effect between endo- and exo-cellulases. When the CBMs are fused to endo-cellulases, the affinity of these enzymes for amorphous cellulose will be enhanced, which would allow the enzymes to focus on binding to amorphous cellulose while avoiding the unproductive binding onto crystalline cellulose. This targeted binding of endo-cellulases would further free exo-cellulases to target chain ends, avoiding competition with the endo-cellulases. Consequently, the complementary interaction between endo- and exo-cellulases is expected to be facilitated (synergistic effect).

The aim of this research was to investigate this hypothesis on a specific cellulase couple: a recombinant endo-cellulase Cel5B, from a rumen bacterium *Ruminococcus albus,* bearing a family 6 CBM, which is reported to have a specific high affinity toward amorphous cellulose (Sakka *et al.*, 1996), and a new exo-cellulase CBH1 from *Trichoderma harzianum* FP108. Cel5B without its fused CBM was also evaluated to prove the enhanced targeting by the presence of CBM. Avicel, which is one of the most frequently used commercial cellulose for studying synergy, was used for the hydrolysis substrate. The effect of pH and temperature, and the need for the addition of β-glucosidase to remove product inhibition during hydrolysis

were also investigated.

Chapter 3 - Material and Methods

3.1 Material

Cellulose

A microcrystalline cellulose, Avicel PH 101 (Sigma-Aldrich Canada Ltd. Oakville, Ontario) was used as a cellulose substrate throughout this study.

Cellulases

Three kinds of recombinant cellulases were used: an endo-cellulase IV (Cel5B) from a rumen bacterium *Ruminococcus albus*, its fusion enzyme with the carbohydrate-binding module (CBM6) of *Clostridium stercorarium* xylanases A (Cel5B+CBM6), and an exo-cellulase from *Trichoderma harzianum* FP108 (CBH1). They were kindly provided by Dr. Hyeun-Jong Bae (Chonnan National University, South Korea). A β-glucosidase enzyme solution (NS50010) was kindly provided from Novozymes North America Inc. (Franklinton, NC). Cel5B, Cel5B+CBM6, and CBH1 were suspended in 50 mM potassium phosphate buffer (pH 7.0), and they were assayed for their protein content, and stored at 4°C until used. The molecular weight of each enzyme is 36,000, 50,000, and 94,000 Da for Cel5B, Cel5B+CBM6 (Bae et al., 2003), and CBH1, respectively (personal communication with Dr Hyeun-Jong Bae, 2005). The purity of enzyme protein for Cel5B was checked using SDS-PAGE method (Barker, 2003) (Figure 6).

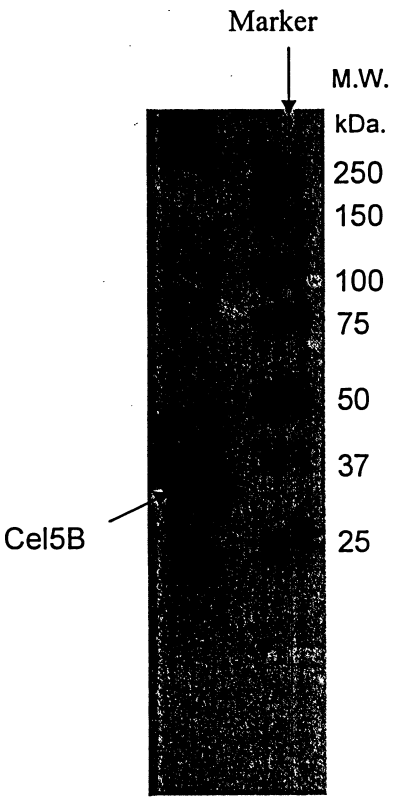


Figure 6. SDS-PAGE for Cel5B

3.2 Enzymatic Hydrolyses

Avicel was suspended in 1.0 ml buffer to give a final concentration of 10 mg/ml in 1.5 ml microcetrifuge tubes (UltiDent Scientific, QC, Canada). Sodium acetate buffer (50 mM) and potassium phosphate buffer (50 mM) were used for pH ranges 3-5.0 and 5.5-8, respectively. The buffer contained 0.04% (w/v) sodium azide to prevent undesirable microbial growth. The suspended cellulase solutions individually or in mixtures were then added. Microcentrifuge tubes were placed in a temperature-control water bath (ORS-200 Shaking Water Bath Boekel Scientific, Feasterville, PA) with linear shaking of 125 strokes per minute. Each microcentrifuge tube represented a sample was recovered and centrifuged at

6000 rpm for 3 minutes at room temperature. The supernatant was refrigerated at – 14 °C until determination of reducing sugars, described below. The hydrolysis yield was reported as described below.

- Hydrolytic time course of individual enzymes

  Individual enzyme solutions (Cel5B, Cel5B+CBM6, CBH1) were added to give a final concentration of 0.36 μM. The reaction took place in 50 mM potassium phosphate buffer at pH 7.0 and 40 °C. Samples were recovered after 1, 2, 4, 12, and 24 hours of incubation.

- Elimination of product inhibition by β-glucosidase

  Individual enzyme solutions were used in similar conditions as above. β-Glucosidase was added to give a final concentration of 1.6, 3.2, 16, 25.7, 32, 64, 96, and 128 μg/ml. Samples were recovered after 24 hours of incubation.

- Hydrolytic time course of individual enzymes with β-glucosidase

  Individual enzyme solutions were used in similar conditions as above in the presence of 64 μg/ml β-glucosidase. Hydrolysis was performed at 45 °C and pH 7.0 for Cel5B and Cel5B+CBM6, and pH 5.0 for CBH1. Samples were recovered after 24 hours of incubation.

- Effect of pH and/or temperature on individual enzymes

  Individual enzyme solutions were added in similar conditions as above, in varying pH. Hydrolysis was performed at 45 °C for Cel5B and Cel5B+CBM6, and at 40, 45, 50, and 60 °C for CBH1. Samples were recovered after 24 hours of incubation.

- Evaluation of potential synergistic effect between endo- and exo-cellulases

  The endo-/exo-enzyme ratio was varied as shown in Table 8 by keeping constant the sum of the concentrations of endo- and exo-cellulases at 0.36 μM. Each enzyme in the mixture was also individually evaluated at its concentration in the mixture, in identical conditions. All reaction mixtures contained 64 μg/ml β-glucosidase, and pH was set at 5.0, 5.5, 6.0 and 6.5. The hydrolysis yield was evaluated after 24 hours at 45 °C.

Table 8. Endo-/exo-cellulase ratios for examination of potential synergy during Avicel hydrolysis

	Mole percentage of individual enzymes					
End-[1]	0%	20%	40%	60%	80%	100%
Exo-	100%	80%	60%	40%	20%	0%
[Total]	0.36μM					

[1]Cel5B or Cel5B+CBM6

3.3 Analyses

Protein concentration

The concentration of soluble protein in each cellulase solution was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard protein. Bradford dye reagent was obtained from Bio-Rad Laboratories Ltd. (Ontario, Canada). The standard curve is shown in Figure A1 of the Appendices.

Reducing sugars concentration

The amount of reducing sugars released from the enzymatic hydrolysis of cellulose (Avicel) was determined by a modified Dinitrosalicylic Acid (DNS) method (Miller *et al.*, 1959). The volume of samples and the standard glucose solution to be assayed was 0.5 ml instead of 1 ml. Glucose was used to generate a standard curve, shown in Figure B1 of the Appendices.

3.4 Hydrolysis Yield

The hydrolysis yield compared the amount of reducing sugars experimentally released by the enzymatic hydrolysis of the cellulose in Avicel to the theoretical amount of glucose expected from the complete degradation of cellulose.

The complete hydrolysis of cellulose to glucose can be expressed by the following equation:

	Cellulose			→	Glucose
	$(C_6H_{10}O_5)n$	+	H_2O	→	$nC_6H_{12}O_6$
M.W.	164				180

Assuming that 10 mg/ml (1%, w/v) Avicel is pure cellulose, and that it is completely hydrolyzed into glucose, then 10.98 mg/ml of glucose is theoretically expected to be produced:

$$\frac{10 \text{ mg cellulose}}{\text{ml solution}} \times \frac{\text{n(180 g glucose)}}{\text{n(164 g cellulose)}} = \frac{10.98 \text{ mg/ml}}{\text{ml solution}}$$

Consequently, the hydrolysis yield is:

$$\% = \frac{\text{[Experimental reducing sugars] (mg/ml)}}{10.98 \text{ mg/ml}} \quad 100$$

which is reported in this thesis report as hydrolysis yield %

Degree of synergy

Degree of synergy between two cellulases is calculated as:

$$\frac{\text{Hydrolysis yield produced by a mixture of two cellulases}}{\text{Mathematical sum of the hydrolysis yields produced by each cellulase}}$$

3.5 Statistical Analysis

The precision of the data reported in the Results section is represented by error bars defined as:

$$\text{Confidence interval} = \text{mean value} \pm 2 \text{ s.d.}$$

where mean values = average of duplicates for each data,

2 = 95% probability confidence,

s.d. = standard deviation of data used to calculate means.

The greatest confidential intervals from each set of data points are presented in figures.

Chapter 4 - Results and Discussion

The following results report on the experimental steps taken in order to investigate the characteristics of an expected synergistic effect between an endo-cellulase (Cel5B) with and without a carbohydrate-binding module (CBM6), which has a high affinity for amorphous cellulose, and an exo-cellulase (CBH1) during the hydrolysis of Avicel cellulose.

4.1 Temporal Hydrolytic Time Course for Individual Enzymes

Percentages of hydrolysis yield obtained from Avicel by each of Cel5B, Cel5B+CBM6 and CBH1 are shown on Figure 7. Cel5B+CBM6 achieved about 35% hydrolysis yield from 1% (w/v) Avicel in the first hour. Between 12 to 24 hours, almost no increase in the yield was detected, resulting in a maximum yield of around 41%. Compared to this fusion enzyme, Cel5B showed a similar type of curve featuring a relatively high increase in the yield for the initial first hour followed by an almost horizontal curve from 2 to 24 hours. But Cel5B's maximum of about 11% yield is only about 25% of the maximum yield achieved by Cel5B+CBM6. In comparison to the two endo-cellulases, CBH1 only produced a yield of about 2%, and the reaction stopped shortly after 2 hours of incubation. The shape of the curves observed for Cel5B and Cel5B+CBM6 are reminiscent of those seen when highly crystalline cellulose was hydrolyzed by endo-cellulases (Lemos *et al.*, 2003). The initial stage of a relatively high increase in the hydrolysis yield is probably due to the hydrolysis of mainly amorphous regions of the substrate because they are more easily hydrolysable than crystalline cellulose (Lynd *et al.*, 2002). The subsequent slowing down of the rate is said to be exhaustion of the amorphous cellulose; the recalcitrance of the remaining crystalline

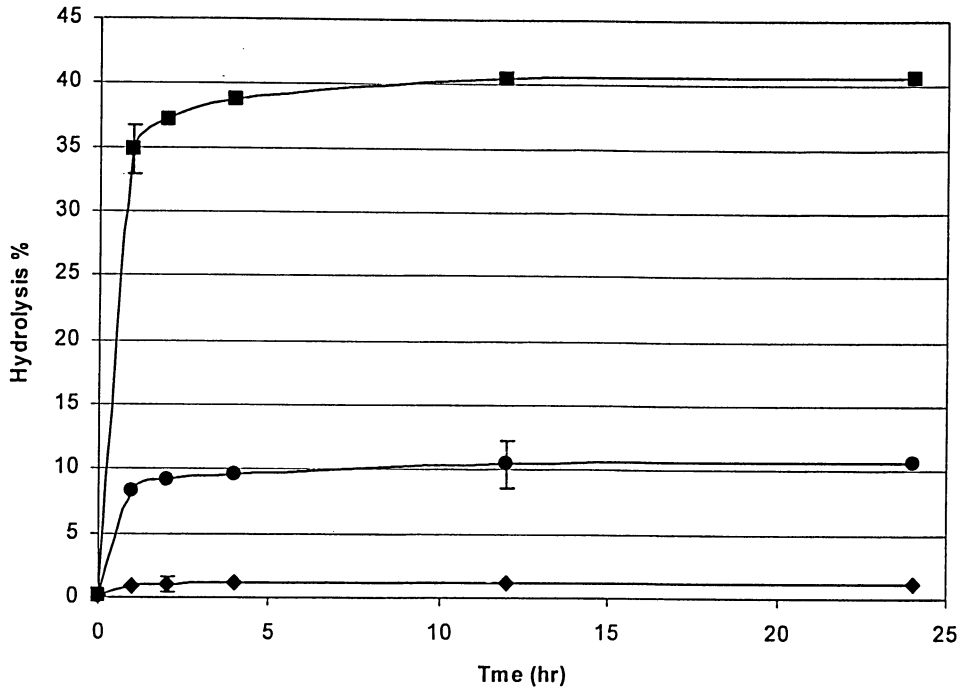


Figure 7. Avicel hydrolysis by cellulases at pH 7.0 and 40 °C: Avicel 10 mg/ml, (■) Cel5B+CBM6, (●) Cel5B, (♦) CBH1.

