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The effect of high intensity mixing on the enzymatic hydrolysis of concentrated cellulose fiber suspensions

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ABSTRACT

Enzymatic hydrolysis of lignocellulosic biomass in a high shear environment was examined. The conversion of cellulose to glucose in samples mixed in a torque rheometer producing shear flows similar to those found in twin screw extruders was greater than that of unmixed samples. In addition, there is a synergistic effect of mixing and enzymatic hydrolysis; mixing increases the rate of cellulose conversion while the increased conversion facilitates mixing. The synergy appears to result in part from particle size reduction, which is more significant when hydrolysis occurs during intense mixing.

1. Introduction

An increasing global demand for liquid fuels combined with an appreciation for the consequences of global warming as a result of petroleum-derived fuel use is motivating the search for abundant, more carbon–neutral liquid fuels. Bio-fuels such as ethanol that are produced from renewable feedstocks are desirable as an alternative to petroleum-derived fuels. Lignocellulosic biomass is an abundant, widely available, renewable carbon source and a likely candidate for use in the production of fuel ethanol. Ethanol produced from lignocellulosic biomass is one of a small number of renewable fuels that have the potential to replace a large fraction of the petroleum-derived fuel used globally (Sheehan and Himmel, 1999). The National Renewable Energy Laboratory (NREL) has proposed a process for converting lignocellulosic biomass to ethanol (Aden et al., 2002). The process has tremendous promise but it is currently more costly than ethanol produced from grains and is not yet competitive with the relatively low cost of petroleum-derived fuel.

The economics of the current NREL process can be improved by increasing the solids concentration of biomass in each unit operation. The current process requires approximately 70 wt.% water in the acid-prehydrolysis step and approximately 90 wt.% water in the enzymatic hydrolysis step (Aden et al., 2002). The procedure described for enzymatic hydrolysis is a batch reaction with duration of 3–7 days that is carried out at low insoluble solids content (10–12 wt.%) (Aden et al., 2002). Increasing the solids content, however, creates challenges. At large solids concentrations, the rheology of biomass slurries becomes markedly non-Newtonian, with large apparent viscosities and yield stresses that increase with the volume fraction of insoluble solids (Ehrhardt, 2008; Ehrhardt et al., 2010; Knutsen and Liberatore, 2009; Roche et al., 2009a; Stickel et al., 2009). Mixing biomass slurries becomes more difficult under these conditions, which can make heat transfer less efficient and create mass transfer limitations on various length scales in both dilute-acid prehydrolysis and enzymatic hydrolysis (Carrasco and Roy, 1992; Hodge et al., 2008, 2009).

Enzymatic hydrolysis alters the rheological properties of biomass. Ehrhardt (2008) and Ehrhardt et al. (2010) measured the flow resistance of dilute-acid-hydrolyzed corn stover in a torque rheometer. The resistance decreased with time after the addition of enzymes. Roche et al. (2009a) found that the yield stress of (acid-hydrolyzed) corn stover decreased with time during enzymatic hydrolysis. The decrease in yield stress was largely explained by the decrease in insoluble solids concentration that occurred as the enzymatic hydrolysis proceeded.

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Mixing processes themselves can alter enzymatic hydrolysis reactions. Cavaco-Paulo and Almeida (1994) examined the effect of mechanical agitation on the partial enzymatic hydrolysis of cotton fibers and fabrics. Here, the objective of the hydrolysis reaction was to soften the cotton fibers and fabric, as opposed to completely converting cellulose to glucose. The authors found that mechanical agitation enhanced the rate of hydrolysis (as measured by the fiber weight loss), but did not alter the fiber length. The degree of mechanical agitation was not quantified. Lenting and Warmoeskerken (2001) hypothesized that the role of the mechanical action is to increase the amount of amorphous cellulose, which is more susceptible to enzyme attack than crystalline cellulose. Ingesson et al. (2001) and Roche et al. (2009b) also found an enhancement in the rate of enzymatic hydrolysis of corn stover when samples were mixed. Effects of hydrolysis on particle structure were not discussed. Roche et al. speculated that the hydrolysis rate increased because the enzymes were more uniformly distributed initially when the samples were thoroughly mixed.

