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Influence of Mixing Regime on Enzymatic Saccharification of Steam-Exploded Softwood Chips

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Abstract

In an attempt to elucidate the effect of reduced mixing on the enzymatic hydrolysis of lignocellulosic feedstocks, a pretreated softwood substrate was hydrolyzed under various mixing regimes using a commercial cellulase mixture. The substrate was generated by SO_2-catalyzed steam explosion of Douglas fir wood chips followed by alkali-peroxide treatment to remove lignin. Three mixing regimes were tested; continuous mixing at low (25 rpm) and high (150 rpm) speeds, and mixing at low-speed interspersed with 5-min intervals of high-speed agitation at 150 rpm. At both substrate concentrations (7.5 and 10% [w/w]), the mixed-speed mixing was able to produce sufficiently high conversion rates and yields (93% after 96 h), close or slightly better than those obtained under vigorous mixing (150 rpm). The low-speed shaking produced appreciably lower conversion yields at both levels of substrate concentration. Therefore, the mixed-speed regime may be a viable process option, because it does not seem to have an adverse impact on the cellulose conversion yield and can be an effective means of reducing the mixing energy requirements of an enzymatic hydrolysis process.

Index Entries: Enzymatic hydrolysis; mixing; softwoods; Douglas fir; bioconversion.

Introduction

In the bioconversion of lignocellulosic feedstocks to fuel-grade alcohol, enzymatic hydrolysis can be used to convert solid cellulose to a

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readily fermentable stream of glucose monomers. In comparison with acid-catalyzed processes, enzymatic saccharification offers definite advantages by avoiding glucose degradation that can easily occur in acidic environments, and by eliminating the need for expensive, corrosion-resistant equipment required for the acid-based operations *(1)*. However, enzymes, in addition to being costly, act in a much slower fashion than acids in hydrolyzing cellulose and, therefore, require relatively long residence times. Research is being conducted worldwide to reduce the operational cost of an enzymatic hydrolysis unit.

Mixing is an important process design factor that can influence the hydrolysis operation in several ways. Considering the heterogeneity of the hydrolysis reaction environment, in which a liquid enzyme acts on a solid substrate, adequate mixing is required to ensure sufficient contact between the reactants, as well as to promote heat and mass transfer within the reaction vessel. Moreover, it has been shown that excessive mixing can deactivate the enzyme and reduce the conversion yield, owing to the shear force generated by the mixer and the entrapment of air bubbles into the medium at the air-liquid surface *(2,3)*. For example, Mukataka et al. *(3)* showed that excessively high mixing speeds (>200 rpm) lowered the extent of cellulose conversion (Avicel and paper pulp) while moderate mixing speeds (100–200 rpm) provided a good combination of fast initial hydro-lysis rates and high conversion yields. Studies conducted by other research-ers *(4)* showed that while mixing speeds (paddle mixer) as high as 340 rpm enhanced the conversion of steam-pretreated spruce wood, higher mixing speeds (340–510 rpm) only increased the initial rate of hydrolysis and not the final conversion yield. Therefore, one way of improving the economy of the overall process is to determine the optimum level of mixing, so as to reduce the extent of shear-induced enzyme deactivation and lower the mixing energy costs.

We as well as others have shown that low-speed mixing regimes interspersed with short intervals of high-speed agitation could achieve comparable sugar yields during the hydrolysis of cellulose *(5,6)*. Such mixed-speed regimes can reduce the energy requirements of the hydrolysis operation and avoid the denaturation of enzyme caused by high-shear mixing, while allowing acceptable levels of substrate conversion. In the present study, we assessed the effectiveness of a mixed-speed mixing regime, in comparison with continuous low- and high-speed mixing, for enzymatic saccharification of a softwood-derived substrate, as a potential feedstock for a wood-to-ethanol bioconversion process.

