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Short Communication

Effects of enzyme loading and β-glucosidase supplementation on enzymatic hydrolysis of switchgrass processed by leading pretreatment technologies

Venkata Ramesh Pallapolu [a], Y.Y. Lee [a,*], Rebecca J. Garlock [b,c], Venkatesh Balan [b,c], Bruce E. Dale [b,c], Youngmi Kim [d], Nathan S. Mosier [d], Michael R. Ladisch [d], Matthew Falls [e], Mark T. Holtzapple [e], Rocio Sierra-Ramirez [f], Jian Shi [g], Mirvat A. Ebrik [g], Tim Redmond [g], Bin Yang [g], Charles E. Wyman [g], Bryon S. Donohoe [h], Todd B. Vinzant [h], Richard T. Elander [h], Bonnie Hames [i], Steve Thomas [i], Ryan E. Warner [j]

[a] Department of Chemical Engineering, Auburn University, 212 Ross Hall, Auburn, AL 36849, USA
[b] Department of Chemical Engineering and Materials Science, Michigan State University, 3900 Collins Road, Lansing, MI 48910, USA
[c] Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, USA
[d] LORRE, Department of Agricultural and Biological Engineering, Purdue University, 500 Central Dr., West Lafayette, IN 47907, USA
[e] Department of Chemical Engineering, Texas A&M University, 3122 TAMU, College Station, TX 77843-3122, USA
[f] Universidad de los Andes Chemical Engineering Department Grupo de Conversion de Energia, Bogotá, Colombia
[g] Center for Environmental Research and Technology, Department of Chemical and Environmental Engineering, Bourns College of Engineering, University of California at Riverside, 1084 Columbia Avenue, Riverside, CA 92507, USA
[h] Chemical and Biosciences Center, National Renewable Energy Laboratory, 1617 Cole Blvd., Golden, CO 80401, USA
[i] Ceres, Inc., 1535 Rancho Conejo Blvd., Thousand Oaks, CA 91320, USA
[j] Genencor, A Danisco Division, 925 Page Mill Road, Palo Alto, CA 94304, USA

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ABSTRACT

The objective of this work is to investigate the effects of cellulase loading and β-glucosidase supplementation on enzymatic hydrolysis of pretreated Dacotah switchgrass. To assess the difference among various pretreatment methods, the profiles of sugars and intermediates were determined for differently treated substrates. For all pretreatments, 72 h glucan/xylan digestibilities increased sharply with enzyme loading up to 25 mg protein/g-glucan, after which the response varied depending on the pretreatment method. For a fixed level of enzyme loading, dilute sulfuric acid (DA), SO_2, and Lime pretreatments exhibited higher digestibility than the soaking in aqueous ammonia (SAA) and ammonia fiber expansion (AFEX). Supplementation of Novozyme-188 to Spezyme-CP improved the 72 h glucan digestibility only for the SAA treated samples. The effect of β-glucosidase supplementation was discernible only at the early phase of hydrolysis where accumulation of cellobiose and oligomers is significant. Addition of β-glucosidase increased the xylan digestibility of alkaline treated samples due to the β-xylosidase activity present in Novozyme-188.

1. Introduction

Lignocellulosic biomass is currently considered the most promising long-term feedstock for production of bioethanol. The recalcitrance of the feedstock is the major hurdle in the bioconversion process. In the plant cell structure, cellulose fibrils are embedded in a matrix of lignin and hemicellulose. This forms a chemical and structural barrier for enzymatic degradation of cell wall sugars and subsequent microbial fermentation to ethanol (Lynd et al., 2002; Kumar and Wyman, 2009). For cellulase enzymes to be able to effectively access the cellulose, the cell wall structure needs to be disrupted. Various pretreatment methods are employed by in order to remove these barriers. The pretreatment mode of action varies depending upon the method applied. Pretreatment often results in a change of composition, alteration of physical properties including cellulose crystallinity. The CAFI team has put forward a collaborative effort to examine some of the promising pretreatment technologies including: soaking in aqueous ammonia (SAA),

Abbreviations: AFEX, ammonia fiber expansion; CAFI, consortium for applied fundamentals and innovation; CBU, cellobiase unit; DA, dilute sulfuric acid; DSG, Dacotah switchgrass; FPU, filter paper unit; HPLC, high-performance liquid chromatography; LHW, liquid hot water; N 188, Novozyme-188; NREL, National Renewable Energy Laboratory; SAA, soaking in aqueous ammonia; SCP, Spezyme-CP; SH, secondary hydrolysis; SO_2, sulfur dioxide.