In this report, we describe the effects of intense mechanical mixing on the particle size, rheology, and rate of enzymatic hydrolysis of concentrated biomass suspensions. The intense mechanical mixing is achieved with a torque rheometer. Biomass materials investigated include dilute-acid-hydrolyzed corn stover, as well as suspensions of cotton fibers. The cotton fiber suspensions represent model biomass systems with monomodal fiber length distributions. This model biomass system is employed so that effects of intense mixing and enzymatic hydrolysis on fiber length can be quantified. We find that both mechanical mixing and enzymatic hydrolysis tend to reduce the average fiber length. In addition, there is a synergistic effect in that mixing increases the rate of enzymatic hydrolysis, while hydrolysis reduces biomass viscosity and facilitates mixing.

2. Methods

Whatman #1 filter paper was used as a model biomass for this study. This filter paper was used because it has a monomodal fiber length distribution and has been used in previous studies to determine enzyme activity (Adney and Baker, 1996). The fibers are cotton linters with a cellulose content of approximately 99 wt.% and a weight-average fiber length of 0.60 mm as measured with a Kajaani FS-100 fiber length analyzer. The biomass was prepared by forming an aqueous slurry of dispersed fibers from disintegrated Whatman filter paper and then forming a wet pad from the slurry using vacuum filtration. Pads were washed in pH 4.8 citrate buffer and the final solids concentration of the formed pad was determined by measuring weight loss upon drying for 1 h at 150 °C.

Ensiled corn stover was hammer milled to obtain samples with particle sizes on the order of 3 cm. The milled stover was dilute-acid hydrolyzed according to NREL Laboratory Analytical Procedure 007 (LAP 007) (Hsu, 1995). The reaction was carried out with a sulfuric acid concentration of 1.0 wt.%, a total solids content of 10 wt.% and an effective reaction temperature (Ehrhardt et al., 2010) of 160 °C for 30 min (Ehrhardt, 2008). Reaction products were neutralized with $NaHCO_3$ and then vacuum washed and filtered. The cellulose content of the acid-hydrolyzed stover was assumed to be 50% by weight (Zhu et al., 2009).

We note that because both the cotton and acid-hydrolyzed corn stover were washed thoroughly prior to mixing and enzymatic hydrolysis experiments, the fraction of soluble solids in these materials was 0 wt.% (within experimental uncertainty).

Enzymatic hydrolysis was carried out using Celluclast (Novozymes Corp.). This cellulase was produced from *Trichoderma reesei* and has an activity of 50 FPU/mL as measured following the NREL enzyme activity assay protocol (Adney and Baker, 1996). Additional cellobiase (Novozymes Corp.) produced from *Aspergillus niger* was added to maximize conversion of all cellobiose reaction products to glucose. Cellulase was added in the amount of 10 FPU/g cellulose. The two enzyme mixtures were added in a volumetric ratio of 4:1 cellulase to cellobiase. All cellulase solutions were added in a pH 4.8 citrate buffer.

The mixing apparatus was a modified torque rheometer (Bousmina et al., 1999; Goodrich and Porter, 1967; Scott and Zauscher, 1997) with counter-rotating screw elements. The modifications include a machined channel for temperature control via hot and cold water feeds, as well as a clear polycarbonate cover for flow visualization. One of the mixing elements is driven by a shaft connected to a motor. The other is connected to the drive shaft through a gear box and rotates at 2/3 the speed of the first. The volume of the mixing chamber is 100 mL. The torque is measured using a magnetoelastic sleeve torque transducer on the motor drive shaft. The uncertainty of the measurement is 0.056 N m (0.5 in lb), with a maximum reading capability of 11.3 N m (100 in lb). Data were collected at 5 Hz and averaged over 4 s intervals. The sample temperature was measured with a thermocouple positioned at the top of the mixing chamber between the screw elements. The temperature was maintained at 55 ± 2 °C for all experiments.

To quantify the effects of mixing during enzymatic hydrolysis, three types of experiment were performed. In the first type of experiment, biomass was aggressively mixed with enzymes and samples were extracted at prescribed time intervals for further analysis. Different mixing speeds were used to determine the effect of mixing intensity on the hydrolysis reaction. In the second type of experiment, biomass samples were enzymatically hydrolyzed without mixing. In the third type of experiment, biomass was exposed to the same mixing history as in the first type of experiment but with no added enzyme. The pH was confirmed to be 4.8 before and after all enzymatic hydrolysis reactions. Mixing torque and cellulose conversion were measured in each experiment. For experiments with the model system of cotton fibers, the evolution of the average fiber length was also measured.

