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Mitigation of Cellulose Recalcitrance to Enzymatic Hydrolysis by Ionic Liquid Pretreatment

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Abstract

Efficient hydrolysis of cellulose-to-glucose is critically important in producing fuels and chemicals from renewable feedstocks. Cellulose hydrolysis in aqueous media suffers from slow reaction rates because cellulose is a water-insoluble crystalline biopolymer. The high-crystallinity of cellulose fibrils renders the internal surface of cellulose inaccessible to the hydrolyzing enzymes (cellulases) as well as water. Pretreatment methods, which increase the surface area accessible to water and cellulases are vital to improving the hydrolysis kinetics and conversion of cellulose to glucose. In a novel technique, the microcrystalline cellulose was first subjected to an ionic liquid (IL) treatment and then recovered as essentially amorphous or as a mixture of amorphous and partially crystalline cellulose by rapidly quenching the solution with an antisolvent. Because of their extremely low-volatility, ILs are expected to have minimal environmental impact. Two different ILs, 1-*n*-butyl-3-methylimidazolium chloride (BMIMCl) and 1-allyl-3-methylimidazolium chloride (AMIMCl) were investigated. Hydrolysis kinetics of the *IL-treated cellulose* is significantly enhanced. With appropriate selection of IL treatment conditions and enzymes, the initial hydrolysis rates for IL-treated cellulose *were up to 90 times greater* than those of untreated cellulose. We infer that this drastic improvement in the "overall hydrolysis rates" with IL-treated cellulose is mainly because of a significant enhancement in the kinetics of the "primary hydrolysis step" (conversion of solid cellulose to soluble oligomers), which is the rate-limiting step for untreated cellulose. Thus, with IL-treated cellulose, primary hydrolysis rates increase and become comparable with the rates of inherently faster "secondary hydrolysis" (conversion of soluble oligomers to glucose).

Index Entries: Cellulose; enzymatic hydrolysis; ionic-liquid; pretreatment crystallinity index; initial rates; reducing sugars.

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Introduction

Cellulose is the most abundant renewable resource in the world. It is a major fraction of plant biomass, which is the feedstock for "future biorefineries" with the potential to replace the conventional petrochemical refineries in an economy based on renewable resources *(1–3)*. In its natural state, cellulose is highly crystalline in structure with individual cellulose polymer chains held together by strong hydrogen bonding and van der Waals forces. The individual cellulose chains are linear condensation polymer molecules made up of anhydroglucose units joined together by β-1,4 glycosidic bonds *(4)*, with degrees of polymerization (DP) ranging, typically, from 1000 to 15,000 units. The high-crystallinity of cellulose, imparts structural integrity and mechanical strength to the material, and also renders it recalcitrant toward hydrolysis aimed at producing glucose—the feedstock for producing fuels and chemicals—from this polysaccharide. In general, neither the water molecules nor the catalysts for hydrolysis (cellulase enzymes) are able to easily penetrate the crystalline matrix *(1)*.

When the DP exceeds six or seven monomer units, even the individual cellulose chains remain insoluble in water *(4)*. Hence the enzymatic hydrolysis of cellulose is inherently a heterogeneous catalytic process in which the component enzymes of the cellulase system adsorb on cellulose surfaces in order to affect hydrolysis. Pretreatments have been investigated as a means of modifying the cellulose structure in such a way that enzyme hydrolysis can occur at high-yields and improved rates, thus making the overall process economically viable *(3,5–7)*.

The goal of our pretreatment approach is to open the structure of cellulose to make it accessible to the component enzymes of cellulases. In this regard, it is helpful to distinguish between "solvent-swollen cellulose" and "regenerated-cellulose" (RC) *(8–10)*. In solvent-swollen cellulose the degree of crystallinity of cellulose is progressively reduced but not eliminated as the extent of swelling increases, whereas with RC the aim is to render cellulose essentially amorphous. The hydrolysis rates of cellulose are expected to depend on the extent of swelling with the maximal improvement expected with amorphous RC. The reproducible physical properties of RC (viz., DP, essential lack of crystallinity, and so on) make it an ideal substrate in fundamental studies aimed at a quantitative understanding of the mode of action and rates of individual components of a cellulase system. The RC would also make an excellent substrate for a "consolidated bioprocess" where cellulase production, cellulose hydrolysis, and fermentation are carried in a single step, which has the potential to convert lignocellulosic biomass to fuels at a much lower cost *(11)*.

Ionic liquids (ILs) show promise as efficient novel solvents for pretreatment of cellulose. Their ability to dissolve large amounts of cellulose at considerably mild conditions and the feasibility of recovering nearly 100% of the used IL to its initial purity makes them attractive *(12)*. Recently,

cellulose solubilities of up to 39, 25, and 10% (w/w) have been reported for the ILs 3-methyl-N-butylpyridinium chloride *(12)*, 1-*n*-butyl-3-methylimidazolium chloride (BMIMCl) *(13)*, and 1-allyl-3-methylimidazolium chloride (AMIMCl) *(14)*, respectively. ILs are salts that are liquids at or near room temperature and are stable up to temperatures of about 300°C. With their low-volatility, fluidity at ambient temperatures, and unique solvent properties, ILs are a class of prospective solvents that are potentially "green" because of their minimal air emissions.