cellulose to be hydrolyzed brings about a steady state where an almost negligible increase in the yield is observed (Esteghlalian *et al.*, 2001; Lemos *et al.*, 2003; Zhang and Lynd, 2004). The yield achieved by Cel5B+CBM6, 3.8 times higher than that by Cel5B, is evidence that the fused CBM6 enhanced the activity of Cel5B presumably by concentrating the enzyme's binding to amorphous regions of the substrate as was reported by Sakka *et al.* (1996). Also, this result is well in accordance with the values reported by Bae et al. (2003) using similar hydrolysis conditions. However, only a maximum 37% yield was expected from the

hydrolysis of the amorphous regions, based on the degree of crystallinity reported for this commercial Avicel (63-82%; Doelker, 1993). This implies that the maximum yield of approximately 41% achieved by Cel5B+CBM6 may not have resulted solely from the hydrolysis of the amorphous regions, but that Cel5B+CBM6 might be able to hydrolyze part of the crystalline cellulose. The fact that CBH1 produced only 2 % of yield and its hydrolysis stopped after 2 hours was somewhat unexpected since exo-cellulases are reputed to generally have higher activity on crystalline cellulose than endo-cellulases and also that a gradual increase in the yield continues after the initial relatively fast hydrolysis (Irwin et al., 1993; Lemos *et al.*, 2003; Wang *et al.*, 2003; Teeri *et al.*, 1998). This prompted us to investigate the potential effect of an inhibitory factor such as a product (cellobiose) and/or of experimental conditions such as pH and temperature that may have contributed to this behaviour.

4.2 Elimination of Product Inhibition by β-Glucosidase

The relatively low yield of CBH1 might partly be a result of product (cellobiose) feedback inhibition, which can easily be eliminated by the action of β-glucosidase, an enzyme hydrolyzing cellobiose into glucose (Woodward, 1991). Since flat hydrolytic profiles were found for all hydrolysis curves after 4 hours of incubation in Figure 7, the effect of additional β-glucosidase on the hydrolysis yield by each enzyme was examined after 24 hours. Table 9 shows that the presence of 1.6 μg/ml or more of β-glucosidase enhanced the hydrolysis yield obtained through the action of each endo- or exo-cellulase, indicating that inhibition by cellobiose was present in our earlier study. In the case of CBHI, the increased yield continued until the addition of 64 μg/ml of β-glucosidase, achieving a hydrolysis yield almost 4 times higher than obtained when β-glucosidase was not added. No further increase after this point

Table 9. Effect of β-glucosidase on hydrolysis of Avicel with cellulases at 40 °C and pH 7.0

β-Glucosidase (μg/ml)	Hydrolysis yield (%)			
	Cel5B+CBM6	Cel5B	CBH1	Control[1]
0.0	41.58	10.41	1.12	0.00
1.6	46.52	11.90	1.29	0.00
3.2	48.10	14.17	1.63	0.00
16	53.12	14.97	2.91	0.00
24	54.41	15.01	3.69	0.00
32	54.34	15.01	4.09	0.00
64	54.41	15.34	4.27	0.00
96	54.43	15.11	4.26	0.00
128	54.16	15.30	4.25	0.00

[1] β-Glucosidase only

indicated that about 64 μg/ml of β-glucosidase was required to maximize the effect of cellobiose elimination. β-Glucosidase also enhanced the hydrolysis yields of both Cel5B and Cel5B+CBM6 although the minimum amount of β-glucosidase needed to maximize the effect was lower than that for CBH1, 16 μg/ml and 25.7 μg/ml for Cel5B and Cel5B+CBM6, respectively. At these concentrations, the relative increase was about 44% and 31% for Cel5B and Cel5B+CBM6, respectively, compared to the yields by the enzymes without β-glucosidase. No reducing sugars were detected when β-glucosidase alone was added, nor when an Avicel solution devoid of enzymes (data not shown) was incubated. This strongly suggests that cellobiose was produced by the action of each of Cel5B, Cel5B+CBM6 and CBH1. From this result, 64 μg/ml of β-glucosidase was decided to be used when hydrolysis by mixture of endo- and exo-cellulases would be carried out to examine their potential synergistic effect.

4.3 Effect of pH and Temperature on CBH1

The relatively low hydrolysis yield obtained for CBH1 (Figure 7) could also be attributed to the selected experimental conditions since pH 7.0 and 40 °C were conditions reported to be suitable for the endo-cellulases (Bae *et al.*, 2003). No data in the literature were found on this relatively new exo-cellulase. Therefore, the effect of pH ranging from 3.0 to 8.0 on the hydrolysis of Avicel by CBH1 alone was examined at temperatures between 40 and 60 °C (Figure 8). At 50 °C, hydrolysis by CBH1 showed peaks at pH 4.5 and 5.0 with around 10 % yield after 24 hours of incubation. The yield decreased to 9% at pH 4.0. From this point, as the pH was lowered, the yield declined drastically, resulting in 0.2% yield at pH 3.0. Similarly, an increase of pH from 5.0 to 8.0 also resulted in a decrease in the hydrolysis yield, but in a slightly more gradual manner, reaching 0.1 % and a completely zero yield at pH 7.5 and 8.0, respectively. When the temperature was raised to 60 °C, the hydrolysis yield declined at all pH values compared to the hydrolyses at 50 °C. A peak was still seen at pH 4.5 and 5.0 with a yield of 7.1%. From this point, changes in pH in either direction lowered the yields in a similar pattern to the results observed at 50 °C. There was no reducing sugar produced by CBH1 at pH 3.5 and 8.0. On the other hand, decreases in temperature from 50 to 40 °C did not cause significant changes in yields, producing peaks at pH 4.5 and 5.0 with around 9.8% yields. Again, hydrolysis yields at lower and higher pH levels than these peaks showed a similar trend as that seen at 50 °C At pH 7.0 and 40 °C, CBH1 produced about 14 % of the yield obtained at a pH 5.0 and 40 °C, which confirmed that the relatively low yield seen for this exo-cellulase in Figure 7 was largely due to the selected unproductive pH. Also, this result clearly demonstrated that CBH1 maximizes the yield at pH around 5.0. Compared to this, Cel5B and Cel5B+CBM6 were reported to have optimal conditions at pH 7.0 and 45 °C (Karita *et al.*, 1993). Considering that there was almost no change in the

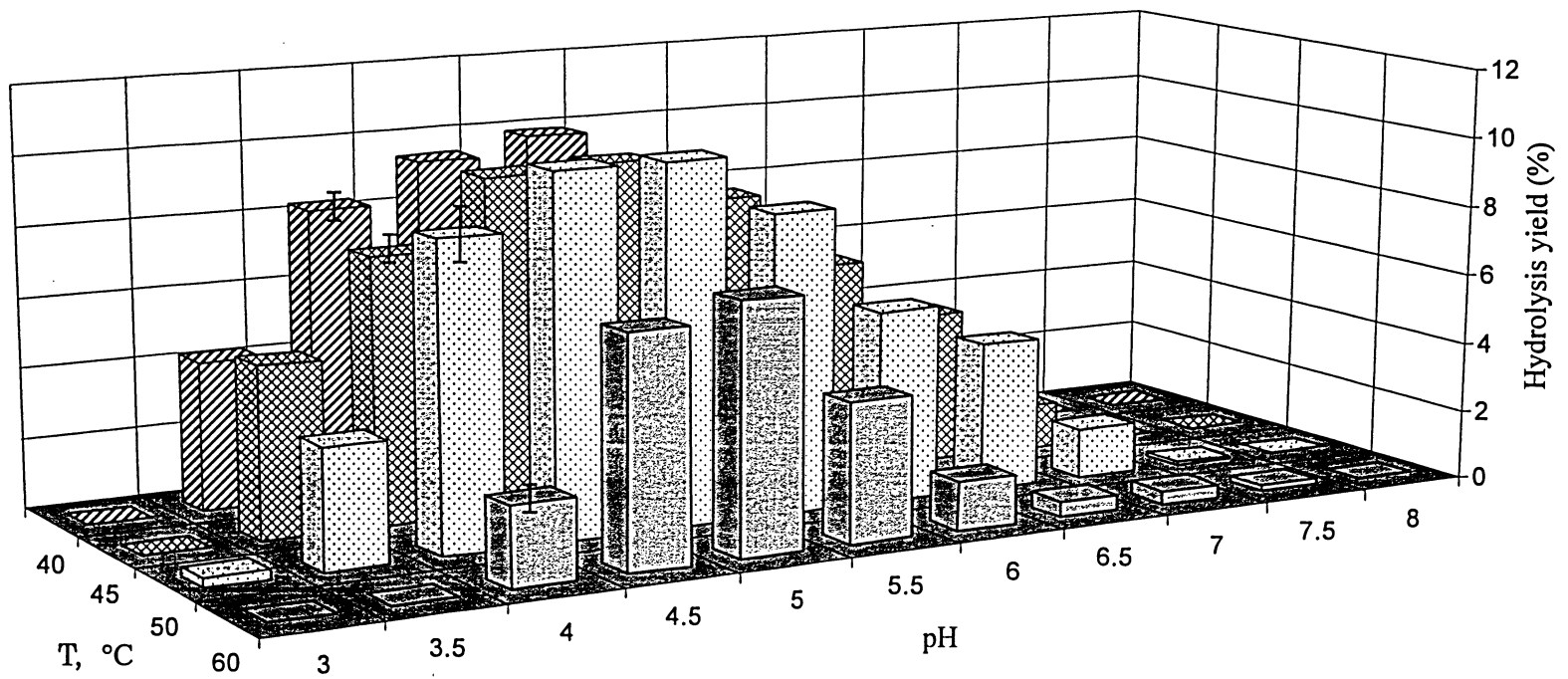


Figure 8. Effect of pH and temperature on hydrolysis of Avicel by CBH1 without β-glucosidase

hydrolysis yield by CBH1 between 40 and 50 °C, and also that endo-cellulases, especially Cel5B+CBM6, showed a remarkably higher yield than CBH1, the temperature for future hydrolyses by mixtures of end- and exo-cellulases was decided to be 45 °C. On the other hand, the pH for future hydrolyses would need to be a compromise between 5.0 and 7.0.

4.4 Determination of Incubation Time for Evaluation of Potential Synergistic Effect between Cel5B (with or without CBM6) and CBH1

The previous experiments indicated that CBH1 maximized the hydrolysis yield at pH around 5.0 and also that β-glucosidase increased the hydrolysis yields from the action of all enzymes. These results were obtained after 24 hours of incubation, which was set based on the profiles of each enzyme seen in Figure 7. However, it was not confirmed yet whether the hydrolysis yields by each enzyme would still reach its respective maximum within 24 hours when the pH was selected at 5.0 for CBH1 and β-glucosidase was added to all enzymes. Therefore, Avicel solutions (10 mg/ml) were re-incubated with each enzyme separately at 45 °C and at pH 7.0 for the endo-cellulases and pH 5.0 for CBH1, with the addition of 64 μg/ml β-glucosidase (Figure 9). Cel5B+CBM6 plus β-glucosidase achieved 52% within the first three hours, and almost no further increase was observed from 12 to 24 hours. The shape of this curve is similar to that obtained for Cel5B+CBM6 alone in Figure 7, but the addition of β-glucosidase produced a higher yield at every sampling point, which resulted in a maximum yield of about 54% yield (approximately 27% higher than without β-glucosidase). β-Glucosidase also increased the hydrolysis yield from Cel5B with a maximum of around 13% yield (approximately 18% higher than without β-glucosidase). On the other hand, the increase in the hydrolysis yield by CBH1 plus β-glucosidase was lower than the endo-cellulases in the first 2 hours, but they achieved almost the same yield as Cel5B plus