Mixing during enzymatic hydrolysis was carried out in the torque rheometer as described above. All experiments were run with the rheometer chamber half full (50 mL) and with an initial biomass concentration of 20 wt.% insoluble solids. The desired mass of dry biomass solids (at a known concentration greater than 20 wt.% solids) was first prepared. The amount of reverse osmosis (RO) water required to obtain a 50 mL sample at 20 wt.% insoluble solids was added to the rheometer chamber. The biomass was then fed to the rheometer with the impellers rotating at 55 rpm over a period of 60 s. The material was mixed for an additional 30 s to homogenize the mixture. For experiments undergoing hydrolysis, enzyme was added at this point. Samples of approximately 0.5 g were extracted from the rheometer at each of the following times: 300 s (210 s after enzyme addition), 600, 900, 1200, 1800, and 2400 s. This study focused on the dynamic behavior of cellulose fiber suspensions during mixed enzymatic hydrolysis. Reaction times longer than 2400 s when the torque response was no longer a function of time were not studied.

Experiments with enzyme and no mixing were carried out in a 500 mL beaker with 20 wt.% solids biomass. The beaker was held in a water bath at 55 °C. A pad of biomass was pressed to a high solids content (~45–50 wt.%) using a hydraulic press. The pad was cut to fit in the bottom of the beaker. The appropriate amount of RO water and enzyme needed to achieve 20 wt.% solids and the same reaction conditions as in the experiments with mixing were added to the heated beaker first. After the liquid temperature reached 55 °C, the biomass pad was added to the bottom of the beaker. When the pad was added, the solution (water plus enzymes) appeared to absorb completely into the pad, making the pad appear uniformly wetted. However, we note that the degree to which

the enzymes were distributed throughout the pad is unknown. Samples were removed from the beaker at the same time intervals employed in the experiments with mixing (the first sample was extracted 210 s after enzyme addition).

Samples extracted during the enzymatic hydrolysis reactions were placed in test tubes containing approximately 6 mL $NaHCO_3$ buffer solution at a pH of 10.5. Cellulase enzymes are rendered inactive at such a high pH, effectively stopping the hydrolysis reaction (Novozymes, 2008). The efficacy of this technique was confirmed by incubating a cellulose/enzyme mixture in the buffer solution at 50 °C for 48 h and measuring glucose concentrations before and after. No cellulase activity was found. The buffer solution also had no effect on the glucose concentrations measured using a glucose analyzer. Glucose concentrations measured for a 50/50 mixture of RO water and a glucose standard and for a 50/50 mixture of $NaHCO_3$ buffer solution and the same glucose standard were equivalent.

Fiber length distributions for all model biomass samples were measured using a Kajaani FS-100 fiber length analyzer. The uncertainty in the measure of weight-average fiber length was 0.02 mm estimated from multiple measurements with the same sample. Due to the wide range of particle sizes in the corn stover samples, fiber length measurements with these materials using the Kajaani fiber length analyzer were not possible. Particle sizes in corn stover samples were examined qualitatively using scanning electron microscopy (SEM). SEM samples were prepared by depositing 0.2 mL of diluted sample (1.5 wt.% solids) onto a 10 mm diameter SEM sample disk. The samples were allowed to air-dry for 48 h before being gold plated.

Cellulose conversion was quantified by measuring the glucose concentration in the samples before and after enzymatic hydrolysis, using a YSI 2700 Select Biochemistry Analyzer (YSI Life Sciences). Following the same calculations as those presented in work by Kristensen et al. (2009), cellulose conversion was defined as the ratio of the measured glucose amount and the theoretical yield of glucose at 100% conversion. The laborious corrections that can be required for determining cellulose conversion at high solids concentrations were avoided by diluting extracted samples by more than 10 times (Kristensen et al., 2009). Duplicate measurements of glucose concentration were made for each sample and the two readings were averaged. The analyzer was calibrated after every five measurements.

3. Results and discussion

3.1. Model biomass

The torque required to mix model biomass systems of cotton fibers with and without enzyme is plotted as a function of time in Fig. 1a. The torque increases rapidly during the initial 60 s loading period after which it begins to decrease. At 90 s, enzyme was added for the hydrolysis runs. At approximately 300 s, the torque required for mixing biomass containing enzyme begins to decrease below that required for biomass containing no enzyme. By 900 s, the torque for the biomass containing enzyme decreases below the sensitivity of the torque transducer (although mixing continues).

