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Point by point analysis: how ionic liquid affects the enzymatic hydrolysis of native and modified cellulose

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New strategies are needed to efficiently convert non-food biomass to glucose as a platform chemical. One promising approach is to use ionic liquids to first dissolve lignocellulose. Yet, in the presence of such solvents, the enzymes that catalyze cellulose hydrolysis become compromised in their activity. However, this decreased cellulase activity has not been examined in detail. Thus, the aim of this study was to investigate how the ionic liquid precisely affects cellulase activity and stability with regard to different cellulose substrates. Hereby, four ionic liquids were screened to identify which one best minimized the loss of enzyme activity. Then, this best ionic liquid was tested on one insoluble and two soluble cellulose substrates. Subsequently, the relevant parameters of solution viscosity and ionic strength were evaluated with respect to enzyme activity and stability. Finally the residual ionic liquid concentration from the precipitation of α-cellulose was varied. The best ionic liquid was found to be 1,3-dimethylimidazolium dimethylphosphate with the highest retained activity of 30% on the α-cellulose substrate in the presence of 10% (v/v) ionic liquid. Most importantly, an increase in viscosity and ionic strength contributed to the decrease in enzyme activity which nonetheless retained their stability. The hydrolysis of precipitated α-cellulose from ionic liquid showed significant higher reaction rates but reduced sugar yields when residual ionic liquid was present. None the less, it should be possible to effectively produce glucose from precipitated cellulose without needing to wash off all residual ionic liquid when optimized cellulase mixtures are used.

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1. Introduction

To economically exploit plant biomass for material or energy use, adequate biomass pretreatment and depolymerization methods are crucial.[1,2] Many of these pretreatment methods are already available and include physical methods, chemical methods such as acid or alkaline treatment, and solvent fractionation such as the organosolv process.[3–7] All these established methods have significant drawbacks, *e.g.*, they consume a considerable amount of energy and form undesired by-products.[8,9] Therefore, new pretreatment methods are continuously explored.[2,10,11] A promising alternative solvent fractionation method involves ionic liquids (IL) that can effectively dissolve cellulose and wood at moderate temperatures to a homogeneous solution.[12–16] Applying ionic liquids for biomass pretreatment comes along with a number of advantages, not provided by other pretreatment methods. Strategies to recover and reuse the ionic liquids are under investigation.[17,18] Furthermore, the wide range of potential ionic liquids available offers a high degree of flexibility in process design. Even though the detailed mechanisms of cellulose dissolution by ionic liquids are still being investigated, it is commonly accepted that the dissolution disrupts the recalcitrant structure of biomass while conserving the polymeric structure of the cellulose chains. Compared to cellulose, the dissolution of lignocellulose poses additional challenges due to the presence of lignin. It has been shown that some ionic liquids also dissolve lignin. Though, the tight linkage of cellulose and lignin poses an additional diffusion barrier to the ionic liquid for dissolution.[19] Due to the factors associated with the lignin in the biomass it is beneficial to first investigate the isolated interaction of cellulose, ionic liquid and cellulase.

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To selectively depolymerize undissolved cellulose to glucose, cellulases are commonly applied in buffered aqueous media.[20,21] However, the highly organized structure of the undissolved cellulose with small pores and crystalline regions is hard to access for the enzymes and leads to low reaction rates.[1,22] The ionic liquid pretreatment of biomass, which dissolves the cellulose, offers promising advantages for enzymatic hydrolysis.[23–25] Following this ionic liquid pretreatment, the dissolved cellulose precipitates out of the solution by adding water.[26] When precipitating the dissolved cellulose from the ionic liquid with water/alcohol mixtures, lignin[27–29] and other biomass components, which negatively influence the enzyme performance, can be selectively removed.[1,30,31] This precipitate now has much larger pores and strongly reduced crystallinity, thereby making it more accessible to enzymatic attack.[29,32–34] This results in shorter reaction times and higher yields of glucose as the desired product.[24,25,29,32,33,35,36] With regard to enzymatic hydrolysis of regenerated cellulose in future industrial processes, one must take into account that there will always be residual ionic liquid present (10–15% (v/v)) in the reaction media. To wash this amount out, however, would be impractical, since it would entail considerable efforts and

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large amounts of water. Therefore, some authors have recently proposed to leave this residual ionic liquid in the reaction medium.[37] Since the cellulases are generally impaired by the presence of ionic liquid, however, it is essential to raise their tolerance to the ionic liquid residues.[35]

Attempts to improve cellulase tolerance to ionic liquid include metagenome screening, thermophilic enzymes or directed evolution.[37–39] In these studies the enzyme activity drops quickly with increasing ionic liquid concentration, whereby enzymatic activity is non existent at ionic liquid concentrations of above 30–50% (v/v). Unfortunately, it is impossible to draw general conclusions about this drop in enzyme activity based on the published results, since there is a large variation in the chosen substrates, enzyme sources and applied hydrolysis conditions.[32,33,37–39] Therefore, a comparative study is required that employs commonly accessible enzymes and carefully selected substrates that allow meaningful conclusions on the effect of ionic liquid on cellulase activity.