Materials and Methods

Substrate

The substrate used was steam-exploded Douglas fir (*Pseudotsuga menziesii*) wood chips. The SO_2-catalyzed steam explosion of the Douglas

fir wood chips was conducted at medium severity conditions (4.5 min, 195°C, 4.5% SO_2 of original dry wood) as described previously *(7)*. The steam-exploded wood chips were further posttreated with an alkaline peroxide solution as described in the next section. The chemical composition of the softwood substrate was determined using a scaled-down version of the Klason protocol of TAPPI Standard method T222 om-88. Briefly, 0.2 g of ground sample (40 mesh) was incubated at 20°C with 3 mL of 72% H_2SO_4 for 2 h with mixing every 10 min. The reaction was then diluted using 112 mL of deionized water. The solution was next autoclaved at 121°C for 1 h and filtered through a medium coarseness sintered glass filter, for the gravimetric determination of acid-insoluble lignin. The concentration of hexose and pentose sugars in the filtrate was determined using a high-performance liquid chromatography (HPLC)-based sugar analysis assay.

Posttreatment

The steam-exploded wood chips, which had been washed with 30-fold (v/w) water, were treated with a 1% (w/w) hydrogen peroxide solution at 2% (dry w/v) substrate concentration. The pH of this solution was adjusted to 11.5 by adding sodium hydroxide, and the posttreatment flasks were incubated in a mixing water bath at 80°C for 45 min. The posttreated substrate was then washed with ample amounts of water (20-fold [v/w]).

Enzymes

Hydrolysis experiments were performed using a complete *Trichoderma reesei* cellulase system (Celluclast) in combination with a commercial β-glucosidase (Novozyme 188) from Novo-Nordisk, Denmark. The Celluclast contained 49 mg of protein/mL as measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA), and contained the following hydrolytic activities: 80 filler paper units (FPU)/mL of filter paper activity, 52 IU/mL of carboxymethylcellulase (CMCase), and 50 IU/mL of β-glucosidase. The protein content and activities of Novozym 188 were as follows: 44 mg/mL, 5 FPU/mL, 34 IU/mL of CMCase, and 500 IU/mL β-glucosidase. All enzyme activities were measured using standard procedures described in ref. *8*.

Enzymatic Hydrolysis

Hydrolysis experiments were conducted in duplicate in 300-mL flasks with a total reaction volume of 50 mL at two substrate concentrations of 7.5 and 10% (w/v). In all experiments, the 50 m*M* citrate buffer (pH 4.8) was supplemented with 40 µg/mL of tetracycline and 30 µg/mL of cycloheximide to prevent microbial contamination. The enzyme loading was 25 FPU/g of cellulose, supplemented with β-glucosidase (β-G IU:FPU = 4:1) to prevent product inhibition by cellobiose. Aliquots of 300 µL taken at various time intervals from the supernatant were immediately chilled on ice and centrifuged at 500 rpm for 10 min and stored for sugar and protein analysis,

prior to which the diluted samples were boiled for 5 min to inactivate the enzyme. The conversion yield at any time point was determined as follows:

$$\text{Conversion yield (\%)} = (\text{g glucose released/g glucose in original substrate}) \times 100$$

Mixing Regimes

The hydrolysis flasks were mixed using a gyratory shaking bath at 45°C for 96 h either by continuous shaking at 25 or 150 rpm or by using the following mixed-speed shaking pattern: During the first 12 h, all the flasks were shaken continuously at 25 rpm and received 5 min of high-speed agitation at 150 rpm every 2 h. After the first 12 h, at which point the mixture viscosity had decreased significantly and less frequent high-speed mixing seemed necessary, the 5 min, high-speed mixing intervals were applied only every 12 h.

