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Effect of Using a Nitrogen Atmosphere on Enzyme Hydrolysis at High Corn Stover Loadings in an Agitated Reactor

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ABSTRACT

[a]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

A comprehensive review of the literature shows that enzyme hydrolysis efficiency decreases with increased solids loadings at constant enzyme:cellulose ratios for pretreated lignocellulosic substrates. In seeking a mechanistic explanation for this phenomenon, we found that a nitrogen atmosphere enhances enzyme hydrolysis and minimizes the decrease in glucose yields as solids loadings are increased in an agitated bioreactor. For liquid hot water pretreated corn stover, at solids loadings of both 100 g/L and 200 g/L and hydrolyzed for 72 hours in a 1L bioreactor at pH 5.0 with 3.6 mg protein per g biomass, glucose yields were 55% in a nitrogen atmosphere vs 45% in air with agitation and about 34% without agitation. While mixing promotes biomass/enzyme contact and disperses sugars released during hydrolysis that would otherwise cause product inhibition, nitrogen gas displaces air, avoiding deactivation of cellulases by oxygen. The nitrogen effect points to a facile approach of enhancing hydrolysis at high solids loadings.

***Keywords*:** cellulases, agitated reactor, corn stover, solids loading effect, nitrogen

Introduction

Pretreatment is needed to disrupt the lignin seal, open up the biomass structure and make the cellulose amendable to enzyme hydrolysis[1]. While acid, alkaline or neutral pH pretreatments may be used, this work addresses liquid hot water (i.e., hydrothermal pretreatment at neutral pH). This represents one of the pretreatments that has been determined to be attractive since no chemicals are added or removed, and since high yields are obtained upon enzyme hydrolysis.[2-15] Lignocellulosic materials are converted into ethanol for use in biofuels through a processing sequence that entails pretreatment, hydrolysis, fermentation to ethanol, and ethanol distillation and drying.[9] Economically viable lignocellulosic ethanol production requires operating at high biomass loadings to minimize sizing of hydrolysis and fermentation vessels, increase product concentrations and decrease recovery costs.[2,10] However, the paste-like properties associated with biomass concentrations of 120 g/L and higher lead to difficulties in material handling and mass transport within unit operations in a biorefinery. The major constraint for high-solids occurs during enzymatic hydrolysis of pretreated biomass to extract fermentable sugars. Sugars yields fall with increased solids loading.

The decrease in conversion with increasing solids concentration i.e., the solids loading effect, has been attributed to a range of phenomena. Geng et al. (2015), Bals et al. (2013), Palmqvist and Linden (2012) and others have observed decreases in glucan conversion with increasing solids loadings even with good mixing to ensure contact between enzymes and lignocellulosic biomass.[16-18] However, at equivalent conditions and constant enzyme:biomass ratios, hydrolysis yields that are independent of solids concentration, while expected, are not observed. Simply adding more enzymes is not an option because they are very expensive. Based on our prior work and a review of the literature, we suspected enzyme instability and / or inhibition to be factors contributing to the solids loading effect and that improving enzyme stability would partially

alleviate this effect.

Many studies have sought to address ways to improve sugar yields at high solids loading. Most have focused on mixing (Table 1)[3-5,16,18-41]. Others have examined the role of glucose inhibition of cellulases (e.g. end product inhibition). Our laboratories as well as others have identified and examined the role of soluble inhibitors, which of course, increase in concentration along with solids. Soluble inhibitors include phenolic compounds and oligosaccharides, including recalcitrant xyliologomers.[1,20,23-26,42,43] Pretreated lignin also inhibits hydrolysis by adsorbing cellulases.[4,5,27] Soluble inhibitors can be removed by washing with hot water,[28] but insoluble inhibitors and lignin residue remain and reduce enzyme activities.[4,24,28,44] Nonspecific binding of cellulases to modified lignin has been addressed by confirming this effect by blocking using BSA [4,45] or a low cost protein source such as soybean[46] . One less studied effect is enzyme deactivation from exposure to the air-liquid interface.[29,30] In this study, we focused in this last effect and propose an inexpensive solution. For this purpose, corn stover was pretreated with liquid hot-water. Liquid hot-water has been widely applied because it avoids the use of a catalyst (e.g. mineral acid) or expensive solvent, which is often toxic to microbes. This study also uses pelletized biomass, which is favored because it allows for more efficient transport and handling[47]. Hydrolysis was conducted in well mixed flasks or using a stirred glass vessel with a marine impeller.

When the hydrolysis reaction fluid is mixed, an air-liquid interface is created, which can lead to foaming and protein denaturation. One possible source of denaturation is oxidation of the enzymes mediated by molecular oxygen. To test for this, hydrolysis was conducted under air and nitrogen. Adding a nitrogen atmosphere improved hydrolysis. Furthermore, conducting hydrolysis under a nitrogen atmosphere gives consistent extents of glucose production at biomass

concentrations of 100 and 200 g/L for enzyme hydrolysis of pretreated corn stover in an agitated vessel. Purchased enzymes would cost $0.83 per gal for corn stover at conditions used in this work (according to Liu et al. (2016))[48] and cost of enzyme continues to be a bottleneck.[49,50] Nitrogen gas costs between $0.2 to 2 per m^3, (https://puritygas.ca/nitrogen-gas-costs/, accessed on 06/17/2020), equivalent to 1.5 to 15¢/gallon, so use of N_2 promises to directly lower production costs. The nitrogen effect indicates a potential approach of enhancing hydrolysis at high solids loadings, while minimizing enzyme loading and associated costs.

Materials and Methods

Each experiment consisted of preprocessing ground corn stover into pellets, adding the pellets into pretreatment tubes containing water, and cooking the pellets under liquid hot water pretreatment conditions.[32,33] The material was then washed, filtered, and added to an agitated reactor containing enzyme and buffer, as described below. Variability between replicate experiments was less than 10%.

Corn stover, milling and densification

Corn stover was harvested in Story County, Iowa in October 2015 using a multi-pass harvest system. The resulting bales were shipped to the full-scale milling facility at DOE's Biomass Feedstock National User Facility (BFNUF) located at Idaho National Laboratory (INL). A Vermeer BG-480 (Pella, IA) with a 50.8-mm screen followed by a Bliss hammer mill (Ponca City, OK) with 6.35-mm screen were used to process the bales into milled corn stover particles. The milled biomass was then pelletized through a 6.35-mm diameter die with a length to diameter ratio of 12 in a Bliss Pioneer Pellet Mill (B35A-75) at the Bliss Industries, LLC testing laboratory (Ponca City, OK). Pellets in sealed 5-gallon plastic buckets, stored at room temperature, were sent to Purdue University for subsequent experiments.

112 ***Liquid Hot Water Pretreatment (LHW)***

113 The pellets were pretreated in 316 stainless steel tubes, as described by Kim et al. (2009)[32]. The
114 tubes have length of 4.5 in., an outside diameter of 1.0 in., wall thickness of 0.065 in., and a
115 measured internal volume of 45 ml. These were filled with water, and submersed for 20 min in a
116 fluidized sand bath preheated to 190°C. Pretreatment consisted of a 5 min heat-up time to
117 190°C, a 15 min hold at 190°C (R_0 = 9.8), and subsequent quenching at 20°C in a bucket of
118 water. The tube was opened, and its contents were placed on Whatman #1 filter paper in a
119 Buchner funnel. Vacuum filtration removed free liquid from the solids and the solids were
120 retained on the filter paper. The ratio of pellet solids to water in these experiments was 15.25 g
121 solids (weighed at 8.35% w/w moisture) to 23.25 g distilled water. Solids content was
122 equivalent to 36% w/w in water. Heat transfer in the sand baths was confirmed by measuring the
123 temperature inside the reactors with a probe. [32] It was similar to the temperatures seen with 10
124 and 15% solids. The pretreated material also had similar composition to pretreated materials at
125 lower solids loadings. One important point here is the pelleted nature of the material was critical
126 to achieve the high solids loading during pretreatment.

