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Enhancing the Enzymatic Hydrolysis of Cellulosic Materials Using Simultaneous Ball Milling

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Abstract

One of the limiting factors restricting the effective and efficient bioconversion of softwood-derived lignocellulosic residues is the recalcitrance of the substrate following pretreatment. Consequently, the ensuing enzymatic process requires relatively high enzyme loadings to produce monomeric carbohydrates that are readily fermentable by ethanologenic microorganisms. In an attempt to circumvent the need for larger enzyme loadings, a simultaneous physical and enzymatic hydrolysis treatment was evaluated. A ball-mill reactor was used as the digestion vessel, and the extent and rate of hydrolysis were monitored. Concurrently, enzyme adsorption profiles and the rate of conversion during the course of hydrolysis were monitored. α-Cellulose, employed as a model substrate, and SO_2-impregnated steam-exploded Douglas-fir wood chips were assessed as the cellulosic substrates. The softwood-derived substrate was further posttreated with water and hot alkaline hydrogen peroxide to remove >90% of the original lignin. Experiments at different reaction conditions were evaluated, including substrate concentration, enzyme loading, reaction volumes, and number of ball beads employed during mechanical milling. It was apparent that the best conditions for the enzymatic hydrolysis of α-cellulose were attained using a higher number of beads, while the presence of air-liquid interface did not seem to affect the rate of saccharification. Similarly, when employing the lignocellulosic substrate, up to 100% hydrolysis could be achieved with a minimum enzyme loading (10 filter paper units/g of cellulose), at lower substrate concentrations and with a greater number of reaction beads during milling. It was apparent that the combined strategy of simultaneous ball milling and enzymatic hydrolysis could improve the rate of saccharification and/or reduce the enzyme loading required to attain total hydrolysis of the carbohydrate moieties.

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Index Entries: Cellulose hydrolysis; cellulase; enzyme adsorption; ball mill reactor; softwood; enzymatic hydrolysis; steam explosion; bioconversion.

Introduction

Bioconversion of low-grade lignocellulosic residues to fuel-grade ethanol is considered to be an environmentally friendly alternative to oil-derived fuels, and is currently being actively pursued by a number of research and commercially driven organizations worldwide. Various feedstocks, including hardwoods, softwoods, and agricultural residues, have been evaluated for their potential in this process, and consequently, several common process variables in the overall wood-to-ethanol operation (i.e., pretreatment, enzymatic hydrolysis, fermentation) have been optimized for the different residues *(1)*. Although significant advances have been made with both hardwoods and agricultural residues, which are currently being used in full-scale operations, the utilization of softwood-derived feedstocks still has several limitations. More specifically, there is the limitation of restricted enzymatic hydrolysis of the cellulosic fractions during downstream processing, which results from the recalcitrance of lignin moieties following pretreatment. For example, it has been shown that complete cellulose hydrolysis of steam-exploded softwood residues can only be achieved when using very high enzyme loadings (~60 filter paper units [FPU]/g cellulose), and after relatively long incubation periods (~96 h) at very low solid concentrations *(2)*. However, these process parameters are industrially unacceptable and not economically viable. Under more realistic conditions, such as shorter incubation times, higher solid concentrations, and/or lower enzyme loadings, the hydrolysis of softwoods is usually much slower and generally incomplete *(2,3)*.

The hydrolysis of cellulose traditionally exhibits a biphasic profile, whereby the rapid initial rate reaction kinetics is quickly followed by an asymptotic, slower phase. It has been suggested that the observed limitations in maintained hydrolytic rates are likely owing to the combined influences of both substrate- and enzyme-related factors, such as the composition and structure of cellulose, as well as the specific activity of the enzymes *(4,5)*.

It has been suggested that one of the major factors limiting complete hydrolysis is the extent of accessible surface area to the cellulase enzymes. To enhance the rate and extent of enzymatic hydrolysis of cellulose, it is necessary to separate and expose the elementary cellulosic microfibrils to the specific enzyme protein. This can be achieved by various pretreatment methods, including the removal of lignin and dissolution of the hemicellulosic fractions *(6–8)*, or by swelling the cellulosic residue using organic-based agents *(9)*. Physical manipulations can also be employed to alter the inherent ultrastructure of lignocellulosics, and consequently, make it more amenable to hydrolytic conversion. To date, a number of physical pretreatments have been evaluated, including grinding and milling, physicochemical (acid-catalyzed steam explosion or chemical delignification), and pure biologic (fungal degradation) methods *(10–12)*. However, each pretreat-

ment process has to be modified according to the nature of the lignocellulosic substrate *(8,11)*.

Previously, milling reactors have been used as a means of pretreatment *(13–15)*, and for simultaneous grinding *(10,16–18)*. For example, Ghose *(18)* found simultaneous milling and heating to be an effective pretreatment method to reduce the particle size of Solka floc. However, because ball milling involves significant energy costs, Henley et al. *(13)* suggested that the use of a low-conversion, continuous stirred tank reactor placed between a ball mill and a hollow-fiber cartridge to reduce the milling time and cost might be more effective. Sidiras et al. *(16)* investigated the acid saccharification of ball-milled straw residues and concluded that ball milling significantly increased the yield and selectivity of saccharification. Additionally, the degree of crystallinity was found to decrease linearly with respect to the time of ball milling *(16)*.