* Corresponding author. Fax: +1 334 844 2063.
E-mail address: yylee@eng.auburn.edu (Y.Y. Lee).

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ammonia fiber expansion (AFEX), calcium hydroxide (Lime), liquid hot water (LHW), dilute sulfuric acid (DA), and SO_2 (Wyman et al., 2005b).

The goal of this research as part of the CAFI project is to assess the enzyme requirement for various pretreatments and seek ways to reduce the enzyme loading. In many cases the required enzyme loading is too high to be economically feasible (Mosier et al., 2005; Kumar and Wyman, 2008a). Since the enzyme is one of the major costs involved in producing cellulosic ethanol, pretreatment advances are still needed to reduce the overall production costs (Wyman et al., 2005a). A typical cellulase enzyme mixture consists of many different enzymes that function synergistically to break down the cell wall polysaccharides. One of the key enzymes in the cellulase system is exo-glucanase which produces cellobiose as an intermediate product (Zhao et al., 2004). It is well known that cellobiose inhibits the action of exo-glucanase (Xiao et al., 2004). If the cellulase complex does not have sufficient β-glucosidase activity, external supplementation is necessary to increase the enzyme efficiency (Lynd et al., 2002). Enzymatic hydrolysis of cellulose and hemicellulose also produces glucose and xylose oligomers as reaction intermediates. Although inhibition of cellulase activity by monomeric sugars and cellobiose is well documented (Kumar and Wyman, 2008a; Quing and Wyman, 2011), limited information is available with regard on the effect of oligomers. More detailed investigations are required to understand how the enzyme loading is influenced by pretreatment method and the type of substrate. The technical objective of this work is to investigate the effects of cellulase loading and β-glucosidase supplementation on production of monomeric and oligomeric sugars. Efforts were put forward to observe data over the entire duration of hydrolysis and to seek explanations for the difference in hydrolysis behavior, if any, among the substrates generated by various CAFI methods.

2. Methods

2.1. Feedstock

DSG was obtained from Ceres, Inc., Thousand Oaks, CA. DSG is of upland variety with thin stem morphology and was harvested in northeast South Dakota. The samples were initially milled to pass through a 0.25 inch screen and shipped to each participating university where they were stored at room temperature. Before the pretreatment, DSG was milled to pass through a 2 mm screen.

2.2. Pretreatment methods

The feedstock was pretreated by the CAFI team by their respective methods. The treatment conditions applied for this work were: AFEX (ammonia, 160 °C, 30 min, chemical loading: 1.35 g/g-dry biomass, solid loading of total volume: 33%); DA (sulfuric acid, 140 °C, 40 min, chemical loading: 0.05 g/g-dry biomass, solid loading of total volume: 10%); for LHW (water, 200 °C, 10 min, chemical loading: 6.70 g/g-dry biomass, solid loading of total volume: 15%); for Lime (calcium hydroxide, 120 °C, 240 min, chemical loading: 1.00 g/g-dry biomass, solid loading of total volume: 6.7%); for SAA (ammonia, 160 °C, 60 min, chemical loading: 1.35 g/g-dry biomass, solid loading of total volume: 10%); and for SO_2 (sulfur dioxide, 180 °C, 10 min, chemical loading: 0.05 g/g-dry biomass, solid loading of total volume: 10%).

2.3. Enzymes

Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was provided by Genencor-Danisco (Palo Alto, CA). The activity of Spezyme-CP, as determined by NREL standard procedure was 59 FPU/mL. The β-glucosidase (Novozyme-188, Cat. No. C-6150) was purchased from Sigma (St. Louis, MO); its activity was 600 CBU/mL. The protein content of Spezyme-CP and Novozyme-188 were measured by the total nitrogen method following TCA precipitation and were found to be 82 mg protein/mL and 67 mg protein/mL, respectively.