127 After vacuum filtration, the wet solids weighed 33 ± 1 g. Two 100 mL aliquots of DI
128 water (temperature ≥ 90°C) were added to the Buchner funnel in two steps. The filtrate
129 contained phenolic and other water-soluble inhibitors. After washing, the weight of the
130 remaining dry solids was 9.0 ± 0.5 g, corresponding to 26.5 ± 1.5% w/w solids content in the
131 pretreated and washed material. Between 36 ± 4% of the starting dry weight was removed by
132 pretreatment and the subsequent washing step, while cellulose and lignin content of the
133 remaining solids increased. The lost weight included partial removal of hemicellulose and lignin
134 (Table 2). The equation of Overend and Chornet (1987)[6]; log Ro ($Ro = t \times \exp((T - 100)/\omega)$)

with a value of ω = 4.6 was used to calculate pretreatment severity, log Ro = 9.8.[51]

Enzymes

Cellic CTEC2 (150 FPU/mL, 180 mg protein/mL, Sigma-Aldrich, St. Louis, MO) gave eight distinct protein bands in an SDS-Page electrophoresis gel. The protein bands were associated with enzyme major activities for lignocellulose hydrolysis (Supp. Figure 1). Identification of activities associated with the bands utilized Uniprot Consortium database.[52-54] Protein concentration was measured using a bicinchoninic acid (BCA) protein assay reagent kit (Thermo-Scientific, Rockford, IL) using bovine serum albumin (BSA) as the standard.[55] All enzymatic assays were performed in triplicate in citrate buffer, 50 mM, pH 4.8, at 50°C. Activity in filter paper units (FPU) was determined using Whatman #1 filter paper (1 × 6 cm, 50 mg) as substrate, according to previously described standard conditions.[56] Total reducing sugars were measured by the dinitro salicylic acid (DNS) method.[57] Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted.

Enzymatic hydrolysis

The pretreated and washed solids were slurried in either 50 mL or 600 mL of 50 mM, pH 4.8 citrate buffer to which an appropriate amount of enzyme was subsequently added into a shake flask or 1 L agitated Bioflo® reactor (Eppendorf North America, Hauppauge, NY), respectively, containing the corn stover slurries. An enzyme loading of 3.6 mg protein/g dry solids (equivalent to 3 FPU/ g pretreated and washed solids, dry basis) was used to carry out hydrolysis (50 mL slurry in 250 mL flasks) at 50ºC for 72 hours in an New Brunswick™ Innova® 44 orbital shaker (Eppendorf North America, Hauppauge, NY (0 or 200 RPM)) or in the 1L stirred reactors (600 mL slurry) with dual marine impellers (Supp. Figure 2) at 0 or 290 rpm, respectively. Solids were loaded into the shake flasks or reactors to obtain 10, 100 and 200 g/L

158 (dry weight basis) with enzyme solutions added to the top of the surface of the corn stover
159 slurries at the beginning of each experiment.

160 For experiments on air-free reactions, the headspace was purged using N_2 flowing at
161 1.5L/hr for 2 hours into each of three 1L glass Bioflo® vessels (Eppendorf North America,
162 Hauppauge, NY) wrapped in heating pads and equipped with dual, marine impellers powered by
163 overhead stirrer motors (Supp. Figure 2). Temperature and mixing (at 290 RPM) were
164 controlled by a Bioflo® 115 control tower. Dissolved oxygen was measured by cutting off the
165 flow of air and inserting, 10 cm into the reactor, an InPro6850i polarography probe (Mettler
166 Toledo, Switzerland), calibrated against 100% saturation levels of oxygen (air) in deionized
167 water in water at equivalent conditions. Since the enzyme formulations contained lytic
168 polysaccharide monooxygenase (LPMO), initial oxygen levels were above 100% saturation since
169 the hydrogen peroxide generated by LPMOs decomposes to oxygen and water and thereby
170 increase dissolved oxygen.[31,58] For hydrolysis in the presence of air, the headspace was purged
171 with air at equivalent conditions. Shake-flask runs in the New Brunswick™ Innova® 44 orbital
172 shaker (0 or 200 RPM) provided a baseline comparison to typical laboratory scale enzyme
173 hydrolysis in the presence of air. Hydrolysis in both shaker flasks (Figure 1A) and 1 L agitated
174 bioreactors (Figure 1B) utilized the same starting materials for these studies.

175 Analyses of the hydrolysates were by Aminex-H87 (Bio-Rad, Hercules, CA) HPLC
176 column with 50 mM H_2SO_4 mobile phase at a flowrate of 0.6 mL/min. Enzymes in 2 mL
177 aliquots of hydrolysate were deactivated by boiling for 5 min. After centrifugation at 13,000
178 rpm for 3 min and at ambient temperature in 1.5 mL microfuge tubes, decanted supernatant was
179 clarified by passing through a 0.2 mm Acrodisc1, nylon syringe filter. Glucan conversion, y,
180 was calculated on a weight basis, using the relation $y = 100 * G/(B * X * 1.11)$ where G

represents measured glucose, B weight of biomass, X fraction of glucan (cellulose), and 1.11 the factor that represents weight gain due to water added across the glycosidic bond upon hydrolysis.

Yield stress measurements

The apparent yield stress of corn stover slurries was determined using an Anton Paar MCR 702 rotational rheometer in controlled shear rate mode with a fixed glass starch cell fixture (inner diameter of 56 mm) in a flexible cup-holder mount. This 24 mm diameter fixture has an active length of 30 mm to minimize particle settling. Samples were placed in the cell and allowed to equilibrate (rest) for 15 min after the fixture was lowered into the cell. The flow behavior of each sample was measured by performing flow curve experiments, in which the magnitude of shear stress was monitored over time as the sample experienced logarithmically increasing applied shear rates from 0.1 to 100 s^{-1}. A single value of yield stress was determined from each flow curve experiment as the local shear stress maximum at applied shear rates less than 5 s^{-1}.[41,59] Flow curve experiments were performed in triplicate to determine an average apparent yield stress value for each corn stover slurry sample.

Statistical analysis

Origin Pro 2020 software (OriginLab Corp., Northampton, MA) with statistical significance set at $\alpha = 0.05$ was used for calculations, data analysis, and graphing.

Results and Discussion

Enzymatic Hydrolysis in Shake Flasks

Shaker flasks agitated using an orbital shaker is the most common apparatus used to conduct hydrolysis experiments at a laboratory scale. Thick slurries mixed in a shaker resist the swirling action, likely resulting in uneven distribution of cellulases and localized pockets of

enzymes and inhibitors that compromise hydrolysis efficiency.[16,60] We gauged the magnitude of this effect by comparing hydrolysis at equivalent compositions of pretreated and washed corn stover slurries at mass loadings of 10, 100, and 200 g/L, respectively.

The difference between mixing (200 RPM) vs. no mixing (0 RPM) is statistically significant at a 10 g/L loading (Figure 1A), with 51% conversion of glucan to glucose obtained after 72 hrs at an enzyme loading of 3.6 mg enzyme/g pretreated and washed solids versus 45% with no mixing (p-value < 0.05). Conversions for 100 and 200 g/L solids loadings in flasks placed in an orbital shaker are similar, whether hydrolysis is carried out by mixing at 200 rpm (Fig 1A). Hence, runs at quiescent conditions (such as in a microfuge tube) suffice for gauging hydrolysis conditions at industrially relevant solids loadings. However, these conversions do not necessarily indicate maximum hydrolysis yields achievable with mixing and are consistent with previous works that used a variety of mixing methods (Table 3).

It should be noted that high enzyme loadings (12 mg protein/g solids) disguise the impact of mixing and inhibitor effects since nearly complete hydrolysis of corn stover occurs (data not shown). Enzyme loadings of 3.6 mg protein/g solids were therefore used in our study to avoid this masking effect. The lower loadings are consistent with previously reported levels of enzyme added to pretreated hardwood, wheat, corn stover, and sugarcane bagasse in shake-flask hydrolysis experiments.[4,44,61]

Yield Stress of Lignocellulose

Yield stress, a key indicator of slurry mixing for dilute acid pretreated corn stover, increases with particle size and/or particle volume fraction in a slurry that exhibits pseudoplastic behavior.[34,41] Through our flow curve experiments, we found the average yield stress for

225 untreated corn stover derived from pellets was 772 ± 96 Pa (Figure 2) compared to literature
226 values of 1,000 to 10,000 Pa for 200 g (initial) solids/L of diluted acid pretreated corn
227 stover.[41,62,63] Yield stress of the untreated slurry gives an important reference point since it has
228 the largest yield stress during mixing. The fluid flows and mixing occurred at stress values
229 greater than the apparent yield stress, as confirmed by visual inspection at 132 rpm with a shear
230 rate of 73.6 s^{-1} and impeller tip speed of 1.17 m/s, corresponding to 290 RPM in the 1 L Bioflo
231 stirred reactor. The shear rate $\dot{y} = \upsilon/h$ is obtained from the velocity υ = RPM (0.10472r); where h
232 is the gap between the tip of the agitator and reactor wall, and r the impeller radius.[64]

233 ***Enzymatic Hydrolysis: Shake Flask vs Stirred Bioreactors***

234 In the absence of mixing and presence of air, glucose yields in shake flasks (Figure 1A,
235 open columns) were higher than in the stirred reactor (compare Figure 1A and 1B) ($p < 0.05$)
236 although the difference was small at equivalent solids loadings. Vigorous mixing in an agitated
237 bioreactor minimizes differences at the lowest loading of 10 g/L.