Kelsey and Shafizadeh have also shown that physical manipulation and enzymatic hydrolysis of various cellulosic substrates, such as pure cellulose powder, newsprint, and white pine heartwood, could be improved by shaking the substrate in small flasks containing sand, glass, or stainless steel beads as grinding elements *(10)*. They found that the rate of saccharification was almost doubled. They also concluded that the simultaneous method constantly generated new, reactive, accessible sites on the cellulosic material, thereby increasing the rate and extent of the bioconversion reaction *(10,19)*. Neilson et al. *(19)* and Ryu and Lee *(9)* investigated a new type of bioreactor, the attrition mill bioreactor (AMR), for its efficacy in improving the rate and extent of hydrolysis. The principle feature of this reactor is an impeller immersed in a bed of stainless steel beads. Through a spinning action, the balls form a moving bed at the bottom of the reactor *(20)*. Neilson et al. *(19)* reported a higher rate and extent of saccharification of a variety of cellulosic substrates with rapid initial hydrolysis using the AMR. However, the hydrolysis leveled off at 70% conversion after 12 h, which was attributed to product inhibition and enzyme deactivation.

Nakao et al. *(21)* showed that a ball-mill reactor had superior performance (e.g., cellulase utilization, productivity, reducing sugar concentration) over a dual packed column reactor in saccharifying cedar, pine, and oak wood particles at the highest possible solid concentration. The synergistic effect of the physical treatment and hydrolysis has also been demonstrated in an intensive mass transfer reactor *(22)*. The basic elements of this reactor are a thermostated vessel containing cellulosic material (microcrystalline cellulose), enzyme solution, ferromagnetic particles, and two ferromagnetic inductors. Using this arrangement, very intensive agitation could be obtained, and the productivity of the enzymatic hydrolysis was enhanced up to 30–50 g/(L·h) at a substrate and an enzyme concentration of 10% (w/v) and 20 FPU/g of substrate, respectively *(22)*.

In the current study, pretreatment using SO_2-catalyzed steam-exploded softwood substrates was employed because it had previously

been shown to significantly enhance the enzymatic digestibility of lignocellulosic residues *(23)*. To further improve the hydrolyzability of this substrate and reduce the enzyme loadings required to attain sufficient degrees of hydrolysis, steam explosion was followed by a combined physical and enzymatic digestion step. This was evaluated using a ball-mill reactor with increasing substrate (Douglas-fir wood chips) concentrations at various relatively low enzyme loadings.

Materials and Methods

Substrates

Medium-severity (4.5 min, 195°C, 4.5% SO_2 of original dry wood) SO_2-catalyzed steam-exploded Douglas-fir (*Pseudotsuga menziesii*) wood chips were used for all experiments *(24)*. Following steam explosion, the residue was further posttreated with an alkaline-peroxide treatment. Briefly, following extensive water washing (30-fold [w/v]), residual lignocellulosic substrate was treated at 2% consistency (w/v) with 1% (w/w) hydrogen peroxide (pH 11.5) at 80°C for 45 min *(25)*.

Enzymes

Hydrolysis experiments were performed using a complete *Trichoderma reesei* cellulase system (Cellulclast) in combination with a commercial β-glucosidase (Novozym 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: filter paper activity of 80 FPU/mL, 52 IU/mL of carboxymethylcellulase (CMCase), 226 IU/mL of xylanase, 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, 94 IU/mL of xylanase, and 500 IU/mL of β-glucosidase. The enzyme activities were measured as described by Ghose *(26)*.

Enzymatic Hydrolysis

Hydrolysis reactions were performed either in the ball-mill reactor or in 300-mL Erlenmeyer flasks with controlled magnetic stirring. The hydrolysis experiments were carried out independently at 5, 7.5, and 10% (w/v) solid concentration in 50 m*M* citrate buffer (pH 4.8) supplemented with 40 μg/mL of tetracycline and 30 μg/mL of cycloheximide to prevent microbial contamination.

In optimization experiments using α-cellulose as a substrate, the enzyme loading was 25 FPU/g of cellulose supplemented with β-glucosidase at a ratio of 4:1, to prevent product inhibition by cellobiose. With the softwood substrate, enzyme loadings were varied between 5 and 25 FPU/g of cellulose at the same ratio (4:1).

The ball-mill reactor employed was a 1.1-L porcelain vessel with a 10-cm volume height and a 14-cm od. Small porcelain beads (100 beads,

approx 400 g) were used as the grinding elements. The reactor was sealed and held in a horizontal position while rotating on a two-spindle rolling base. The ball-mill reactor and the control experiments were incubated concurrently at 45°C in a controlled-temperature incubator. The total volume of the control experiments was 50 mL, while in the ball-mill experiments it was maintained at 200 mL. Aliquots of 0.3 mL were taken at various time intervals, immediately chilled on ice, centrifuged at 2000*g* for 10 min, and then stored for sugar and protein analysis. Prior to analysis, the diluted samples were boiled for 5 min to inactivate the enzyme.

All experiments and reactions were preformed in duplicate. The results depicted in the figures are averages of two independent experiments and generally exhibit a range of ±4%.

Determination of Lignin

The chemical composition of the softwood substrate was determined using a modified Klason lignin method derived from the TAPPI Standard Methods T-222 om-98 *(27)*. 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 with 112 mL of deionized water (final acid concentration of 4% H_2SO_4) and transferred to a serum bottle. The solution was then 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 then determined using high-performance liquid chromatography (HPLC) analysis, while acid-soluble lignin was determined by absorption measurements at 205 nm *(28)*. The amount of sugars released was measured using HPLC. The HPLC system (Dionex DX-300) was equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics). Prior to injection, samples were filtered through 0.45-mm HV filters (Millipore, Bedford, MA), and a volume of 20 µL was loaded. The column was equilibrated with 250 m*M* NaOH and eluted with deionized water at a flow rate of 1.0 mL/min.