Consequently, the aim of this study is to investigate how the ionic liquid precisely affects the cellulase activity and sugar yield. The influence of four ionic liquids on the enzymatic hydrolysis of two soluble and one insoluble cellulose substrate using four different Celluclast® lots were analyzed. To dissect the factors impacting the cellulases, the influence both of ionic liquid and the corresponding viscosity and ionic strength changes on the enzyme activity was analyzed. Additionally, the cellulase stability was investigated both during operation and storage under process conditions. The results are transferred to the hydrolysis of cellulose regenerated from ionic liquid to evaluate up to which ionic liquid concentration the increased reaction rate counterbalances the loss in cellulase activity.

2. Experimental

Materials

Ionic liquids were purchased from the following manufacturers: Merck (Darmstadt, Germany), IoLiTec (Denzlingen, Germany) and BASF (Ludwigshafen, Germany) as specified in Table 1. Before use, the ionic liquids were tested for water concentration by volumetric Karl Fischer Titration (Schott Titroline alpha, Mainz, Germany), whereby the mean water concentration was 0.3% (w/w). The water activities of the ionic liquids were always below 0.015, as measured with the LabMaster-a_w (Novasina Instrument, Laachen, Switzerland). The ionic liquids were employed without further pretreatment. All tested ionic liquids are known to dissolve cellulose and wood.[15]

The cellulose substrates α-cellulose, carboxymethylcellulose (CMC) and p-nitrophenyl-β-cellobioside (pNP-cellobioside) (Sigma, Hamburg, Germany) were tested. The used α-cellulose had a crystallinity index of CrI = 64%.[40] α-Cellulose was gently dried at 80 °C over night, thereby reducing the water concentration from 3.3 to 2.1% (w/w) and the water activity from 0.316 to 0.106.

The commercial enzyme preparation Celluclast® 1.5 L ATCC26621 from Novozyme, Denmark had an enzyme activity of 700 EGU/g and a pH optimum of 4.8 according to manufacturer specifications. The published activities for this preparation according to the filter paper assay (FPA) range from 65–144 U/mL.[41–43] The Celluclast® preparation is the filtrated culture supernatant of *T. reesei* containing *exo*-(cellobiohydrolyase I and II) and *endo*-cellulase (endoglucanase I and II) as well as β–glucosidase.

Cellulase treatment

The off-the-shelf Celluclast has high salt concentration and residual proteins from the media and therefore may lead to systematic errors in the protein concentration measurement. Therefore, the Celluclast® preparation was desalted with a HiPrep 26/10 desalting column (GE Healthcare, Stockholm, Sweden) using an ÄKTA FPLC system (GE Healthcare, Stockholm, Sweden). The culture supernatant was substituted by 0.1 M sodium acetate, pH 4.8 as used for the hydrolysis experiments.

Measurements of cellulase activity and kinetics

The protein concentration of the enzyme preparation was determined with the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturers' protocol. The calibration was performed with bovine serum albumin (BSA) (Thermo Scientific, Waltham, MA, USA).

All reactions were performed with 10 mg mL^{-1} cellulose, incubated in 1 mL 0.1 M sodium acetate buffer (NaAc) at pH 4.8 with 1–10 mg mL^{-1} cellulases in 2 mL Eppendorf test tubes. The mixture was incubated at 45 °C based on information provided by the manufacturer that showed a rapid loss of enzyme stability for temperatures above 50 °C. The suspension was incubated shaking at 1000 rpm on a thermo mixer MHR 23 (HLC Biotech, Bovenden, Germany) for 30 min, unless otherwise specified. Blanks with buffer/enzyme and buffer/substrate were always incubated in parallel to the samples and treated in the same manner. Additionally, a sample with buffer/substrate/enzyme was inactivated immediately at time point zero. The cellulose employed for the enzyme activity measurements was α-cellulose unless otherwise specified. After incubation the mixture was inactivated at 100 °C for 10 min, and the formation of soluble sugars was measured with the dinitrosalicylic acid (DNS) reducing sugar assay.[44,45] Absorbance measurements at 540 nm were performed in a microplate reader Synergy 4 (Biotek, Winooski, VT, USA). Product concentrations were calculated from a calibration with glucose.

