Evaluation of Commercial Cellulase Preparations for the Efficient Hydrolysis of Hydrothermally Pretreated Empty Fruit Bunches

Jungwoo Yang,[a] Ji Eun Kim,[a] Jae Kyun Kim,[a] Sung ho Lee,[b] Ju-Hyun Yu,[c] and Kyoung Heon Kim [a,*]

The performance of cellulase in the enzymatic saccharification of lignocellulose depends on the characteristics of lignocellulosic biomass feedstocks and the pretreatment method used. Efficient hydrolysis of specifically pretreated lignocellulose necessitates the knowledge of the characteristics of the optimal commercial cellulases. In this study, commercial cellulase preparations (Accellerase™ 1000, Accellerase® 1500, and Spezyme® CP from DuPont and Cellic® CTec2 from Novozymes) were evaluated for their hydrolysis efficiency of hydrothermally pretreated empty fruit bunches (EFBs). The highest glucose yields of 91.3% and 84.7% were achieved for 30 FPU of Cellic® CTec2/g glucan with and without Cellic® HTec2, respectively. Of the four cellulases tested, Cellic® CTec2, which showed the highest cellobiohydrolase, xylanase, and β-glucosidase activities, showed the highest glucose yield in the enzymatic hydrolysis of hydrothermally pretreated EFBs. The results of this study are valuable for those who plan to enzymatically hydrolyze hydrothermally pretreated EFBs.

Keywords: Cellulase; Enzymatic hydrolysis; Hydrothermal pretreatment; Empty fruit bunches; Lignocellulose

Contact information: a: Department of Biotechnology, Graduate School, Korea University, Seoul 02841, South Korea; b: Novozymes Korea, Seoul 04342, South Korea; c: Center for Bio-based Chemistry, Korea Research Institute of Chemical Technology, Daejeon 34602, South Korea;
** Corresponding author: khekim@korea.ac.kr*

INTRODUCTION

The increasing dependence on fossil fuels has raised several environmental concerns. Therefore, the production of bio-based fuels or chemicals from lignocellulosic biomass has garnered much attention (Hill *et al*. 2006). The production of fuels and chemicals from lignocelluloses involves three major processing steps: pretreatment of lignocellulose, enzymatic hydrolysis of the pretreated lignocellulose, and microbial fermentation to yield fuels and chemicals (Limayem and Riche 2012). Of these, enzymatic hydrolysis is one of the most critical steps that determine the overall process economics (Yang *et al*. 2011). A typical process of enzymatic hydrolysis of lignocelluloses involves a cooperative effort of several different enzymes, including cellobiohydrolase (CBH), endoglucanase (EG), β-glucosidase (BG), and hemicellulase (Rosgaard *et al*. 2007). Generally, CBH attacks the ends of cellulose chains to produce cellobiose, while EG cleaves in the middle of cellulose chains to reduce the degree of polymerization of cellulose chains. BG then converts cellobiose into glucose. Hemicellulase, mainly composed of endo-xylanase and β-xylosidase, liberates xylose from xylan.

From an industrial viewpoint, the ultimate goal in the enzymatic hydrolysis of lignocellulose is to minimize the enzyme loading and maximize sugar production (Van Dyk and Pletschke 2012). A complete investigation of the characteristics of commercial enzymes is important to accomplish this goal. Currently, commercial cellulases employed for the enzymatic hydrolysis of pretreated lignocellulosic biomass exhibit many obstacles, such as non-productive absorption of enzymes into lignin (Ko *et al*. 2015), decreased enzymatic activity by inhibitors generated during pretreatment (Cantarella *et al*. 2004; Ximenes *et al*. 2011), and product inhibition by monosaccharides, disaccharides, and oligosaccharides during enzymatic hydrolysis (Qing *et al*. 2010; Teugjas and Väljamäe 2013).

In this study, to comparatively understand the performance of industrial-grade cellulase preparations from major manufacturers, four different commercial enzymes were evaluated for the hydrolysis of hydrothermally pretreated empty fruit bunches (EFBs). Through 72 h hydrolysis, the performance and characteristics of each commercial cellulase were compared in terms of the glucose yield, filter paper activity (FPA), and specific activity (U/mg protein). In addition, the possible synergistic effect of hemicellulase on the overall performance of the four cellulase preparations was studied. Therefore, the results in this study are valuable for those who plan to enzymatically hydrolyze hydrothermally pretreated EFBs or similarly prepared lignocellulosic feedstocks.

EXPERIMENTAL

Lignocellulosic Biomass and Hydrothermal Pretreatment

Empty fruit bunches (EFBs), kindly provided by Korindo Group (Jakarta, Indonesia) were finely ground and sieved to obtain particles of approximately 850 μm diameter. Hydrothermal pretreatment was performed at 190 °C for 15 min at a solid-to-liquid ratio of 1:11.5 (w/v) using a 15 L scale reactor. Following pretreatment, EFBs were transferred into a fabric bag with a pore size of approximately 6 μm and washed with tap water. Washed EFBs were dried using a freeze dryer (IlShinBioBase, Dongducheon, Korea) and stored at -20 °C until use.