Determination of Protein and Sugar

Total protein content was measured with the Bio-Rad Protein Assay using bovine serum albumin as standard. The amount of unadsorbed protein in the supernatant was reported as a percentage of the amount of protein present in the substrate blank. The amount of sugars released during enzymatic hydrolysis was determined using a YSI 2700 Select Biochemistry Analyzer (YSI, Yellow Springs, OH).

Results and Discussion

To ensure that end-product inhibition did not limit the rate and extent of cellulose saccharification, all hydrolysis reactions employed a complete

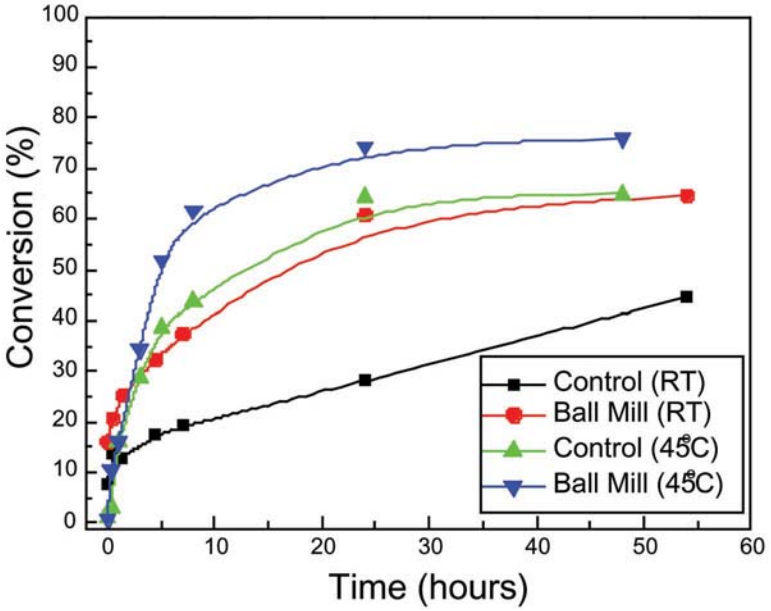

Fig. 1. Effect of temperature (room temperature [RT] and 45°C) on the efficacy of α-cellulose saccharification using simultaneous ball milling and enzymatic hydrolysis at 2.5% (w/v) and 25 FPU/g of cellulose.

cellulase system, Cellulclast (Novo Nordisk), supplemented with β-glucosidase at a ratio of 1:4. The addition of β-glucosidase enzyme to the reaction mixtures ensures that the concentration of cellobiose does not reach inhibiting concentrations, which has previously been shown to limit the efficacy of cellulose conversion by cellulase enzyme complexes deficient in cellobiose-converting enzymes *(29)*.

Optimization of Reaction with α-Cellulose

Initially, the ball-mill reactor was assessed for its capacity to facilitate an environment suitable for efficient cellulose conversion. This was investigated using α-cellulose as a substrate (2.5% concentration) with 25 FPU/g of cellulose enzyme loading, monitored over 24 h. The results clearly indicated that the combined physical/enzymatic reaction environment significantly increased the rate and extent of cellulose hydrolysis during the course of this reaction, at both room temperature (25°C) and 45°C (Fig. 1). During these initial investigations, concurrent, control shake-flask batch reactions were run under the identical conditions, as a benchmark for the ball-mill reaction vessel. It was apparent that increasing the temperature 20°C improved the efficacy of hydrolysis of both the control and ball-mill reactors by 45 and 20%, respectively (Fig. 1). Furthermore, it also demonstrated that by using the ball-mill reaction vessel, the overall conversion of α-cellulose under these conditions could be improved by approx 12%, increasing the hydrolysis from 70 to 82% total conversion. This suggested

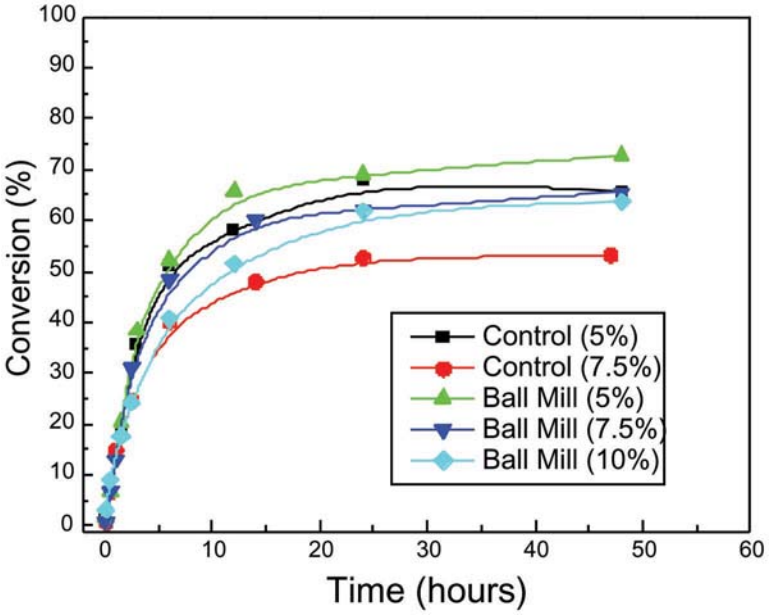

Fig. 2. Simultaneous ball milling and enzymatic hydrolysis of α-cellulose at 5, 7.5, and 10% (w/v).

that the physicomechanical action of the beads in the ball mill did not adversely affect the enzyme in the reaction mixture and could, therefore, improve the overall process. However, the results indicated that this combined action was still not sufficient to drive the hydrolysis reaction to complete conversion.

It is probable that the grinding action facilitated by the ball milling of the substrate increases the number of reactive sites on the cellulose moieties, and continuously disrupts the crystalline structure of the cellulose. Similarly, this physical action will also disrupt lignin-carbohydrate-complexes remaining in the lignocellulosic substrate, and consequently, increase the reactivity of the cellulases with the cellulosic substrate *(9)*.