238 For mixed and unmixed slurries at 10 g/L in the bioreactor with the marine impeller,
239 contact of water and enzyme readily occurs in dilute suspension (i.e., 10 g/L). There is no
240 statistical difference for mixed and unmixed slurries at 10 g/L solids loading (Figure 1B) in the
241 presence of air. Significant decreases in free liquid occur at 100 g/L and very little free liquid is
242 visible at 200 g/L. Hence, at higher loadings the contact between the water-soluble enzyme and
243 insoluble lignocellulosic substrate requires forced mixing. Mixing improves yields from 34%
244 (not mixed) to 45% (mixed). Palmqvist et al. [18,35] showed that a high impeller speed increased
245 glucose yield for 100 g/L (dry basis) acid pretreated corn stover solids. This is consistent with
246 our hydrolysis runs for hydrothermally (e.g. no acid added) pretreated corn stover at 200 g/L in
247 the presence of air (Figures 3A and 3B).

248 Mixing with an impeller changes the interactions among enzymes, substrates, products,
249 inhibitors, and air. Washed, pretreated biomass has lower concentrations of liquid hot water
250 prtreatment-derived inhibitors,[28,32] although insoluble lignin inihibitors are still present.[1,24,36]
251 Also, at high solids and high conversions, glucose, cellobiose, xylose, phenolic compounds and
252 oligomers, all of which are enzyme inhibitors, accumulate locally with higher concentrations
253 than in a well-mixed bulk phase.[20-22] A study at low solids loadings (10 g/L) in shaker flasks
254 using 10 mg/mL Avicel (a lignin-free microcrystalline cellulose) showed that air entrained into
255 the slurry would deactivate cellulolytic enzymes.[30] For our experiments with corn stover, unlike
256 avicel, there is minimal difference between non-agitated and agitated hydrolysis reactions at
257 solids loadings of 10 g/L in the presence of air (Figure 1B, 10 g/L), although differences become
258 apparent at 100 and 200/L suspended in 600 mL volumes. If "free fall mixing" (resembles a
259 cement mixer) is used instead of mechanical agitation of a slurry, a solids loading effect is also
260 observed.[19]

261 ***Combination of air-restricted conditions and improved mixing***

262 Mechanical action promotes contact of a third phase, air, with enzyme and may
263 deactivate cellulolytic enzymes.[30,31] An air-restricted or microaerobic condition is necessary if
264 the air-liquid interface and the air deactivation of cellulases is to be eliminated. Hydrolysis
265 under air-free conditions should enhance yields at the same degree of mixing. When the
266 headspace of agitated reactors (290 RPM) were blanketed with N_2 to remove air, hydrolysis
267 yields were higher at all three solids concentrations and there was no difference in glucan
268 conversion with increasing solids loadings (Figure 3). In the case where hydrolysis and
269 fermentation of the formed glucose occurs simultaneously, the generated CO_2 could displace air
270 and possibly mitigate enzyme deactivation, although the current work did not test this
271 hypothesis. Hence, selection of an optimal mixing strategy for biomass slurries in an agitated

reactor must consider all three phases: solid, liquid, and gas.

Measurement of dissolved oxygen shows that it decreases and then stabilizes during the first 2 hours of hydrolysis and mixing, for reactors not purged with N_2 (Figure 4). The lowest biomass concentration (10 g/L) corresponds to the highest dissolved oxygen (DO) level, and the highest (200 g/L biomass) corresponds to the lowest DO (Figure 4). Bhagia et al. (2018)[30] demonstrated that larger liquid-air interfaces at solids loadings of 10 g/L, lead to greater deactivation of cellulases and lower conversions. This is particularly noticeable at the lower enzyme loadings of 3.6 mg/g solids used in this work and where excess activity would not mask deactivation effects. These data indicate the beneficial effect of using a nitrogen atmosphere occurs within the first two hours at the highest solids loading. Furthemore, monooxygenase (present in the commercial cellulase used here) also affects measurement of dissolved oxygen levels, since this enzyme generates ROS that in turn results in high background readings for the type of DO probe used here (Figure 4).

The LPMO's (i.e., monoxygenases) present in some commercial cellulase preparations[1], such as the one used in this study, utilize oxygen to generate reactive oxygen species (ROS) and enhance cellulose hydrolysis, reducing exposure of cellululytic enzymes to oxygen. However, too much ROS may also deactivate cellulases over time.[31] When nitrogen is purged into a bioreactor fluid that includes LPMO's, the gas displaces oxygen, decreasing both ROS and enzyme deactivation resulting in higher cellulolyic activity.[31] In this sense, our results show the interesting and important finding that controlling deactivation using a nitrogen atmosphere mitigates the solids loading effect for cellulose hydrolysis. Deactivation effects due to air in stirred vessels have also been described for lipases and lysozyme, in addition to cellulases.[30,36] The deactivation coefficient is directly proportional to the mass transfer coefficient k_La

295 consisting of k_L – rate constant for oxygen gas transfer into liquid and a – interfacial area.[65]
296 While we are not aware of other k_La data for lignocellulose slurries, for viscous cassava starch
297 the net value of k_La decreases as solids loading increase, hence suggesting some protective effect
298 could be achieved at high solids.[37,65]

299 **Conclusions**

300 The combination of agitation with a nitrogen atmosphere points to operational factors and
301 mechanisms that mitigate the solids loading effect and possibly enhance enzyme hydrolysis by
302 removing air-based deactivation of enzymes. This effect merits further research for
303 lignocellulosic substrates since it has the potential to simplify enzyme formulations by achieving
304 a decrease in dissolved oxygen using a gas rather than an added enzyme (an oxygenase) to
305 achieve a similar effect. This approach enables the concentration of pretreated solids to be
306 increased, while avoiding a decrease in the extent of enzyme catalyzed cellulose hydrolysis when
307 the enzyme:cellulose ratio is kept constant.

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Literature Cited

1. dos Santos AC, Ximenes E, Kim Y, Ladisch MR. Lignin–Enzyme Interactions in the Hydrolysis of Lignocellulosic Biomass. *Trends Biotechnol*. 2019;37(5):518-531. doi:10.1016/J.TIBTECH.2018.10.010

2. Wingren A, Galbe M, Zacchi G. Techno-Economic Evaluation of Producing Ethanol from Softwood: Comparison of SSF and SHF and Identification of Bottlenecks. *Biotechnol Prog*. 2008;19(4):1109-1117. doi:10.1021/bp0340180

3. Kumar L, Arantes V, Chandra R, Saddler J. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresour Technol*. 2012;103(1):201-208. doi:10.1016/J.BIORTECH.2011.09.091

4. Kim Y, Kreke T, Ko JK, Ladisch MR. Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. *Biotechnol Bioeng*. 2015;112(4):677-687. doi:10.1002/bit.25465

5. Ko JK, Kim Y, Ximenes E, Ladisch MR. Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. *Biotechnol Bioeng*. 2015;112(2):252-262. doi:10.1002/bit.25349

6. Overend RP, Chornet E. Fractionation of lignocellulosics by steam— aqueous pretreatments. *Phil Trans R Soc Lond A*. 1987;321:523-536. Accessed October 16, 2017. http://rsta.royalsocietypublishing.org/content/roypta/321/1561/523.full.pdf

7. Wei W, Jin Y, Wu S, Yuan Z. Improving corn stover enzymatic saccharification via ferric chloride catalyzed dimethyl sulfoxide pretreatment and various additives. *Ind Crops Prod*.