2.4. Composition and enzymatic hydrolysis

Pretreated solids were analyzed for composition using the NREL standard procedures (NREL/TP-510-42618 to TP-510-42622) (Sluiter et al., 2008a). Enzymatic hydrolysis tests were carried out following the NREL standard procedure (NREL/TP 510-4269). The solid loading was based on 1% (w/v) glucan. Enzymatic hydrolysis was conducted in 250 mL Erlenmeyer flasks with 100 mL total working volume. The pH was adjusted between 4.5 and 5.0 with sodium citrate buffer after which antibiotics were added (600 μL of tetracycline and 450 μL of cycloheximide). Hydrolysis was carried out at 50 °C, 150 rpm in a Shaker/Incubator (NBS, Innova-4080). Enzyme loading was applied on the basis of glucan in untreated biomass. Four different enzyme loadings were examined in this study: Spezyme-CP at 4.2, 20.8, 41.7 or 83.4 mg protein/g-glucan; and supplemented with Novozyme-188 at 4.9, 24.2, 48.4 or 96.8 mg total protein/g-glucan. Hydrolysate samples were taken at 12, 24, and 72 h and analyzed for glucose, xylose and cellobiose. To determine the glucose/xylose oligomers, the hydrolysate samples were collected at 6 h and 72 h. The hydrolysate samples were analyzed for monomeric carbohydrates, which was followed by secondary hydrolysis (SH) using 4% sulfuric acid to hydrolyze oligomers (Sluiter et al., 2008b). For all the samples except SAA, HPLC samples were analyzed using a Bio-Rad (Hercules, California, USA) Aminex HPX-87H column. For SAA, an HPLC equipped with a Bio-rad-HPX-87P column was used. The glucose/xylose oligomers were determined using taking the difference between the sugar values before and after SH.

3. Results and discussion

3.1. Composition of pretreated DSG

The composition of treated and untreated DSG samples is as follows:

Pretreatment	Glucan (%)	Xylan (%)	Klason lignin (%)
Untreated	35.0	21.8	21.4
SAA	34.5	13.6	8.3
AFEX	35.8	22.4	24.4
Lime	34.5	14.6	10.3
LHW	30.0	1.8	19.4
DA	30.8	2.59	17.8
SO_2	33.6	2.5	15.8

All the samples were washed with cold water before analysis. Composition was calculated on the basis of untreated DSG. The highest percentage of delignification was achieved with SAA treatment (62%) among all the pretreatment methods. The DSG treated with alkaline reagents retained most of the cellulose in the biomass. SAA and Lime treatments retained 62% and 67% of initial xylan, respectively. AFEX pretreatment does not produce any separate liquid stream during pretreatment and retained all of the solids, thus the composition of the substrate unchanged. All pretreatments that primarily remove xylan from biomass (SO_2, DA, and LHW) show more than 80% glucan digestibility. Among the pretreatment methods that primarily removed lignin (SAA

and Lime), samples treated with Lime showed higher digestibility (91%) than SAA, in spite of the higher delignification of the SAA treated samples. Although lignin is a major barrier to enzymatic hydrolysis, the gross amount of it is not to be taken as the sole factor determining the efficiency of enzymatic hydrolysis. The changes of structure and surface conditions of the biomass components taking place by pretreatment may play a significant role in the enzymatic hydrolysis. As an example, the physical and chemical conditions of lignin surface are significantly altered by chemical treatments (sulfonation, ammoniation, etc.). This in turn may cause significant difference in the way it interacts with cellulase enzyme. Non-productive binding of cellulase enzyme with lignin is one such example. Further investigation is needed to delineate this phenomenon.

3.2. Effect of enzyme loading and β-glucosidase supplementation on digestibility

The effects of cellulase enzyme loading with external β-glucosidase were studied with DSG samples generated from CAFI pretreatments. The 72 h digestibility calculated based on untreated biomass was plotted against the total protein number of Spezyme-CP and/or Novozyme-188 (Fig. 1). In some cases, the glucan digestibility and cellobiose were interpolated from the initial experimental data for comparison purposes. The glucan digestibility and cellobiose accumulation for different pretreated samples measured at four different enzyme loadings are summarized in Table 1. The enzyme loading is expressed as total combined protein of Spezyme-CP and Novozyme-188. The enzyme loadings are abbreviated to Low (10 mg protein/g-glucan), Moderate (25 mg protein/g-glucan), High (50 mg protein/g-glucan), and Very High (80 mg protein/g-glucan).