As a direct extension of these initial experiments, the solid concentration of the hydrolysis reaction was altered, since this variable has been identified as one of the major restrictions of scaling up this type of bioconversion process. Therefore, the reaction conditions in the next series of simultaneous ball milling and enzymatic hydrolysis (SBMH) experiments were modified by only altering the concentration of α-cellulose from 5 to 10% (w/v). It was apparent that the low concentration of 5% (w/v) yielded the highest conversion (73%), while higher substrate concentration (7.5 or 10%) limited the bioconversion to 66 and 64%, respectively, after 48 h (Fig. 2). However, in all cases, the conversion in the ball-mill reactor was consistently higher than the corresponding controls in the shake flasks.

Previously it had been suggested that shear forces and air-liquid interfaces cause inactivation of enzyme preparations employed during hydroly-

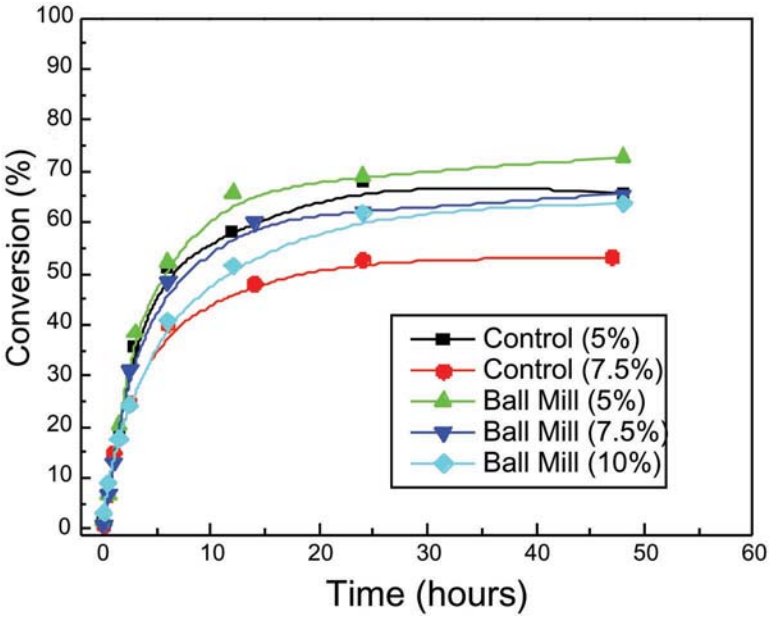

Fig. 3. Ball milling of 5% (w/v) α-cellulose over time with different numbers of porcelain beads.

sis experiments, and consequently, they were implicated in contributing to the incompleteness of the bioconversion process *(17,30)*. Similarly, Reese and Ryu *(30)*, observed significant deactivation owing to shear effects; however, they found that such effects were more apparent for cellobiohydrolase components than for the endoglucanase components of a cellulase preparation *(30)*. To investigate the impact of shear force and the influence of air-liquid interface in the SBMH reactor, altered numbers of porcelain beads and reaction volumes were used to evaluate their effect on hydrolysis. When no beads were used in the ball-mill reactor with an α-cellulose concentration of 5% (w/v), the conversion after 6 h was approx 40%. When 50 beads were incorporated, the extent of conversion increased to 47%, demonstrating an 18% improvement in the same time frame under identical conditions (Fig. 3). By doubling the number of beads to 100, a 52% conversion of the cellulose after 6 h was observed. This provided a 30% increase over a zero-bead treatment, and an 11% improvement over the 50-bead treatment. The conversion yields after 48 h were 67, 66, and 73% with 0, 50, and 100 beads, respectively (Fig. 3). These results clearly demonstrated that a greater degree of hydrolysis was attainable by incorporating more beads into the reaction vessel, but, only under limited reaction times (i.e., 6 h). Subsequent experiments evaluated the influence of substrate concentration, with or without (0–100) beads. Using 100 beads at a substrate concentration of 7.5% resulted in an improvement in hydrolysis yield after 6 h, from 32 to 48%. After 48 h, this improved from 58 to 66%, an increase of approx 14% (Fig. 4). Similar results were found when a 10%

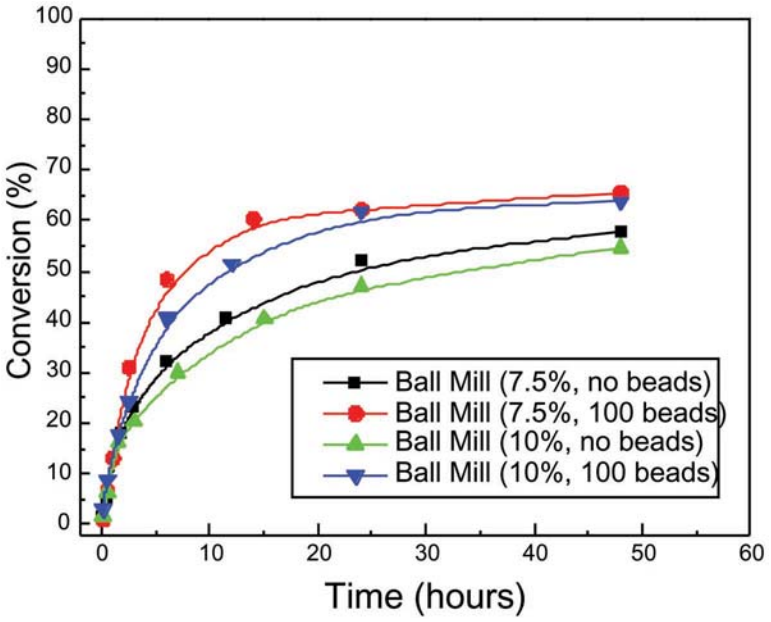

Fig. 4. Ball milling of 7.5 and 10% (w/v) α-cellulose over time with (100 beads) or without beads at low reaction volume.

substrate concentration was used. After 6 h the control reaction (no beads) only demonstrated a 28% cellulose conversion, whereas a 41% conversion was attained with 100 beads. After 48 h of hydrolysis, the conversion was 64%, representing a 16% increase compared with the no-beads control (Fig. 4).