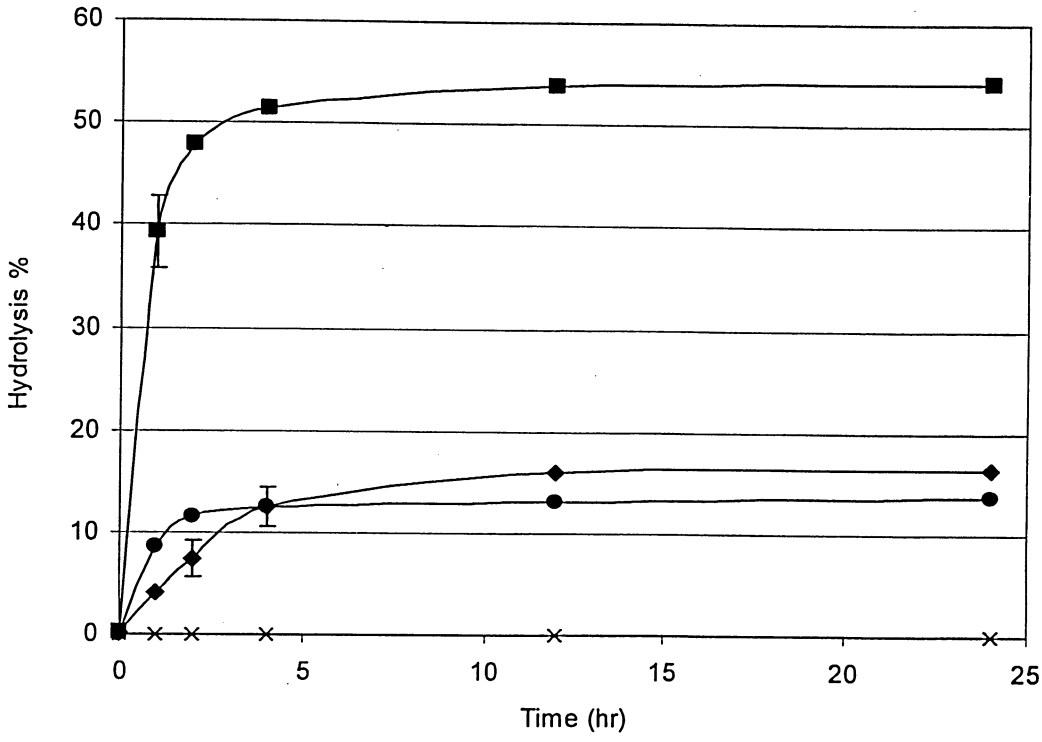


Figure 9. Hydrolysis of Avicel by cellulases supplemented with β-glucosidase at 45 °C: (■) Cel5B+CBM6, (●) Cel5B, (♦) CBH1, and (×) Control (β-glucosidase alone)

β-glucosidase did after 5 hours. Then, after around 12 hours of incubation, the curve plateaued at a yield of about 16%, which was approximately 15 times higher than the value obtained when Avicel was hydrolyzed by CBH1 alone at pH 7.0 and 40 °C (Figure 7). As stated in section 4.1, the hydrolysis of endo-cellulases said to be mostly resulting from amorphous cellulose in the substrate. However, the improved hydrolysis yield for Cel5B+CBM6 by added β-glucosidase made it more difficult to attribute the obtained 52% yield entirely to amorphous parts of Avicel, which may imply that Cel5B+CBM6 might have the ability to hydrolyze part of crystalline cellulose on its own. On the other hand, no increase

in the hydrolysis yield by CBH1 after the initial state is similar to the curve in Figure 7, which indicates that CBH1 may not be very effective in hydrolyzing crystalline cellulose. While this result left a concern in terms of the effectiveness of CBH1 on hydrolyzing crystalline cellulose, observed steady states for all enzymes after 12 hours made it easier to confirm the incubation time for hydrolyses by mixtures of endo- and exo-cellulases to be 24 hours.

4.5 Evaluation of Potential Synergy between Cel5B (with or without CBM6) and CBH1 during Hydrolysis of Avicel

Figure 10 presents hydrolysis yields achieved by mixtures of CBH1 and either one of endo-cellulases as well as by each cellulase separately at 45 °C and pH varying from 5.0 to 6.5, which also shows calculated degree of synergy between the enzymes from the yields. β-Glucosidase (64 μg/ml) was added to every hydrolysis solution. When Avicel was hydrolyzed by Cel5B alone at pH 6.5 ((□) in Figure 10 a), increasing the concentration of the enzyme from the left-hand side of the graph (0 μM) to the right-hand side (0.36 μM) resulted in an almost linear increase in the yield from 0 to 14%. Similarly, hydrolysis yields by Cel5B+CBM6 alone (■) increased up to 55% with enzyme concentration. The concentration of CBH1 alone (×) was increased from the right-hand side of the graph (0 μM) to the left-hand side (0.36 μM), the yield increased from 0 to around 10 %. When Avicel was hydrolyzed by mixtures of Cel5B and CBH1 (△) or of Cel5B+CBM6 and CBH1 (▲), their total concentration was kept constant at 0.36. Hydrolysis yield produced by mixtures (referred to subsequently as YBM) of Cel5B and CBH1 (△) were higher than those by individual Cel5B (□) and CBH1 (×).

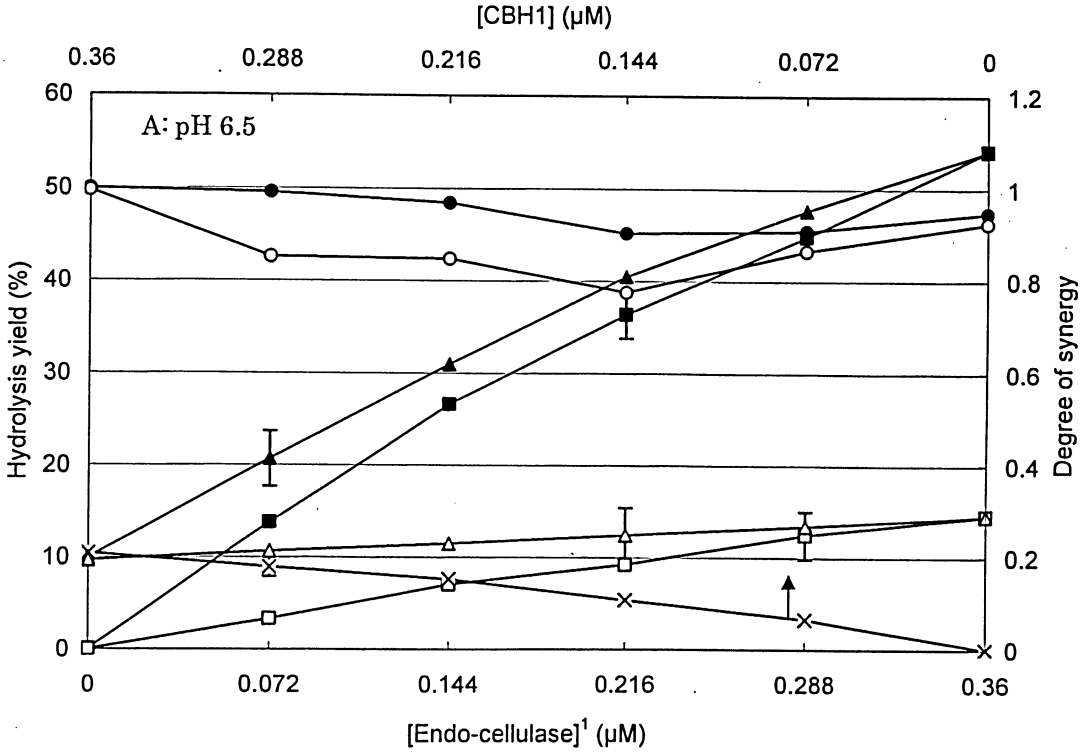
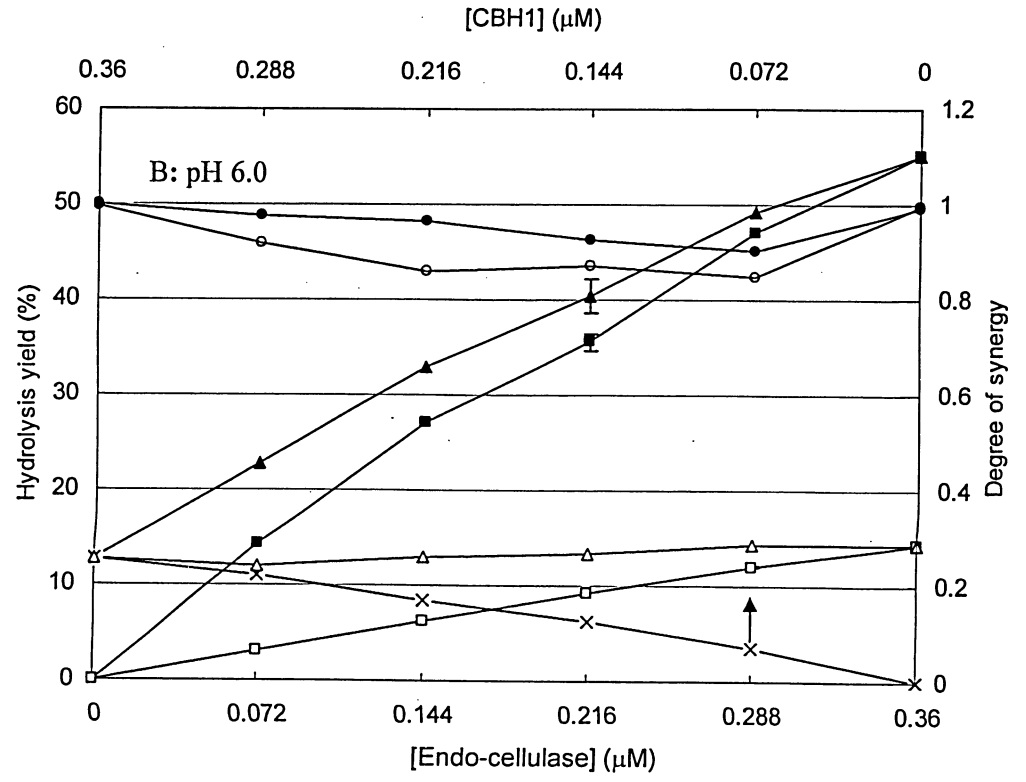


Figure 10 Evaluation of synergistic effect between Cel5B (with or without CBM6) and CBH1 at 45 °C: (A) pH 6.5, (B) pH 6.0, (C) pH 5.5, (D) pH 5.0; (●) degree of synergy between Cel5B+CBM6 and CBH1, (○) degree of synergy between Cel5B and CBH1, (▲) Cel5B+CBM6 and CBH1, (■) Cel5B+CBM6, (△) Cel5B and CBH1, (□) Cel5B, (×) CBH1
[1]Cel5B or Cel5B+CBM6