In our approach, ILs were used to dissolve cellulose followed by rapid precipitation with an antisolvent such as water or alcohol. The structure of the RC was examined using X-ray powder diffraction (XRD) and found to lack the crystallinity of untreated cellulose. The hydrolysis kinetics of the RC was studied using a commercial cellulase system, with and without additional β-glucosidase to gain insights into the mechanism of hydrolysis of RC vs untreated cellulose.

An important aspect of our approach is that the IL is able to instantly reject (precipitate) all the dissolved cellulose in presence of antisolvents such as water, methanol, and ethanol through a preferential solute-displacement mechanism. Once the cellulose is precipitated, the antisolvent used for displacement can easily be stripped off the nonvolatile IL through flash distillation and the IL recovered for subsequent reuse. Two different ILs, BMIMCl (melting point 70°C) and AMIMCl (melting point 35°C) *(15)*, displaying appreciable solubility for cellulose were investigated.

Materials and Methods

Microcrystalline cellulose, Avicel PH-101 (FMC Corp.) was obtained from Sigma Aldrich (Philadelphia, PA). Citric acid monohydrate, sodium citrate, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, sodium potassium tartarate (Rochelle salt), phenol, sodium metabisulfite, methanol, and ethanol were obtained from Fisher Scientific (Hanover Park, IL). BMIMCl was purchased from Lancaster Synthesis (Alfa Aesar, Pelham, NH) and used without further purification. AMIMCl was prepared according to published procedures *(16)*.

Celluclast 1.5L, a *Trichoderma reesei* cellulase (Novozyme Corp., Bagsvaerd, Denmark), was used in all enzyme hydrolysis experiments. Cellulase activity was determined by the standard filter paper assay and expressed as filter paper units (FPU) per gram of glucan *(17)*. Novozyme 188, a β-glucosidase, was added with Celluclast 1.5L for some hydrolysis experiments. Cellobiase activity was determined by a cellobiose hydrolysis assay *(17)* and expressed as cellobiose units (CBU) per gram of glucan.

Cellulose Pretreatment and Regeneration

Avicel and BMIMCl (or AMIMCl) mixtures containing 5, 10, 15, and 30% (w/w) cellulose were incubated in a 5-mL autoclave vial. The vial and

the contents were heated in a block heater to either 130°C for 10 min or to 120°C for 30 min. The samples were gently stirred by placing the block heater on an orbital shaker. Deionized water was used as an antisolvent for precipitating cellulose from the ILs, BMIMCl, and AMIMCl. About 2 mL of antisolvent was added to the cellulose/IL mixture. Immediately a precipitate was formed. The sample was briefly centrifuged and supernatant was removed. The precipitated sample was washed twice with additional 2-mL aliquots of deionized water followed by the cellulose hydrolysis buffer solution. The resultant cellulose is referred to as RC. It should be noted that, with samples containing initial cellulose weight percent more than its solubility limit in the IL, only partial dissolution of cellulose occurs during incubation. Subsequent antisolvent treatment provides a cellulose mix of RC and partially crystalline cellulose (PCC). In what follows, cellulose samples recovered following antisolvent treatment are referred to as IL-treated cellulose irrespective of whether the resulting cellulose is RC or a mixture of RC and PCC.

XRD Measurements

Smooth films were cast at room temperature on microscopic slides from untreated and IL-treated cellulose samples. XRD data for these films were generated at 25°C with an XPERT' PRO powder diffractometer with Xcelerator' detector (PANalytical, Almelo, The Netherlands) using Nickel filtered CuKα radiation. Samples were scanned over the angular range 6–45°, 2θ, with a step size of 0.05°, and step time of 10 s.

Enzymatic Hydrolysis

Batch enzymatic hydrolysis of IL-treated and untreated cellulose was carried out at 50°C with 50 m*M* citric acid buffer (pH 4.8) in a reciprocating shaker bath. Two different sets of hydrolysis experiments were conducted. In the first set, the effect of augmenting cellulase with additional β-glucosidase (Novozyme 188) on the rates of hydrolysis of untreated cellulose and RC was investigated. A batch volume of 3 mL with a cellulose concentration of 16.7 mg/mL was used with both untreated cellulose and RC (recovered from 5% [w/w] cellulose–IL mixture). The enzyme loadings were varied from 8 to 32 FPU/g glucan of Celluclast 1.5L and 0 to 83 CBU/g glucan Novozyme 188. The second set of experiments was aimed at investigating the effect of crystallinity of cellulose on hydrolysis. In these experiments, batch volumes were adjusted for IL-treated cellulose samples to achieve the same cellulose concentration of 16.7 mg/mL used with untreated cellulose. The resulting volumes were 3, 6, 9, and 18 mL, respectively, for cellulose samples recovered from IL–cellulose mixtures of 5, 10, 15, and 30% (w/w) cellulose. A constant enzyme loading of 16 FPU/g glucan of Celluclast 1.5L and 83 CBU/g glucan Novozyme 188 was used.