Table 1 List of ionic liquids

Ionic liquid	Abbreviation	Manufacturer
1,3-Dimethylimidazolium dimethylphosphate	[MMIM] [DMP]	Merck and IoLiTec
1-Allyl-3-methylimidazolium chloride	[AMIM] [Cl]	Merck
1-Butyl-3-methylimidazolium chloride	[BMIM] [Cl]	Merck
1-Ethyl-3-methylimidazolium acetate	[EMIM] [Ac]	BASF and IoLiTec

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To prepare p-nitrophenyl-β-cellobioside substrate a stock solution of 100 mg mL^{-1} was prepared with 40% (v/v) isopropanol in buffer (0.1 M NaAc, pH 4.8). For the experiments, the stock solution was diluted to 4 mg mL^{-1} p-nitrophenyl-β-cellobioside with buffer or with the buffer/IL mixture. The incubation was performed in microplates with 200 μL substrate solution and 1–10 mg mL^{-1} cellulase at 45 °C for 30 min in a Synergy 4 microplate reader (Biotek, Winooski, VT, USA). Absorbance due to release of 4-nitrophenol were measured at 380 nm in 1 min intervals. The cellulase activity was calculated from the linear slope of the conversion curves.

For measurements of cellulose hydrolysis in presence of ionic liquids, a specified amount of ionic liquid was added to the buffer. It is important to note that at the investigated ionic liquid concentrations, the ionic liquid concentration was always too low to cause any cellulose dissolution by the ionic liquid during enzyme hydrolysis.[26] To avoid effects on the enzyme due to pH changes in the buffer/IL mixture, the pH-value was adjusted to 4.8 with 1 M H_2SO_4 before addition of cellulose and enzyme. To ensure that the sugars measured after enzymatic hydrolysis are not distorted due to hydrolysis of cellulose by the ionic liquid, the cellulose was incubated in buffer with the respective ionic liquid concentration without cellulase in parallel to the hydrolysis experiments. The viscosity of the buffer/IL mixture was measured with a MCR 301 rheometer (Anton Paar, Graz, Austria).

Cellulose dissolution and regeneration

For experiments regarding regenerated cellulose, 100 mg α-cellulose was dissolved in 1 mL [MMIM] [DMP] at 100 °C until a clear solution was formed (~1 h). Afterwards, 7 mL buffer (0.1 M NaAc, pH 4.8) was added to precipitate the dissolved cellulose out of solution while vigorously agitating. To reach the desired concentration of residual ionic liquid for the enzymatic hydrolysis buffer or ionic liquid was added to the reach the appropriate concentration of ionic liquid and a cellulose concentration of 10 mg mL^{-1}. The pH-value was adjusted to 4.8 with small amounts of 1 M H_2SO_4.

Regarding the experiments with regenerated cellulose in the absence of any residual ionic liquid, the regenerated cellulose was washed excessively after precipitation with at least 100-fold buffer volume (0.1 M NaAc, pH 4.8). The regenerated cellulose was stored wet until use. A fraction of the washed gel-like precipitate was dried at 104 °C for 48 h, and the dry weight of the precipitated cellulose was found to be 6% (w/w). Based on the dry weight, the wet regenerated cellulose was weighed such that 10 mg mL^{-1} solid cellulose was present for the hydrolysis experiments, in which the glucose yield was monitored by means of the reducing sugar assay.

3. Results and discussion

Influence of ionic liquid, enzyme and substrate

It is challenging to understand the exact effect that the ionic liquid, from the lignocellulose pretreatment, has on the enzyme activity because of the large variability of all reaction system components such as substrate, enzyme preparation, and solvent. To dissect the key parameters that influence enzyme activity, type and manufacturer of the ionic liquid, lot-to-lot-variability of the enzyme preparation and the type of substrate were all varied, respectively, while the other parameters were kept constant. Thus, the cellulase activities were compared with respect to the different applied ionic liquids listed in Table 1. The respective enzyme activities of one Celluclast® lot were measured for α-cellulose using the various types of ionic liquid in a concentration of 10% (v/v); Fig. 1 illustrates the relative enzyme activity as a function of the ionic liquid used.