The 72 h glucan digestibility for all the pretreated samples increased sharply up to Moderate enzyme loading, after that point, the response varied: continued gradual increase throughout for alkaline treated samples (SAA, AFEX, and Lime), leveling-off for acid treated samples (DA and SO_2), and a sharp increase up to High enzyme loading for LHW treated samples (Fig. 1). The highest glucan digestibilities at Low, Moderate, High, and Very High enzyme loadings achieved were: 66.1% (SO_2), 83.1% (SO_2), 90.8% (LHW),

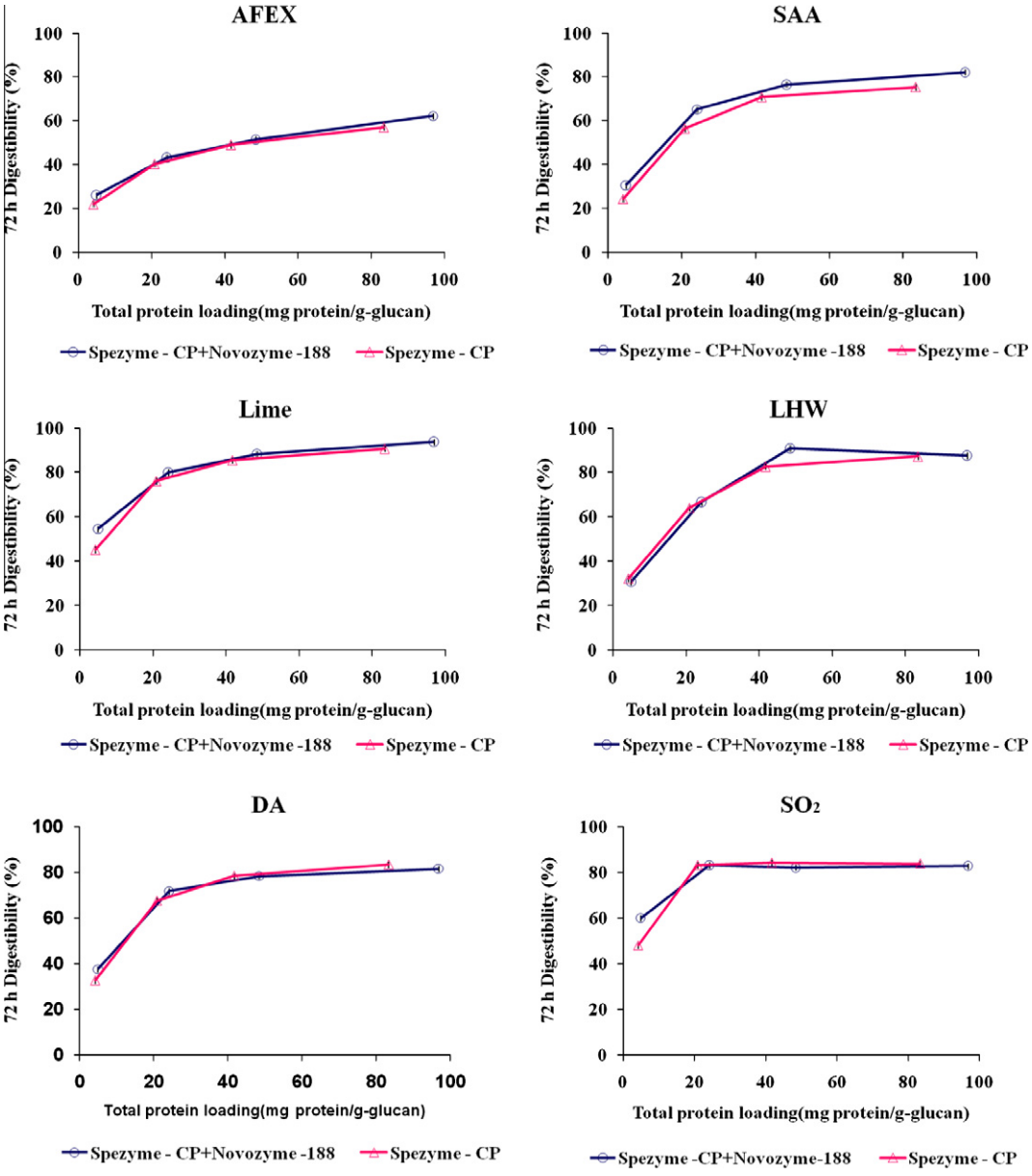

Fig. 1. Seventy two hours glucan digestibility of all the pretreated samples with and without addition of β-glucosidase.