The enzyme reaction was monitored by withdrawing 20 μL of samples from the supernatant periodically. Withdrawn samples were diluted

10 times. Untreated and IL-treated cellulose were hydrolyzed using the same cellulase and β-glucosidase (Novozyme 188) stock solutions. The untreated-cellulose controls were run concurrently with all the IL-treated cellulose hydrolysis experiments to eliminate potential differences in temperature history or enzyme loading. The released reducing sugars were measured by the DNS method using D-glucose as a standard *(18)*. Released glucose was determined separately by high-performance liquid chromatography (HPLC) using a HPX-87 P column (Bio-Rad Laboratories Inc., Hercules, CA) at 80°C equipped with a refractive index detector. The mobile phase was deionized water with a flow rate of 0.6 mL/min.

Results and Discussion

Dissolution and Regeneration of Cellulose, and its Crystallinity

During the incubation of Avicel in ILs at 120°C and 130°C, complete dissolution was observed for 5% (w/w) Avicel solutions in BMIMCl and AMIMCl. Ten percent solutions were completely dissolved in BMIMCl and almost completely dissolved in AMIMCl. For 15 and 30% (w/w) Avicel in ILs, only partial dissolution occurred. The maximum solubility of Avicel observed visually at 120°C was 9% in AMIMCl and 13% in BMIMCl. The dissolution mechanism of cellulose in BMIMCl and AMIMCl can be attributed to the nature of the bulky imidazolium cation and the relatively strong electronegativity and small size of the chloride ion. BMIMCl and AMIMCl have high hydrogen bond basicity and the anion plays a key role in the dissolution of cellulose. The chloride ion attacks the free hydroxyl groups and deprotonates cellulose. The imidazolium cation with its electron rich aromatic π system interacts with cellulose hydroxyl oxygen atoms through nonbonding or π electrons, and in addition prevents cross linking of the cellulose molecules *(16,19)*. Following dissolution in BMIMCl or AMIMCl, Avicel was precipitated from the IL by adding water as antisolvent, which forces the IL to reject dissolved cellulose through a preferential solute-displacement mechanism. During this displacement, the IL is extracted into the antisolvent through hydrogen bonding, dipolar, and columbic interactions between the IL and antisolvent *(20)*.

Samples of cellulose recovered following antisolvent treatment of IL–cellulose mixtures and untreated cellulose were examined by XRD to gain insight into the structural changes resulting from IL-treatment (Figs. 1 and 2). Crystallinity index (CrI) was determined from XRD *(21)* data and calculated using the formula: $\mathrm{CrI} = [(I_{020} - I_{am})/I_{020}] \times 100$, where I_{020} is the intensity above baseline at the 020 peak maximum near 2θ 22.5° and I_{am} is the minimum in peak intensity near 2θ of 18°*(21)*. CrI was reduced for all samples incubated in the ILs (Table 2). For cellulose concentrations less than the solubility limit in the IL (5 and 10% [w/w]), the cellulose recovered