Compositional Analysis of Lignocellulosic Biomass

Carbohydrate and lignin contents of EFBs were determined according to the Laboratory Analytical Procedures (LAP) of the National Renewable Energy Laboratory (NREL) (Sluiter *et al*. 2008). Briefly, 0.3 mg EFBs were treated with 3 mL of 72% sulfuric acid (H_2SO_4) at 30 °C for 1 h, followed by treatment with 87 mL of 3% H_2SO_4 at 121 °C for 1 h to achieve complete hydrolysis. Following the acidic digestion, the concentrations of monomeric sugars in the liquid fraction were measured by a high-performance liquid chromatography (HPLC; Agilent Technologies, Waldbronn, Germany), with use of an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The HPLC column temperature was set at 65 °C, and 0.5 mM H_2SO_4 solution was used as the mobile phase at a flow rate of 0.6 mL/min. Glucose, galactose, xylose, arabinose, and mannose were detected by using a refractive index detector. These sugars were quantified according to their own calibration curves. The contents of each monomeric sugar were used to express the composition of glucan and hemicellulose. The glucan content was directly converted from the glucose content, and the hemicellulose content was obtained by summing up the contents of xylan,

galactan, arabinan, and mannan, which were converted from the monomeric sugar contents of xylose, galactose, arabinose, and mannose, respectively.

The acid-soluble lignin content was determined at 205 nm using a microplate spectrophotometer (xMark™, Bio-Rad, Hercules, CA). Acid-insoluble lignin in the liquid fraction was filtered and incubated at 575 °C for 3 h; the weight loss of the solids was expressed as the percentage (w/w) of the total weight of the initial biomass. The ash content was determined as previously described (Sluiter *et al*. 2005).

Enzymes and their Assays

Enzymes Accellerase™ 1000, Accellerase® 1500, and Spezyme® CP were a kind gift from DuPont (DuPont Danisco, WI), while Cellic® CTec2 and Cellic® HTec2 (234.5 mg/mL) were kindly provided by Novozymes Korea (Seoul, Korea).

The cellulase activity expressed as filter paper unit (FPU)/mL for each enzyme was determined, as previously described (Adney and Baker 2008). Briefly, the diluted enzyme solution (0.5 mL) was incubated with 1 mL of 50 mM citrate buffer and 50 mg of Whatman No. 1 filter paper strip at 50 °C for 60 min. The enzymatic reaction was stopped with the addition of 3 mL of 3,5-dinitrosalicylic acid (DNS), and the mixture heated at 100 °C for 5 min in a water bath. The amount of glucose released was measured at 540 nm wavelength using a microplate spectrophotometer. The activity of 1 FPU/mL was defined as the amount of enzyme releasing 2.0 mg of glucose per minute.

The BG activity was measured by treating 0.5 mL of diluted enzyme solution with 1 mL of 2 mM of *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich, St. Louis, MO) at 50 °C for 10 min, as previously described (Grover *et al*. 1977). The enzymatic reaction was stopped with the addition of 2 mL of 1 M sodium carbonate (Na_2CO_3) solution. The amount of *p*-nitrophenol released was measured at 410 nm wavelength using a microplate spectrophotometer (xMark™, Bio-Rad, Hercules, CA). One enzyme unit of β-glucosidase (U/mL) was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute.

The xylanase activity was determined by incubating 0.5 mL of diluted enzyme solution in the presence of 0.5 mL of 1% (w/v) beachwood xylan (Sigma-Aldrich) at 50 °C for 5 min, as previously described (Bailey *et al*. 1992). The reaction was stopped using DNS. The amount of released xylose was measured at 540 nm wavelength using a microplate spectrophotometer. One enzyme unit of xylanase (U/mL) was defined as the amount of enzyme releasing 1 μmol of xylose per minute. Protein concentrations in each enzyme solution were determined using a bicinchoninic acid (BCA) protein kit (Thermo Scientific, Rockford, IL).

The EG activity was determined by incubating 0.5 mL of 2% (w/v) carboxymethyl cellulose suspension with 0.5 mL of diluted enzyme solution with (CMC; Sigma-Aldrich) at 50 °C for 30 min, as previously described (Ghose 1987). The reaction was stopped using DNS. The amount of released glucose was measured at 540 nm wavelength using a microplate spectrophotometer. One enzyme unit of endoglucanase (U/mL) was defined as the amount of enzyme releasing 1 μmol of glucose per minute.

The CBH activity was determined by incubating 1.6 mL of 1.25% (w/v) Avicel (Sigma-Aldrich) suspension with 0.4 mL of diluted enzyme solution at 50 °C for 30 min (Zhang *et al*. 2009), as previously described. The amount of released glucose was measured by HPLC. One enzyme unit of cellobiohydrase (U/mL) was defined as the amount of enzyme releasing 0.5 mg of glucose per minute.

Enzymatic Hydrolysis of Pretreated EFBs

The enzymatic hydrolysis of pretreated EFBs was performed in triplicate as previously described (Selig *et al*. 2008). Briefly, pretreated EFBs were transferred into a 30 mL glass bottle with a biomass loading equivalent to 1% (w/v) glucan and treated with 10 mL of a solution containing cellulase (10 FPU/g to 30 FPU/g glucan), 0.05 M sodium citrate buffer (pH 4.8), and sterilized distilled water. The reaction mixture was incubated at 50 °C for 72 h. Following enzymatic hydrolysis, the concentration of glucose was estimated using an HPLC equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). Glucose yield (%) was calculated as the amount of released glucose per amount of glucose in the initial biomass.