Table 1
Glucan digestibility and cellobiose of different pretreatment samples at Low, Moderate, High, and Very High enzyme loadings.

Enzymes	Glucan digestibility (%)						Cellobiose (%)					
	SAA	AFEX	Lime	LHW	DA	SO_2	SAA	AFEX	Lime	LHW	DA	SO_2
10 mg protein/g-glucan												
12 h												
SCP	20.0	13.8	27.4	23.2	19.3	23.4	3.8	3.2	16.3	4.3	20.9	28.0
SCP + N 188	22.4	16.9	37.1	27.6	29.1	36.7	2.3	0.2	5.2	0.0	2.3	1.9
24 h												
SCP	27.5	19.0	38.1	36.1	32.6	44.8	3.2	2.6	16.5	2.2	18.9	28.9
SCP + N 188	31.5	22.2	47.8	36.5	40.9	59.6	1.3	0.0	5.4	0.0	1.8	2.1
72 h												
SCP	35.5	28.4	56.0	43.3	44.9	60.3	1.6	0.9	10.4	0.8	9.7	15.2
SCP + N 188	39.7	30.5	61.3	40.4	46.7	66.1	0.6	0.0	4.1	0.0	1.7	3.8
25 mg protein/g-glucan												
12 h												
SCP	33.0	24.9	46.5	40.2	34.2	42.6	2.6	2.0	17.0	6.6	17.8	26.6
SCP + N 188	36.3	28.1	59.5	49.3	45.1	57.2	1.2	0.0	6.6	0.0	2.8	0.0
24 h												
SCP	48.2	31.6	60.6	60.5	55.7	73.0	1.9	1.1	14.1	2.0	18.2	17.8
SCP + N 188	54.1	34.4	71.4	63.2	64.7	83.0	0.8	0.0	6.1	0.0	1.7	1.3
72 h												
SCP	59.3	42.0	78.0	67.7	69.7	83.3	0.9	0.0	5.3	0.2	4.5	4.8
SCP + N 188	65.5	43.8	80.3	67.5	72.0	83.1	0.2	0.0	3.7	0.0	1.9	3.4
50 mg protein/g-glucan												
12 h												
SCP	42.7	32.3	60.3	64.0	44.5	57.2	1.4	1.1	12.3	6.1	11.7	17.7
SCP + N 188	43.5	35.9	70.0	76.5	52.1	69.9	0.6	0.0	6.1	0.0	1.5	0.0
24 h												
SCP	58.3	39.4	73.7	80.2	67.4	82.7	0.9	0.0	8.3	0.7	10.9	7.1
SCP + N 188	62.3	42.8	79.8	87.2	73.3	86.1	0.3	0.0	5.3	0.0	2.1	1.5
72 h												
SCP	71.6	50.6	86.5	83.6	79.4	84.1	0.4	0.0	2.8	0.0	2.1	1.3
SCP + N 188	76.7	51.7	88.5	90.8	78.4	82.1	0.1	0.0	2.7	0.0	1.6	2.0
80 mg protein/g-glucan												
12 h												
SCP	42.7	38.0	69.5	77.7	50.3	65.8	0.5	0.1	8.2	3.9	6.8	9.7
SCP + N 188	46.4	41.8	74.5	81.9	55.3	71.7	0.4	0.0	5.5	0.0	1.2	0.0
24 h												
SCP	62.7	45.9	80.6	83.9	73.0	85.8	0.2	0.0	5.2	0.1	5.1	3.0
SCP + N 188	66.1	49.7	83.3	88.3	76.1	85.3	0.1	0.0	4.6	0.0	1.7	1.5
72 h												
SCP	74.9	56.4	90.3	86.9	83.0	83.8	0.0	0.0	1.8	0.0	1.2	1
SCP + N 188	80.1	58.4	91.9	88.8	80.5	82.6	0.0	0.0	2.2	0.0	1.2	1.7

Note: glucan digestibility and cellobiose were calculated at combined protein loading of SCP (Spezyme-CP) or/and N 188 (Novozyme-188).