Although supplementation of additional porcelain beads to the reaction vessel significantly enhanced the initial rates of enzymatic hydrolysis, the extent of total hydrolysis was only slightly improved. It is probable that the higher number of porcelain beads provokes more impacts and initially increases the number of accessible sites on the lignocellulosic substrates. However, it seems that this treatment is unable to generate the required sites since hydrolysis proceeds. It has been suggested that these observed effects are a result of an initial decrease in cellulose crystallinity *(10)*. However, the biphasic nature of the cellulose hydrolysis reaction was still observed and there was a leveling off in reaction rate after about 12 h of reaction time, representing a conversion rate of approx 65–70%. As discussed later, it has been suggested that this may be related to enzyme limitations *(19)*.

A second parameter was assessed: the influence of the air-liquid interfaces present in the ball-mill reactor. Using the same number of beads in all experiments (100 beads), the reaction volumes were altered, either to one-third of the reactor volume or completely full. In the latter case, no air-liquid interfaces were present, which precluded the deactivation of enzymes owing to exposure to air. Regardless of the substrate consistency

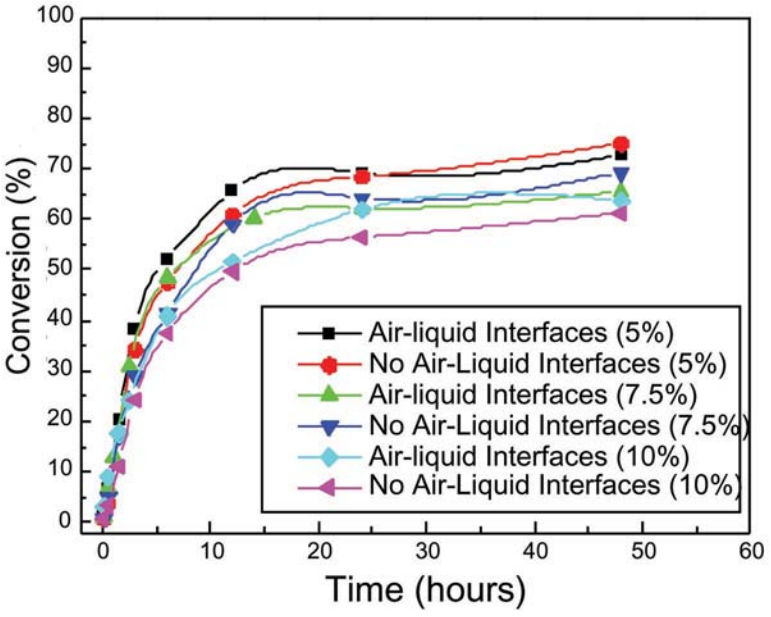

Fig. 5. Simultaneous ball milling and enzymatic hydrolysis with and without air-liquid interface using 5, 7.5, and 10% (w/v) of α-cellulose.

used (5, 7.5, or 10%), the results of the enzymatic hydrolysis with or without air-liquid interface were similar (Fig. 5). Using a 5% substrate concentration, the conversion with air-liquid interfaces was slightly higher (52%) than that without (47%) after 6 h, but reached the same level after 48 h (73–75%) of reaction time. Similarly, at 7.5 and 10% substrate concentrations, the amount of sugar released after 6 h of reaction time was only slightly higher than at lower reaction volume (Fig. 5). These results suggested that the presence of air-liquid interfaces had little or no effect on the total sugars released from the substrate at the termination of the reaction. This is in agreement with Jones and Lee *(20)*, who found that at a low impeller speed (200 rpm), the presence of an air-liquid interface did not cause any significant enzyme deactivation in the AMR *(20)*. However, at higher impeller speeds (700 rpm), the absence of air-liquid interfaces was required to minimize deactivation of the enzymes. Therefore, it appeared that the main factors that influenced the enzymatic hydrolysis reaction in the ball-mill reactor were the concentration of the cellulosic substrate and the number of beads present in the reaction vessel.

Simultaneous Ball Milling with Delignified Steam-Exploded Douglas-fir Wood Chips

The initial experiments demonstrate that simultaneous ball milling and hydrolysis significantly improved the extent of saccharification for α-cellulose. Having established the optimal parameters (100 beads and low volume), we conducted subsequent experiments with a "real" lignocellu-

Table 1
Percentage of Chemical Composition (by weight)
of Original, Steam-Exploded, and Posttreated Douglas-fir Wood[a]

Component	Original (%)	Steam exploded	Posttreated steam-exploded (%)
Acid-insoluble lignin	30.0	38.1	5.1
Glucose	48.9	61.3	88.8
Mannose	13.5	1.26	0.6
Galactose	3.9	0.05	0.04
Xylose	2.7	0.33	0.4
Arabinose	1.2	0	0
Total	99.3	101.04	94.9

[a]Conditions for steam explosion and posttreatment are described in Materials and Methods.

losic substrate, derived from Douglas-fir wood chips. The wood chips were first steam exploded and then further extracted with an alkaline hydrogen peroxide solution to extract and isolate the lignin in the substrate (to be used in other applications). The chemical composition of the starting material and the steam-exploded and alkaline peroxide delignified Douglas-fir wood is shown in Table 1. The lignin content was reduced from ~38% after steam explosion to ~5% following posttreatment with alkaline (NaOH) peroxide at pH 11.5. The major sugar component in the substrate was glucose (89%), while the total amount of other sugars (mannose, galactose, and xylose) made up only 1%. Therefore, conversion was expressed based on the amount of glucose measured in the supernatant. Various experiments with different substrate consistencies and enzyme loadings were performed to determine the minimum amount of enzyme required for optimal conversion of the cellulosic fraction.