The source of noise in the data shown in Fig. 1a is a product of both the material properties and of the geometry of the rheometer. The offset rotation rate and irregular geometry of the mixing elements result in variable mixing element positions over three consecutive rotations. Although the variations in torque appear to be noise, results from duplicate experiment are repeatable within the 0.11 N m sensitivity of the transducer. There is a notable decrease in torque variations that occurs over the first 300 s of mixing for the experiments containing enzyme. This behavior corresponds with a rapid change in sample texture over that same time period. By 900 s the biomass undergoing hydrolysis resembles a homogeneous paste with a texture comparable to toothpaste.

Both the forces exerted on the fibers during mixing and the action of the enzymes can alter the fibers. The weight-average fiber length of the model biomass systems is plotted as a function of time for all three types of experiment in Fig. 2. Samples exposed to enzyme but with no mixing exhibit little if any change in fiber length over the 2400 s studied. Samples that are mixed in the absence of enzyme exhibit a decrease in fiber length over the first 600 s, after which the length no longer changes with time. Samples that are mixed in the presence of enzyme exhibit a more significant decrease in fiber length, which continues over the duration of the experiment. The decrease in fiber length of the mixed biomass exposed to enzyme is concurrent with the decrease in torque required for mixing shown in Fig. 1a. Since the stresses generated within a fiber suspension are a function of the fiber lengths (Dalpke and Kerekes, 2005), this behavior suggests that the decrease in fiber length is a major contributor to the decrease in viscosity observed during mixed enzymatic hydrolysis.

Evidence for how the enzymes are accelerating fiber size reduction during mixing is apparent in Fig. 2. Mixing in the presence of enzymes reduces average fiber size to less than half the starting length. On the other hand, enzyme activity without mixing does not cause a significant decrease in fiber length. It is likely that the enzymes are damaging the outer surfaces of fibers without hydrolyzing through entire fibers on the time scale of the experiments in this study. Cavaco-Paulo and Almeida (1994) produced SEM images of fiber surface damage caused by enzymatic hydrolysis. The authors found surface fissures in cotton fiber samples exposed to enzyme that were not present in virgin fibers. Damage of this type, which would leave fibers more susceptible

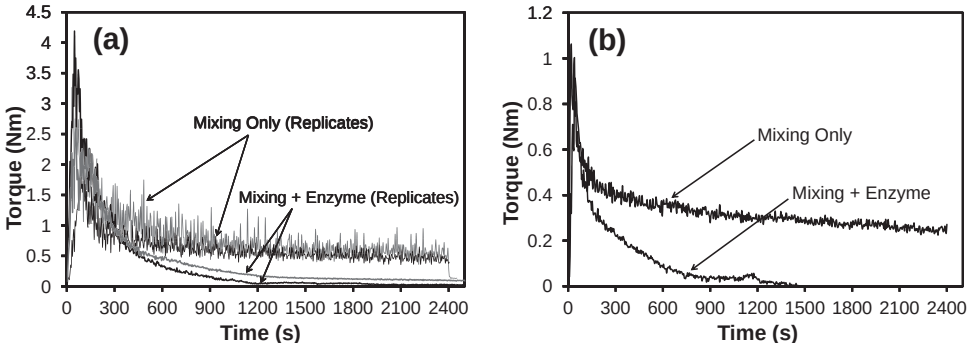

Fig. 1. The torque required for mixing model biomass samples consisting of concentrated cotton fiber suspensions (20 wt.% solids) as a function of time. Replicate results for samples with and without added enzyme are shown (a). The torque required for mixing concentrated corn stover samples (20 wt.% solids) as a function of time (b). Mixing speed: 55 rpm.

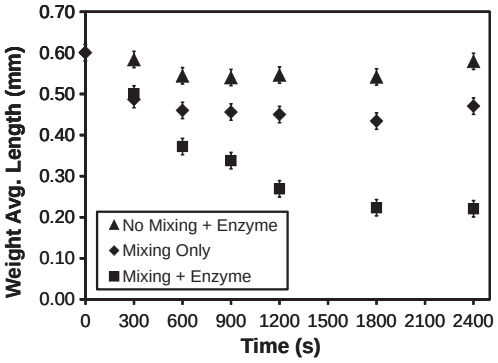

Fig. 2. Weight-average fiber length for model biomass samples as a function of time in the three different types of experiment. Mixing speed: 55 rpm.

to shear-induced breakage, could explain the decrease in the weight-average fiber length of samples that are mixed in the presence of enzymes.