RESULTS AND DISCUSSION

Effect of Hydrothermal Pretreatment on the Composition of EFBs

The unpretreated EFBs comprised 37.1% (w/w) glucan, 24.7% hemicellulose, and 22.2% lignin (Table 1). Although the composition of EFBs may vary with their harvest locations, the EFBs in this study showed similar composition with their glucan, hemicellulase, and lignin content ranging from 36.8 to 41.7%, 19.9 to 28.2%, and 20.3 to 31.8%, respectively (Hong *et al*. 2013; Jung *et al*. 2013; Ho *et al*. 2014). Hydrothermal pretreatment was performed at 190 °C for 10 min on EFBs to solubilize hemicellulose. As shown in Table 1, hemicellulose solubilization up to 79.3% (w/w) was achieved, which was attributable to the hydrothermal pretreatment. Similar results have been reported in other studies using various biomass (Suryawati *et al*. 2008; Nitsos *et al*. 2013). However, the glucan recovery from the insoluble solids was 100.0% (w/w) after pretreatment (Table 1), indicating that cellulose was merely solubilized by hydrothermal pretreatment in this study. These results are in agreement with another study that showed high glucan recovery of 84.9 to 95.7% obtained from the hydrothermal pretreatment of EFBs at 200 °C to 210 °C for 10 min (Zakaria *et al*. 2015). Therefore, the hydrothermal pretreatment was highly effective for solubilization of hemicellulose and to achieve maximum glucan recovery in this study.

Table 1. Composition of EFBs before and after Hydrothermal Pretreatment*

Biomass Component	Composition of EFBs**	
	Unpretreated	Pretreated
Glucan	37.1 ± 0.1	55.4 ± 0.5
Hemicellulose	24.7 ± 0.1	7.6 ± 0.2
Lignin	22.2 ± 0.2	31.1 ± 0.1
Ash	1.3 ± 0.0	1.1 ± 0.1

*The total recovery yield of insoluble solids after pretreatment and washing with water was 67.0% (w/w).
**Experimental data are expressed as mean ± standard deviation.

Comparison between the Hydrolysis of Pretreated EFBs with Four Different Commercial Enzymes

Four different commercial enzymes were compared for the hydrolysis of the hydrothermally pretreated EFBs at 1% glucan loading (Fig. 1). In general, the glucose yield increased with an increase in the enzyme loading for all four enzymes. The lowest glucose

yields at three different enzyme loadings were 37.8% and 37.9% with 10 FPU of Accellerase™ 1000 and Accellerase®1500/g glucan, respectively (Fig. 1). In spite of an increase in the enzyme loading to 30 FPU for Accellerase™ 1000 and Accellerase® 1500/g glucan, the glucose yields were still the lowest values of 73.4% and 67.6%, respectively.

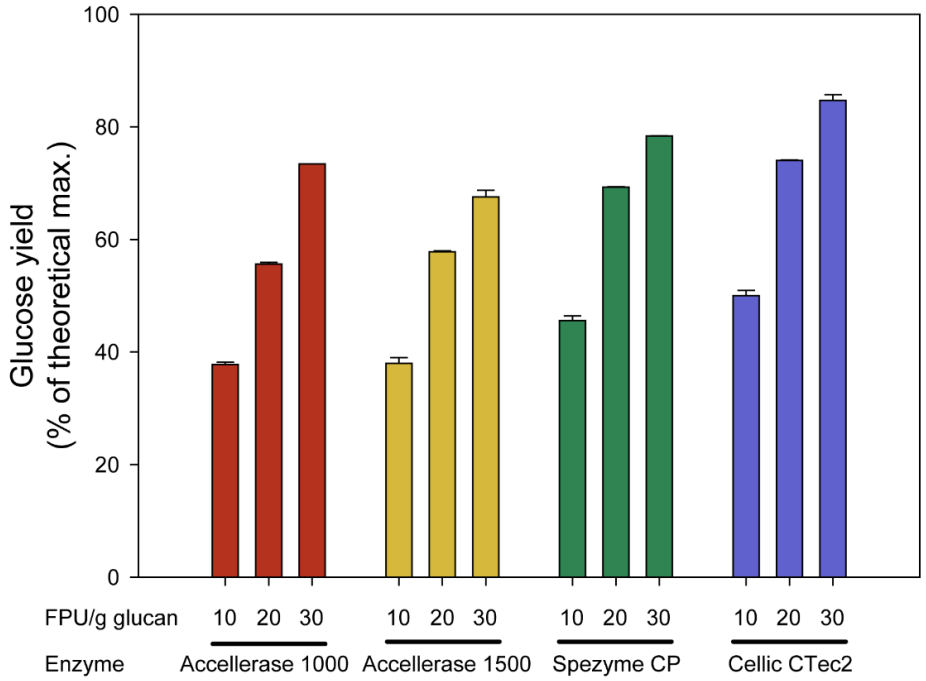


Fig. 1. Hydrolysis of hydrothermally pretreated EFBs by four different commercial enzymes. Enzymatic hydrolysis was performed using 10 to 30 FPU of the commercial cellulase preparations/g glucan at 50 °C for 72 h at 1% (w/v) glucan loading in 0.05 M citrate buffer (pH 4.8). The glucose yields were determined by measuring glucose concentrations by HPLC.