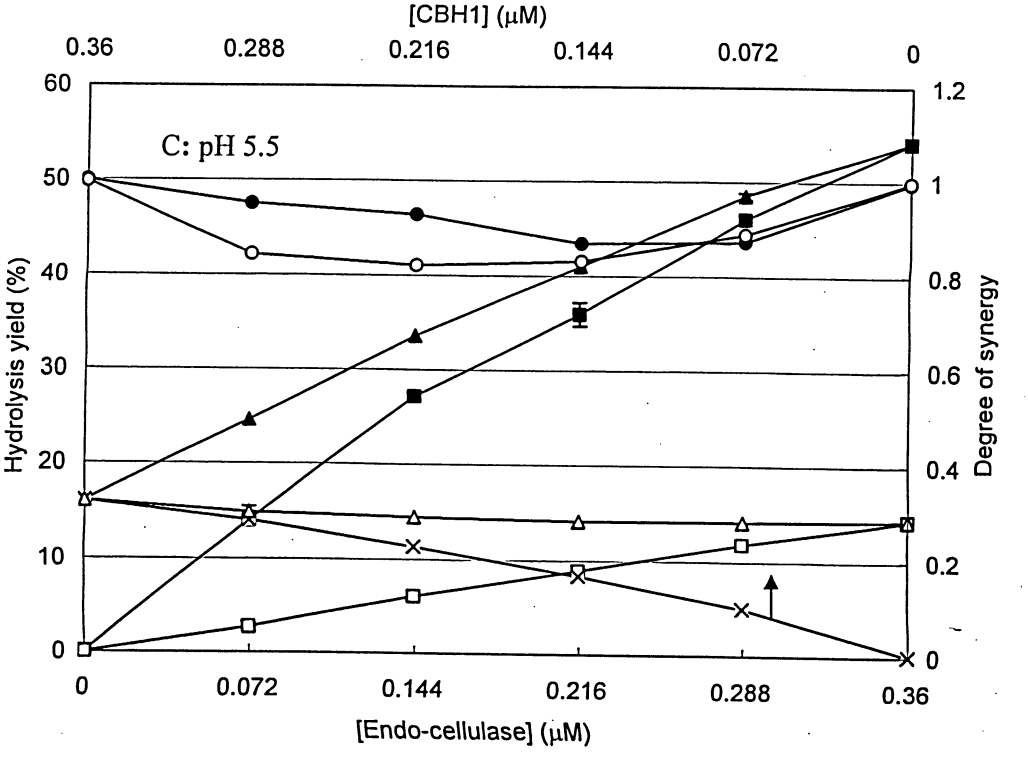


Figure 10 Continued

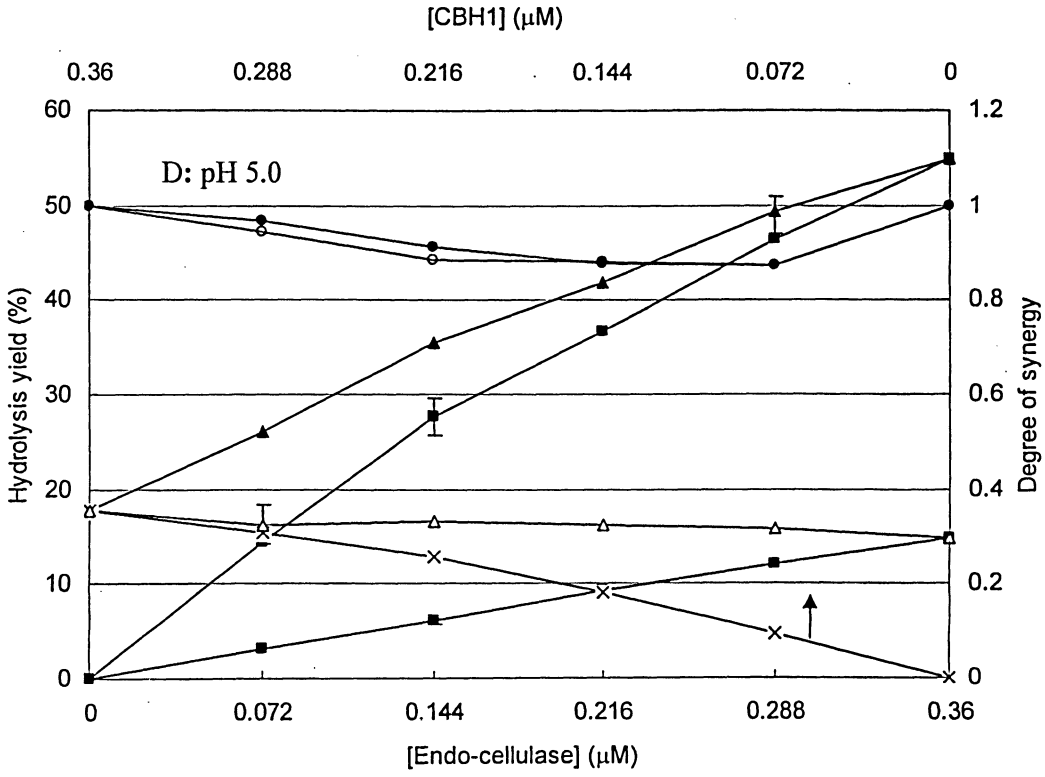


Figure 10 Continued

YBMs of Cel5B+CBM5 and CBH1 (▲) were also higher than with either of Cel5B+CBM6 (■) or CBH1 (×). From the hydrolysis yields determined for both individual enzymes and the enzyme mixtures, degrees of synergy (DS) between CBH1 and either Cel5B or Cel5B+CBM6 were calculated and shown as the symbols (○) and (●), respectively. The DSs for both combinations of enzyme were less than 1 at any enzyme concentration proving that, despite our expectations, synergistic effect was not present. Similar results were obtained when pH was varied from6.5 to 5.0 in 0.5 unit intervals (pH 6.0, 5.5 and 5.0 in Figure 10 B, C, and D, respectively). This unanticipated outcome prompted us to investigate what sort of factors contributed to no synergism between endo- and exo-cellulases. If they exist, can they

be attributed to endo-cellulases or to CBH1, or to both?

The hydrolysis curve observed for CBH1 in Figure 9 indicated a possible fundamental problem by showing that the hydrolysis by CBH1 produced approximately 16% yield after 24 hours, which is not significantly different from the hydrolysis yield achieved by Cel5B. Since Cel5B is an endo-cellulase and reported to have little activity against crystalline cellulose (Karita *et al.*, 1993; Bae *et al.*, 2003), most of the yield obtained by Cel5 is expected to be from the amorphous parts of Avicel, which may imply that the hydrolysis yield by CBH1 is also predominantly from amorphous parts of the substrate. Furthermore, one can not neglect the fact that the appeared hydrolysis curve for CBH1 in Figure 9 resembles the ones for the endo-cellulases in a way that the hydrolysis stopped the initial stage of relatively high increases in hydrolysis yield. These results indicate that CBH1 is not very effective in hydrolyzing crystalline cellulose. Consequently, CBH1 might not have been unable to proceed with further hydrolysis of cellulose chains provided by the action of endo-cellulases, which could present a possible reason why synergistic effect between endo- and exo-cellulase did not take place.

The possible low activity of CBH1 against crystalline cellulose, however, fails to provide an adequate explanation for one aspect of the results, which is that the hydrolysis yields by cellulase mixtures resulted in below the values of mathematical sum of the yields by the two enzymes working separately. As presented in Table 3 and 4, this type of negative synergy (DS < 1) is commonly seen between two same type of cellulases; endo- and endo-cellulases, or exo- and exo-cellulases. And such negative synergistic effect is typically explained as a result of competitive interactions or redundant actions because the cellulases hydrolyze the same spots on cellulose (Baker *et al.*, 1995; Irwin *et al.*, 1993). Since endo- and exo-cellulases are classified based on their estimated different mode of action, it may seem

unreasonable to consider that such a competitive interaction would take place between them unless their total concentration is high enough to saturate the substrate, which is probably not the case for the reason mentioned above. However, as shown in Table 2, studies indicated that some endo-cellulases possess a relatively high exo-type activity. For example, endo-cellulases from *Clostiridium thermocellum* (Gilad *et al.*, 2003), *Cellulomonas fimi* (Tomme *et al.*, 1996), *Thermobifida fusca* (Wilson, 2004; Sakon *et al.*, 1997) were demonstrated to have a relatively high activity against crystalline cellulose, and also described as being able to move in successive manner along the cellulose chains while still being able to randomly cleave the bonds in amorphous regions.

Presence of any degree of exo-type activity is not verified neither for Cel5B nor Cel5B+CBM6 in addition to their endo-type activity. However, as shown in Figure 9, Cel5B+CBM6 alone achieved almost 55% of hydrolysis yield, which is higher than a hydrolysis yield expected from hydrolysis of amorphous cellulose in Avicel. This is because Avicel should contain at most 37% amorphous cellulose, given that the reported values of the DC for Avicel are 63-82% (Doelker, 1993). Thus, in order to obtain 55% of yield from Avicel, Cel5+CBM6 must hydrolyze some part of crystalline cellulose. This aspect of Cel5B+CBM6 resembles an endo-cellulase from *T. fusca* in that the enzyme alone achieved 87% of theoretically obtainable hydrolysis yield when the amorphous cellulose comprised at most 65% of the used substrate, indicating that the endo-cellulase also hydrolyze some crystalline cellulose. Based on this finding as well as the fact that the endo-cellulase is slower in reducing the DP of cellulose compared to other endo-cellulases from the same organism, researchers concluded that the endo-cellulase has dual activity; both endo- and exo-type activity, which implies that that Cel5B+CBM6 may also possesses exo-type activity. This hypothesis could be further supported by other studies reported by Riedel *et al.*, (1997) and

Irwin *et al.*, (1993).

A cellulase (Avicellase I) from *C. stercorarium* was classified as endo-cellulase due to its high activity in terms of reducing the DP of cellulose (Bronnenmeier and Staudenbauer, 1990). However, an additional exo- type activity was suggested since the endo-cellulase had four times higher activity against Avicel than another typical exo-cellulase from the same organism (Riedel *et al.*, 1997). Between these two cellulases a synergistic effect was confirmed with a DS of 1.4 using Avicel as a substrate. However the synergistic effect was only observed when the two cellulases were simultaneously present in a hydrolysis solution, but not when Avicel was hydrolyzed by these cellulases in a sequential manner; Avicel was initially hydrolyzed by either one of the cellulases and followed by a secondary hydrolysis of the washed remaining Avicel by the other cellulase. Such simultaneous presence is not necessary for endo-exo synergism (Valjamae *et al.*, 1999; Nidetzky *et al.*, 1994a), but is a suggested feature for exo-exo type synergism (Tomme *et al.*, 1990) although mechanisms of synergism between pairs of exo-cellulases is still poorly understood (see section 2.3). Therefore their synergistic interaction was concluded as exo-exo type synergism. This is an evidence that exo-exo type synergism could take place even between enzymes classified as endo- and exo-cellulases. Furthermore, the successive endo-cellulase from *T. fusca* also exhibited synergistic effects with both an endo- and an exo-cellulase from the same organism, giving the DS of 1.7 and 1.45, respectively. However, these vales of the DS were significantly lower than a DS of 4.5 exhibited between endo- and exo- cellulases from *T. fusca* with presumably more typical endo- and exo type activities (Irwin *et al.*, 1993), which implies that the dual type of activity of the successive endo-cellulase caused a redundancy or competitive action with both endo- and exo-cellulase and resulted in small DSs.

Assuming that Cel5B+CBM6 had an exo-type activity, the observed DS < 1 could be

explained by a competitive interaction between the two cellulases with similar type of action, which further explains the negative DS between Cel5 and CBH1 as well. The distinction of endo- or exo-type activity appeared to be attributed to their distinct protein structures of the catalytic domain (see subsection 2.5.2). Hence, the hypothesized exo-activity should not be procured for Cel5B+CBM6 as a result of the fused CBM6 but be originated from its catalytic domain, Cel5B. Therefore if Cel5B+CBM6 has exo-type activity, Cel5B must also have the same exo-type activity. Thus, a competitive interaction could also take place between Cel5B and CBH1 to result in a negative DS. Therefore the author speculates that both Cel5B and Cel5+CBM6 have exo-type activity, which caused competitive interactions with CBH1 and resulted in the negative DS.

4.6 Effect of pH on Avicel hydrolysis by Cel5B and Cel5B+CBM6

During the course of studying potential synergistic effect between the endo- and exo-cellulases, it appeared that changes in pH values did not affect the hydrolysis yields by Cel5B and Ce5B+CBM6. This contradicted the previous studies that found that both endo-cellulases had an optimal pH of 7.0 for hydrolysis of carboxymethyl-cellulose (CMC), and that a change in the pH significantly affected their activity. The reported relative activities for both endo-cellulases were 40%, 80% and 50-60% at pH 5.0, 6.0 and 6.5, respectively, compared to the highest activity obtained at pH 7.0 (Bae, 2002). Therefore, effect of pH on the yield from Avicel by these enzymes was re-investigated (Figure 11). At a pH of 3.0 a hydrolysis yield by Cel5B alone was less than 1% of the theoretically obtainable reducing sugar