Initially, an enzyme loading of 25 FPU/g of cellulose was evaluated for its capacity to saccharify substrates at 5, 7.5, and 10% (w/v) solid concentrations during simultaneous ball milling and hydrolysis of delignified substrates. At a 5% concentration, hydrolysis proceeded rapidly. After 6 h >75% of the original substrate was hydrolyzed to monomeric glucose (Fig.6). At higher consistencies, 7.5 and 10%, the conversion rate was lower, but gave 55 and 47% total conversion, respectively. Clearly, the initial lower solid concentration performed best, resulting in complete saccharification after approx 12 h. At a 7.5% solid concentration, 100% conversion was obtained after roughly 24 h, whereas with the 10% solid concentration the maximum hydrolysis achieved was only 88% after 48 h under the same conditions and enzyme loading. In control experiments carried out in shake flasks with magnetic stirring under the same reaction conditions, the conversion rate and extent of hydrolysis were significantly lower, demonstrating 20–25% lower hydrolysis yields (data not shown). Clearly, the rate of enzymatic hydrolysis provided by the SBMH proved to be fast and very

effective. Comparison of these results with the data obtained from α-cellulose experiments under the same reaction parameters (Fig. 2) indicated that the conversion of the lignocellulosic substrate was higher. For example, at 5, 7.5, and 10% substrate concentration, an increase in conversion of 37, 52, and 38%, respectively, was detected in the "real" substrate over the control "ideal" substrates (α-cellulose). This is likely owing to an increase in accessible surface area, resulting from steam explosion and posttreatment.

The extent of enzymatic hydrolysis was then compared at different enzyme loadings, and at different substrate concentrations concurrently. At 10 FPU/g of cellulose, the initial hydrolysis rate was observed to be slower than at an enzyme loading of 25 FPU/g cellulose. After 6 h, approx 35, 25, and 20% of the substrate was digested at 5, 7.5, and 10% solid concentration, respectively (Fig. 6). At 5 and 7.5% substrate concentrations, more than half of the substrate was hydrolyzed (57 and 53%, respectively) after 12 h at 10 FPU/g of cellulose. When compared to the 25 FPU/g of cellulose enzyme loading, the extent of saccharification was slightly lower after 48 h, with 96, 88, and 85% of the substrate hydrolyzed at 5, 7.5, and 10% consistency, respectively. At 5 FPU/g of cellulose, the conversion after 48 h was 67, 62, and 55% for 5, 7.5, and 10% concentrations, respectively (Fig. 6). Although it required longer incubation times to obtain complete hydrolysis, it is clear that the amount of cellulase required to achieve effective hydrolysis of cellulose could be reduced from 25 to 10 FPU/g of cellulose (corresponding to 65% savings in enzyme). At the 10% substrate concentration, enzyme loadings of 25 and 10 FPU led to similar end results after 24 h, although the reaction rate in the first 12 h was higher at a loading of 25 FPU/g of cellulose. However, the yield obtained using 5 FPU/g of cellulose decreased the amount of glucose by 37%.

From these results it can be concluded that an enzyme loading of 10 FPU/g of cellulose was sufficient to obtain an acceptable conversion of cellulose with a notable reduction in cellulase loading at all of the substrate concentrations studied.

Adsorption and Desorption

To determine the adsorption/desorption characteristics of the supplemented enzymes during ball milling of the softwood substrate, the total amount of protein in the supernatant was monitored over the reaction time. Adsorbed enzyme is defined as the percentage of adsorbed enzyme to the total amount of enzyme added to the initial reaction vessel (Fig. 7). At 5 and 10 FPU/g of cellulose a rapid adsorption of the added protein was observed in the initial phase of the reaction (Fig. 7A,B), with approx 70% of the added enzyme being adsorbed in the first 2 h of the reaction. The adsorption maximum when using the 5% solid concentration was reached after approx 30 min. However, the highest maximal adsorption (77 and 69%) was obtained at a 10% substrate concentration (5 and 10 FPU/g of cellulose, respectively) after 1.5 h of incubation. It was also evident that a fast initial absorption was followed by a relatively rapid desorption into the