Notably, the FPAs of Accellerase™ 1000 and Accellerase® 1500 were higher than that of Spezyme® CP but slightly lower than that of Cellic® CTec2 (Table 2), indicating that the overall performance in the enzymatic hydrolysis may be attributed to factors other than the FPA of cellulase. More specifically, lower glucose yields with Accellerase™ 1000 and Accellerase® 1500 may be related to their low xylanase, cellobiohydrolase, and extremely low β-glucosidase activities as compared to those of Spezyme® CP and Cellic® CTec2 (Table 2). The hemicellulose content of the pretreated EFBs used in this study was as lower as 5% (w/w). However, xylan is mainly localized on the surface of cellulose, and xylanase may have removed xylan, which may have hindered the access of cellulase (Kumar and Wyman 2009). For instance, xylanase supplementation to stream-pretreated corn stover containing 9.8% hemicellulose significantly increased the glucose yield in the enzymatic hydrolysis of the corn stover (Hu *et al.* 2011). Meanwhile, β-glucosidase alleviates the product inhibition caused by cellobiose (Teugjas and Väljamäe 2013) and exhibits the highest non-productive binding to lignin in hydrothermally pretreated biomass (Ko *et al*. 2015). In this study, specific activities of xylanase and β-glucosidase for Accellerase™ 1000 were measured to be 7.6 and 2.9 U/mg protein, respectively, (Table 2) and were comparable to those reported in a previous study (Fujii *et al.* 2009).

Table 2. Protein Concentrations and Specific Activities of the Commercial Cellulases Analyzed by Others and in this Study[a]

Enzyme	Protein[b] (mg/mL)	FPA (FPU[c] mg^{-1} protein)	Specific activity (U mg^{-1} protein)				Reference
			CBH[d]	EG[e]	Xylanase	β-Glucosidase	
Accellerase™ 1000	NM[f] 105.1±13.7	0.44±0.01 0.51±0.00	0.25±0.01 0.18±0.02	6.75±0.34 14.1±0.51	8.86±0.85 7.56±0.12	2.85±0.06 1.18±0.02	Fujii *et al*. 2009 This study
Accellerase® 1500	114 89 78.4±7.6	0.50 0.87 0.45±0.01	NM NM 0.31±0.00	NM NM 14.9±0.10	NM NM 6.25±0.08	NM NM 1.48±0.11	Ju *et al*. 2014 Zhu *et al*. 2015 This study
Spezyme® CP	123±10 135 126 117.3±15.8	0.48 0.36 0.56 0.37±0.00	NM NM NM 0.20±0.01	NM NM 18.33±5.77 14.1±0.44	NM 3.37 NM 12.79±0.11	NM NM 0.41 11.09±0.04	Kumar and Wyman 2009 Kabel *et al*. 2006 Nieves *et al*. 1998 This study
Cellic® CTec2	190 257 161 237.2±7.0	0.62 0.46 0.75 0.54±0.04	NM NM NM 0.48±0.06	NM NM NM 11.8±0.47	NM NM NM 38.53±0.48	NM NM 16.96 10.99±0.16	Ko *et al*. 2015 Ju *et al*. 2014 Cannella *et al*. 2012 This study

[a]Experimental data are expressed as mean ± standard deviation.
[b]Protein concentration (mg/mL) was determined by BCA method.
[c]FPU: Filter paper unit
[d]CBH: Cellobiohydrolase
[e]EG: Endoglucanse
[f]NM: Not measured

In addition, the highest glucose yield by Cellic CTec2 can be explained by the highest CBH activity of Cellic® CTec2 among the enzymes tested in this study (Table 2). It is because the apparent synergic interaction between EG and CBH is one of most important factors in enzymatic hydrolysis of cellulose (Van Dyk and Pletschke 2012).

The glucose yields obtained by 10 FPU to 30 FPU of Spezyme® CP/g glucan were measured as 45.5% to 78.3% (Fig. 1). Although Spezyme® CP showed the lowest FPA among the four enzymes (Table 2), the higher glucose yield obtained with Spezyme® CP as compared with Accellerase™ 1000 or Accellerase® 1500 may be attributed to the higher specific activities of xylanase and β-glucosidase (approximately two and ten times, respectively). The highest glucose yield obtained in this study was 84.7% with 30 FPU of Cellic® CTec2/g glucan (Fig. 1). At 10 FPU concentration, the highest glucose yield was obtained with Cellic® CTec2 (*i.e.*, 50.0%, Fig. 1). In spite of the highest protein concentration of Cellic® CTec2, its specific enzyme activities (U/mg protein) were the highest among the four enzymes (Table 2). The protein concentration and FPA of Cellic® CTec2 were measured to be 237.2 mg/mL and 0.54 FPU/mg protein, respectively (Table 2) and were comparable to those previous reported (161 mg/mL to 257 mg/mL and 0.46 FPU/mg to 0.75 FPU/mg protein, respectively).