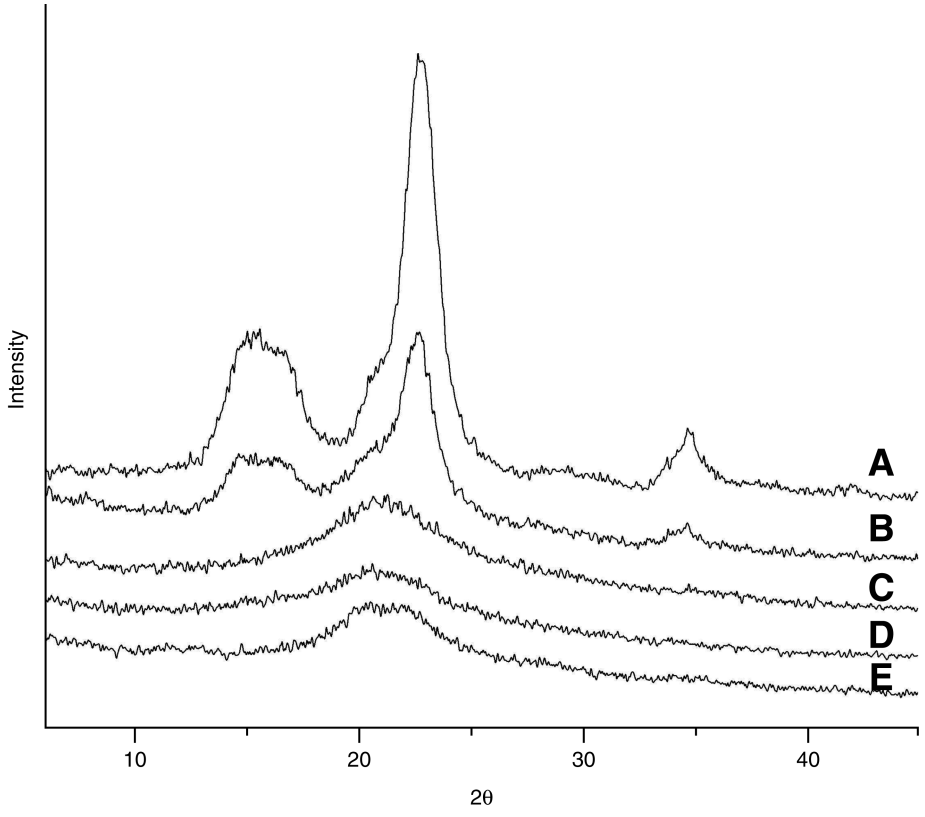

Fig. 1. XRD patterns for IL-treated and untreated-Avicel. Untreated Avicel **(A)**, exhibited a significantly greater degree of crystallinity than that of regenerated samples **(B–E)**. Avicel samples were incubated in AMIMCl at 120°C for 30 min and precipitated with deionized water. Samples B–E corresponds to 30, 15, 10, and 5% (w/w) Avicel incubated in AMIMCl. The crystallinity of B is significantly higher than that of C–E (CrI is listed in Table 2).

following antisolvent addition is essentially amorphous. Accordingly, the reduction in the measured CrI was greatest for 5 and 10% (w/w) samples and remains essentially the same for both cases. However, with samples containing initial cellulose weight percent more than its solubility limit in the IL (15 and 30% [w/w]), only partial dissolution of cellulose occurs during incubation. Subsequent antisolvent treatment provides a cellulose mix of RC and PCC. The proportion of PCC is expected to rise as the initial weight percent of cellulose incubated in IL is increased. This gradual increase in PCC will lead to a corresponding increase in CrI as was observed from the CrI obtained from XRD measurement (Table 2).

Effect of IL-Treatment on Rate of Hydrolysis of Cellulose

As noted, cellulose was incubated in BMIMCl or AMIMCl and recovered by quenching with antisolvent water. The IL-treated and untreated

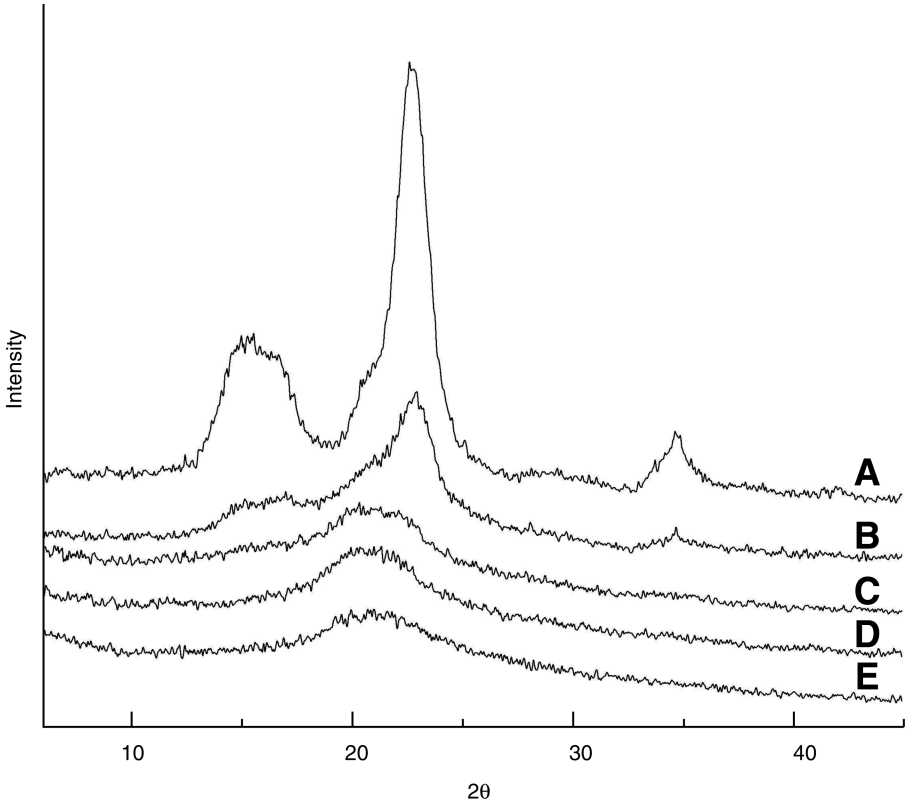

Fig. 2. XRD patterns for IL-treated and untreated-Avicel. Untreated Avicel **(A)**, exhibited a significantly greater degree of crystallinity than that of regenerated samples **(B–E)**. Avicel samples were incubated in BMIMCl at 120°C for 30 min and precipitated with deionized water. Samples B–E correspond to 30, 15, 10, and 5% (w/w) Avicel incubated in BMIMCl. The crystallinity of B is significantly higher than that of C–E (CrI is listed in Table 2).

cellulose samples were then enzymatically hydrolyzed using the Celluclast 1.5L cellulase system with and without additional β-glucosidase (Novozyme 188). Total soluble reducing sugars and glucose concentrations were monitored during the course of hydrolysis. The initial rate of formation of total soluble reducing sugars (a measure of hydrolysis) was higher for IL-treated cellulose compared with untreated cellulose for all incubation conditions and enzyme loadings examined (Tables 1 and 2). The concentrations of total soluble sugars and glucose are shown as functions of time in Figs. 3 and 4, respectively.