2019;140:111663. doi:10.1016/j.indcrop.2019.111663

8. Yuan Z, Li G, Wei W, Wang J, Fang Z. A comparison of different pre-extraction methods followed by steam pretreatment of bamboo to improve the enzymatic digestibility and ethanol production. *Energy*. 2020;196:117156. doi:10.1016/j.energy.2020.117156

9. Ladisch M, Mosier N, Kim Y, Ximenes E, Hogsett D. Converting Cellulose to Biofuels. *Chem Eng Prog*. 2010;106(3):56-63. Accessed July 9, 2020. https://www.cheric.org/research/tech/periodicals/view.php?seq=988578

10. Zacchi G, Axelsson A. Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions. *Biotechnol Bioeng*. 1989;34(2):223-233. doi:10.1002/bit.260340211

11. Ximenes E, Farinas CS, Kim Y, Ladisch MR. Hydrothermal Pretreatment of Lignocellulosic Biomass for Bioethanol Production. In: *Hydrothermal Processing in Biorefineries*. Springer International Publishing; 2017:181-205. doi:10.1007/978-3-319-56457-9_7

12. Sannigrahi P, Ragauskas AJ. Fundamentals of Biomass Pretreatment by Fractionation. In: *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. John Wiley & Sons, Ltd; 2013:201-222. doi:10.1002/9780470975831.ch10

13. Mosier NS. Fundamentals of Aqueous Pretreatment of Biomass. In: *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. John Wiley & Sons, Ltd; 2013:129-143. doi:10.1002/9780470975831.ch7

14. Wyman CE, Dale BE, Balan V, Elander RT, Holtzapple MT, Ramirez RS, Ladisch MR,

Mosier NS, Lee YY, Gupta R, Thomas SR, Hames BR, Warner R, Kumar R. Comparative Performance of Leading Pretreatment Technologies for Biological Conversion of Corn Stover, Poplar Wood, and Switchgrass to Sugars. In: *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*. John Wiley & Sons, Ltd; 2013:239-259. doi:10.1002/9780470975831.ch12

15. Ladisch M. *Final Scientific/Technical Report DE-FC36-08GO18103*.; 2017.

16. Geng W, Jin Y, Jameel H, Park S. Strategies to achieve high-solids enzymatic hydrolysis of dilute-acid pretreated corn stover. *Bioresour Technol*. 2015;187:43-48. doi:10.1016/J.BIORTECH.2015.03.067

17. Bals BD, Gunawan C, Moore J, Teymouri F, Dale BE. Enzymatic hydrolysis of pelletized AFEX™-treated corn stover at high solid loadings. *Biotechnol Bioeng*. 2014;111(2):264-271. doi:10.1002/bit.25022

18. Palmqvist B, Lidén G. Torque measurements reveal large process differences between materials during high solid enzymatic hydrolysis of pretreated lignocellulose. *Biotechnol Biofuels*. 2012;5(1):57. doi:10.1186/1754-6834-5-57

19. Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnol Bioeng*. 2007;96(5):862-870. doi:10.1002/bit.21115

20. Kristensen JB, Felby C, Jørgensen H. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol Biofuels*. 2009;2(1):11. doi:10.1186/1754-6834-2-11

21. Hong J, Ladisch MR, Gong C, Wankat PC, Tsao GT. Combined product and substrate inhibition equation for cellobiase. *Biotechnol Bioeng*. 1981;23(12):2779-2788.

384 doi:10.1002/bit.260231212

385 22. Ladisch MR, Gong C-S, Tsao GT. Cellobiose hydrolysis by endoglucanase (glucan
386 glucanhydrolase) fromTrichoderma reesie: Kinetics and mechanism. *Biotechnol Bioeng*.
387 1980;22(6):1107-1126. doi:10.1002/bit.260220602

388 23. Ladeira Ázar RIS, Morgan T, dos Santos ACF, de Aquino Ximenes E, Ladisch MR,
389 Guimarães VM. Deactivation and activation of lignocellulose degrading enzymes in the
390 presence of laccase. *Enzyme Microb Technol*. 2018;109:25-30.
391 doi:10.1016/J.ENZMICTEC.2017.09.007

392 24. Michelin M, Ximenes E, de Lourdes Teixeira de Moraes Polizeli M, Ladisch MR. Effect
393 of phenolic compounds from pretreated sugarcane bagasse on cellulolytic and
394 hemicellulolytic activities. *Bioresour Technol*. 2016;199:275-278.
395 doi:10.1016/j.biortech.2015.08.120

396 25. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M. Deactivation of cellulases by phenols.
397 *Enzyme Microb Technol*. 2010;48:54-60. doi:10.1016/j.enzmictec.2010.09.006

398 26. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M. Inhibition of cellulases by phenols.
399 *Enzyme Microb Technol*. 2010;46:170-176. doi:10.1016/j.enzmictec.2009.11.001

400 27. Ko JK, Ximenes E, Kim Y, Ladisch MR. Adsorption of enzyme onto lignins of liquid hot
401 water pretreated hardwoods. *Biotechnol Bioeng*. 2015;112(3):447-456.
402 doi:10.1002/bit.25359

403 28. McMillan JD, Jennings EW, Mohagheghi A, Zuccarello M. Comparative performance of
404 precommercial cellulases hydrolyzing pretreated corn stover. *Biotechnol Biofuels*.
405 2011;4(1):29. doi:10.1186/1754-6834-4-29

406 29. Kim MH, Lee SB, Ryu DDY, Reese ET. Surface deactivation of cellulase and its
407 prevention. *Enzyme Microb Technol*. 1982;4(2):99-103.
408 doi:10.1016/0141-0229(82)90090-4

409 30. Bhagia S, Dhir R, Kumar R, Wyman CE. Deactivation of Cellulase at the Air-Liquid
410 Interface Is the Main Cause of Incomplete Cellulose Conversion at Low Enzyme
411 Loadings. *Sci Rep*. 2018;8(1):1350. doi:10.1038/s41598-018-19848-3

412 31. Scott BR, Huang HZ, Frickman J, Halvorsen R, Johansen KS. Catalase improves
413 saccharification of lignocellulose by reducing lytic polysaccharide monooxygenase-
414 associated enzyme inactivation. *Biotechnol Lett*. 2016;38(3):425-434.
415 doi:10.1007/s10529-015-1989-8

416 32. Kim Y, Hendrickson R, Mosier NS, Ladisch MR. Liquid Hot Water Pretreatment of
417 Cellulosic Biomass. In: Biofuels: Methods and Protcols, J.R. Mielenz, ed., Humana Press,
418 Totowa, NJ; 2009:93-102. doi:10.1007/978-1-60761-214-8_7

419 33. Kim Y, Kreke T, Hendrickson R, Parenti J, Ladisch MR. Fractionation of cellulase and
420 fermentation inhibitors from steam pretreated mixed hardwood. *Bioresour Technol*.
421 2013;135:30-38. doi:10.1016/j.biortech.2012.10.130

422 34. Roche CM, Dibble CJ, Knutsen JS, Stickel JJ, Liberatore MW. Particle concentration and
423 yield stress of biomass slurries during enzymatic hydrolysis at high-solids loadings.
424 *Biotechnol Bioeng*. 2009;104(2):290-300. doi:10.1002/bit.22381

425 35. Palmqvist B, Wiman M, Lidén G. Effect of mixing on enzymatic hydrolysis of steam-
426 pretreated spruce: a quantitative analysis of conversion and power consumption.
427 *Biotechnol Biofuels*. 2011;4(1):10. doi:10.1186/1754-6834-4-10

36. Ghadge RS, Sawant SB, Joshi JB. Enzyme deactivation in a bubble column, a stirred vessel and an inclined plane. *Chem Eng Sci*. 2003;58(23-24):5125-5134. doi:10.1016/j.ces.2003.08.008

37. Müller G, Várnai A, Johansen KS, Eijsink VGH, Horn SJ. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. *Biotechnol Biofuels*. 2015;8(1):187. doi:10.1186/s13068-015-0376-y

38. Zhang J, Chu D, Huang J, Yu Z, Dai G, Bao J. Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor. *Biotechnol Bioeng*. 2009;105(4):718-728. doi:10.1002/bit.22593