The total protein concentration of Accellerase™ 1000, Accellerase® 1500, Spezyme® CP, and Cellic® CTec2 corresponding to 10 FPU/g glucan used for the hydrolysis was calculated to be 19.6, 22.2, 27.0, and 18.5 mg/g glucan, respectively (Table 2). Thus, Cellic® CTec2 was deemed as the optimal enzyme to achieve the highest glucose yield from hydrothermally pretreated EFBs, in terms of its higher specific enzyme activities and the lowest protein loading.

Furthermore, the soluble components of the four commercial cellulase preparations were analyzed to identify possible major additives or stabilizers in the cellulase preparations (Table 3). As a result, three major components were detected HPLC. Glycerol, a well-known enzyme stabilizer, was detected in all of the industrial-grade cellulase preparations at approximately 100 g/L. Sorbitol was the dominant additive only in Spezyme® CP at 206.2 g/L which was approximately 2 times higher than that of a previous reported value (Nieves *et al*. 1998). Glucose was the dominant additive in Cellic® CTec2 at 220.4 g/L, which may have originated from culture broth for enzyme production.

Table 3. Three Major Soluble Components (g/L) in the Compositions of the Four Commercial Cellulase Preparations*

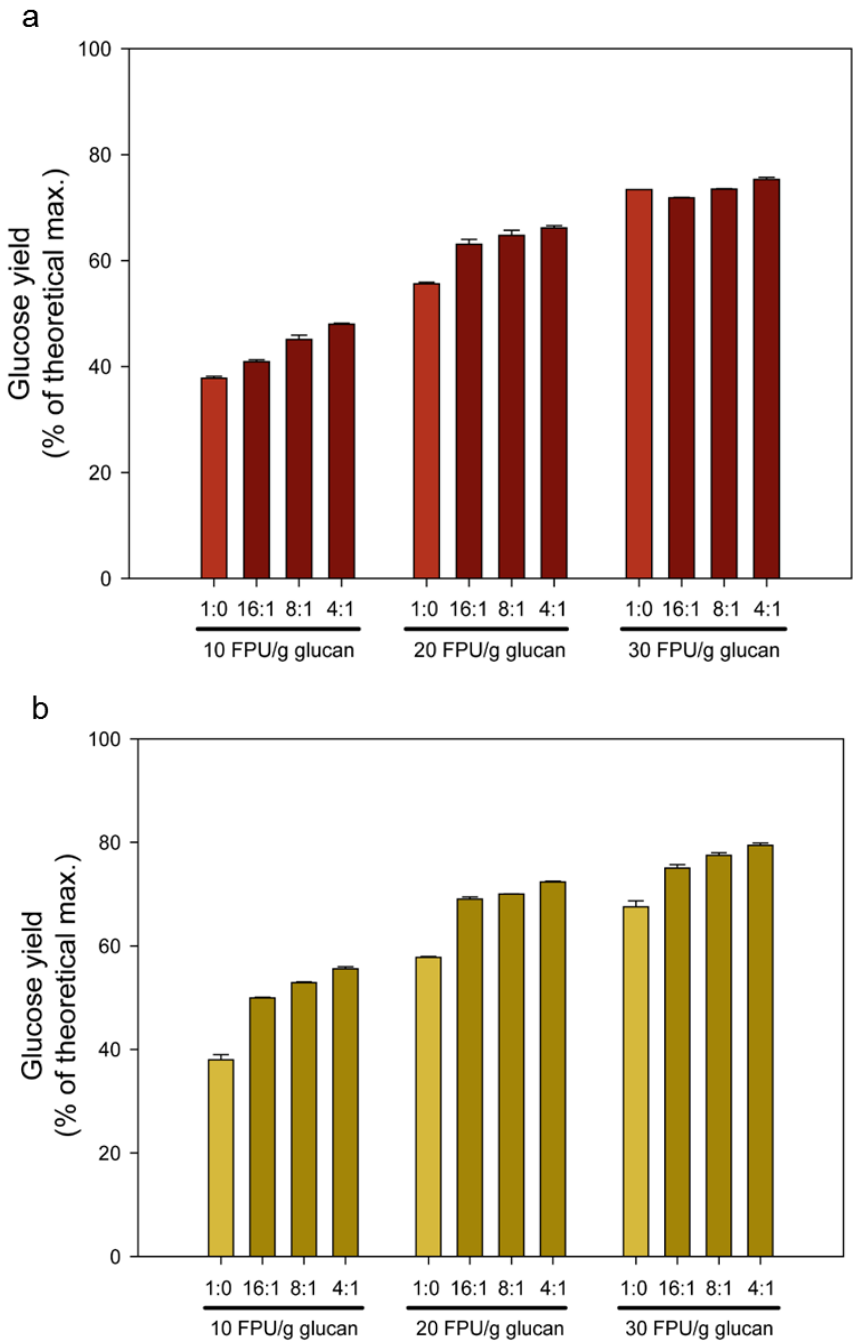
	Glucose	Sorbitol	Glycerol
Acellerase™ 1000	2.4	ND	102.0
Acellerase® 1500	0.9	ND	100.3
Spezyme® CP	2.2	206.2	107.5
Cellic® CTec2	220.4	ND	104.5

*Soluble components were analyzed from the liquid fraction of ultra-filtered (10kDa membrane) of 10 fold diluted enzyme preparations.
**ND: Not detected