and 91.9% (Lime), respectively. Supplementation of β-glucosidase increased glucan digestibility for SAA pretreated samples at all levels of enzyme loading throughout the hydrolysis. For other pretreated samples, the increase was seen only until 24 h (Table 1). For SAA and AFEX treated samples, 34% and 56% of glucan remained unhydrolysed after 72 h with Moderate enzyme loading. Upon increasing the total enzyme loading to High level, only 9–12% increase in glucan digestibility was observed. Previous studies along these line has proven that alkaline pretreatments including SAA and AFEX retain much of the xylan following pretreatment, therefore, supplementation of xylanase enzyme increases glucan digestibility (Gupta et al., 2008; Bals et al., 2010; Kumar and Wyman, 2009).

For AFEX treated samples, with Low and Moderate enzyme loadings discernible increase in glucan digestibility was observed with supplementation of β-glucosidase, but only at the early phase of hydrolysis (Table 1). With High and Very High enzyme loadings, however, β-glucosidase supplementation showed only a slight increase in the glucan digestibility. Cellobiose was absent throughout the hydrolysis for all the enzyme loadings. This indicates that at High and Very High enzyme loadings, the β-glucosidase activity in the Spezyme-CP is sufficient to convert all cellobiose to glucose. Obviously, no additional β-glucosidase is required at this point to improve glucan hydrolysis. For LHW treated samples, with Low and Moderate enzyme loadings, β-glucosidase supplementation increased glucan digestibility only at early stage of hydrolysis (12 h), but with High and Very High enzyme loadings, 5–7% increase in digestibility was observed until 24 h of enzymatic hydrolysis (Table 1). The effect of β-glucosidase supplementation on DA treated samples decreased as the enzymatic hydrolysis progressed, and this effect was further decreased with increase in enzyme loading (Table 1). Our data agree with Kumar and Wyman that β-glucosidase supplementation has a significant effect on total sugar yields in the first 24 h of hydrolysis of DA treated corn stover, after which its effect was insignificant (Kumar and Wyman, 2009). Much like DA treated samples, the β-glucosidase effect on glucan digestibility for SO_2 pretreated samples was only observed at the early phase of hydrolysis and with Low enzyme loading (Table 1).

The highest xylan digestibility achieved for SAA, AFEX and Lime treated samples with Spezyme-CP alone (at 60 FPU/g-glucan) were 56%, 64%, and 84%, respectively. With Novozyme-188 supplementation at 120 CBU/g-glucan, the digestibility increased to 62%, 70%, and 93%, respectively. The increase in xylan digestibility with β-glucosidase supplementation is due to the β-xylosidase activity existing in Novozyme-188 (Dien et al., 2008). SAA, AFEX and Lime pretreated samples had sharp increase of xylan digestibility up to Moderate enzyme loading followed by a gradual increase with higher enzyme loading. Xylanase activity in Spezyme-CP and/or

Table 2
Glucose and xylose oligomers produced during enzymatic hydrolysis of DSG of different pretreatments.

	Glucose oligomers (%)[a,c]				Xylose oligomers (%)[b,c]			
	Spezyme-CP		Spezyme-CP + Novozyme-188		Spezyme-CP		Spezyme-CP + Novozyme-188	
	6 h	72 h	6 h	72 h	6 h	72 h	6 h	72 h
SAA								
3 FPU or/and 6 CBU	16.9	7.8	11.7	5.1	28.1	16.2	26.1	14.7
15 FPU or/and 30 CBU	11.0	6.1	8.7	3.2	25.0	12.9	23.0	12.1
AFEX								
3 FPU or/and 6 CBU	4.5	3.7	1.5	0.7	31.1	26.9	30.6	20.2
15 FPU or/and 30 CBU	6.4	1.1	2.2	0	27.1	20.9	26.2	14.3
Lime								
3 FPU or/and 6 CBU	6.6	7.2	3.2	5.0	12.2	14.8	13.8	14.2
15 FPU or/and 30 CBU	9.1	18.4	0.9	14.6	21.7	27.0	13.7	14.5
LHW								
3 FPU or/and 6 CBU	9.1	3.9	0.0	6.1	–[d]	–	–	–
15 FPU or/and 30 CBU	10.8	12.6	0.0	5.2	–	–	–	–
DA								
3 FPU or/and 6 CBU	10.6	4.8	0.1	4.0	–	–	–	–
15 FPU or/and 30 CBU	8.2	3.4	1.4	0.0	–	–	–	–
SO_2								
3 FPU or/and 6 CBU	22.8	31.7	4.8	16.4	–	–	–	–
15 FPU or/and 30 CBU	26.8	20.8	9.2	19.2	–	–	–	–