39. Zhang X, Qin W, Paice MG, Saddler JN. High consistency enzymatic hydrolysis of hardwood substrates. *Bioresour Technol*. 2009;100(23):5890-5897. doi:10.1016/J.BIORTECH.2009.06.082

40. Ramachandriya KD, Wilkins M, Atiyeh HK, Dunford NT, Hiziroglu S. Effect of high dry solids loading on enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar. *Bioresour Technol*. 2013;147:168-176. doi:10.1016/J.BIORTECH.2013.08.048

41. Viamajala S, McMillan JD, Schell DJ, Elander RT. Rheology of corn stover slurries at high solids concentrations – Effects of saccharification and particle size. *Bioresour Technol*. 2009;100(2):925-934. doi:10.1016/J.BIORTECH.2008.06.070

42. Kumar R, Wyman CE. Strong cellulase inhibition by Mannan polysaccharides in cellulose conversion to sugars. *Biotechnol Bioeng*. 2014;111(7):1341-1353. doi:10.1002/bit.25218

43. Kumar R, Wyman CE. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading

technologies. *Biotechnol Bioeng*. 2009;102(2):457-467. doi:10.1002/bit.22068

44. Zanchetta A, dos Santos ACF, Ximenes E, da Costa Carreira Nunes C, Boscolo M, Gomes E, Ladisch MR. Temperature dependent cellulase adsorption on lignin from sugarcane bagasse. *Bioresour Technol*. 2018;252:143-149. doi:10.1016/J.BIORTECH.2017.12.061

45. Brethauer S, Studer MH, Yang B, Wyman CE. The effect of bovine serum albumin on batch and continuous enzymatic cellulose hydrolysis mixed by stirring or shaking. *Bioresour Technol*. 2011;102(10):6295-6298. doi:10.1016/j.biortech.2011.02.016

46. Florencio C, Badino AC, Farinas CS. Soybean protein as a cost-effective lignin-blocking additive for the saccharification of sugarcane bagasse. *Bioresour Technol*. 2016;221:172-180. doi:10.1016/j.biortech.2016.09.039

47. Schill S. Stover Pellets Pack in the Pounds. *Ethanol Prod Mag*. 2014;20(19).

48. Liu G, Zhang J, Bao J. Cost evaluation of cellulase enzyme for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling. *Bioprocess Biosyst Eng*. 2016;39(1):133-140. doi:10.1007/s00449-015-1497-1

49. Druzhinina IS, Kubicek CP. Genetic engineering of *Trichoderma reesei* cellulases and their production. *Microb Biotechnol*. 2017;10(6):1485-1499. doi:10.1111/1751-7915.12726

50. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng*. 2012;109(4):1083-1087. doi:10.1002/bit.24370

51. Kim Y, Kreke T, Mosier NS, Ladisch MR. Severity factor coefficients for subcritical

liquid hot water pretreatment of hardwood chips. *Biotechnol Bioeng*. 2014;111(2):254-263. doi:10.1002/bit.25009

52. Uniprot Consortium. UniProt: the universal protein knowledgebase. *Nucleic Acids Res*. 2017;45(D1):D158-D169. doi:10.1093/nar/gkw1099

53. Himmel ME, Adney WS, Fox JW, Mitchell DJ, Baker JO. Isolation and characterization of two forms of β-d-glucosidase fromAspergillus niger. *Appl Biochem Biotechnol*. 1993;39-40(1):213-225. doi:10.1007/BF02918991

54. Kittl R, Kracher D, Burgstaller D, Haltrich D, Ludwig R. Production of four Neurospora crassa lytic polysaccharide monooxygenases in Pichia pastoris monitored by a fluorimetric assay. *Biotechnol Biofuels*. 2012;5(1):79. doi:10.1186/1754-6834-5-79

55. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150(1):76-85. doi:10.1016/0003-2697(85)90442-7

56. Ghose TK. Measurement of cellulase activities. *Pure Appl Chem*. 1987;59(2):257-268. doi:10.1351/pac198759020257

57. Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal Chem*. 1959;31(3):426-428. doi:10.1021/ac60147a030

58. Watts RJ, Foget MK, Kong SH, Teel AL. Hydrogen peroxide decomposition in model subsurface systems. *J Hazard Mater*. 1999;69(2):229-243. doi:10.1016/S0304-3894(99)00114-4

59. Derakhshandeh B, Kerekes RJ, Hatzikiriakos SG, Bennington CPJ. Rheology of pulp fibre

suspensions: A critical review. *Chem Eng Sci*. 2011;66(15):3460-3470. doi:10.1016/j.ces.2011.04.017

60. Lavenson DM, Tozzi EJ, Karuna N, Jeoh T, Powell RL, McCarthy MJ. The effect of mixing on the liquefaction and saccharification of cellulosic fibers. *Bioresour Technol*. 2012;111:240-247. doi:10.1016/J.BIORTECH.2012.01.167

61. Cannella D, Hsieh CWC, Felby C, Jørgensen H. Production and effect of aldonic acids during enzymatic hydrolysis of lignocellulose at high dry matter content. *Biotechnol Biofuels*. 2012;5(1):26. doi:10.1186/1754-6834-5-26

62. Ehrhardt MR, Monz TO, Root TW, Connelly RK, Scott CT, Klingenberg DJ. Rheology of Dilute Acid Hydrolyzed Corn Stover at High Solids Concentration. *Appl Biochem Biotechnol*. 2010;160(4):1102-1115. doi:10.1007/s12010-009-8606-z

63. Pimenova N V., Hanley TR. Measurement of Rheological Properties of Corn Stover Suspensions. *Appl Biochem Biotechnol*. 2003;106(1-3):383-392. doi:10.1385/ABAB:106:1-3:383

64. Mezger T. *The Rheology Handbook, 3rd Revised Ed.* 3rd ed. Vincentz Network; 2011.

65. Saptoro A, Herng MTH, Teng ELW. Oxygen transfer to cassava starch solutions in an aerated, well-mixed bioreactor: Experimental and mass transfer studies. *Korean J Chem Eng*. 2014;31(4):650-658. doi:10.1007/s11814-013-0251-0

66. Roche CM, Dibble CJ, Stickel JJ. Laboratory-scale method for enzymatic saccharification of lignocellulosic biomass at high-solids loadings. *Biotechnol Biofuels*. 2009;2(1):28. doi:10.1186/1754-6834-2-28

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514

Figure Captions

Figure 1. (A) % glucan conversion for 50 mL slurry of pretreated corn stover in 250 flasks; with or without shaking (B) % glucan conversion for 600 mL pretreated corn stover slurry in 1 L agitated reactor. Conditions: 72 h, pH 4.8 (50 mM citrate buffer), 3.6 mg (Cellic CTEC2) protein/g dry solids). Columns are averages of triplicates and error bars show 95% confidence interval.

Figure 2. Yield stress as shear rate increases: 200 g solids/L of untreated corn stover compared to estimated impeller speeds in the rheometer and stirred reactor. Error bars indicate 95% confidence interval of triplicate measurements.

Figure 3. Glucan conversions of pretreated corn stover in a 1L bioreactor with N_2 purged mixed reactor compared to air exposed reactions with or without mixing after 72 hours and 50°C. Total volume: 600 mL, at pH 4.8 and impeller speed of 290 RPM. Conditions the same as Figure 1. Columns are averages of triplicates and error bars show 95% confidence interval. Data for experiments with air is the same as shown in Figure 1.

Figure 4. DO in a 1L bioreactor with dual marine impellers (290 RPM) at different solids loadings. Hydrolysis at 50°C, pH 4.8, 3.6 mg (Cellic CTEC2) protein/g dry solids.100% DO, base level equivalent to DI water, saturated with air at 50°C and 290 RPM. Trend lines are the best model fit identified by OriginPro 2020. For 10 and 200 g/L, the model used is the nonlinear Boltzmann equation. For 100 g/L, the model used is a modified Gaussian model.

Supplemental Figure 1. Cellic CTEC2: SDS-PAGE showing MW of 6.5 to 200 kDa. Enzyme components correspond to Uniprot Consortium data (2017).

Supplemental Figure 2. Bioreactor showing impeller configuration with dimensions.