Effects of Hemicellulase on the Enzymatic Hydrolysis of Pretreated EFBs Using Four Different Enzymes

The effect of hemicellulase Cellic® HTec2 on the enzymatic hydrolysis of the hydrothermally pretreated EFBs was studied in the presence of 10 FPU to 30 FPU of commercial enzymes/g glucan. An increase in the glucose yield was observed with

increased Cellic® HTec2 loading for all four enzymes (Fig. 2). This trend was more noticeable with lower cellulase loading (*e.g.*, 10 FPU of cellulase/g glucan) as compared to the higher cellulase loading (*e.g.*, 30 FPU, Fig. 2). For instance, at 10 FPU/g glucan, 40.9% and 48.0% glucose yield was achieved with Accellerase™ 1000 and Cellic® HTec2 at the total protein ratios of 16:1 and 4:1, respectively (Fig. 2a). However, at 20 and 30 FPU/g glucan, an increase in the Cellic® HTec2 loading from 16:1 to 4:1 enhanced the glucose yield from 63.1% to 66.1% and from 71.8% to 75.3%, respectively (Fig. 2a). Similar trends were observed with other enzymes (Figs. 2b, 2c, and 2d). This phenomenon may be associated with the high loadings of commercial cellulases, which are thought to contain hemicellulases represented by the xylanase activity (Table 2).



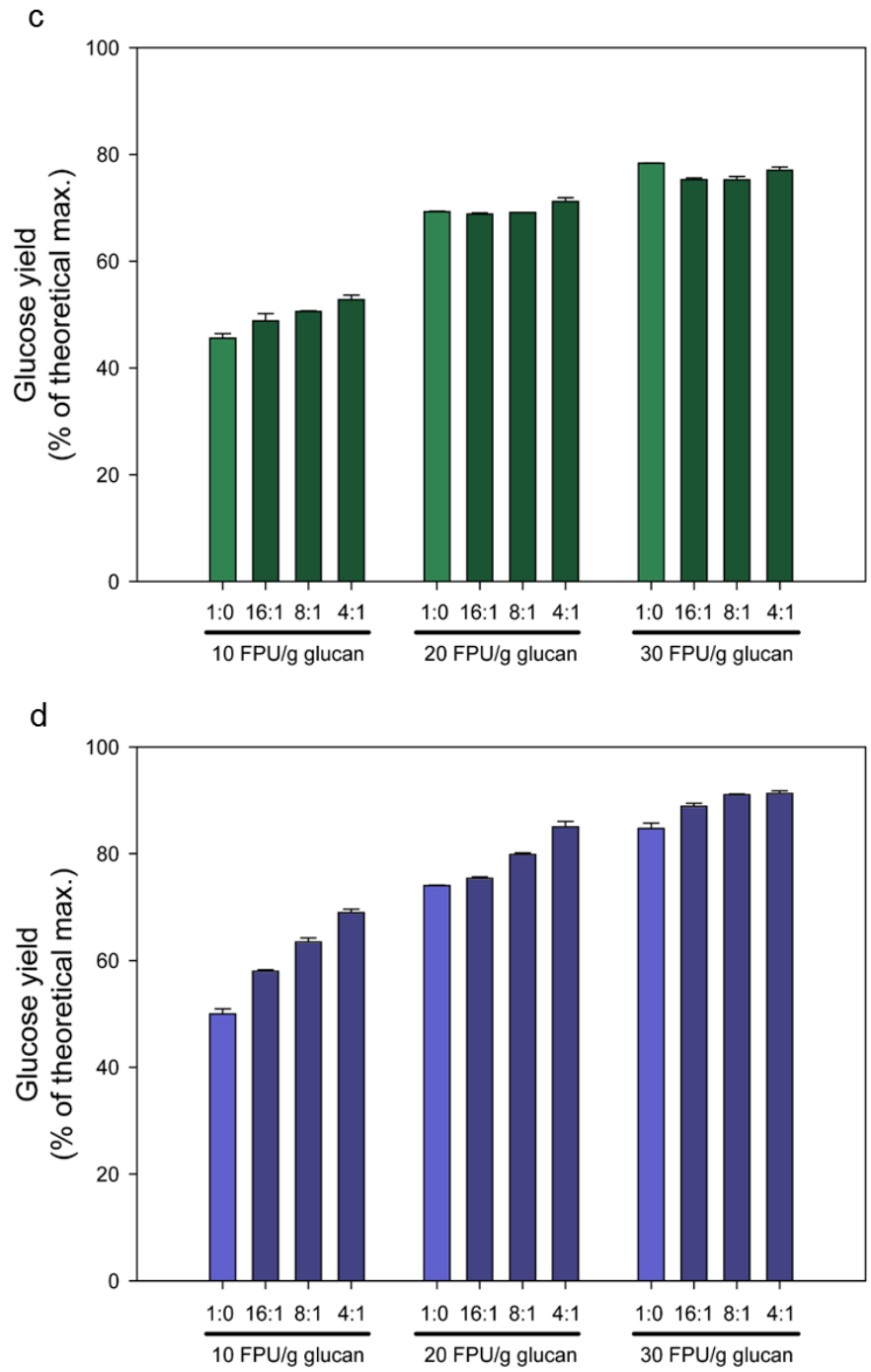


Fig. 2. Effects of hemicellulase on the enzymatic hydrolysis of pretreated EFBs using four different enzymes. Enzymatic hydrolysis was performed using 10 FPU to 30 FPU of the commercial cellulase preparations/g glucan at 50 °C for 72 h with the addition of the commercial hemicellulase, Cellic® HTec2 at 1% (w/v) glucan loading. Cellic HTec2 was added at the total protein ratio of the commercial enzymes and Cellic® HTec2 (wt/wt in protein). The glucose yields were determined by measuring glucose concentrations by HPLC. (a) Accellerase™ 1000; (b) Accellerase® 1500; (c) Spezyme® CP; (d) Cellic® CTec2.