Note: [a]The percentage yield of glucose oligomers containing two or more glucose units.
[b]The percentage yield of xylose oligomers containing two or more xylose units.
[c]The glucose and xylose oligomers were calculated based on initial glucan and initial xylan present.
[d]DA, LHW and SO_2 contain very low initial amount of xylan. Level of xylose oligomers produced during hydrolysis is negligible.

Novozyme-188 is not high enough to hydrolyze all of the xylan of SAA and AFEX treated samples even at High loading of enzymes. DA, LHW and SO_2 treated samples contain very low initial amount of xylan. Level of xylose produced from these samples is therefore negligible. Spezyme-CP and/or Novozyme-188 were not able to hydrolyze all of the xylan from SAA and AFEX treated samples, which may be because of the low xylanase activity of these enzymes (Dien et al., 2008).

3.3. Cellobiose

Increase of Spezyme-CP loading from Low to Very High level without β-glucosidase addition caused decrease of cellobiose for all the pretreted samples. Supplementation of β-glucosidase further reduced the cellobiose for all the pretreated samples, the extent reduction varying with individual pretreatments (Table 1). The DSG treated with Lime, DA and SO_2 produced more cellobiose without β-glucosidase supplementation at Low enzyme loading than the other treated samples. Cellobiose in these sample was reduced considerably with increase in cellulase loading, which undoubtedly contributed to increase in glucan digestibility. For AFEX treated samples, cellobiose was observed only at Low cellulase loading. Cellobiose formation was uniformly low in SAA, AFEX and LHW regardless of enzyme loading or the presence of external β-glucosidase, yet the glucan digestibility of LHW was much higher than the other two pretreated samples. This may be due to the fact that a large amount of xylose oligomers, a known inhibitor to cellulase and β-glucosidase are produced during hydrolysis of SAA and AFEX treated samples (Kumar and Wyman, 2008b).

3.4. Oligomers

Significant amount of glucose and xylose oligomers were formed as intermediate products during enzymatic hydrolysis of all pretreated samples. The glucose and xylose oligomers formed were as high as 32% (SO_2) and 27% (AFEX) of initial glucan and xylan, respectively (Table 2). Previous studies have reported that glucose and xylose oligomers inhibit cellulase activity (Kumar and Wyman 2008b; Gupta et al., 2008). With SAA, Lime, LHW, SO_2 treated sample, β-glucosidase supplementation reduced glucose and xylose oligomers, but complete conversion of the oligomers did not occur even after 72 h of enzymatic hydrolysis. Our data concurs with Gupta et al. (2008) that high DP glucose oligomers are generated in the early phase of hydrolysis and cannot be hydrolyzed by cellulase and β-glucosidase.

For alkaline treated samples, the xylose oligomers after 72 h of enzymatic hydrolysis were much higher than glucose oligomers. According to Kumar and Wyman (2008b) xylose oligomers reduce the cellulase as well as β-glucosidase activity. Low xylanase activity in commercial enzymes is to be blamed for accumulation of significant amount of xylose oligomers hindering enzymatic hydrolysis of glucan and xylan in biomass. Supplementation of external xylanase or β-xylosidase could reduce this inhibition and increase the digestibility (Quing and Wyman, 2011).

4. Conclusion

For a fixed enzyme loading, DA, SO_2, and Lime methods rendered higher 72 h digestibilities than the SAA or AFEX. Digestibility of DSG from acidic treatments sharply increase with enzyme loading up to 25 mg/g-glucan and leveled off. In alkaline and neutral treatments, sharp rise of digestibility was followed by further gradual increase with higher enzyme loading. Supplementation of β-glucosidase: (a) improved the glucan digestibility for SAA, (b) reduced the cellobiose and glucose oligomers at the early phase, but did not improve the terminal digestibility for other pretreatments, (c) improved xylan digestibility of alkaline treatments due to β-xylosidase activity in β-glucosidase.

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