Role of Additional β-Glucosidase on Cellulose Hydrolysis

The initial rate of soluble reducing sugar formation of untreated cellulose and cellulose regenerated from a 5% cellulose/BMIMCl mixture is shown in Table 1 for various enzyme loadings, with and without

Table 1
Effect of β-Glucosidase on Hydrolysis. Initial Rate of Formation of Total Reducing Sugars, Measured by DNS Assay During the Enzymatic Hydrolysis of Approx 17 mg/mL of Regenerated or Untreated Avicel Samples With Cellulase Loadings Varying From 8 to 32 FPU/g Glucan and 0 to 83 CBU/g Glucan

Enzyme activity per gram of glucan		Initial rate (mg/mL/min)		Rate enhancement[a]
Cellulase (FPU)	β-Glucosidase (CBU)	Untreated cellulose	Regenerated cellulose	RC
8	0	0.0004	0.0047	12
8	83	0.0004	0.0320	71
16	0	0.0043	0.0427	10
16	83	0.0044	0.3915	89
32	0	0.0110	0.3953	36
32	83	0.0140	0.5030	36

Rates are calculated from analysis of supernatant sampled during the first 20 min of hydrolysis. RC was formed by incubating samples of 5% cellulose in ILs (BMIMCl) at 130°C for 10 min followed by precipitation with water.

[a]Rate enhancement is defined as the ratio of initial rate of reducing sugars released for RC divided by that of untreated cellulose.

Table 2
Effect of CrI of IL-Treated Avicel on Hydrolysis. Initial Rate of Formation of Total Soluble Reducing Sugars, Measured by DNS Assay During the Enzymatic Hydrolysis of Approx 17 mg/mL Avicel (With a Cellulase Activity of 16 FPU/g Glucan and β-Glucosidase Activity of 83 CBU/g Glucan)

Concentration of Avicel in IL	Initial rate (mg/mL/min)	Rate enhancement[a]	Crystallinity index
Untreated	0.0046	–	76.4
5% in AMIMCl	0.3274	71	12.9
10% in AMIMCl	0.3397	74	11.7
15% in AMIMCl	0.2304	50	15
30% in AMIMCl	0.1263	27	47.0
5% in BMIMCl	0.3412	74	11.5
10% in BMIMCl	0.3763	82	11.6
15% in BMIMCl	0.289	63	14.2
30% in BMIMCl	0.2140	46	43.4

Rates are calculated from analysis of supernatant sampled during the first 20 min of hydrolysis. RC or a mixture of RC and PCC was formed by incubating cellulose in ILs (AMIMCl/BMIMCl) at 120°C for 30 min followed by contact with water. A mixture of RC and PCC formed at Avicel concentrations in IL more than 10% (w/w).

[a]Rate enhancement is defined as the ratio of initial rate of reducing sugars released for RC divided by that of untreated cellulose.