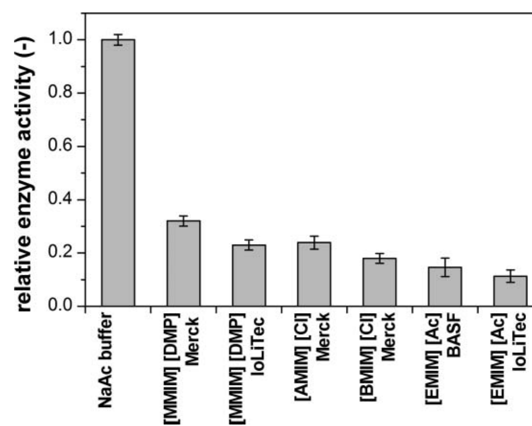

Fig. 1 Effect of different types and manufacturers of ionic liquids (10% (v/v)) on the relative cellulase activity. The relative cellulase activities were calculated based on soluble sugars formed from 10 mg mL^{-1} α-cellulose at 45 °C using 8.8 mg mL^{-1} desalted cellulase after 30 min. The error bars reflect the standard deviation of triplicate experiments.

For all tested ionic liquids the enzyme activity dropped to values of 15–30% of that in buffer. Using [MMIM] [DMP] the cellulases retained the highest relative cellulase activity of nearly 30%. The cellulase activity in the presence of [AMIM] [Cl] was comparable to that with [MMIM] [DMP]. The least cellulase friendly ionic liquids were [BMIM] [Cl] and [EMIM] [Ac] that both showed almost the same enzyme activity within the experimental error of 10%. These results show that all the four tested ionic liquids, even at a very low concentration of 10% (v/v), had an enormous impact on cellulase activity – decreasing it up to 85%.

Not only were different relative cellulase activities obtained for different ionic liquids but also with the same type of ionic liquid from different manufacturers. In the case of [MMIM] [DMP], there were significant differences in the relative enzyme activity depending on the manufacturer, whereas for [EMIM] [Ac] the variation remained within the experimental error (Fig. 1). Even though the different tested ionic liquids had the same purity grade according to manufacturer specification, inherent production impurities of ionic liquids may affect the enzyme activities. The role of ionic liquid impurities for biocatalysis has been comprehensively discussed in previous studies.[46,47] In particular, chloride and acid impurities have been shown to negatively influence lipase activity.[48,49] Recent studies have investigated the acidolysis of wood in ionic liquids.[50] Therefore, acid impurities in the ionic liquid could potentially also influence cellulose hydrolysis. This effect could be excluded for the results in Fig. 1 as incubation of cellulose in buffer with the respective

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Table 2 Comparison of various Celluclast® lots in buffer and in 10% (v/v) [MMIM] [DMP][a]

	Specific Celluclast activity		Relative activity in 10% (v/v) [MMIM] [DMP]	
Celluclast lot #	off-the-shelf (U/mg)	desalted (U/mg)	off-the-shelf (–)	desalted (–)
128K1301	0.064	—	0.24	—
058K1200	0.076	0.091	0.17	0.26
074K156	0.082	0.100	0.21	0.17
077K1156	0.071	—	0.22	—

[a] Substrate concentration: 10 mg mL^{-1} α-cellulose; T = 45 °C.

ionic liquid concentration without cellulase did not result in any sugar formation.

To further study the impact of ionic liquid on the enzymatic activity, four Celluclast® lots were compared, using 10% (v/v) of only one ionic liquid (Table 2). For these experiments [MMIM] [DMP] was selected, because with this ionic liquid the highest relative cellulase activity was observed in the previous experiments.

The specific enzyme activities of the various off-the-shelf Celluclast® lots in buffer deviated by 12% whereas the typical experimental error of the individual specific cellulase activity measurement amounted to 5%. The specific enzyme activity of the desalted Celluclast® was higher than that of untreated ones. This higher enzyme activity of the desalted Celluclase® lots is attributed to fewer protein impurities being present. Therefore, the desalted lots showed consistent catalytic activities. The results indicate that the commercial Celluclast® preparation is reproducibly standardized for the typical assay conditions using semi-amorphous α-cellulose in buffer. A further purification to individual enzymes, which would require a series of purification steps,[40,51–54] was not deemed necessary since this better represents the technical enzyme mixture. In the presence of 10% (v/v) [MMIM] [DMP] the relative enzyme activities of the four tested Celluclast® lots deviates by up to 20% from the average (Table 2). This relatively large deviation suggests that it would be best to just use one Celluclast® lot to specifically analyze the actual effect that the ionic liquid exerts on the cellulase activity.