The maximal synergistic effect of Cellic® HTec2 on the enzymatic hydrolysis of EFBs occurred with Cellic® CTec2 (Fig. 2d). The glucose yield increased from 58.0% to 69.0%, when the protein ratio of 10 FPU of Cellic® CTec2/g glucan and Cellic® HTec2 was changed from 16:1 to 4:1. The highest glucose yield of 91.3% was obtained with 30 FPU of Cellic® CTec2/g glucan and Cellic® HTec2 at the protein ratio of 4:1 (Fig. 2d).

The maximal synergistic effect of Cellic HTec2 on the enzymatic hydrolysis of EFBs occurred with Cellic® CTec2 (Fig. 2d). The glucose yield increased from 58.0% to 69.0%, when the protein ratio of 10 FPU of Cellic® CTec2/g glucan and Cellic® HTec2 was changed from 16:1 to 4:1. The highest glucose yield of 91.3% was obtained with 30 FPU of Cellic® CTec2/g glucan and Cellic® HTec2 at the protein ratio of 4:1 (Fig. 2d).

CONCLUSIONS

1. In this study, several key enzyme activities and specific cellulase activities of the four commercial cellulase preparations were analyzed. These cellulase preparations were applied for the hydrolysis of the hydrothermally pretreated empty fruit bunches (EFBs) with or without the hemicellulase, Cellic® HTec2. The analysis of specific enzyme activities of the four cellulases indicated that the endoglucanase (EG), xylanase, and β-glucosidase (BG) activities, not the filter paper activity (FPA), determined the important enzyme activities for the enzymatic hydrolysis of pretreated EFBs.
2. Of the four enzymes tested, Cellic® CTec2 showed the highest cellobiohydrolase (CBH), xylanase, and BG activities and achieved the highest glucose yields from the hydrolysis of pretreated EFBs.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministry of Trade, Industry & Energy, Korea (10049674). The support of page charges by Korea University BK21 PLUS for School of Life Sciences and Biotechnology is also acknowledged. This research was performed at the Korea University Food Safety Hall for the Institute of Biomedical Science and Food Safety.

REFERENCES CITED

Adney, B., and Baker, J. (2008). *Measurement of Cellulase Activities* (NREL/TP-510-42628), National Renewable Energy Laboratory, Golden, CO.

Bailey, M. J., Biely, P., and Poutanen, K. (1992). "Interlaboratory testing of methods for assay of xylanase activity," *J. Biotechnol*. 23(3), 257-270. DOI: 10.1016/0168-1656(92)90074-J

Cannella, D., Hsieh, C. C., Felby, C., and Jørgensen, H. (2012). "Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content," *Biotechnol*. *Biofuels* 5, 26. DOI: 10.1186/1754-6834-5-26

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F. (2004). "Effects of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF," *Biotechnol. Prog.* 20(1), 200-206. DOI: 10.1021/bp0257978

Fujii, T., Fang, H., Inoue, H., Murakami, K., and Sawayama, S. (2009). "Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials," *Biotechnol. Biofuels* 2, 24. DOI: 10.1186/1754-6834-2-24

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268. DOI: 10.1351/pac198759020257

Grover, A. K., Macmurchie, D. D., and Cushley, R. J. (1977). "Studies on almond emulsin beta-D-glucosidase. I. Isolation and characterization of a bifunctional isozyme," *Biochim. Biophys. Acta* 482(1), 98-108.

Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. (2006). "Environmental, economic, and energetic costs and benefits of biodiesel and ethanol fuels," *Proc. Natl. Acad. Sci. USA* 103 (30), 11206-11210. DOI: 10.1073/pnas.0604600103

Ho, L. A., Carvalheiro, F., Duarte, L. C., Roseiro, L. B., and Charalampopoulos, D. (2014). "Production and purification of xylooligosaccharides from oil palm empty fruit fibre by a non-isothermal process," *Bioresour. Technol.* 152, 526-529. DOI: 10.1016/j.biortech.2013.10.114

Hong, J. Y., Kim, Y. S., and Oh, K. K. (2013). "Fractionation and delignification of empty fruit bunches with low reaction severity of high sugar recovery," *Bioresour. Technol.* 156, 176-183. DOI: 10.1016/j.biortech.2013.07.058

Hu, J., Arantes, V., and Saddler, J. N., (2011). "The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?," Biotechnol Biofuels 4, 36. DOI:10.1186/1754-6834-4-36