Analytical Methods

The total protein content in enzyme adsorption experiments was measured using Bio-Rad Protein Assay (Bio-Rad) with bovine serum albumin as standard. The unadsorbed protein in the samples was quantified using an automatic plate reader (Molecular Devices, Menlo Park, CA), which was then subtracted from the total protein added to each tube to determine the amount of substrate-bound protein. The amount of glucose released during hydrolysis was analyzed using a YSI 2700 Select Biochemistry Analyzer (YSI, Yellow Springs, OH). Since the presence of mixed sugars in the samples could compromise the accuracy of this procedure, an HPLC system was initially used to identify the type and concentration of different sugars. No appreciable level of any sugar other than glucose was detected, therefore, the YSI system was found suitable for glucose analysis.

The HPLC system (Dionex DX-300) was equipped with an ion-exchange PA1 column (Dionex) and a gold electrode for pulsed amperometric detection. Prior to injection, samples were filtered through 0.45-µm HV filters (Millipore, Bedford, MA) and a volume of 20 µL was injected. The column was equilibrated with 250 m*M* NaOH and eluted with deionized water at a flow rate of 1 mL/min.

Results and Discussion

The substrate used for the hydrolysis studies was shown to be primarily composed of cellulose, since glucose constituted the majority of the sugars in the substrate (88%), and the total amount of all other sugars (mannose, galactose, and xylose) was only 1% (Table 1). In addition, it was apparent that the alkaline peroxide posttreatment effectively removed the majority of lignin from the steam-exploded wood chips, reducing the lignin content from 38 to 5%. It is probable that extractives, some residual soluble lignin and ash make up the balance of the composition.

Table 1
Chemical Composition of Delignified, Steam-Exploded Douglas Fir as Determined by Klason Lignin Method

Component	%
Acid-insoluble lignin	5.10
Glucose	88.80
Mannose	0.60
Galactose	0.04
Xylose	0.40
Arabinose	ND[a]
Total	94.9

[a] Not detected.

We hydrolyzed the delignified, steam-exploded wood chips under two relatively high substrate concentrations: 7.5 and 10% (w/v). The conversion yields were, in general, higher when a lower solid concentration was used (Figs. 1, 2, and Table 2).

At a consistency of 7.5%, near complete hydrolysis was achieved after 96 h under all three mixing regimes. The mixed-speed regime consisted of continuous mixing at 25 rpm with periodic, high-speed agitation at 150 rpm for 5 min. These high-speed pulses were applied every 2 h during the first 12 h, and only once every 12 h after the initial 12 h. At a consistency of 7.5%, the initial reaction rates and conversion yields for the mixed-speed and continuous mixing regimes were almost identical (Fig. 1). Therefore, it was apparent that the use of sporadic intervals of fast mixing could be effective in enhancing the substrate-enzyme interactions and in avoiding local accumulation of reaction products (glucose and cellobiose) within a high-solid reaction mixture. We also observed that mixing at 25 rpm did not appear to be a vigorous enough treatment to produce sufficient contact between the substrate and the enzyme. Consequently, it resulted in conversion yields that were 5–15% lower than those obtained under high- and mixed-speed mixing, depending on the time when the samples were taken. During the latter part of the reaction (at and after 72 h), conversion yields approached those obtained under high- and mixed-speed mixing. This was probably due to the substantial decrease in the viscosity of the reaction mixture and better interaction between the enzymes and the remaining substrate. We as well as others observed similar results during the enzymatic hydrolysis of α-cellulose under the mixing conditions used in the present study *(5,6)*.

The conversion yields were in general lower at the higher substrate concentration (10% [w/v]) (Fig. 2), because of higher mass transfer limitations within the reaction medium. The conversion yields under low-speed mixing were always lower than those obtained under high- and mixed-speed regimes. The relatively high initial reaction rate and conversion yields