After the ionic liquids and the Celluclast® lots were varied subsequently the substrates were varied next. Here, a total of three conventional cellulose substrates were used: p-nitrophenyl-β-cellobioside (pNP-cellobioside), carboxymethylcellulose (CMC) and, finally, α-cellulose. The first two substrates are soluble in buffer, whereas α-cellulose is not. This insoluble α-cellulose was selected, because its crystallinity and porosity are close to cellulose in wooden biomass.[21] Here, the respective effects of increasing concentrations of one ionic liquid ([MMIM] [DMP] up to 50% (v/v)) were analyzed with respect to the three different substrates. Please note that increasing concentration of [MMIM] [DMP] up to 50% (v/v) did not cause any dissolution or structural changes in α-cellulose. Hence, any changes in the relative enzyme activity have to be caused exclusively by the ionic liquid (Fig. 2).

As Fig. 2 illustrates, an increase in the concentration of the ionic liquid [MMIM] [DMP] caused a reduction in relative enzyme activity on all tested substrates. At an [MMIM] [DMP] concentration of 30% (v/v), the relative cellulase activity

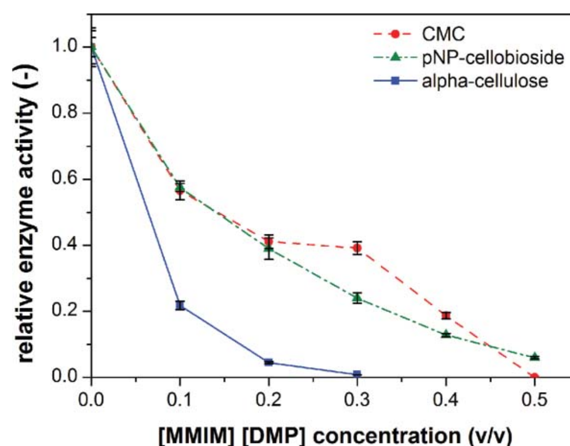

Fig. 2 Effect of increasing [MMIM] [DMP] concentration and different substrates on the relative cellulase activity in relation to the enzyme activity in buffer. Enzyme activity calculated based on soluble sugars formed by using 1.2 mg mL^{-1} desalted cellulase at 45 °C and 10 mg mL^{-1} α-cellulose after 30 min, 10 mg mL^{-1} CMC after 10 min, and 4 mg mL^{-1} pNP-cellobioside after 5 min, respectively.

approached zero for insoluble α-cellulose. Analogously, at an [MMIM] [DMP] concentration of 50% (v/v) the relative cellulase activity also dropped to zero for both soluble substrates. The soluble substrates exhibited nearly the same relative enzyme activity decrease with increasing ionic liquid concentration. The results of the current study concur with other studies that used cellulases from metagenome screening and CMC as substrate.[55] Additionally, similar trends of relative enzyme activity as a function of [BMIM] [Cl] concentration were shown with cellulases from *P. janithellum* on both soluble and insoluble substrates.[39] Recent results for cellulases from extremophile organisms retained nearly 90% relative enzyme activity in 20% (v/v) [EMIM] [Ac] but also completely lost enzyme activity at 50% (v/v) ionic liquid.[37]

Fig. 2 also shows that for soluble substrates, the relative enzyme activity at a particular [MMIM] [DMP] concentration amounted up to 40% higher than that for insoluble α-cellulose. A possible reason for the difference between soluble and insoluble substrate could be the variable assay duration of 5 to 30 min, which could reduce enzyme stability in the ionic liquid. However, this assumption is disproved by the cellulase stability investigations as seen and discussed later in Fig. 4 and 5. Most likely, the ionic liquid influences the individual enzymes contained in the cellulase preparation to a different extent. Soluble and insoluble substrates require different mixtures of *endo*- and *exo*- cellulase

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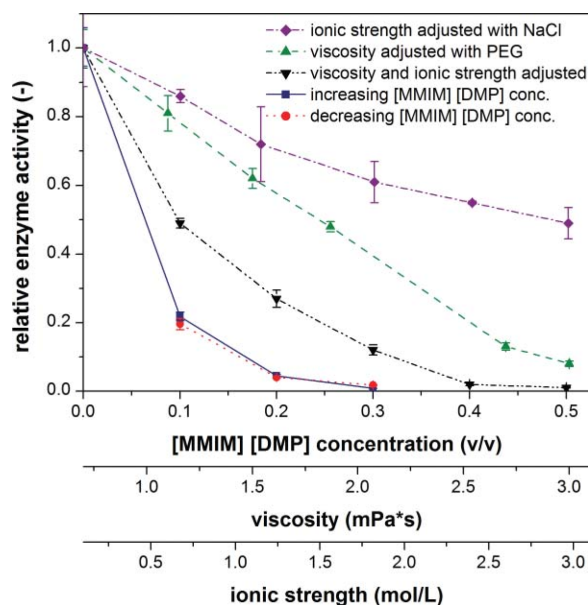