540 Table 1. Deleterious factors of lignocellulose enzymatic hydrolysis.

541

Factor	Solutions	References
Soluble inhibitors	Hot water washing*	Kim et al., 2009, 2013[32,33] McMillan et al., 2011[28] Ximenes et al., 2010b[26]
Insoluble inhibitors	Protein blocking	Kim et al., 2015[4] Ko et al., 2015a, 2015b[5,27] Ximenes et al., 2010a[25] Kumar et al., 2012[3]
Product inhibition	Simultaneous saccharification and fermentation	Jørgensen et al., 2007[19] Kristensen et al., 2009[20] Hong et al., 1981[21] Ladisch et al., 1980[22]
Absence of complete Mixing	Different mixing methods and geometries*	Jørgensen et al., 2007[19] Roche et al., 2009a[34] J. Zhang et al., 2009[38] X. Zhang et al., 2009[39] Geng et al., 2015[16] Ramachandriya et al., 2013[40] Palmqvist et al., 2011[35] Palmqvist and Lidén, 2012[18]
Enzyme deactivation by air/oxygen	Anaerobic conditions* Amphiphilic additives	M. H. Kim et al., 1982[29] Scott et al., 2016[31] Bhagia et al., 2018[30]

542 *indicate methods used in this work.

543

544 Table 2. Composition (%) of pelletized corn stover before and after liquid hot water pretreatment
545 at 190°C, 20 min.

	Untreated Pelletized Corn Stover	**Pretreated Pelletized Corn Stover**
Cellulose	35 ± 2	50 ± 1
Hemicellulose	20.2 ± 1	10 ± 2
Insoluble lignin	16.5 ± 0.5	33.5 ±0.5
Soluble lignin	2.2 ± 0.1	1.7 ± 0.3
Total lignin	18.5 ± 0.5	35.0 ± 0.4
Extractives	15.5 ± 1.5	-
Ash	3 ± 1	5.1 ± 0.1
Total	91 ± 1	99 ± 3

546

Table 3. Summary of previous reports on hydrolysis of pretreated biomass at increasing solids loadings.

Biomass	Pretreatment	Washing?	Enzyme (loading)	Mixing process	Solids loading	Glucan Conversion			Reference
						48 hours	72 hours	96 hours	
Wheat straw	Steam pretreated 80°C for 6 mins followed 195-205°C for 6-12 min	No	Celluclast 15L + Novozyme 188 (7 FPU/g solids)	Horizontal reactor	20 g/L	-	-	87.5%	Jørgensen et al., 2007[19]
					200 g/L	-	-	60%	
					400 g/L	-	-	40%	
Corn stover	Diluted acid 48 mg acid/g dry biomass 190°C, 1 min	Yes	GC220 (20 mg protein/g glucan)	Shaker flask	150 g/L	-	60%	65%	Roche et al., 2009b[66]
					200 g/L	-	40%	45%	
					300 g/L	-	30%	25%	
				Roller bottles	150 g/L	-	80%	85%	
					200 g/L	-	70%	70%	
					300 g/L	-	50%	55%	
Corn stover	Diluted acid 0.8% sulfuric acid 158°C, 10 min	Yes	Cellic CTEC2 + Cellic HTEC2 (5 FPU/g solids, 7.21 mg protein/g solids)	Conical tube rotated in a hybridization reactor with added steel balls	50 g/L	-	-	78.4%	Geng et al., 2015[16]
					100 g/L	-	-	76.3%	
					150 g/L	-	-	73.2%	
Pelletized Corn stover	AFEX 1 kg ammonia/kg dry biomass, > 80°C	No	Cellic CTEC3 + HTEC3 (10 FPU/g glucan)	Incubator shaker (150 RPM)	30 g/L	-	73%	-	Bals et al., 2014[17]
					180 g/L	-	68%	-	

Biomass	Pretreatment	Washing?	Enzyme	Mixing	Solids	Glucan Conversion			Reference

			(loading)	process	loading				
						48 hours	72 hours	96 hours	
Hardwood pulp	Kraft pulped from mill	NA	Celluclast 15L + Novozyme 188 (20 FPU/g glucan)	Peg Mixer	20 g/L	-	100%	100%	X. Zhang et al., 2009[39]
					200 g/L	-	80%	80%	
Eastern redcedar	Diluted acid 3.75% sulfuric acid 20% sodium bisulfite 90°C, 3 hours, 150 RPM followed by 200°C, 10 min	Yes	Accelerase® 1500 (46 FPU/g glucan)	Shaker flasks at 250 RPM with added steel balls	20 g/L	-	-	82.4%	Ramachandriya et al., 2013[40]
					160 g/L	-	-	82.5%	
					200 g/L	-	-	75.2%	
Arundo donax	Steam explosion 2.5% sulfur dioxide 210°C, 5 min	Yes	Cellic CTEC2 (0.1g solution/g solids)	Anchor impeller (controlled power input)	100 g/L	40.8%	-	-	Palmqvist and Lidén, 2012[18]
					150 g/L	35.1%	-	-	
					200 g/L	30.3%	-	-	
Norway spruce					100 g/L	44.0%	-	-	
					150 g/L	34.4%	-	-	
					200 g/L	27.6%	-	-	

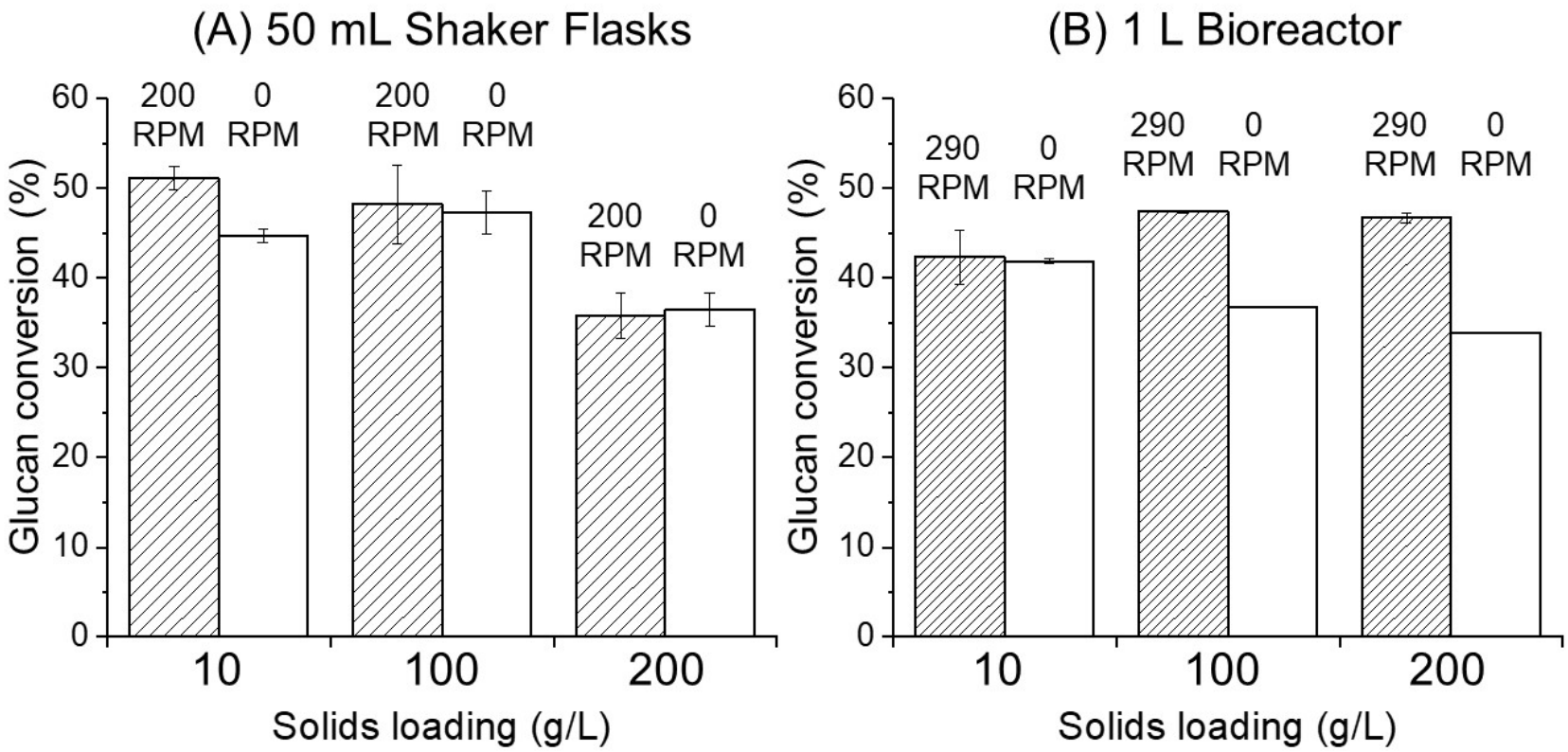

(A) 50 mL Shaker Flasks
(B) 1 L Bioreactor
Glucan conversion (%)
Solids loading (g/L)
200 RPM
0 RPM
290 RPM
0 RPM
10
100
200
0
10
20
30
40
50
60