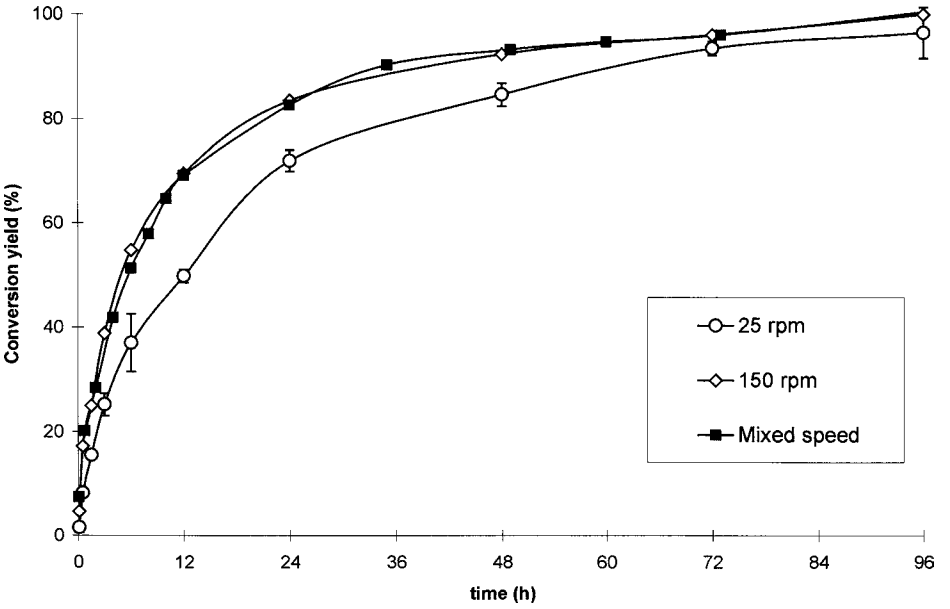

Fig. 1.Enzymatic hydrolysis of delignified, steam-exploded Douglas fir wood chips at 7.5% (w/v) solid concentration using low-speed (25 rpm), high-speed (150 rpm), and mixed-speed mixing regimes.

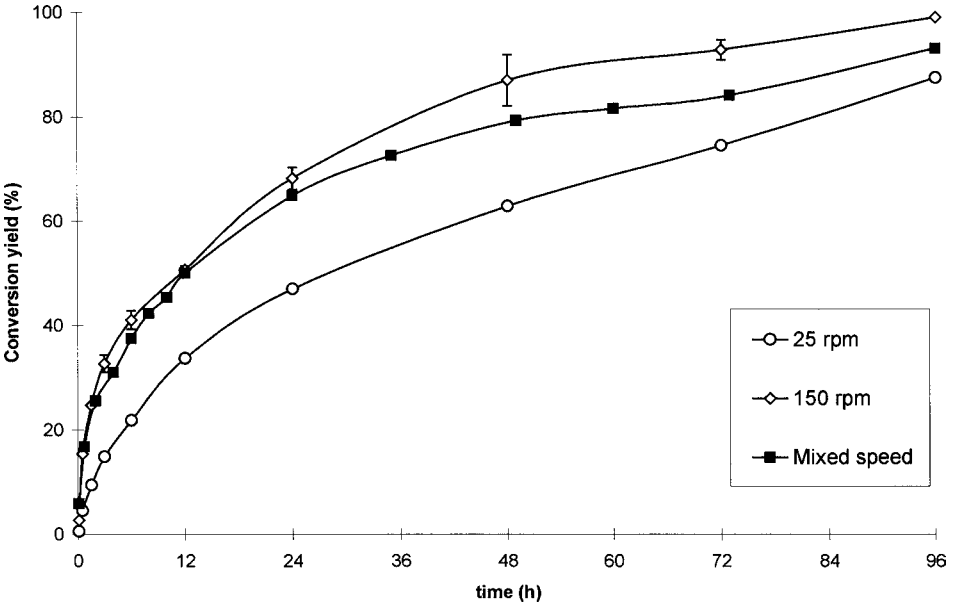

Fig. 2. Enzymatic hydrolysis at 10% (w/v) solid concentration using low-speed (25 rpm), high-speed (150 rpm), and mixed-speed mixing regimes.