SEM images of the model biomass samples were used to qualitatively evaluate changes in fiber morphology and to confirm the results of Kajaani fiber length analysis. The images are included with the online version of the article as supplementary material. The image of the sample exposed to mixing with enzyme contains many fibers with lengths on the order of 0.2 mm. The relative fiber lengths observed in these SEM images are consistent with the quantitative results obtained from Kajaani analysis. The Kajaani results also indicate that samples mixed in the absence of enzymes have a shorter weight-average fiber length than unmixed samples in the absence of enzymes. Although the fiber length distributions of these two samples are different, it is difficult to reach that conclusion from a comparison of their SEM images. Even in a model biomass sample with a relatively uniform fiber composition, the use of a single analysis technique such as SEM to quantify particle size and structure is insufficient. In complex fibrous biomass samples that cannot be characterized solely with a fiber length distribution, better particle size analysis techniques are required.

Since glucose monomer is the major product of the enzymatic hydrolysis of pure cellulose, the measured glucose concentrations in the samples are used to determine the cellulose conversion, as seen in Fig. 3a. Samples mixed in the absence of enzyme contain no glucose and are not shown. The mixed sample exposed to enzyme for 1800 s has a 50% greater cellulose conversion than the corresponding unmixed sample exposed to enzyme. This trend suggests that improved conversion with mixing may continue beyond the time examined here.

More rapid conversion in mixed samples may be caused by several mechanisms: reduction of mass transfer limitations, improved dispersion of the enzyme initially throughout the biomass, or an increase in surface area of the cellulose substrate arising from the fiber length decrease. Accelerated cellulose conversion due to mixing occurs in this study, by whatever mechanism, while mixing is made easier due to the activity of cellulase enzymes. This synergy suggests that mechanical mixing of concentrated biomass suspensions during enzymatic hydrolysis may be exploited to simultaneously accelerate cellulose conversion to glucose and reduce mixing power requirements.

It is unclear if mechanical mixing should be performed continuously, or on an intermittent schedule of mixing periods of perhaps varying intensity. Determining optimum schedules for mixing is a topic for future research.

The effects of mixing speed on the fiber length and cellulose conversion in model biomass systems exposed to enzyme are shown in Fig. 4 for two reaction times. The cellulose conversion is not a function of mixing speed over the range of speeds examined. Data at slower speeds were not obtained since the mixing device stalled below 25 rpm. Weight-average fiber length in samples with and without enzyme is also not a function of mixing speed over the range of speeds examined. The data presented is representative of the behavior observed at other sampling times. This result indicates that high speed mixing is not necessary to obtain more rapid cellulose conversion. The most beneficial improvements in cellulose conversion and torque reduction were observed at the lowest mixing speed. The most important consequence of this discovery is that high solids enzymatic hydrolysis carried out in a mixed bioreactor would be optimally effective at the lowest speeds where the least amount of energy input would be required. We note however, that the mechanical work exerted on the biomass depends not only on the speed but also on the mixer geometry as well as the biomass composition. The optimum mixing speeds for other materials and geometries must therefore be determined independently.

3.2. Acid-hydrolyzed corn stover

The behavior of dilute-acid-hydrolyzed corn stover is similar to that of the model biomass system of cotton fibers. Torque and cellulose conversion as a function of time for corn stover samples are shown in Figs. 1b and 3b, respectively. The torque decreases with mixing time for all samples but the torque decreases faster for the biomass with enzyme added. Cellulose conversion increases for all samples exposed to enzyme but the rate is higher for the mixed systems. Both of these trends are observed in the model biomass system.

Qualitative changes in particle morphology that occur during each type of experiment were observed in SEM images of corn stover samples. These images are also included with the online version of the article as supplementary material. Prior to mixing or