Figure 1

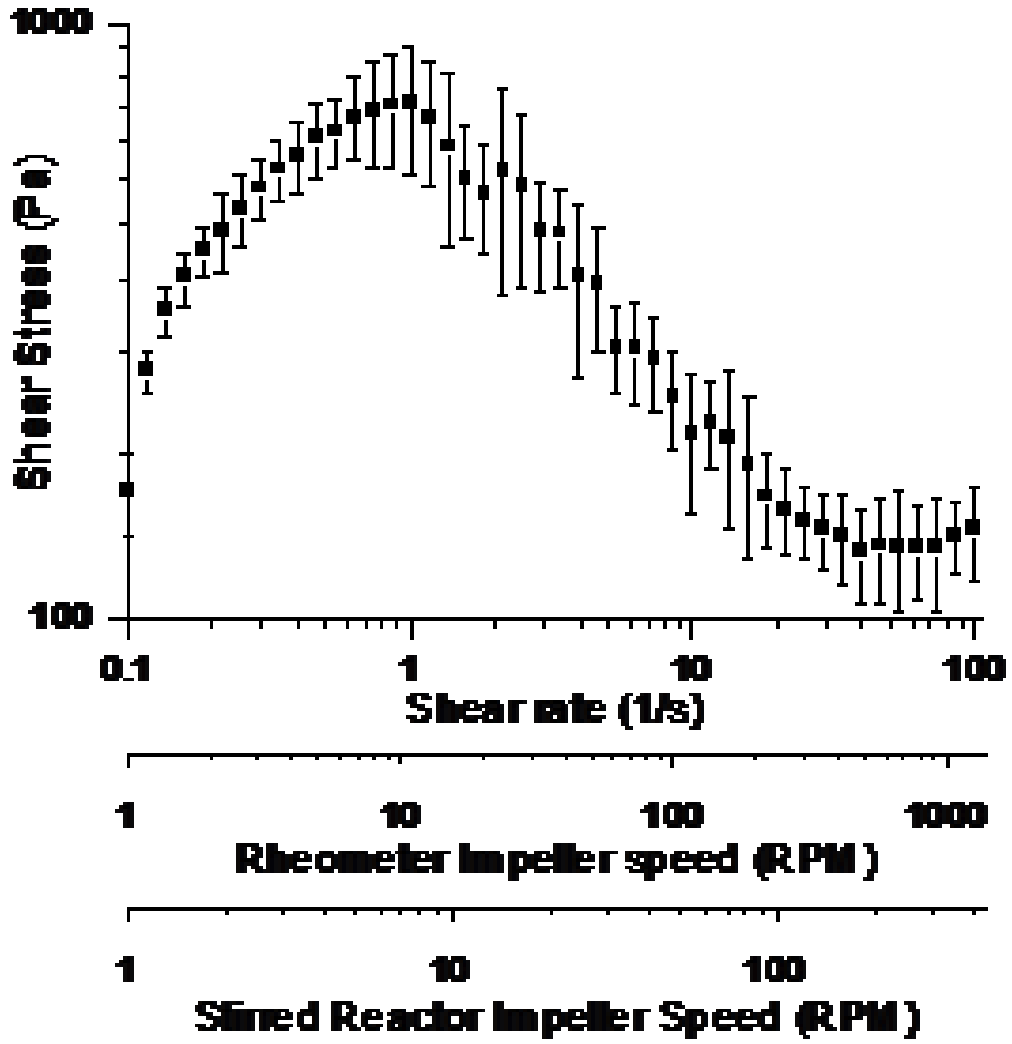

1000
100
Shear Stress (Pa)
0.1
1
10
100
Shear rate (1/s)
1
10
100
1000
Rheometer Impeller speed (RPM)
1
10
100
Stirred Reactor Impeller Speed (RPM)

Figure 2

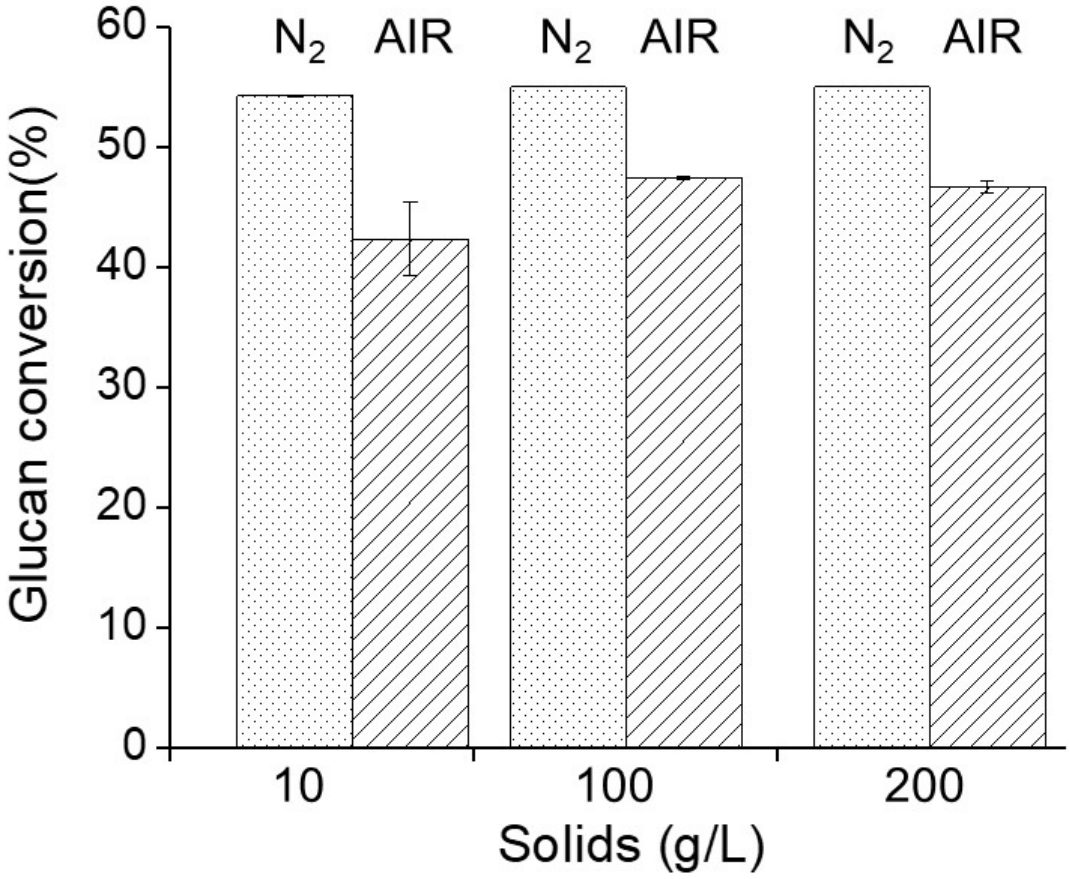

60
50
40
30
20
10
0
Glucan conversion(%)
N2
AIR
N2
AIR
N2
AIR
10
100
200
Solids (g/L)