Fig. 3 Analysis of physicochemical parameters of [MMIM] [DMP] influencing the relative cellulase activity. The enzyme activities were calculated based on soluble sugars formed from 10 mg mL^{-1} α-cellulose at 45 °C using 1.8 mg mL^{-1} desalted cellulase after 30 min. Viscosity and ionic strength were adjusted to the values corresponding to the [MMIM] [DMP] volume fraction. To produce decreasing [MMIM] [DMP] concentration, the enzymes were incubated with 30% (v/v) [MMIM] [DMP] for 30 min and diluted back to the respective concentration of [MMIM] [DMP].

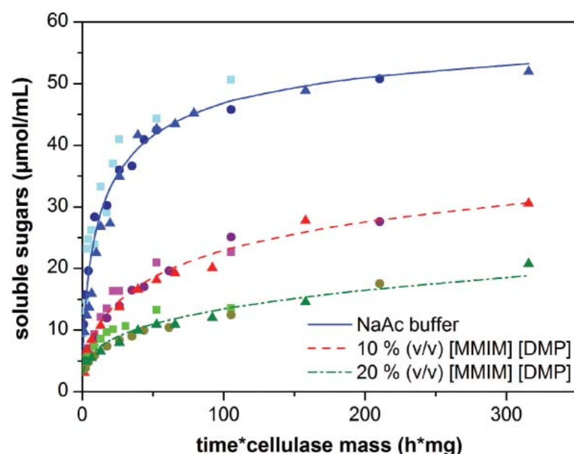

Fig. 4 Selwyn test for enzyme inactivation by [MMIM] [DMP]. Soluble sugars formed from 10 mg mL^{-1} α-cellulose using desalted cellulase were measured at 45 °C. The symbols represent varying enzyme concentrations (squares: 2.2 mg mL^{-1}; circle: 4.4 mg mL^{-1}; triangle: 6.6 mg mL^{-1}). The lines indicate regression curves for the different volume fractions of ionic liquid.

activities.[56–59] In particular, endoglucanase activity is prominent in the hydrolysis of soluble substrates. Therefore, the choice of the soluble or insoluble substrate leads to different effects of the ionic liquid on the relative enzyme activity. Consequently, even though soluble substrates are commonly used in the laboratory because of their easy handling, they do not reflect the realistic situation in industrial applications that always deal with insoluble substrates. Thus, since an apt cellulosic substrate

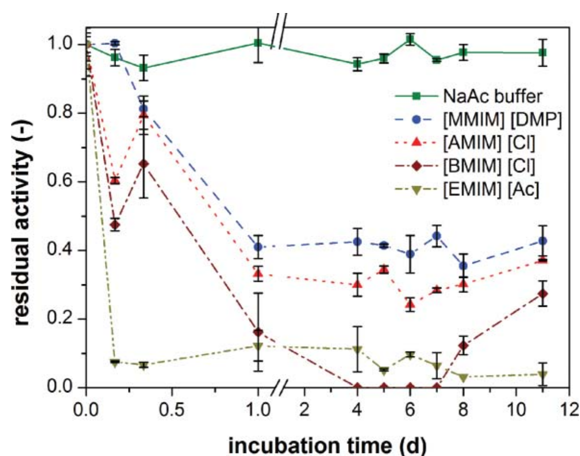

Fig. 5 Cellulase storage stability under process conditions (45 °C) with 10% (v/v) ionic liquid over 11 days. Buffer or buffer/IL mixtures was incubated with 1.8 mg mL^{-1} desalted cellulase at 45 °C over various number of days and enzyme activity monitored by measuring formation of soluble sugars at 45 °C from 10 mg mL^{-1} α-cellulose after 30 min.

should mimic the cellulose present in native lignocellulose, α-cellulose is the best substrate to use in laboratory experiments.

Physicochemical parameters, enzyme activity and stability

After it was established that the catalytic activity decreases depending on the type of ionic liquid and substrate, the following experiments aimed to explain how the chosen [MMIM] [DMP] ionic liquid actually decreases the cellulase activity. Therefore, the parameters pH, viscosity, and ionic strength of the reaction medium were measured as a function of increasing [MMIM] [DMP] concentration (Table 3).