Table 2
Percentage of Substrate Conversion During the Enzymatic Hydrolysis of Delignified, Steam-Exploded Douglas Fir Wood Chips at 7.5 and 10% (w/v) Substrate Concentrations and Under Low- (25 rpm), High- (150 rpm), and Mixed-Speed Mixing Conditions.

	Conversion yield (%)					
	7.5% (w/v) Solid concentration			10% (w/v) Solid concentration		
Time (h)	25 rpm	150 rpm	Mixed speed	25 rpm	150 rpm	Mixed speed
12	50	69	69	34	51	50
24	72	83	83	47	68	65
48	85	92	93	63	87	79
72	95	96	96	74	93	84
96	96	99	99	88	99	93

obtained during the first 12 h under mixed-speed mixing (Fig. 2) revealed the beneficial effect of the intermittent, fast mixing intervals. After the initial 12 h period, when the high-speed pulses were applied less frequently (once every 12 h), the rate and extent of hydrolysis began to decrease, and the mixed-speed profile started to deviate from that of the high-speed mixing (Fig. 2). The final conversion yields (at 96 h) for the high-, mixed-, and low-speed mixing were relatively close: 88, 93, and 99%, respectively. However, under shorter, and perhaps more realistic, hydrolysis times, the effect of mixing was more pronounced (Table 2). The mixed-speed regime was clearly superior to the continuous, low-speed mixing in producing higher conversion yields at 10% substrate concentration, although not as effective as fast mixing (150 rpm). Our results are somewhat different from those reported by others *(4)*. In hydrolyzing pretreated spruce wood in a vessel equipped with a paddle mixer, Tengborg et al. *(4)* found that the conversion yields under mixed-speed agitation were similar to those obtained under continuous low-speed mixing at 170 rpm, and lower than those resulting from high-speed agitation at 340 and 510 rpm. The difference between the results of these two studies could be attributed to the considerably higher mixing speeds used by Tengborg et al. *(4)*.

It is well recognized that the adsorption of enzyme onto the solid substrate is the first step in initiating the hydrolysis reaction *(9)*. To confirm the previous observations, we monitored the amount of protein that was adsorbed to the substrate during the course of hydrolysis (Fig. 3). When the reaction mixture containing 7.5% solids was shaken at 150 rpm, the amount of adsorbed enzyme peaked at the 6th h of incubation, where 70% of the added enzyme was bound to the wood. Over the same period but under mixed- and low-speed mixing conditions, only 45%and 38% of the added enzyme was adsorbed, respectively. It was interesting to observe that the faster and higher enzyme adsorption obtained when fast mixing