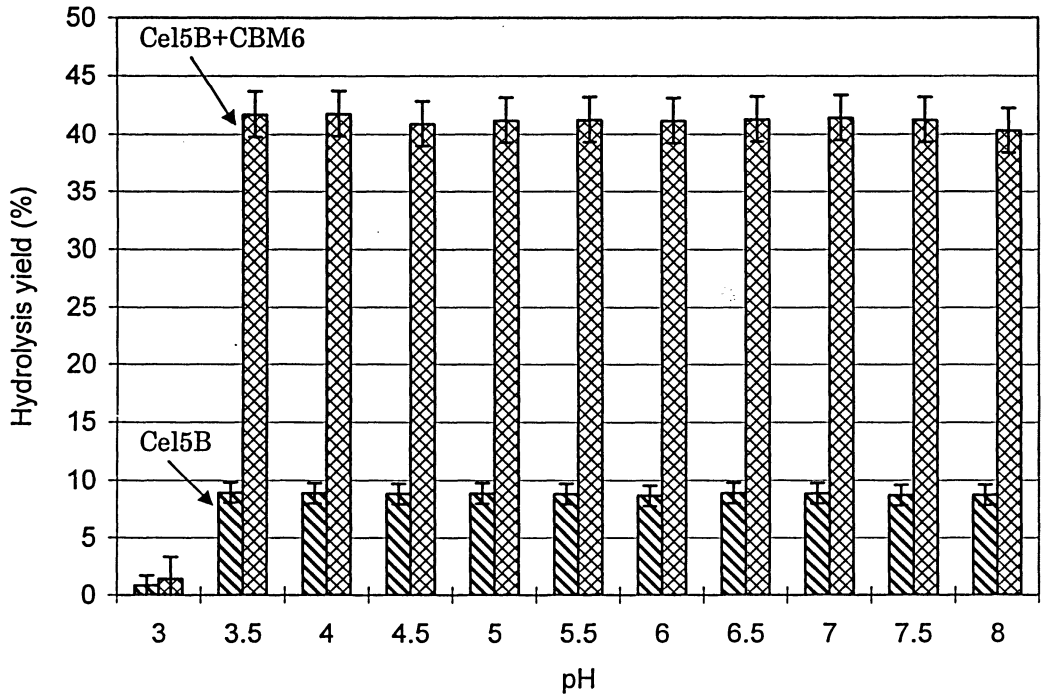


Figure 11. Effect of pH on Avicel hydrolysis by endo-cellulases at 45°C, in the absence of β-glucosidase

from 10 mg/ml Avicel. When the pH value was increased to 3.5, the yield rose to about 9%. However, further increases in pH did not have a major impact on the yield by Cel5 up to pH 8.0. Similarly, hydrolysis yield by Cel5B+CBM6 was only 1.4 % at pH 3.0, which increased to around 42% at pH 3.5. However, pH values between 3.5 and 8.0 did not cause any change in the yield by Cel5B+CBM6 either, which completely conflicts with the previous report. A major difference between this experiment and the previous one is that the previous experiment was carried out with CMC as a substrate whereas in our case Avicel was used, instead. CMC is a cellulose derivative with a number of hydrophilic functional substitutions such as hydroxyl groups (e.g. CH_2COOH) on glucose units, so that CMC can easily dissolve

in water (Zhang, 2001). A change in pH generally affects the enzymatic reaction through directly altering the stability of enzyme or through altering the electronic charges of the enzyme protein and the substrate which results in increasing or decreasing the affinity between them. For the reaction between cellulases and Avicel, a change in the pH is unlikely to cause significant surface characteristics of Avicel because of its chemically inert property resulting from the highly ordered linear chain structures (Dourado *et al.*, 1999). Thus, in our experiment, a change of pH probably mostly affected the enzymes. Since the cellulases did not change the hydrolysis yield from Avicel with pH from 3.5 to 8.0, it is assumed that the enzymes could hold the affinity for Avicel with those pH ranges. But at pH 3.0, the enzyme might lose its stability or affinity, which may result in a reduction of the yield. On the other hand, it is assumed that because of the polar substitutions, a change in pH alters the electronic property of CMC easily, which may have resulted in a decrease in the affinity between the enzymes and the substrate. Consequently, the enzymes lost activities against CMC. A change of the optimal pH for cellulases to hydrolyze different types of cellulose substrate was also seen when CMC and a microcrystalline cellulose (Sigmacel) were hydrolyzed by an endo-cellulase from *T. reesei* (Ortega *et al.*, 2001). This study found optimal pH values at 4.0, and 5.0 for the hydrolysis of CMC and Sigmacell, respectively. Furthermore, changes in pH drastically affected the activity of the endo-cellulase when CMC was used, whereas the enzyme activity did not show a significant change when hydrolyzing Sigmacell with the same pH variation used for CMC hydrolysis, from 4.0 to 6.0. This report suggests our assumption that activities of the endo-cellulases was largely due to the altered electronic characteristics of CMC.

CMC is a material which is most frequently used for classification of cellulase into endo- or exo-cellulase (Wood and Bhat, 1988; Zhang and Lynd, 2004), and when the activity

of a cellulase is measured their optimal conditions including pH are also often determined using CMC. However, our finding of a possible change in optimal pH with respect to different types of substrate alarms the use of determined enzyme's optimal pH using a particular substrate for the hydrolysis of other types of cellulose substrate.

Chapter 5 - Conclusion and Recommendations

Despite our great expectations, a synergistic effect was not observed between CBH1 and either Cel5B or Cel5B+CBM6. This contradicts our hypothesis that the high affinity of CBM6 for amorphous cellulose would facilitate complementary interaction between endo- and exo-cellulase, leading to a high DS. Although decisive evidence was not obtained from the experiment, we speculate that the endo-cellulases have exo-type activity to hydrolyze crystalline cellulose, which caused a competitive interaction with the exo-cellulase and resulted in negative degree of negative synergy.

Further investigations of the endo-cellulase's characteristics are of great interest because such an endo-cellulase with dual activity may present a new possible approach to achieve hydrolysis of cellulose. That is, the hydrolysis of cellulose is currently considered to require a presence of both endo- and exo-cellulases with the help of β-glucosidase's action. However, if one enzyme can possess both types of activity and be effective in both reducing the DP and hydrolyzing crystalline cellulose, the necessity of having both endo- and exo-cellulase might be eliminated, which may further lead to a cost reduction of the enzymatic hydrolysis.

The unobserved synergistic effect was regrettable. However, the thought investigation of the potential factors gave us an important insight into the current classification endo- and exo-cellulase. Although the classification is very useful to describe enzyme characteristics and understand interactions between the enzymes and the substrate, it is important to keep in mind that the real distinction between the two types of cellulases is not as sharp as it seemed to be. That is, the cellulases' actions are more or less continuous from strictly endo-type to strictly exo-type. Therefore, an adequate caution needs to be paid when enzymes are characterized and selected as endo- or exo-cellulase.

Finally, the potential of CBM6 to enhance a complementary interaction between endo- and exo-cellulases still remains to be investigated. From this study, we learned that for such investigation, CBMs with a high affinity for amorphous cellulose need to be fused to a catalytic domain of cellulase with a strict endo-type activity. And a choice of exo-cellulase to be paired with the endo-cellulase should also have the distinct exo-type activity.

Appendices

Appendix A - Determination of protein concentration

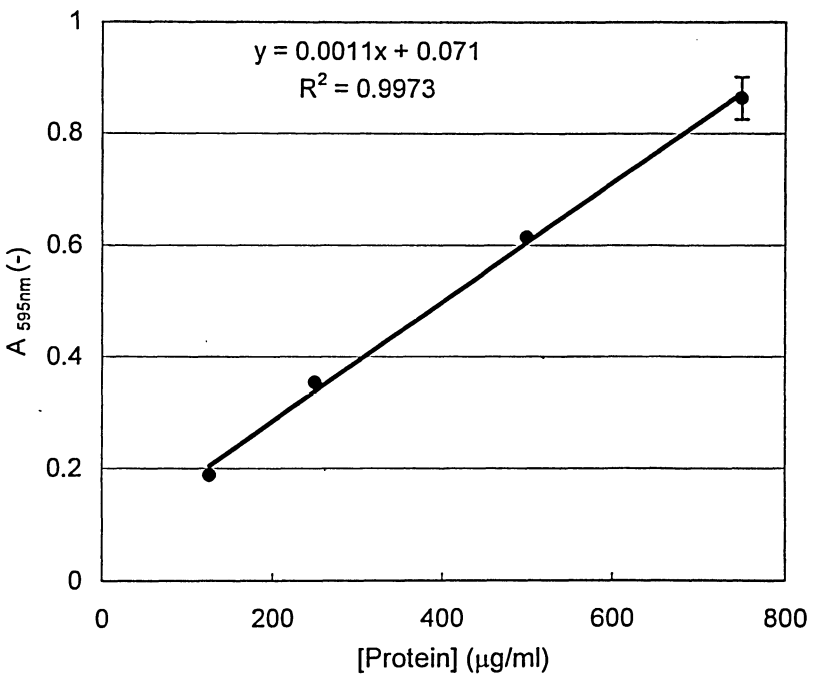


Figure A1. Standard curve for determination of protein concentration

This standard curve was made based on the data on Table A1, and the dots in the figure are the mean values of absorbance.

Table A1 Data for protein determination standard curve

	Absorbance measured for standard protein (BSA)				
	125 µg/ml	250 µg/ml	500 µg/ml	750 µg/ml	1000 µg/ml
1st set	0.1828	0.3575	0.6276	0.8927	1.1032
2nd set	0.1929	0.3505	0.6008	0.8331	1.0632
Mean value	0.1879	0.354	0.6142	0.8629	1.0832

Table A2 Protein concentration for Cel5B+CBM6

Dilution Factor	Absorbance at 570 nm	Protein conc. of sample	Protein conc. of enzyme solution (μg/ml)	Determined protein conc. of enzyme solution (μg/ml)
X1	0.6214	525.9491	525.9491	532.68
X1	0.6304	534.7751	534.7751	
X2	0.3587	268.3297	536.6595	
X2	0.3570	266.6626	533.3252	

1: Protein concentration of the samples was calculated by the equation obtained from the above standard curve:

[Protein] (μg/ml) = 980.66 x absorbance - 83.433

2: The original protein concentration estimated by each sample was obtained by multiplying the calculated protein concentrations by the dilution factors.

3: Protein concentration of original enzyme solution was determined as a mean value of estimated concentration by each sample.

Table A3. Protein concentration for Cel5B

Dilution Factor	Absorbance at 570 nm	Protein conc. of sample (μg/ml)	Protein conc. of enzyme solution (μg/ml)	Determined protein conc. of enzyme solution (μg/ml)
X 1	1.1004	995.6853	995.6853	988.01
X 1	1.0873	982.8386	982.8386	
X 2	0.5911	496.2645	992.5291	
X 2	0.5874	492.6067	985.2134	
X 4	0.3376	247.6574	990.6297	
X 4	0.3352	245.2940	981.1762	

Table A4. Protein concentration for CBH1

Dilution Factor	Absorbance at 570 nm	Protein conc. of sample (μg/ml)	Protein conc. of enzyme solution (μg/ml)	Determined protein conc. of enzyme solution (μg/ml)
X1				2091.87
X1				
X2				
X2				
X4	0.6169	521.5362	2086.1446	
X4	0.6197	524.2820	2097.1280	
X8	0.3532	262.9361	2103.4889	
X8	0.3503	260.0922	2080.7376	

Table A5. Protein concentration for β-glucosidase

Dilution Factor	Absorbance at 570 nm	Protein conc. of sample (μg/ml)	Protein conc. of enzyme solution (μg/ml)	Determined protein conc. of enzyme solution (μg/ml)
X10				32156.13
X10				
X20				
X20				
X40	0.9089	807.8889	32315.5550	
X40	0.9046	803.6720	32146.8814	
X80	0.4960	402.9744	32237.9488	
X80	0.4920	399.0517	31924.1376	

Appendix B - Reducing sugar concentration and percentages of hydrolysis yield determination by DNS method

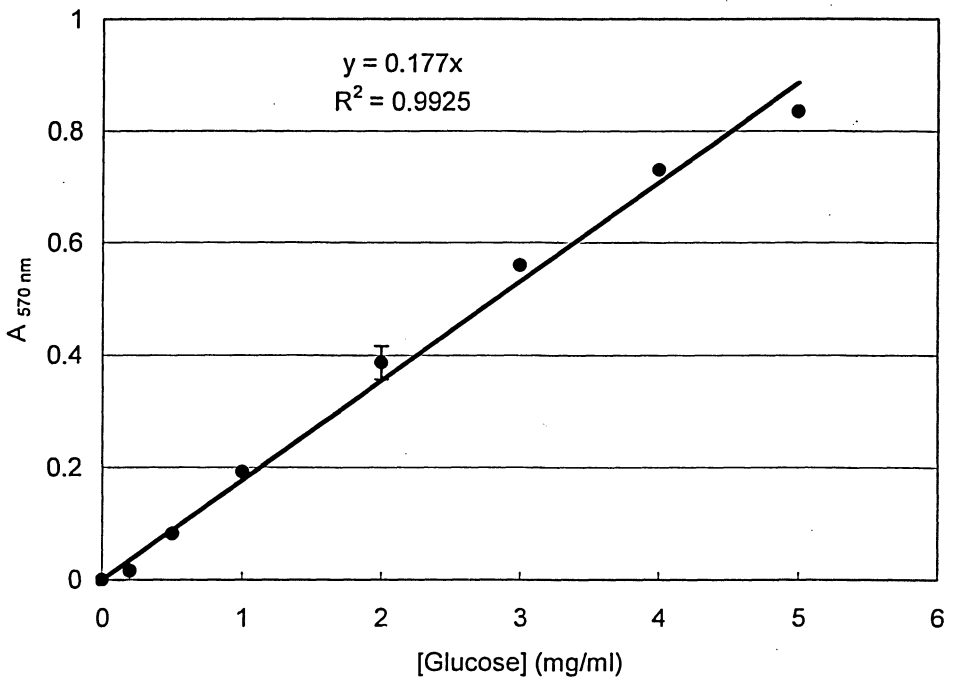


Figure B1. An example of DNS standard curve for reducing sugar concentration

The standard curve was produced from the experimental data shown Table B1. The dots in the figure are corresponding to the mean value of the duplicates shown in the table.