In particular, the pH-value increased drastically with increasing [MMIM] [DMP] concentration. With 50% (v/v) [MMIM] [DMP] the pH-value was already more than 1.5 pH units above the enzyme optimum of 4.8. Thus, all cellulase activity measurements in this study were performed with pH-values adjusted to 4.8. Viscosity and ionic strength increase approximately linearly with ionic liquid concentration. At 50% (v/v) [MMIM] [DMP] the viscosity of the reaction solution amounted to 4-fold the buffer viscosity; here, the ionic strength rose even 30-fold compared to the buffer ionic strength. To specially investigate the effect of the parameters viscosity and ionic strength on cellulase activity, it was hereby necessary to exclude using any ionic liquid. Thus, PEG 2000 and NaCl were applied to artificially adjust

Table 3 Physicochemical parameters of the reaction mixture relative to increasing [MMIM] [DMP] concentration

Concentration [MMIM] [DMP]	pH (not adjusted)	Viscosity (45 °C)	Ionic strength
(v/v)	(–)	(mPa*s)	(M)
0.0	4.8	0.7	0.1
0.1	5.0	1.1	0.7
0.2	5.2	1.5	1.2
0.3	5.5	1.9	1.8
0.4	6.0	2.7	2.4
0.5	6.5	3.0	3.0

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viscosity and ionic strength, respectively, without the presence of ionic liquid in the medium (Fig. 3).

As illustrated in Fig. 3, at the viscosity and ionic strength values corresponding to 30% (v/v) [MMIM] [DMP], the relative cellulase activity dropped by 60% and 40%, respectively. However, this decrease in relative cellulase activity was moderate compared to the decrease in relative cellulase activity in the presence of ionic liquid where almost no relative cellulase activity was detected in the presence of 30% [MMIM] [DMP]. At higher viscosities, the relative enzyme activity continued to drop and at a viscosity of 3 mPa*s, corresponding to 50% (v/v) [MMIM] [DMP], the relative enzyme activity was less than 10%. The linear decrease in relative cellulase activity with increasing viscosity suggests considerable advantages in applying low-viscosity ionic liquids. A reduced mass transfer rate due to the higher viscosity has been previously described for biocatalysis with α-chymotrypsin in the two ionic liquids [EMIM] [Tf2N] and [MTOA] [Tf2N].[60,61]

Similar to the viscosity, the relative enzyme activity decreased with increasing ionic strength. This decrease, however, was less pronounced than for viscosity; even at an ionic strength of 3 M, corresponding to 50% (v/v) [MMIM] [DMP], the relative enzyme activity was 50%. The combined increase in viscosity and ionic strength resulted in a stronger decrease in catalytic activity due to the additive effects. With the ionic strength and viscosity values corresponding to 40–50% (v/v) [MMIM] [DMP], almost no relative enzyme activity was measured. In contrast to the drop caused by the ionic liquid, the combined change of viscosity and ionic strength was not as prominent. A possible explanation for the stronger decrease due to the ionic liquid could be the solvent polarity as a potential structural unfolding and deactivation due to solvent polarity has been reported for lipases (CaLB) in different ionic liquids.[62] Consequently, even though the parameters of viscosity and ionic strength are important in influencing enzymatic activity, other molecular effects of the ionic liquid also play a significant role for the cellulase activity.

Another question to address is if the decrease in relative cellulase activity as a function of ionic liquid concentration is reversible or irreversible. Therefore, the Celluclast® enzyme mixture was incubated with 30% (v/v) [MMIM] [DMP] for 30 min and diluted to 10–20% (v/v) [MMIM] [DMP] before the cellulase activity measurement. Fig. 3 shows that the catalytic activity was regained when the ionic liquid was diluted with buffer. Therefore, the enzymes in the Celluclast® mixture were not irreversibly inactivated by [MMIM] [DMP]. To further confirm this, the enzymes' operational stability was analyzed using the Selwyn test for enzyme inactivation.[63] According to Selwyn, product formation measured at different enzyme concentrations is plotted *versus* time multiplied by enzyme concentration (Fig. 4).

Here, the reaction curves for three different cellulase concentrations at varying ionic liquid concentration of up to 20% (v/v) [MMIM] [DMP] coincided, indicating that no enzyme inactivation is caused by [MMIM] [DMP] in a concentration up to 20% (v/v). This finding was confirmed for the other ionic liquids listed in Table 1, by testing storage stability under process conditions (45 °C, pH 4.8 at a protein concentration of 1.8 mg mL^{-1}) with 10% (v/v) ionic liquid for 11 days as depicted in Fig. 5.