Figure 3

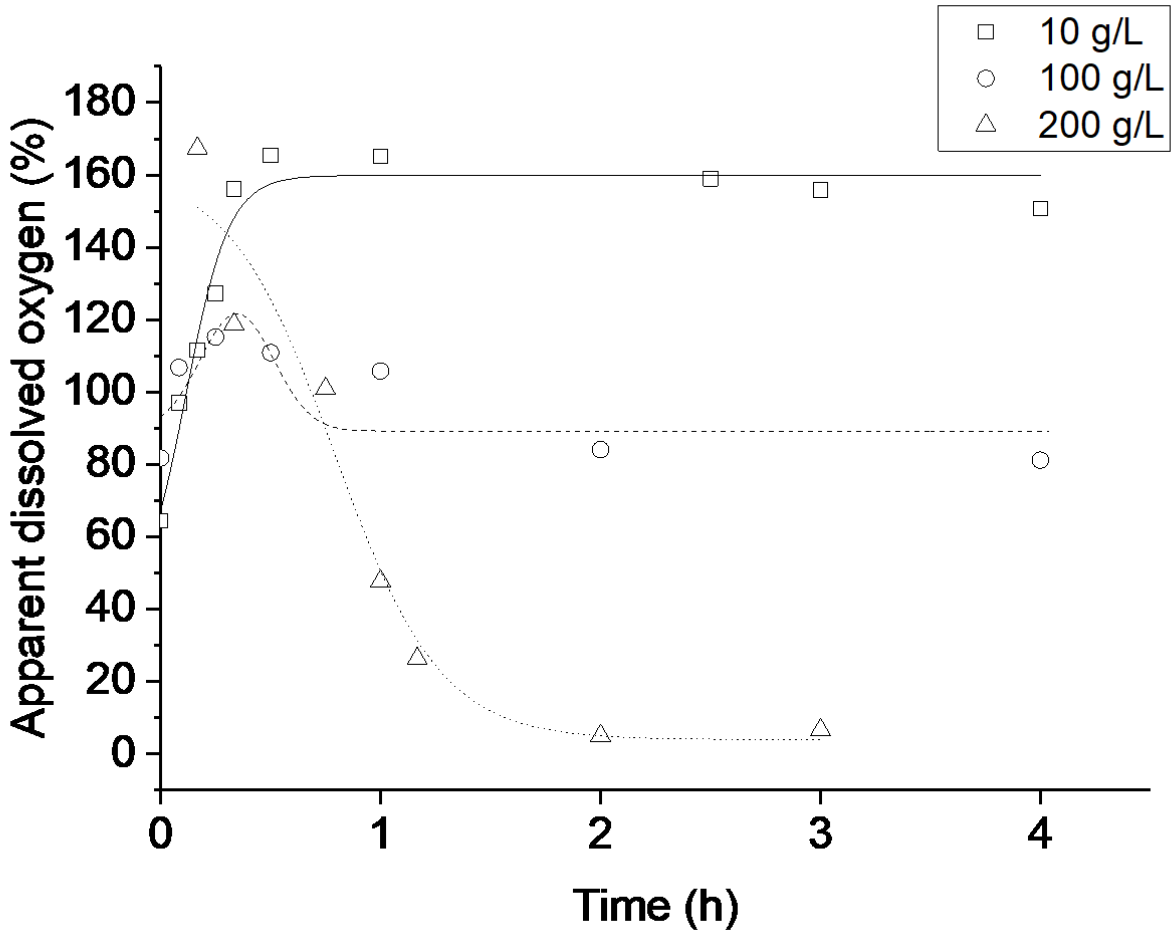
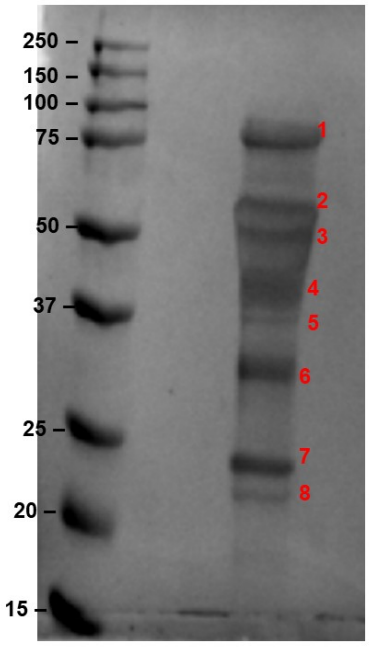
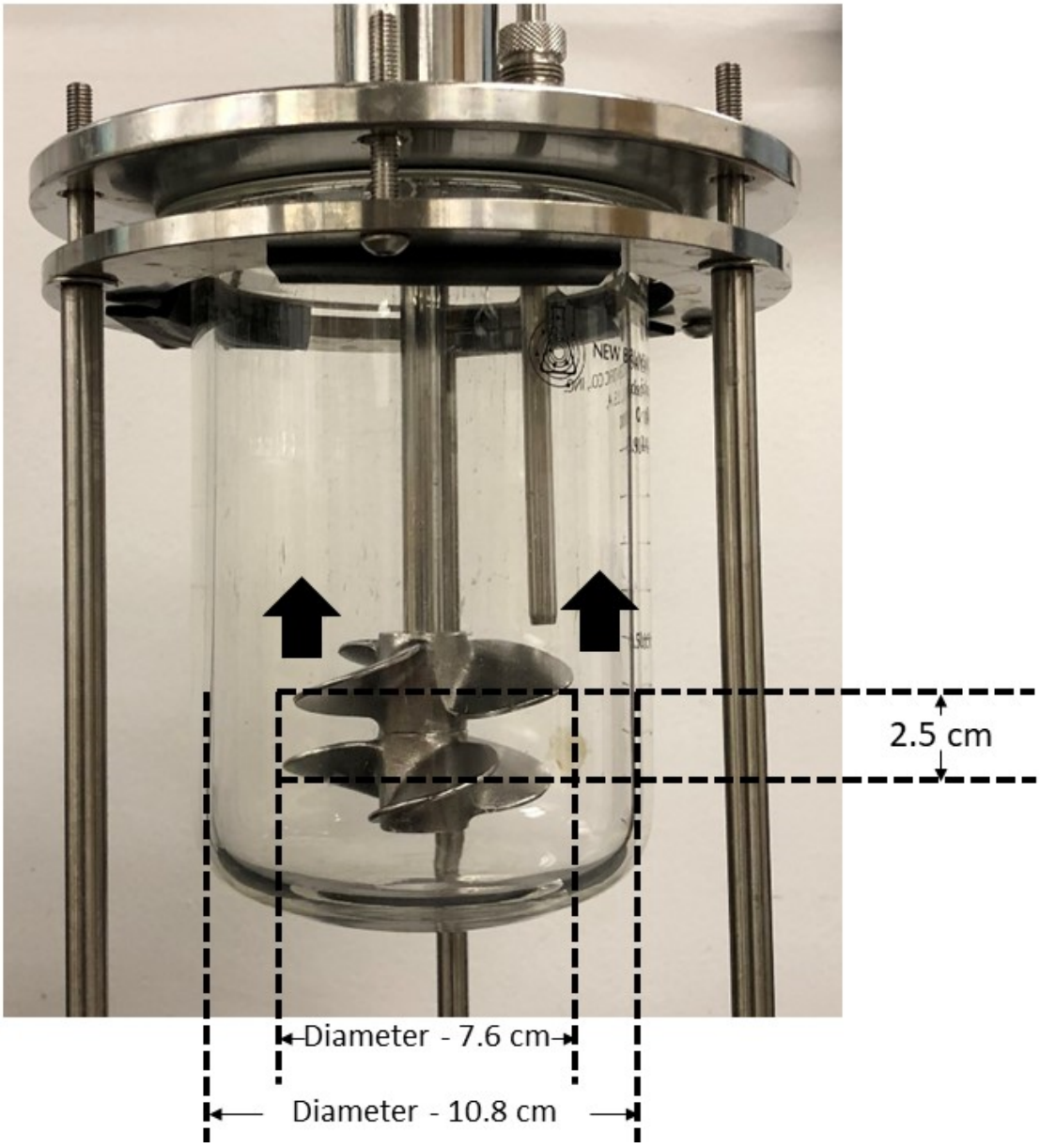

10 g/L
100 g/L
200 g/L
Apparent dissolved oxygen (%)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
Time (h)

Figure 4



Band	Enzyme	Measured activity (U/mL)
1	β-glucosidase	7022 ± 151
2	Exoglucanase	3582 ± 110
3	Exoglucanase	
4	Endoglucanase	976 ± 50
5	Xylanase	2037 ± 20
6	Lytic Polyssacharide Monooxygenase	-
ND	β-xylosidase	166 ± 5

Supplemental Figure 1



Supplemental Figure 2