Ju, X., Bowden, M., Engelhard, M., and Zhang, X. (2014). "Investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates," *Appl. Microbiol. Biotechnol.* 98(10), 4409-4420. DOI: 10.1007/s00253-013-5450-4

Jung, Y. H, Kim, I. J, Kim, H. K, and Kim, K. H. (2013). "Dilute acid pretreatment of lignocellulose for whole slurry ethanol fermentation," *Bioresour. Technol.* 132, 109-114. DOI: 10.1016/j.biortech.2012.12.151

Kabel, M. A, van der Maarel, M. J. E. C., Klip, G., Voragen, A. G. J., and Schols, H. A. (2006). "Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials," *Biotechnol. Bioeng.* 93(1), 56-63. DOI: 10.1002/bit.20685

Ko, J. K., Ximenes, E., Kim, Y., and Ladisch, M. R. (2015). "Adsorption of enzyme onto lignins of liquid hot water-pretreated hardwoods," *Biotechnol. Bioeng.* 112(3), 447-456. DOI: 10.1002/bit.25359

Kumar, R., and Wyman, C. E. (2009). "Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies," *Biotechnol. Bioeng.* 102 (2), 457-467. DOI: 10.1002/bit.22068

Limayem, A., and Riche, S. C. (2012). "Lignocellulosic biomass for bioethanol production: current perspectives, potential issues, and future prospects," *Prog. Energ. Combust. Sci*. 38(4), 449-467. DOI: 10.1016/j.pecs.2012.03.002

Nieves, R. A., Ehrman, C. I., Adney, W. S., Elander, R. T., and Himmel, M. E. (1998). "Technical communication: Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol," *World J. Microbiol. Biotechnol*. 14(2), 301-304. DOI: 10.1023/A:1008871205580

Nitsos, C. K., Matis, K. A., and Triantafyllidis, K. S. (2013). "Optimization of hydrothermal pretreatment of lignocelluosic biomass in the bioethanol production process," *ChemSusChem* 6(1), 110-122. DOI: 10.1002/cssc.201200546

Qing, Q., Yang, B., and Wyman, C. E. (2010). "Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes," *Bioresour. Technol*. 101(24), 9624-9630. DOI: 10.1016/j.biortech.2010.06.137

Rosgaard, L., Pedersen, S., Langston, J., Akerhielm, D., Cherry, J. R., and Meyer, A. S. (2007). "Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates," *Biotechnol. Adv*. 23(6), 1270-1276. DOI: 10.1021/bp070329p

Selig, M., Weiss, N., and Ji, Y. (2008). *Enzymatic Saccharification of Lignocellulosic Biomass* (NREL/TP-510-42629), National Renewable Energy Lab, Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. (2005). *Determination of Ash in Biomass* (NREL/TP-510-42622), National Renewable Energy Laboratory, Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO.

Suryawati, L., Wilkins, M. R., Bellmer, D. D., Huhnke, R. L., Maness, N. O., and Banat, I. M. (2008). "Simultaneous saccharification and fermentation of kanlow switchgrass pretreated by hydrothermolysis using *Kluyveromyces marxianus* IMB4," *Biotechnol. Bioeng*. 101(5), 894-902. DOI: 10.1002/bit.21965

Teugjas, H., and Väljamäe, P. (2013). "Product inhibition of cellulases studied with 14C-labeled cellulose substrates," *Biotechnol. Biofuels* 6(1), 104. DOI: 10.1186/1754-6834-6-104

Van Dyk, J. S., and Pletschke, B. I. (2012). "A review of lignocellulosic bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy," *Biotechnol. Adv*. 30(6), 1458-1480. DOI: 10.1016/j.biotechadv.2012.03.002

Ximenes, E., Kim, Y., Mosier, N., Dien, B., and Ladisch, M. (2011). "Deactivation of cellulases by phenols," *Enzyme Microb. Technol*. 48(1), 54-60. DOI: 10.1016/j.enzmictec.2010.09.006

Yang, B., Dai, Z., Ding, S., and Wyman, C. E. (2011). "Enzymatic hydrolysis of cellulosic biomass," *Biofuels* 2(4), 421-450. DOI: 10.4155/bfs.11.116

Zakaria, M. R., Hirata, S., and Hassan, M. A. (2015). "Hydrothermal pretreatment enhanced enzymatic hydrolysis and glucose production from oil palm biomass," *Bioresour. Technol*. 176, 142-148. DOI: 10.1016/j.biortech.2014.11.027

Zhang, Y. H. B., Hong, J., and Ye, X. (2009). "Cellulase activity," *Methods Mol. Biol*. 581, 213-231. DOI: 10.1007/978-1-60761-214-8_14

Zhu, J., Qin, L., Li, W., Zhang, J., Bao, J., Huang, Y., Li, Z., and Yuan, Y. (2015). "Simultaneous saccharification and co-fermentation of dry diluted acid pretreated corn stover at high dry matter loading: Overcoming the inhibitors by non-tolerant yeast," *Bioresour*. *Technol*. 198, 39-46. DOI: 10.1016/j.biortech.2015.08.140

Article submitted: April 1, 2017; Peer review completed: June 19, 2017; Revised version received: July 28, 2017; Accepted: August 5, 2017; Published: September 7, 2017.
DOI: 10.15376/biores.12.4.7834-7840