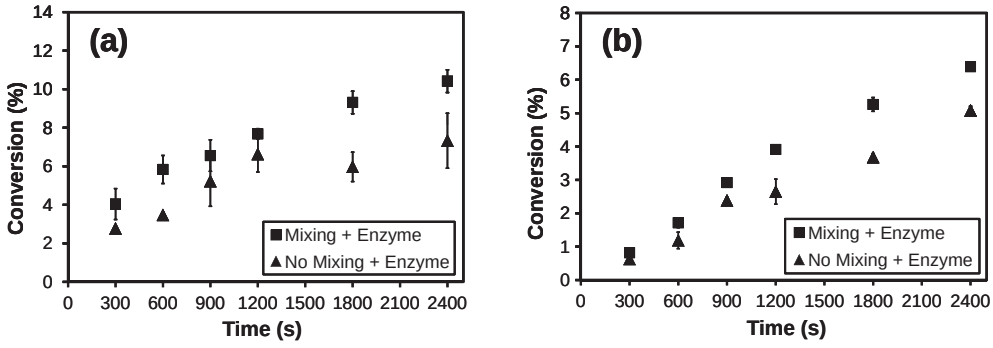

Fig. 3. Cellulose conversion for mixed and unmixed model biomass samples exposed to enzyme as a function of time. Note: a conversion of 10% corresponds with 0.099 g of glucose released for every 1 g of starting solid material. (a) Cellulose conversion for mixed and unmixed corn stover samples exposed to enzyme as a function of time. Note: a conversion of 10% corresponds with 0.05 g of glucose released for every 1 g of starting solid material. (b) Mixing speed: 55 rpm.

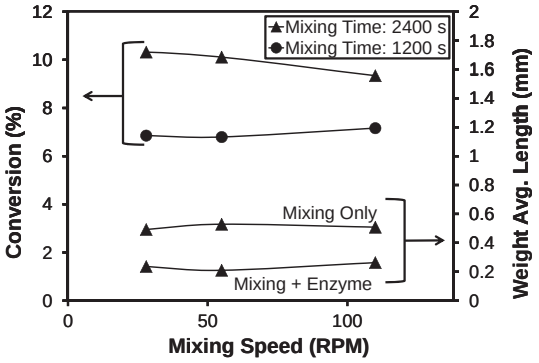

Fig. 4. Cellulose conversion and weight-average fiber length of model biomass samples at different times as a function of mixing speed.

enzymatic hydrolysis, dilute-acid-hydrolyzed corn stover contains a variety of anatomical components that vary in size, shape and aspect ratio. Enzymatic hydrolysis without mixing has little effect on the particle morphology over the duration of reaction times used here. Mixing without enzyme results in a more homogeneous material composed of small diameter, individual fibers with few of the larger structures still present. Mixing in combination with enzymatic hydrolysis results in an even more homogeneous material composed of very short individual fibers and a small number of larger, more complex structures. Major differences in the structure of the model biomass and the structure of the corn stover exist. The cotton linters used as a model biomass are far less complicated structurally than the corn stover which contains cell walls of vascular and structural tissues in a complex arrangement. Although the changes in particle morphology occurring during mixing and enzymatic hydrolysis in stover samples are complicated, it is apparent that the combination of mixing and hydrolysis significantly reduces individual fiber lengths in corn stover samples.

3.3. Effect of mixing on enzyme stability

Shear-induced enzyme deactivation may occur when enzymes are exposed to a shear-intensive environment. A number of publications have found that very high shear rates and shear imposed for long durations have the ability to inhibit or deactivate enzymes in solution (Kaya et al., 1994, 1996; Kim et al., 1982). The improved conversions seen in this study during mixing indicate that any enzyme inhibition or deactivation that might be present during the trials as a result of mixing is outweighed by the benefits. To consider the effect of the shear used on the long term activity of the cellulase enzyme, samples of enzymatically hydrolyzed corn stover (both mixed and unmixed) were incubated with enzyme for an additional 48 h without mixing (mixed samples were removed from the high-shear environment and allowed to react in unmixed flasks). The extent of conversion for the mixed and unmixed samples was 38 ± 3% and 33 ± 3%, respectively. We note that these conversions compare favorably with those reported by Roche et al. (2009b), who obtained approximately 33% conversion for unmixed corn stover samples at an enzyme loading of 12 FPU/g cellulose (20 wt.% insoluble solids starting material). The mixing used in this study does not deactivate the enzymes or render them less active. Kaya et al. (1994) calculated effective shear rates or "G-factors" in their study of cellulase binding and deactivation in high shear fields. The effective shear rates where they observed cellulase un-binding and deactivation occurring are more than double those calculated for the mixing used here at the highest mixing speed of 110 rpm.

4. Conclusions

The results of this study indicate a synergistic relationship between mixing and enzyme activity during enzymatic hydrolysis: the rate of cellulose conversion is improved while mixing is made easier due to particle size reduction. Increased cellulose conversion rates for mixed systems compared to unmixed systems may be caused by reduction of mass transfer limitations, improved distribution of enzyme, or increased particle surface area that results from particle size reduction (or a combination thereof). The synergy between mixing and the action of enzymes suggests that aggressive mixing of concentrated biomass suspensions during enzymatic hydrolysis may be beneficial.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.11.117.

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