Table B1. Data for DNS standard curve

	Absorbance 570 nm for [Glucose] (mg/ml)							
	0	0.2	0.5	1	2	3	4	5
1st set	0.0173	0.0742	0.1874	0.4009	0.538	0.7453	0.8339	1.0167
2nd set	0.0147	0.0885	0.1973	0.372	0.5828	0.7171	0.8347	0.9972
Mean values	0.016	0.08135	0.19235	0.38645	0.5604	0.7312	0.8343	1.00695

The tables below contain experimentally obtained data for each result presented in section 4.0. Each set of data is presented in the order of measured values of absorbance, calculated reducing sugar concentration, and determined percentage of hydrolysis.

Equations used to convert absorbance to concentration of reducing sugar were reproduced several times during the course of research by remaking standard curves similar to the example presented above, and are provided along with the tables. Conversion from reducing sugar concentration to percentage of hydrolysis yield was done by the equation:

Hydrolysis % = [Reducing sugar] / 10.98 mg/ml X 100,

which is describe in section 3.4.

- Hydrolytic time course of individual enzymes (Figure 7)

Table B2. Absorbance measured for samples prepared for producing hydrolytic time course of individual enzymes

	First run			Second run		
	Absorbance at 570 nm					
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0024	0.0025	0.0022	0.0018	0.0030	0.0020
1	0.6638	0.1393	0.0170	0.6288	0.1716	0.0133
2	0.7014	0.1593	0.0253	0.6776	0.1828	0.0143
4	0.7313	0.1675	0.0262	0.7090	0.1892	0.0159
12	0.7633	0.1769	0.0230	0.7422	0.2109	0.0198
24	0.7659	0.1859	0.0226	0.7433	0.2107	0.0202

Table B3. Calculated concentrations of produced reducing sugar for samples prepared for producing hydrolytic time course of individual enzymes

	First run			Second run		
	[Reducing sugar] (mg/ml)					
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0142	0.0148	0.0130	0.0106	0.0177	0.0118
1	3.9209	0.8228	0.1007	3.7141	1.0136	0.0786
2	4.1430	0.9409	0.1494	4.0024	1.0797	0.0845
4	4.3194	0.9894	0.1548	4.1879	1.1175	0.0939
12	4.5086	1.0449	0.1359	4.3840	1.2457	0.1170
24	4.5239	1.0981	0.1335	4.3905	1.2445	0.1193

[Reducing sugar] (mg/ml) = 5.9067 x $A_{570\,nm}$ (R^2 = 0.992)

Table B4. Determined percentages of hydrolysis yield for hydrolytic time course of individual enzymes

	First run			Second run		
	Hydrolysis yield (%)					
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.1291	0.1345	0.1183	0.0968	0.1614	0.1076
1	35.7092	7.4937	0.9167	33.8263	9.2312	0.7155
2	37.7319	8.5696	1.3610	36.4515	9.8337	0.7693
4	39.3387	9.0107	1.4094	38.1407	10.1780	0.8553
12	41.0618	9.5164	1.2373	39.9267	11.3454	1.0651
24	41.2017	10.0005	1.2158	39.9859	11.3346	1.0867
	Standard deviation					
0	0.01614	0.01345	0.00538			
1	0.94141	0.86879	0.10060			
2	0.64016	0.63209	0.29587			
4	0.59901	0.58368	0.27704			
12	0.56754	0.91452	0.08607			
24	0.60788	0.66706	0.06455			

- Elimination of product inhibition by β-glucosidase (Table 9)

Table B5. Absorbance for samples prepared for examination cellobiose inhibition

First run				
β-Glucosidase (μg/ml)	Absorbance at 570 nm			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	0.7445	0.1770	0.0235	0.0001
1.6	0.8370	0.2058	0.0253	0.0001
3.2	0.8885	0.2385	0.0241	0.0002
16.0	0.9689	0.2599	0.0580	-0.0001
24	0.9922	0.2707	0.0727	0.0001
32	1.0001	0.2717	0.0770	-0.0001
64	1.0121	0.2759	0.0810	0.0003
96	1.0050	0.2765	0.0811	0.0001
128	1.0070	0.2804	0.0809	0.0000
Second run				
β-Glucosidase (μg/ml)	Absorbance at 570 nm			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	0.8013	0.2100	0.0180	0.0000
1.6	0.8925	0.2367	0.0227	0.0002
3.2	0.8997	0.2882	0.0365	0.0001
16	1.0059	0.2968	0.0503	0.0001
24	1.0308	0.2875	0.0645	-0.0001
32	1.0200	0.2864	0.0749	0.0002
64	1.0109	0.2945	0.0778	-0.0001
96	1.0186	0.2852	0.0773	-0.0002
128	1.0065	0.2883	0.0772	0.0001

Table B6. Calculated concentration of reducing sugars for samples prepared for examination of cellobiose inhibition

First run				
β-Glucosidase (μg/ml)	[Reducing sugar] (mg/ml)			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	4.3975	1.0455	0.1388	0.0006
1.6	4.9439	1.2156	0.1494	0.0006
3.2	5.2481	1.4087	0.1424	0.0012
16	5.7230	1.5352	0.3426	-0.0006
24	5.8606	1.5990	0.4294	0.0006
32	5.9073	1.6049	0.4548	-0.0006
64	5.9782	1.6297	0.4784	0.0018
96	5.9362	1.6332	0.4790	0.0006
128	5.9480	1.6562	0.4779	0.0000
Second run				
β-Glucosidase (μg/ml)	[Reducing sugar] (mg/ml)			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	4.7330	1.2404	0.1063	0.0000
1.6	5.2717	1.3981	0.1341	0.0012
3.2	5.3143	1.7023	0.2156	0.0006
16	5.9415	1.7531	0.2970	0.0006
24	6.0886	1.6982	0.3810	-0.0006
32	6.0248	1.6917	0.4424	0.0012
64	5.9711	1.7395	0.4595	-0.0006
96	6.0166	1.6846	0.4566	-0.0014
128	5.9451	1.7029	0.4558	0.0006

[Reducing sugar] (mg/ml) = 5.9067 x A_{570nm} (R^2 = 0.992)

Table B7. Determined hydrolysis percentages for samples prepared for examination of cellobiose inhibition

First run				
β-Glucosidase (μg/ml)	Hydrolysis yield (%)			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	40.0504	9.5217	1.2642	0.0054
1.6	45.0265	11.0710	1.3610	0.0054
3.2	47.7969	12.8301	1.2965	0.0108
16	52.1221	13.9813	3.1201	-0.0054
24	53.3755	14.5629	3.9109	0.0054
32	53.8005	14.6161	4.1422	-0.0054
64	54.4460	14.8421	4.3574	0.0161
96	54.0641	14.8743	4.3628	0.0054
128	54.1716	15.0841	4.3520	0.0000
Second run				
β-Glucosidase (μg/ml)	Hydrolysis yield (%)			
	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0.0	43.1060	11.2970	0.9683	0.0000
1.6	48.0121	12.7333	1.2211	0.0108
3.2	48.3994	15.5037	1.9635	0.0054
16	54.1125	15.9664	2.7054	0.0054
24	55.4520	15.4661	3.4698	-0.0054
32	54.8710	15.4069	4.0293	0.0108
64	54.3814	15.8427	4.1853	-0.0054
96	54.7957	15.3424	4.1584	-0.0124
128	54.1448	15.5091	4.1514	0.0054
	Standard deviation			
0.0	1.5278	0.8876	0.1479	0.00
1.6	1.4928	0.8311	0.0699	0.00
3.2	0.3013	1.3368	0.3335	0.00
16.0	0.9952	0.9925	0.2074	0.00
25.7	1.0382	0.4516	0.2206	0.00
32.0	0.5353	0.3954	0.0565	0.00
64.0	0.0323	0.5003	0.0861	0.00
96.0	0.3658	0.2340	0.1022	0.00
128.0	0.0134	0.2125	0.1003	0.00

- Examination of effect of pH and temperature on hydrolysis yield by CBH1(Figure 8)

Table B8. Absorbance measured for samples prepared for examination of effect of pH and temperature on hydrolysis yield by CBH1

	First run				Second run			
	Absorbance at 570 nm							
pH	40	45	50	60	40	45	50	60
3	0.0015	0.0022	0.0039	0.0006	0.0010	0.0004	0.0068	0.0005
3.5	0.0805	0.0904	0.0623	0.0002	0.0998	0.1160	0.0812	0.0002
4	0.1880	0.1469	0.1732	0.0411	0.1639	0.1751	0.1943	0.0545
4.5	0.2199	0.2061	0.1919	0.1322	0.1796	0.1971	0.2420	0.1446
5	0.2097	0.2165	0.2240	0.1695	0.2103	0.1929	0.2105	0.1306
5.5	0.1883	0.1857	0.1846	0.0862	0.1643	0.1694	0.1788	0.0831
6	0.1112	0.1233	0.1015	0.0420	0.1160	0.1384	0.1311	0.0171
6.5	0.0857	0.0745	0.1063	0.0166	0.0697	0.1107	0.0735	0.0015
7	0.0223	0.0206	0.0457	0.0085	0.0366	0.0346	0.0143	0.0067
7.5	0.0032	0.0011	0.0009	0.0042	0.0260	0.0024	0.0021	0.0032
8	0.0028	0.0011	0.0011	0.0015	0.0010	0.0010	0.0022	0.0014

Table B9. Calculated concentrations of samples prepared for examination of effect of pH and temperature on hydrolysis yield by CBH1

	First run				Second run			
	[Reducing sugars] (mg/ml)							
pH	40	45	50	60	40	45	50	60
3	0.0078	0.0114	0.0202	0.0031	0.0052	0.0021	0.0352	0.0026
3.5	0.4170	0.4682	0.3227	0.0009	0.5169	0.6008	0.4206	0.0010
4	0.9738	0.7609	0.8971	0.2129	0.8489	0.9069	1.0064	0.2823
4.5	1.1390	1.0675	0.9940	0.6847	0.9303	1.0209	1.2535	0.7490
5	1.0862	1.1214	1.1602	0.8779	1.0893	0.9991	1.0903	0.6765
5.5	0.9753	0.9619	0.9562	0.4465	0.8510	0.8774	0.9261	0.4304
6	0.5760	0.6386	0.5258	0.2175	0.6008	0.7169	0.6790	0.0886
6.5	0.4439	0.3859	0.5506	0.0860	0.3610	0.5734	0.3807	0.0078
7	0.1155	0.1067	0.2367	0.0440	0.1896	0.1792	0.0741	0.0347
7.5	0.0166	0.0057	0.0047	0.0218	0.1347	0.0124	0.0109	0.0166
8	0.0145	0.0057	0.0057	0.0078	0.0052	0.0052	0.0114	0.0073

[Reducing sugar] (mg/ml) = 5.1796 x A_{570nm} (R^2 = 0.997)

Table B10. Determined hydrolysis yield for samples prepared for examination of effect of pH and temperature on hydrolysis yield by CBH1

	First run				Second run			
	Hydrolysis yield (%)							
pH	40	45	50	60	40	45	50	60
3	0.0708	0.1038	0.1840	0.0283	0.0472	0.0189	0.3208	0.0236
3.5	3.7974	4.2644	2.9389	0.0080	4.7079	5.4721	3.8305	0.0094
4	8.8685	6.9297	8.1704	1.9388	7.7317	8.2600	9.1657	2.5709
4.5	10.3734	9.7224	9.0525	6.2363	8.4723	9.2978	11.4159	6.8212
5	9.8922	10.2130	10.5668	7.9958	9.9205	9.0997	9.9299	6.1608
5.5	8.8827	8.7600	8.7081	4.0663	7.7505	7.9911	8.4345	3.9201
6	5.2456	5.8164	4.7885	1.9813	5.4721	6.5287	6.1844	0.8067
6.5	4.0427	3.5144	5.0145	0.7835	3.2880	5.2221	3.4672	0.0708
7	1.0520	0.9718	2.1558	0.4010	1.7265	1.6322	0.6746	0.3161
7.5	0.1510	0.0519	0.0425	0.1981	1.2265	0.1132	0.0991	0.1510
8	0.1321	0.0519	0.0519	0.0708	0.0472	0.0472	0.1038	0.0660

pH	Standard deviation			
3	0.0118	0.0425	0.0684	0.0000
3.5	0.4552	0.6038	0.4458	0.0000
4	0.5684	0.6651	0.4977	0.3161
4.5	0.9505	0.2123	1.1817	0.2925
5	0.0142	0.5566	0.3184	0.9175
5.5	0.5661	0.3845	0.1368	0.0731
6	0.1132	0.3562	0.6979	0.5873
6.5	0.3774	0.8538	0.7736	0.3564
7	0.3373	0.3302	0.7406	0.0425
7.5	0.5378	0.0307	0.0283	0.0236
8	0.0425	0.0000	0.0259	0.0000