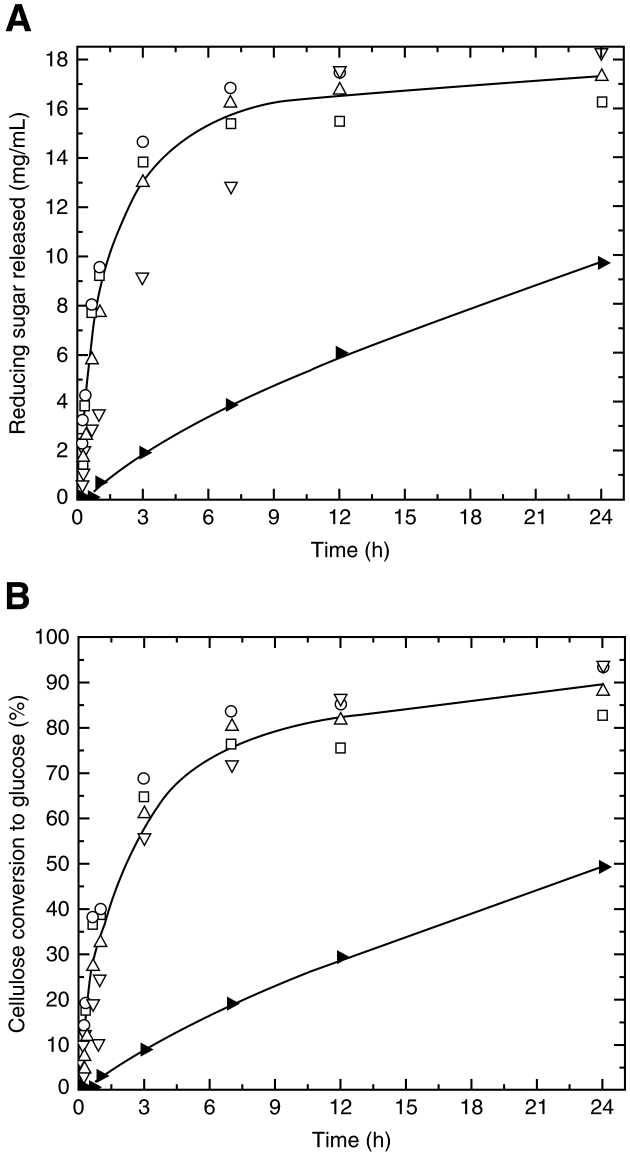

Fig. 3. Avicel samples of 5% (□), 10% (○), 15% (△), and 30% (▽) were incubated for 30 min in AMIMCl at 120°C, and precipitated with deionized water. Hydrolysis rates of IL incubated samples are compared with that of untreated Avicel (▶). Conversion of cellulose to sugars for batch samples of approx 17 mg/mL Avicel hydrolyzed with *T. reesei* cellulase activity of 16 FPU/g glucan and 83 CBU/g glucan at 50°C is shown as a function of time for **(A)** total soluble sugars (measured using a DNS assay) and **(B)** as *percent cellulose conversion to* glucose (measured by HPLC).

β-glucosidase addition. In the discussion that follows, the rate of formation of soluble reducing sugars will be referred to as the hydrolysis rate. This hydrolysis rate somewhat underestimates the true hydrolysis rate because hydrolysis of insoluble cellulose into smaller but insoluble fragments is

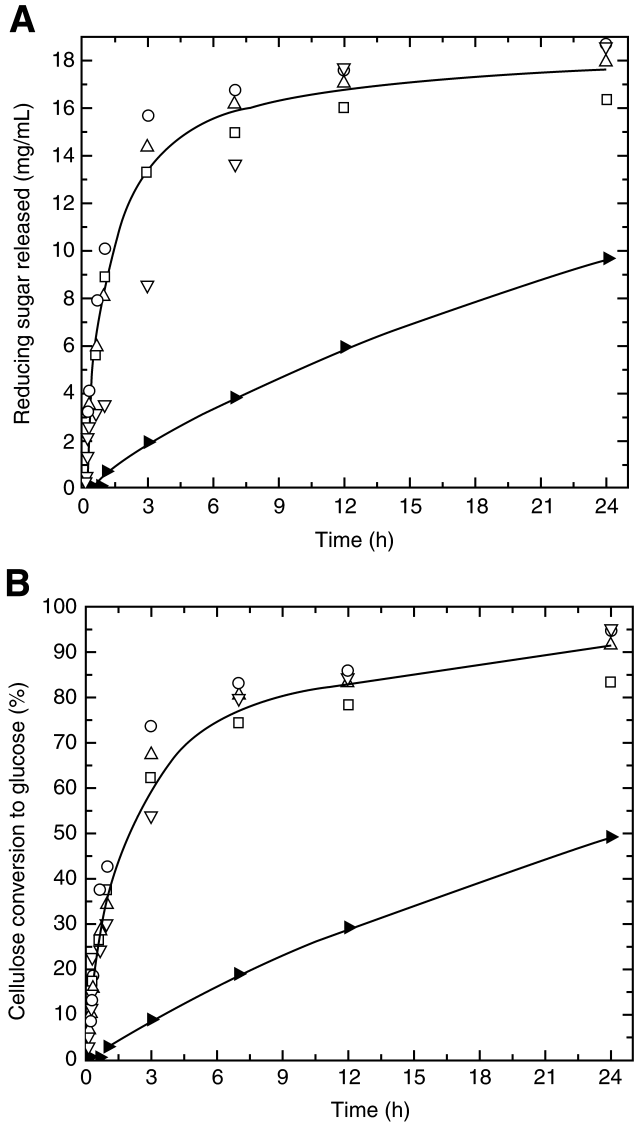

Fig. 4. Avicel samples of 5% (□), 10% (○), 15% (△), and 30% (▽) were incubated for 30 min in BMIMCl at 120°C, and precipitated with deionized water. Hydrolysis rates of IL incubated samples are compared with that of untreated Avicel (▶). Conversion of cellulose to sugars for batch samples of 17 mg/mL Avicel hydrolyzed with *T. reesei* cellulase activity of 16 FPU/g glucan and 83 CBU/g glucan at 50°C is shown as a function of time for **(A)** total soluble sugars (measured using a DNS assay) and **(B)** as *percent cellulose conversion to* glucose (measured by HPLC).

not taken into account in the DNS assay of soluble reducing sugars. The rate enhancement, defined as the ratio of initial hydrolysis rate of IL-treated cellulose to that of untreated cellulose, appears highest for an enzyme loading of 16 FPU/g glucan with addition of β-glucosidase at 83 CBU/g glucan. At these enzyme loadings the hydrolysis rate of RC is nearly two

orders of magnitude more than that of untreated cellulose. For modest cellulase activities (8 and 16 FPU/g glucan), the hydrolysis rates of RC increased significantly with addition of β-glucosidase (by six- to ninefold). This increase was not seen in untreated cellulose samples at similar cellulase activities (Table 1).