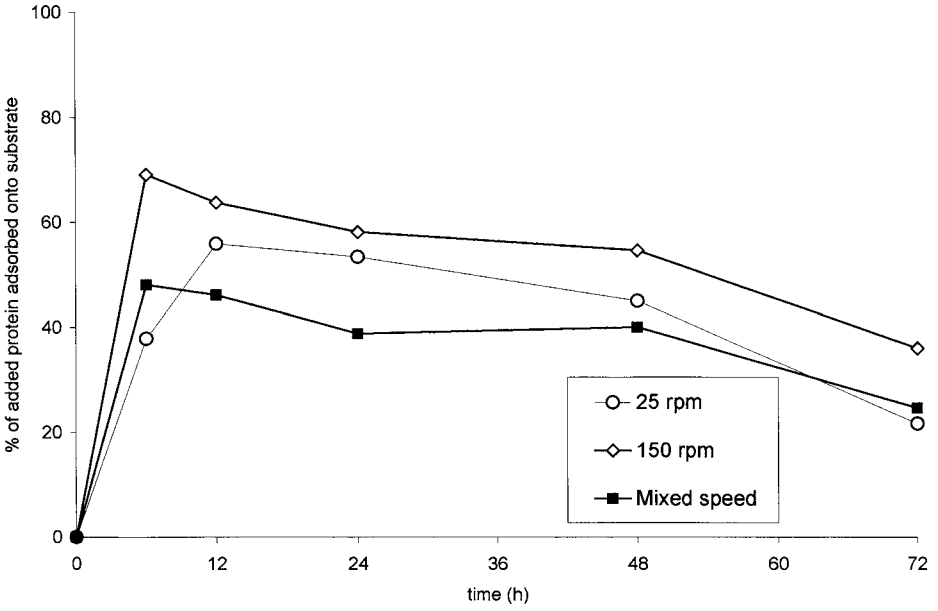

Fig. 3. Protein adsorption onto substrate during enzymatic hydrolysis of delignified, steam-exploded Douglas fir wood chips at 7.5% (w/v) consistency and by using low-speed (25 rpm), high-speed (150 rpm), and mixed-speed mixing regimes.

was employed did not translate into faster hydrolysis (Fig. 1). Wang and Converse *(9)* suggested that at high enzyme concentrations,where may be the case in our experiments, overly extensive coverage of the substrate surface by the large enzyme molecules might cause steric hindrance, which will prevent access to the chain ends. It is possible, therefore, that a high enzyme-to-substrate ratio does not necessarily improve the reaction rate, and at some point, may even inhibit the reaction. While the nonspecific binding of cellulases to lignin can increase enzyme adsorption on a substrate, the presence of lignin-bound enzymes does not improve hydrolysis *(10)*. It is not known, however, how differently the mixing speed influences enzyme adsorption onto lignin as compared to cellulose.

In the case of low-speed mixing, the amount of adsorbed enzyme continued to increase after the first 6 hours and peaked at 12 h, in which about 55% of the added enzyme was found to be bound to the substrate. Therefore, it was apparent that the speed and frequency of mixing had a direct influence on the rate and extent of enzyme adsorption. A similar trend of protein adsorption was observed at 10% substrate concentration (data not shown), although with greater fluctuation, which was perhaps the result of uneven distribution of proteins within the highly dense reaction mixture.

Under all three mixing conditions, after the initial adsorption phase, most of the adsorbed enzyme was released back into the reaction medium (Fig. 3). The desorption phenomenon continued to occur throughout the course of hydrolysis, although a portion of the enzymes remained bound

to the solid residues until the end of the reaction. Others have reported similar adsorption/desorption behavior during the course of hydrolysis *(11,12)*. Previously, we have observed that in using a lignified substrate (kraft pulp), enzymes are desorbed more slowly than during hydrolysis of a relatively pure cellulose (Avicel) *(11)*. The substrate used in the present study, which contained about 5% lignin, exhibited a relatively slow enzyme desorption phase. After 72 h and with all three mixing regimes, 35, 25, and 20%, respectively, of the added enzyme remained associated with the lignaceous residue.

In general, the amount of protein bound to the substrate under the mixed-speed mixing regime was lower than that achieved under the high- and low-speed mixing conditions, even at shorter reaction times. For instance, at 24 h, when the mixed-speed agitation was used, 40% of the enzyme remained bound to the substrate. After the same period, but under low- and high-speed mixing, 55 and 60% of the total enzyme was still adsorbed to the solids, respectively. This may have important implications for the recovery and reuse of enzymes to reduce the cost of enzymatic hydrolysis operations *(13)*.

Conclusion

At a relatively high substrate concentration (7.5% [w/v]), a mixed-speed mixing regime was found to be as effective as high-speed mixing in producing relatively high conversion yields. When the substrate concentration was raised to 10% (w/v), the mixed-speed regime was still superior to low-speed mixing in producing higher conversion yields, however, it did not appear to be as effective as continuous, high-speed mixing. Therefore, it is apparent that under moderate levels of substrate concentration, using a continuous, low-speed mixing interspersed with short periods of vigorous agitation could be a feasible mixing option, as it can reduce the energy requirements of the hydrolysis reactor and still produce reasonably high conversion yields.

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