- Hydrolytic time course of each enzyme with 64 μg/ml of β-glucosidase (Figure 9)

Table B11. Absorbance measured for samples prepared for hydrolytic time course of each enzyme with 64 μg/ml of β-glucosidase

	First run			
	Measured Absorbance at 570 nm			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.0034	0.0012	0.0011	0.0002
1	0.9365	0.1893	0.1119	-0.0001
2	1.1089	0.2483	0.1892	0.0003
4	1.1945	0.2638	0.3036	0.0005
12	1.2335	0.2911	0.3654	-0.0003
24	1.2415	0.2916	0.3662	-0.0001
	Second run			
	Measured Absorbance at 570 nm			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.0016	0.0021	0.0036	0.0004
1	0.8567	0.2003	0.0757	0.0006
2	1.0769	0.2775	0.1493	0.0004
4	1.1552	0.3069	0.2698	0.0003
12	1.2255	0.3118	0.3650	0.0005
24	1.2338	0.3314	0.3799	0.0004

Table B12. Calculated reducing sugar concentration for samples prepared for hydrolytic time course of each enzyme with 64 μg/ml of β-glucosidase

	First run			
	[Reducing sugar] (mg/ml)			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.0163	0.0058	0.0053	0.0010
1	4.4989	0.9094	0.5376	-0.0005
2	5.3272	1.1928	0.9089	0.0014
4	5.7384	1.2673	1.4585	0.0024
12	5.9257	1.3984	1.7554	-0.0014
24	5.9642	1.4008	1.7592	-0.0005
	Second run			
	[Reducing sugar] (mg/ml)			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.0077	0.0101	0.0173	0.0019
1	4.1156	0.9622	0.3637	0.0029
2	5.1734	1.3331	0.7172	0.0019
4	5.5496	1.4743	1.2961	0.0014
12	5.8873	1.4979	1.7535	0.0024
24	5.9272	1.5920	1.8250	0.0019

[Reducing sugar] (mg/ml) = 4.804 x A_{570nm} (R^2 = 0.995)

Table B13. Determined hydrolysis yields for samples prepared for hydrolytic time course of each enzyme with 64 μg/ml of β-glucosidase

	First run			
	Hydrolysis yield (%)			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.1488	0.0525	0.0481	0.0088
1	40.9740	8.2823	4.8959	-0.0044
2	48.5169	10.8637	8.2779	0.0131
4	52.2621	11.5419	13.2832	0.0219
12	53.9684	12.7363	15.9871	-0.0131
24	54.3185	12.7582	16.0221	-0.0044
	Second run			
	Hydrolysis yield (%)			
Time (hr)	Cel5B+CBM6	Cel5B	CBH1	β-Glucosidase
0	0.0700	0.0919	0.1575	0.0175
1	37.4826	8.7636	3.3120	0.0263
2	47.1168	12.1413	6.5322	0.0175
4	50.5426	13.4276	11.8044	0.0131
12	53.6184	13.6420	15.9696	0.0219
24	53.9816	14.4995	16.6215	0.0175
Time (hr)	Standard deviation			
0	0.0394	0.0197	0.0547	0.0044
1	1.7457	0.2406	0.7919	0.0153
2	0.7000	0.6388	0.8729	0.0022
4	0.8597	0.9429	0.7394	0.0044
12	0.1750	0.4528	0.0088	0.0175
24	0.1684	0.8707	0.2997	0.0109

- Evaluation of potential synergy during Avicel hydrolysis at pH 6.5 (Figure 10A)

Table B14. Absorbance measured for samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 6.5

Individual enzymes	First run			Second run		
	Absorbance at 570 nm					
[Enzyme] µM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0013	0.0002	0.0004	0.0010	0.0011	0.0004
7.2	0.3498	0.0841	0.0958	0.2892	0.0716	0.0572
14.4	0.6570	0.1755	0.1436	0.5723	0.1523	0.1072
21.6	0.9003	0.2267	0.1919	0.7821	0.1988	0.1618
28.8	1.0862	0.3255	0.2294	0.9839	0.2503	0.1838
36	1.3036	0.3684	0.2622	1.1969	0.3054	0.2202

	First run		Second run	
Mixtures	Absorbance at 570 nm			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	0.2357	0.2389	0.2230	0.2086
20	0.5276	0.2773	0.4298	0.2154
40	0.7189	0.2641	0.7083	0.2631
60	0.9925	0.2653	0.8794	0.3041
80	1.1623	0.3450	1.0412	0.2750
100	1.2840	0.3522	1.2151	0.3181

Table B15. Calculated reducing sugar concentration for samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 6.5

Individual enzymes	First run			Second run		
	[Reducing sugar] (mg/ml)					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0060	0.0009	0.0018	0.0049	0.0054	0.0020
7.2	1.6014	0.3850	0.4386	1.4312	0.3543	0.2831
14.4	3.0077	0.8034	0.6574	2.8322	0.7537	0.5305
21.6	4.1216	1.0378	0.8785	3.8705	0.9838	0.8007
28.8	4.9726	1.4901	1.0502	4.8691	1.2387	0.9096
36	5.9679	1.6865	1.2004	5.9232	1.5114	1.0897

	First run		Second run	
Mixtures	[Reducing sugar] (mg/ml)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	1.0790	1.0937	1.1036	1.0323
20	2.4154	1.2695	2.1270	1.0660
40	3.2911	1.2090	3.5052	1.3020
60	4.5437	1.2145	4.3520	1.5049
80	5.3210	1.5794	5.1527	1.3609
100	5.8782	1.6124	6.0133	1.5742

[Reducing sugar] (mg/ml) = 4.578 x A_{570nm} (R^2 = 0.997) for first run

[Reducing sugar] (mg/ml) = 4.9488 x A_{570nm} (R^2 = 0.996) for second run

Table B16. Determined hydrolysis yields of samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 6.5

Individual enzymes	First run			Second run		
	Hydrolysis yield (%)					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0542	0.0083	0.0167	0.0451	0.0496	0.0180
7.2	14.5846	3.5065	3.9943	13.0345	3.2271	2.5781
14.4	27.3930	7.3173	5.9873	25.7942	6.8643	4.8316
21.6	37.5371	9.4520	8.0011	35.2501	8.9601	7.2925
28.8	45.2880	13.5714	9.5646	44.3454	11.2813	8.2841
36	54.3523	15.3601	10.9322	53.9455	13.7647	9.9246

	First run		Second run	
Mixtures	Hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	9.8273	9.9607	10.0508	9.4018
20	21.9977	11.5617	19.3715	9.7083
40	29.9738	11.0114	31.9238	11.8582
60	41.3813	11.0614	39.6355	13.7061
80	48.4609	14.3844	46.9280	12.3945
100	53.5351	14.6846	54.7658	14.3371

	Standard deviation				
	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
	0.0042	0.0208	0.0021	0.5083	0.4831
	0.7734	0.1376	0.7067	1.4153	0.7800
	0.7984	0.2251	0.5775	1.1537	0.5808
	1.1424	0.2439	0.3523	1.7213	0.8238
	0.4711	1.1445	0.6400	1.1794	1.8532
	0.2022	0.7964	0.5024	0.2027	0.7970

From the determined hydrolysis percent by individual enzymes, mathematical sum of the yield by separately working one of the endo-cellulases and the exo-cellulase were calculated.

Table B17. Mathematical sum of the hydrolysis yield by separately working one of the endo-cellulases and the exo-cellulase at pH 6.5

	First run		Second run	
	Calculated hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1
0	10.9864	10.9405	9.9697	9.9742
20	24.1492	13.0711	21.3186	11.5111
40	35.3940	15.3184	33.0866	14.1568
60	43.5244	15.4393	40.0817	13.7917
80	49.2823	17.5657	46.9235	13.8593
100	54.3690	15.3767	53.9636	13.7827

- Evaluation of potential synergy during Avicel hydrolysis at pH 6.0 (Figure 10B)

Table B18. Absorbance measured for samples prepared for evaluation of synergistic effect during Avicel hydrolysis at pH 6.0

Individual enzymes	First run			Second run		
	Absorbance at 570 nm					
[Enzyme] µM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0024	0.0017	0.0008	0.0015	0.0003	0.0009
7.2	0.3159	0.0651	0.0699	0.3149	0.0733	0.0841
14.4	0.5929	0.1376	0.1358	0.6080	0.1397	0.1444
21.6	0.7841	0.2071	0.1781	0.7965	0.2051	0.1996
28.8	1.0504	0.2636	0.2428	1.0364	0.2693	0.2432
36	1.2171	0.3170	0.2857	1.2270	0.3180	0.2762

	First run		Second run	
Mixtures	Absorbance at 570 nm			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	0.2857	0.2856	0.2762	0.2762
20	0.4994	0.2692	0.5058	0.2601
40	0.7327	0.2819	0.7298	0.2886
60	0.8847	0.3026	0.9082	0.2921
80	1.1006	0.3198	1.0833	0.3108
100	1.2171	0.3170	1.2270	0.3179

Table B19. Calculated reducing sugar concentration for samples prepared for evaluation of synergistic effect during Avicel hydrolysis at pH 6.0

Individual enzymes	First run			Second run		
	[Reducing sugar] (mg/ml)					
[Enzyme] µM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0118	0.0083	0.0039	0.0075	0.0015	0.0045
7.2	1.5477	0.3190	0.3425	1.5755	0.3667	0.4208
14.4	2.9049	0.6742	0.6653	3.0419	0.6989	0.7224
21.6	3.8416	1.0147	0.8726	3.9850	1.0261	0.9986
28.8	5.1463	1.2915	1.1896	5.1852	1.3473	1.2168
36	5.9631	1.5531	1.3998	6.1388	1.5910	1.3819

	First run		Second run	
Mixtures	[Reducing sugar] (mg/ml)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	1.3998	1.3993	1.3819	1.3819
20	2.4468	1.3189	2.5306	1.3013
40	3.5898	1.3811	3.6513	1.4439
60	4.3345	1.4826	4.5438	1.4614
80	5.3923	1.5668	5.4199	1.5550
100	5.9631	1.5531	6.1388	1.5905

[Reducing sugar] (mg/ml) = 4.8994 x A_{570nm} (R^2 = 0.995) for first run

[Reducing sugar] (mg/ml) = 5.0031 x A_{570nm} (R^2 = 0.996) for second run

Table B20. Determined hydrolysis yields of samples prepared for evaluation of synergistic effect during Avicel hydrolysis at pH 6.0

Individual enzymes	First run			Second run		
	Hydrolysis yield (%)					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.1071	0.0759	0.0357	0.0683	0.0137	0.0410
7.2	14.0958	2.9048	3.1190	14.3486	3.3400	3.8321
14.4	26.4559	6.1399	6.0595	27.7039	6.3655	6.5797
21.6	34.9874	9.2410	7.9470	36.2930	9.3455	9.0949
28.8	46.8700	11.7621	10.8340	47.2242	12.2708	11.0815
36	54.3084	14.1449	12.7483	55.9090	14.4899	12.5852

	First run		Second run	
Mixtures	Hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	12.7483	12.7438	12.5852	12.5852
20	22.2838	12.0120	23.0471	11.8516
40	32.6939	12.5787	33.2538	13.1502
60	39.4763	13.5024	41.3827	13.3097
80	49.1100	14.2698	49.3612	14.1618
100	54.3084	14.1449	55.9090	14.4853

	Standard deviation				
	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
	0.0194	0.0311	0.0000	0.1009	0.1126
	0.1264	0.2176	0.3565	0.2502	0.3413
	0.6240	0.1128	0.2601	1.1979	0.6868
	0.6528	0.0522	0.5739	0.9128	0.3123
	0.1771	0.2543	0.1238	0.5336	0.6109
	0.8003	0.1725	0.0815	0.8029	0.1751

From the determined hydrolysis percent by individual enzymes, mathematical sum of the yield by one of the endo-cellulases and the exo-cellulase were calculated.