Effect of CrI of Cellulose on Hydrolysis

A constant enzyme loading of 16 FPU/g glucan with addition of β-glucosidase at 83 CBU/g glucan was used in all experiments conducted with various IL-treated cellulose samples (incubation of 5, 10, 15, and 30% cellulose in the ILs). The initial rates of hydrolysis for untreated and IL-treated cellulose are shown in Table 2. Initial rates of enzymatic hydrolysis of IL-treated cellulose were at least fifty times that of untreated cellulose. Initial rates of enzymatic hydrolysis of completely dissolved and RC samples (5 and 10%) were higher than the IL-treated cellulose samples that were partially dissolved (15 and 30%) as seen in Table 2. Samples containing initial cellulose concentrations less than 10% are within the solubility limit in IL, and more than 10% are above the solubility limit in IL. Addition of antisolvent to IL–cellulose mixtures produced RC when the cellulose concentration is within the solubility limit and produced a mixture of RC and PCC above the solubility limit. Mixture of RC and PCC samples have residual crystallinity (Table 2), which accounted for lower initial rates compared with RC samples.

For both RC (5 and 10%) and a mixture of RC and PCC (15 and 30%) samples, conversion to glucose after 7 h of hydrolysis was about 80–85%, whereas it is only 20% for untreated cellulose (Figs. 3 and 4). Higher conversions were expected for RC samples as the crystallinity of cellulose is almost eliminated in RC samples. Higher conversions obtained for mixtures of RC and PCC (15 and 30%) are somewhat surprising. In spite of the residual crystalliniy of these cases, the conversions were higher and comparable with that of RC samples (5 and 10%) (Figs. 3 and 4). This implies that the crystallinity of 15 and 30% cellulose samples treated in IL was reduced sufficiently to provide enough accessible sites for cellulase enzyme adsorption and activity. This is a promising observation as it suggests that it is not necessary to "totally eliminate" the crystallinity of cellulose to achieve significant enhancement in hydrolysis rates, and even with appreciable residual crystallinity most of the recalcitrance to hydrolysis can be mitigated. This also offers the possibility to process larger amounts of cellulose rapidly (i.e., up to 30 wt% of cellulose can be incubated in the IL-treatment step). All IL-treated cellulose samples reached almost 95% conversions to glucose within 24 h, whereas untreated cellulose only reached 50% conversion in that time period (Figs. 3 and 4).

The hydrolysis rates of IL-treated cellulose samples within the solubility limit (5 and 10%) were comparable for cellulose incubated in either

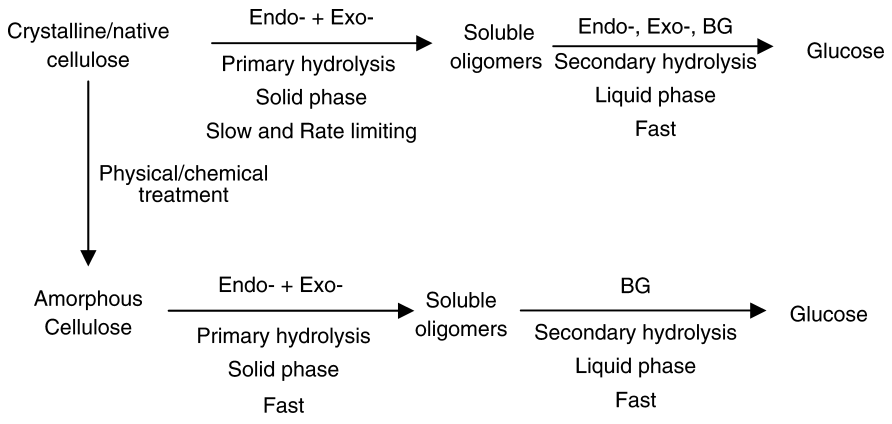

Fig. 5. Mechanism of enzymatic hydrolysis of cellulose by *T. reesei* cellulase *(3)*. Endo refers to endoglucanase. Exo, exoglucanase (cellobiohydrolase); BG, β-glucosidase.

AMIMCl or BMIMCl. With IL-treated cellulose samples above the solubility limit (15 and 30%), the hydrolysis rates appear to differ according to the dissolving capabilities of the IL. The hydrolysis rates of IL-treated cellulose prepared from 15% to 30% Avicel incubation in BMIMCl were higher than those prepared with AMIMCl (Table 2).

Mechanism of Enzymatic Hydrolysis of RC

The hydrolysis of insoluble cellulose can be divided into primary and secondary steps *(3)*. Primary hydrolysis (of the solid phase) involves the release of glucose and soluble intermediates such as cellobiose, cellotriose, and soluble cellodextrins from insoluble substrates. Secondary hydrolysis (in the liquid phase) involves the hydrolysis of soluble intermediates to lower molecular weight intermediates and glucose (Fig. 5). Exoglucanases and endoglucanases can hydrolyze solid cellulose substrates by adsorbing on cellulose surface (primary hydrolysis). Exoglucanases act on chain ends and predominantly release cellobiose (a glucose dimer). Endoglucanases hydrolyze cellulose by random chain scission and reduce DP of cellulose more effectively than the exoglucanases. Both exo and endoglucanases are inhibited by cellobiose. β-glucosidase converts cellobiose and soluble oligomers to glucose and only acts on substrates in the liquid phase (secondary hydrolysis). Primary hydrolysis of native crystalline cellulose is much slower than secondary hydrolysis, because of the macro and microstructure and extensive network of inter- and intrahydrogen bonding of cellulose fibrils and crystalline microfibrils, which greatly limits the permeability of enzymes and water to hydrolysis sites *(8,22)*.