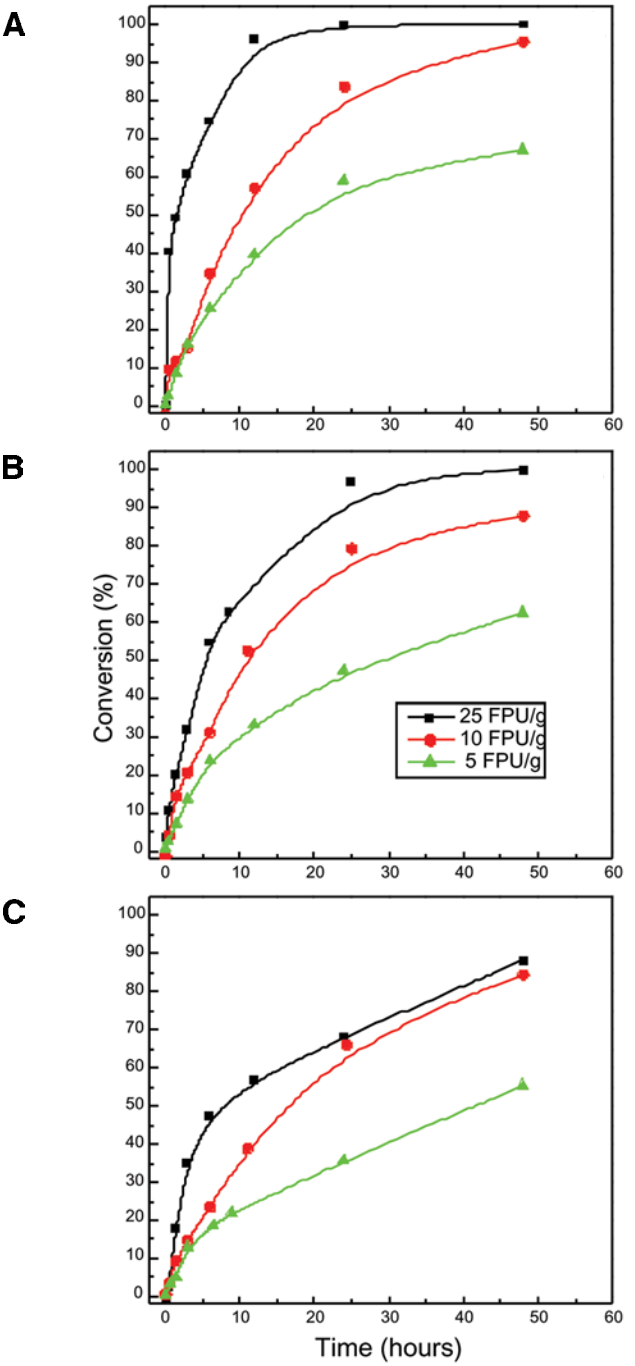

Fig. 6. Simultaneous ball milling and enzymatic hydrolysis of delignified steam-exploded Douglas-fir wood chips at various enzyme loadings (5, 10, and 25 FPU/g of cellulose) at **(A)** 5, **(B)** 7.5, and **(C)** 10% (w/v).

supernatant. After 12 h, a large percentage of the protein was found in the reaction supernatant. The transition between adsorbed and desorbed protein occurred much more quickly at the higher concentrations than at lower concentrations. For example, at 5 FPU/g of cellulose and a 5% substrate concentration, <15% of the original enzyme remained adsorbed on the substrate after 24 h. However, at a 10% solid concentration, 45% of the total protein remained bound to the lignocellulosic substrate (Fig. 7A). This may be owing to the lower conversion rate, and the higher viscosity of the reaction medium. The adsorption behavior for 10 FPU/g of cellulose was similar (Fig. 7B) to that observed for 5 FPU/g of cellulose. At a 5% substrate concentration, the amount of enzyme adsorbed to the substrate was only 5%, whereas at a 10% solid concentration, 45% of the protein was associated with the residual substrate.

By contrast, the adsorption profiles obtained at the 25 FPU/g of cellulose enzyme loading were significantly different (Fig. 7C). The added protein was adsorbed more gradually, and the maximal absorption was not reached until after 9–12 h (depending on substrate concentration). With the 10% substrate concentration, the protein adsorption rate onto the substrate was slower than obtained at the 5% substrate concentration. After 24 h, almost 50% of the protein was still bound to the cellulosic substrate. This may be owing to protein-protein interactions at the higher protein concentrations. When these results were compared with the data obtained from the preliminary experiments that used α-cellulose as a substrate, under the same reaction conditions and 25 FPU/g of cellulose (Fig. 8), the adsorption profiles were similar to those using enzyme loadings of 5 and 10 FPU/g of cellulose. A fast initial adsorption of cellulase in the early phase of the hydrolysis followed by a fast desorption was common to both substrates. At a 10% substrate consistency, the decline in protein leveled after approx 12 h, and demonstrated a 35% residual adsorption after 48 h of incubation. At a 5% concentration, the protein continued to decline gradually throughout the hydrolysis, with approx 25% of the original protein still bound after 48 h.

A considerable amount of work has attempted to determine the way in which the different components of the cellulase system are involved in "model" cellulose (Avicel, microcrystalline cellulose) biodegradation *(4,31)*. It has been suggested that a constant adsorption/desorption of cellulases takes place throughout hydrolysis *(32)*. This phenomenon has been attributed to different regions of the substrate (amorphous, crystalline) being exposed to the cellulase enzymes as different crystalline cellulose faces are eroded during hydrolysis from polysaccharide to monosaccharide *(4,8,33)*. However, with lignocellulosic materials, such as birch or softwood substrates, it was found that the enzyme remains tightly associated with the substrate during the logarithmic hydrolysis phase of hydrolysis *(8,34)*, and that the lignin significantly influenced the hydrolysis reaction as cellulases were irreversibly adsorbed *(35–37)*. It has also been suggested that the rate of hydrolysis decreases when lignin was present (38).