Table B21. Mathematical sum of the hydrolysis yield by separately working one of the endo-cellulases and the exo-cellulase at pH 6.0

	First run		Second run	
	Calculated hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1
0	12.8553	12.8241	12.6536	12.5989
20	24.9298	13.7388	25.4301	14.4215
40	34.4029	14.0869	36.7988	15.4604
60	41.0470	15.3006	42.8726	15.9252
80	49.9891	14.8811	51.0562	16.1029
100	54.3441	14.1806	55.9500	14.5309

- Evaluation of potential synergy during Avicel hydrolysis at pH 5.5 (Figure 10C)

Table B22. Absorbance measured for samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 5.5

Individual enzymes	First run			Second run		
	Absorbance at 570 nm					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0007	0.0012	0.0005	0.0017	0.0003	0.0019
7.2	0.3193	0.0547	0.1178	0.3251	0.0685	0.1095
14.4	0.6245	0.1375	0.1953	0.6211	0.1392	0.1868
21.6	0.8042	0.2038	0.2649	0.8389	0.2046	0.2565
28.8	1.0753	0.2762	0.3269	1.0362	0.2662	0.3186
36	1.2592	0.3296	0.3781	1.2193	0.3284	0.3593

	First run		Second run	
Mixtures	Absorbance at 570 nm			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	0.3781	0.3781	0.3593	0.3593
20	0.5652	0.3355	0.5615	0.3483
40	0.7694	0.3357	0.7669	0.3278
60	0.9346	0.3237	0.9452	0.3257
80	1.1221	0.3246	1.1010	0.3249
100	1.2592	0.3296	1.2193	0.32843

Table B23. Calculated reducing sugar concentration for samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 5.5

Individual enzymes	First run			Second run		
	[Reducing sugar] (mg/ml)					
[Enzyme] µM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0034	0.0057	0.0024	0.0082	0.0014	0.0091
7.2	1.5286	0.2619	0.5639	1.5600	0.3287	0.5254
14.4	2.9897	0.6583	0.9350	2.9803	0.6680	0.8964
21.6	3.8499	0.9757	1.2682	4.0255	0.9818	1.2308
28.8	5.1478	1.3223	1.5650	4.9722	1.2774	1.5288
36	6.0282	1.5779	1.8101	5.8508	1.5758	1.7241

	First run		Second run	
Mixtures	[Reducing sugar] (mg/ml)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	1.8101	1.8101	1.7241	1.7241
20	2.7058	1.6061	2.6944	1.6713
40	3.6833	1.6071	3.6800	1.5729
60	4.4742	1.5496	4.5355	1.5629
80	5.3718	1.5540	5.2831	1.5590
100	6.0282	1.5779	5.8508	1.5760

[Reducing sugar] (mg/ml) = 4.7873 x A_{570nm} (R^2 = 0.996) for first run

[Reducing sugar] (mg/ml) = 4.7985 x A_{570nm} (R^2 = 0.996) for second run

Table B24. Determined percentage of hydrolysis yields of samples prepared for evaluation of potential synergistic effect during Avicel hydrolysis at pH 5.5

Individual enzymes	First run			Second run		
	Hydrolysis yield (%)					
[Enzyme] µM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0305	0.0523	0.0218	0.0743	0.0131	0.0830
7.2	13.9215	2.3849	5.1361	14.2076	2.9936	4.7854
14.4	27.2283	5.9950	8.5151	27.1434	6.0833	8.1636
21.6	35.0633	8.8857	11.5497	36.6618	8.9415	11.2096
28.8	46.8833	12.0424	14.2529	45.2842	11.6335	13.9235
36	54.9013	14.3706	16.4852	53.2861	14.3518	15.7022

	First run		Second run	
Mixtures	Hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	16.4852	16.4852	15.7022	15.7022
20	24.6428	14.6279	24.5388	15.2215
40	33.5460	14.6366	33.5152	14.3256
60	40.7487	14.1134	41.3073	14.2338
80	48.9238	14.1526	48.1161	14.1988
100	54.9013	14.3706	53.2861	14.3531

	Standard deviation				
	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
	0.0219	0.0196	0.0306	0.3696	0.4111
	0.1430	0.3043	0.1754	0.0217	0.1396
	0.0424	0.0442	0.1758	0.2125	0.1259
	0.7992	0.0279	0.1700	0.6235	0.1479
	0.7995	0.2044	0.1647	0.9749	0.3798
	0.8076	0.0094	0.3915	0.7770	0.0212

From the determined hydrolysis percent by individual enzymes, mathematical sum of the yield by one of the endo-cellulases and the exo-cellulase were calculated.

Table B25. Mathematical sum of the hydrolysis yield by separately working one of the endo-cellulases and the exo-cellulase at pH 5.5

	First run		Second run	
	Calculated hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1
0	16.5157	16.5375	15.7765	15.7153
20	28.1744	16.6378	28.1311	16.9171
40	38.7780	17.5447	38.3530	17.2930
60	43.5784	17.4008	44.8253	17.1050
80	52.0194	17.1785	50.0696	16.4189
100	54.9231	14.3924	53.3691	14.4348

- Evaluation of potential synergy during hydrolysis of Avicel at pH 5.0 (Figure 10D)

Table B26. Absorbance measured for samples prepared for evaluation of potential synergistic effect during hydrolysis of Avicel at pH 5.0

Individual enzymes	First run			Second run		
	Absorbance at 570 nm					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0009	0.0014	0.0013	0.0010	0.0010	0.0005
7.2	0.3228	0.0745	0.1191	0.3416	0.0750	0.0988
14.4	0.6266	0.1301	0.2105	0.6507	0.1481	0.2103
21.6	0.8138	0.2039	0.2986	0.8768	0.2196	0.2959
28.8	1.0397	0.2747	0.3577	1.0961	0.2799	0.3543
36	1.2440	0.3337	0.4174	1.2886	0.3503	0.4023

	First run		Second run	
Mixtures	Absorbance at 570 nm			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	0.4174	0.4174	0.4023	0.4023
20	0.6135	0.3530	0.5895	0.3969
40	0.8028	0.3759	0.8356	0.3921
60	0.9388	0.3632	0.9882	0.3820
80	1.1008	0.3549	1.1732	0.3737
100	1.2440	0.3337	1.2886	0.3503

Table B27. Calculated reducing sugar concentration for samples prepared for evaluation of potential synergistic effect during hydrolysis of Avicel at pH 5.0

Individual enzymes	First run			Second run		
	[Reducing sugar] (mg/ml)					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0043	0.0066	0.0062	0.0048	0.0048	0.0024
7.2	1.5319	0.3536	0.5652	1.6311	0.3581	0.4718
14.4	2.9737	0.6174	0.9990	3.1070	0.7072	1.0042
21.6	3.8621	0.9677	1.4171	4.1866	1.0486	1.4129
28.8	4.9342	1.3037	1.6976	5.2338	1.3365	1.6917
36	5.9038	1.5837	1.9809	6.1529	1.6726	1.9209

	First run		Second run	
Mixtures	[Reducing sugar] (mg/ml)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	1.9809	1.9809	1.9209	1.9209
20	2.9115	1.6753	2.8148	1.8952
40	3.8099	1.7839	3.9899	1.8722
60	4.4554	1.7237	4.7186	1.8240
80	5.2242	1.6843	5.6019	1.7844
100	5.9038	1.5837	6.1529	1.6726

[Reducing sugar] (mg/ml) = 4.7458 x A_{570nm} (R^2 = 0.997) for first run

[Reducing sugar] (mg/ml) = 4.7749 x A_{570nm} (R^2 = 0.999) for second run

Table B28. Determined hydrolysis yields of samples prepared for evaluation of potential synergistic effect during hydrolysis of Avicel at pH 5.0

Individual enzymes	First run			Second run		
	Hydrolysis yield (%)					
[Enzyme] μM	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM6	Cel5B	CBH1
0	0.0389	0.0605	0.0562	0.0435	0.0435	0.0217
7.2	13.9521	3.2201	5.1478	14.8552	3.2615	4.2965
14.4	27.0830	5.6232	9.0983	28.2972	6.4405	9.1454
21.6	35.1742	8.8130	12.9062	38.1296	9.5498	12.8679
28.8	44.9381	11.8731	15.4606	47.6664	12.1721	15.4075
36	53.7684	14.4233	18.0410	56.0377	15.2336	17.4949

	First run		Second run	
	Hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Cel5B+CBM6 and CBH1	Cel5B and CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
0	18.0410	18.0410	17.4949	17.4949
20	26.5168	15.2574	25.6357	17.2601
40	34.6988	16.2472	36.3379	17.0514
60	40.5770	15.6983	42.9741	16.6121
80	47.5790	15.3396	51.0192	16.2512
100	53.7684	14.4233	56.0377	15.2336

	Standard deviation				
	Cel5B+CBM6	Cel5B	CBH1	Cel5B+CBM and CBH1	Cel5B and CBH1
	0.0023	0.0085	0.0172	0.2707	0.2815
	0.4516	0.0207	0.4256	0.4250	0.0058
	0.6071	0.4086	0.0235	0.5879	0.3895
	1.4777	0.3684	0.0191	1.5012	0.3919
	1.3641	0.1495	0.0265	0.9385	0.2761
	1.1346	0.4052	0.2730	1.1174	0.3879

From the determined hydrolysis percent by individual enzymes, mathematical sum of the yield by one of the endo-cellulases and the exo-cellulase were calculated.

Table B29. Mathematical sum of the hydrolysis yield by separately working one of the endo-cellulases and the exo-cellulase at pH 5.0

	First run		Second run	
Mixtures	Calculated hydrolysis yield (%)			
Ratio of endo-cellulase (%)	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1	Sum of Cel5B+ CBM6 and CBH1	Sum of Ce5B and CBH1
0	18.0799	18.1015	17.5384	17.5384
20	29.4127	18.6806	30.2628	18.6691
40	39.9892	18.5294	41.1650	19.3083
60	44.2725	17.9113	47.2750	18.6952
80	50.0859	17.0209	51.9629	16.4686
100	53.8246	14.4794	56.0594	15.2553

- Effect of pH on hydrolysis yield by Cel5B+CBM6 and Cel5B

Table B30. Measured absorbance for samples prepared for examining effect of pH on hydrolysis by Cel5B+CBM6 and Cel5B

	First run		Second run	
	Absorbance at 570 nm			
pH	Cel5B+CBM6	Cel5B	Cel5B+CBM6	Cel5B
3	0.0364	0.0228	0.0287	0.0159
3.5	0.9730	0.1902	0.9290	0.2161
4	0.9950	0.1871	0.9095	0.2163
4.5	0.9499	0.1955	0.9142	0.2054
5	0.9174	0.1929	0.9612	0.2097
5.5	0.9574	0.195	0.9236	0.2055
6	0.9471	0.1884	0.9303	0.2051
6.5	0.9596	0.1894	0.9230	0.2143
7	0.9844	0.1931	0.9044	0.2095
7.5	0.9461	0.1881	0.9349	0.2074
8	0.9428	0.1849	0.8958	0.2120

Table B31. Calculated reducing sugar concentration for samples prepared for examining effect of pH on hydrolysis by Cel5B+CBM6 and Cel5B

	First run		Second run	
	[Reducing sugar] (mg/ml)			
pH	Cel5B+CBM6	Cel5B	Cel5B+CBM6	Cel5B
3	0.1753	0.1098	0.1382	0.0766
3.5	4.6864	0.9161	4.4744	1.0408
4	4.7923	0.9011	4.3805	1.0418
4.5	4.5751	0.9416	4.4032	0.9893
5	4.4186	0.9291	4.6295	1.0100
5.5	4.6112	0.9392	4.4484	0.9898
6	4.5616	0.9074	4.4807	0.9878
6.5	4.6218	0.9122	4.4455	1.0320
7	4.7413	0.9300	4.3560	1.0090
7.5	4.5568	0.9060	4.5029	0.9989
8	4.5409	0.8906	4.3145	1.0211

[Reducing sugar] (mg/ml) = 4.8164 x A_{570nm} (R^2 = 0.997)

Table B32. Determined hydrolysis percentage for samples prepared for examining effect of pH on hydrolysis by Cel5B+CBM6 and Cel5B

	First run		Second run	
	Hydrolysis yield (%)			
pH	Cel5B+CBM6	Cel5B	Cel5B+CBM6	Cel5B
3	1.5967	1.0001	1.2589	0.6975
3.5	42.6808	8.3432	40.7508	9.4793
4	43.6459	8.2072	39.8954	9.4880
4.5	41.6676	8.5756	40.1016	9.0099
5	40.2419	8.4616	42.1632	9.1985
5.5	41.9966	8.5537	40.5139	9.0143
6	41.5447	8.2642	40.8078	8.9968
6.5	42.0931	8.3081	40.4876	9.3986
7	43.1809	8.4704	39.6717	9.1898
7.5	41.5009	8.2510	41.0096	9.0976
8	41.3561	8.1107	39.2945	9.2994

	Standard deviation	
pH	Cel5B+CBM6	Cel5B
3	0.1689	0.1513
3.5	0.9650	0.5681
4	1.8752	0.6404
4.5	0.7830	0.2171
5	0.9606	0.3685
5.5	0.7413	0.2303
6	0.3685	0.3663
6.5	0.8027	0.5452
7	1.7546	0.3597
7.5	0.2456	0.4233
8	1.0308	0.5944

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