For RC, because of the amorphous nature of the cellulose, many β-glucosidic sites are available for endoglucanases, which hydrolyze cellulose rapidly to form short glucose oligomers *(8)*. Within 3–4 h of enzymatic hydrolysis, the RC solutions appeared transparent, whereas

untreated cellulose had a distinct solid phase. This indicates that RC was rapidly hydrolyzed to soluble oligomers of DP <6 (or slightly soluble oligomers of DP <13) *(4,8,23)*. This rapid formation of soluble cellulose oligomers may result in comparable primary and secondary hydrolysis rates for RC.

The hydrolysis rates of RC are increased by about one order of magnitude over those for untreated cellulose for samples hydrolyzed with 8, 16, and 32 FPU/g glucan with no additional β-glucosidase added (Table 1). With the addition of 83 CBU/g glucan, the enzymatic hydrolysis rates increased by almost another order of magnitude for RC samples hydrolyzed with 8 and 16 FPU and to a lesser extent for samples hydrolyzed with 32 FPU/g glucan. The increase in hydrolysis rate with β-glucosidase addition observed for RC can be attributed in part to the relatively fast primary hydrolysis of amorphous cellulose and accumulation of cellobiose which inhibits exo and endoglucanase activity. β-glucosidase addition alleviates this accumulation and also increases the rate of secondary hydrolysis. In contrast, the hydrolysis rates of untreated cellulose were unaffected by β-glucosidase addition at 8 and 16 FPU/g glucan (rates increased modestly at 32 FPU/g glucan similar to the increase seen for RC). For untreated cellulose with modest cellulase loadings, primary hydrolysis appears to be rate limiting in contrast to relatively fast primary hydrolysis for RC. Hence, β-glucosidase addition does not seem to enhance the soluble sugar formation for untreated crystalline cellulose as the endo- and exoglucanase activity is not inhibited during the initial stages of enzymatic hydrolysis, owing to low initial concentrations of cellobiose/soluble oligomers (Table 1).

Conclusions

In a novel technique, the microcrystalline cellulose was incubated with an IL and then recovered as essentially amorphous or as a mixture of amorphous and PCC, by rapidly quenching the IL–cellulose mixture with an antisolvent. When the incubation samples contained initial cellulose weight percent more than its solubility limit in the IL, subsequent antisolvent treatment provides a cellulose mix of amorphous RC and PCC. The crystallinity index (CrI) obtained from XRD measurement of the IL-treated samples displayed a corresponding increase in CrI when the initial weight percent of cellulose was more than the solubility limit in the IL.

The IL-treated cellulose samples were hydrolyzed to sugars using Celluclast 1.5L and Novozyme 188. IL-treated cellulose exhibited improved hydrolysis kinetics with optically transparent solutions formed after first few hours of reaction, indicating relatively fast hydrolysis kinetics. With optimal IL-treatment conditions and enzyme loadings, initial rates of hydrolysis of IL-treated cellulose were *two orders of magnitude* higher than those observed with untreated cellulose. Among IL-treated cellulose

preparations, the *initial rates* observed with samples containing only RC were higher than the initial rates for the samples that were mixtures of RC and PCC. In spite of the observed differences in the initial rates and CrI, all IL-treated cellulose preparations showed significantly higher glucose conversions compared with untreated cellulose: about 80–85% conversions to glucose were observed for IL-treated cellulose samples in 7 h of hydrolysis where as it was only 20% for untreated cellulose. Thus, it seems that it is not really necessary to completely eliminate the crystallinity of cellulose in order to achieve significant enhancement in hydrolysis rates; even with some residual crystallinity most of the recalcitrance to hydrolysis can be mitigated. This offers the possibility to hydrolyze large amounts of cellulose rapidly using the proposed IL-pretreatment technique.

In the proposed technique, dissolution of cellulose in the IL and its subsequent precipitation with antisolvent, allows separation of the IL/antisolvent solution from cellulose by a simple filtration or centrifugation step. The IL and antisolvent can then be recovered, easily separated, and recycled. Because of the nonvolatility of the IL, antisolvent can be easily stripped from the IL/antisolvent solution for recovery and recycle of both the IL and antisolvent. The synthesis of ILs generally consists of a few steps with relatively high-yields *(16,24)*. The price of ILs is expected to dramatically decrease as commercial uses of ILs are developed and manufacturing scale increases. These considerations point to the promise of the proposed technique in dealing with the recalcitrance of cellulose to hydrolysis.

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