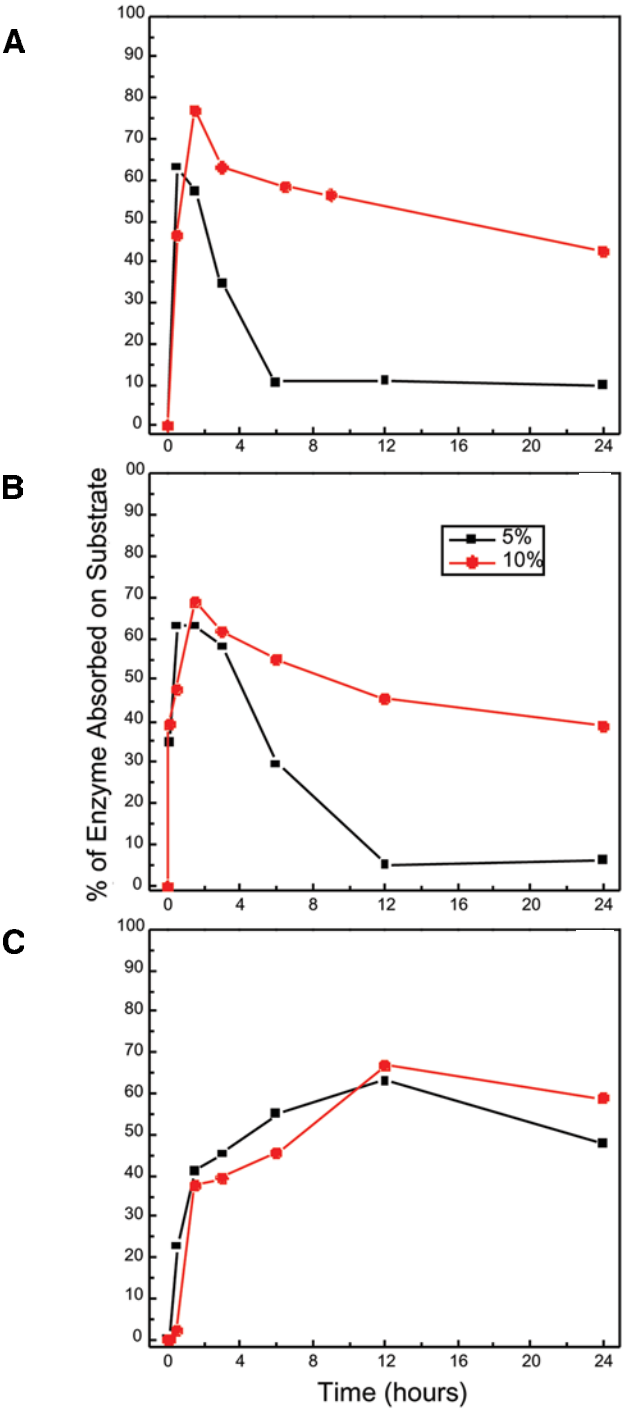

Fig. 7. Adsorption of enzyme to substrate (percentage of adsorbed enzyme) for **(A)** 5, **(B)** 10, and **(C)** 25 FPU/g of cellulose at 5 and 10% (w/v).

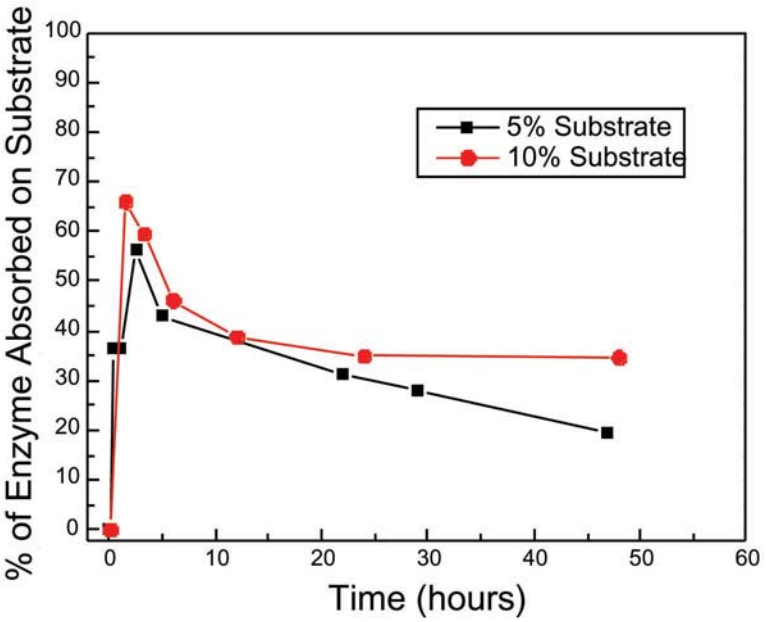

Fig. 8. Adsorption of enzyme to α-cellulose (percentage of adsorbed enzyme) at 5 and 10% (w/v) and 25 FPU/g of cellulose.

The results of the present study clearly indicate that the limitations of either residual lignin or substrate recalcitrance can at least be partially resolved by the use of physical manipulation, such as a ball-mill reactor; this combined treatment appeared to increase the efficacy of hydrolysis at lower enzyme concentrations over a range of substrate concentrations.

Conclusions

Simultaneous ball milling and hydrolysis significantly improved the extent of lignocellulosic saccharification. In preliminary experiments employing α-cellulose as the substrate, the number of beads supplemented to the reaction vessel had a positive correlation with the rate and extent of the hydrolysis reaction (i.e., the higher the number of beads, the better the hydrolysis). Contrary to previous work *(20)*, and in support of the results of Furcht and Silla *(17)*, the presence of an air-liquid interface did not seem to dramatically impact the efficiency of hydrolysis. In experiments with Douglas-fir wood chips that were steam exploded and posttreated with an alkaline hydrogen peroxide solution prior to enzymatic conversion, a 30% higher conversion rate was observed when compared to using α-cellulose as the substrate under similar reaction conditions. The lignin removal step (pre- and posttreatment) seemed to make the surface of the substrate more accessible to the enzymes, and therefore, more amenable to conversion. An enzyme loading of 10 FPU/g of cellulose performed nearly as well as using 25 FPU/g of cellulose for 5–10% initial solid concentration in a ball-mill reactor, a result that is not observed in traditional shake-flask reactions.

Therefore, it can be concluded that employing a ball-mill reactor for slightly longer incubation times can attain substantial savings in enzyme.

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