In buffer, no significant cellulase inactivation was detected over the entire time period. In the presence of 10% (v/v) ionic liquid, the enzyme activity dropped to 10–40% of the enzyme activity at time zero within the first day and remained at this level for at least 10 days. The observed behavior for the different ionic liquids relative to each other is in good agreement with the trends observed in Fig. 1. The decrease in catalytic activity within the first day seems to be attributed to an adaptation of the enzymes to the new ionic liquid environment. The results are in good agreement with previous results for cellulase mutants from *Penicillium janthinellum*, which showed good stability for five days in [BMIM] [Cl].[39] Interestingly, the change in enzymatic activity within the first day is not visible in the Selwyn assay (Fig. 4). The Selwyn test reliably indicates time-dependent deactivation processes, but not instantaneous ones. Instantaneous deactivation of enzymes has been shown previously for alcohol dehydrogenases in the presence of several immiscible organic solvents.[64] The observed cellulase stability in mixtures with imidazolium-based ionic liquids was also observed for alcohol dehydrogenases in electrochemical synthesis.[65] Hence, in the future, only a few experiments will be needed to select suitable ionic liquids for cellulose pretreatment and hydrolysis. These experiments should involve relative enzyme activity measurements at a few ionic liquid concentrations and include verification of cellulase stability.

Based on the detailed analysis it is now possible to highlight several important factors that determine the effect of ionic liquid on enzymatic cellulose hydrolysis. The reduced cellulase activity is linked to increased viscosity and increased ionic strength of the reaction medium. Moreover, the molecular effects of the ionic liquid inhibit the cellulases but not irreversibly inactivate them. The implications for hydrolysis of cellulose regenerated from an ionic liquid solution and therefore of cellulose with altered structure are handled in the next section.

Regenerated cellulose hydrolysis

When characterizing the effect of residual ionic liquid on the hydrolysis of regenerated cellulose, two opposing factors mask the effect that the ionic liquid has on the catalytic activity and cannot be easily differentiated from one another. On the one hand, the reaction rate is significantly bolstered due to increased porosity and reduced crystallinity of the substrate. On the other hand, the hydrolysis rate is reduced due to the ionic liquid. With our aforementioned findings for untreated cellulose, the actual influence of the ionic liquid could be isolated while neglecting structural changes of α-cellulose precipitated out of the ionic liquid. As the reaction rates for untreated and regenerated α-cellulose were determined at different [MMIM] [DMP] concentrations, the initial reaction rates and the relative changes in the enzyme activity are summarized in Fig. 6.

As depicted by the bars in Fig. 6, the initial reaction rate for the regenerated α-cellulose increased up to 20-fold compared to that of the untreated α-cellulose. Even with 30% (v/v) residual ionic liquid, the initial reaction rate for regenerated α-cellulose was higher than for untreated α-cellulose without any ionic liquid. However, the decrease in relative enzyme activity with increasing [MMIM] [DMP] concentration for the two substrates seemed to be very similar. Therefore, the ionic liquid indeed influences

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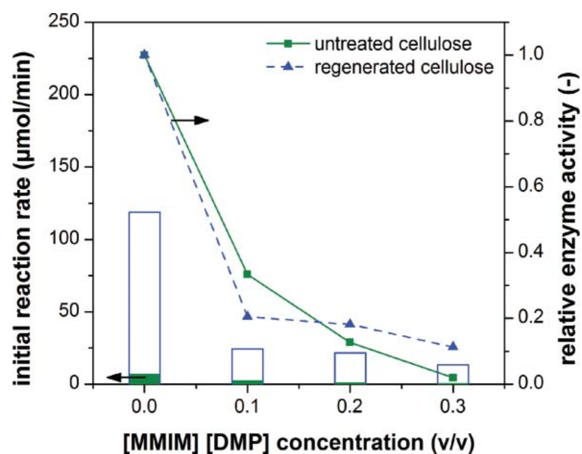

Fig. 6 Initial reaction rate and relative enzyme activity of untreated and regenerated cellulose at different [MMIM] [DMP] concentrations. Bars denote the initial reaction rate of untreated cellulose (full bar) and regenerated cellulose (open bar), whereas curves indicate relative enzyme activity as a function of [MMIM] [DMP] concentration. Soluble sugars formed from 10 mg mL^{-1} substrate at 45 °C by using 1.8 mg mL^{-1} desalted cellulase were measured.

the hydrolysis catalyzed by cellulases regardless of the cellulose structure. This confirms the previously established suitability of α-cellulose as the best test substrate to analyze the effect of ionic liquid on the cellulase activity. Nevertheless, by observing product yields after 48 h, decreased sugar yields are observed with increasing concentrations of [MMIM] [DMP] (Fig. 7).

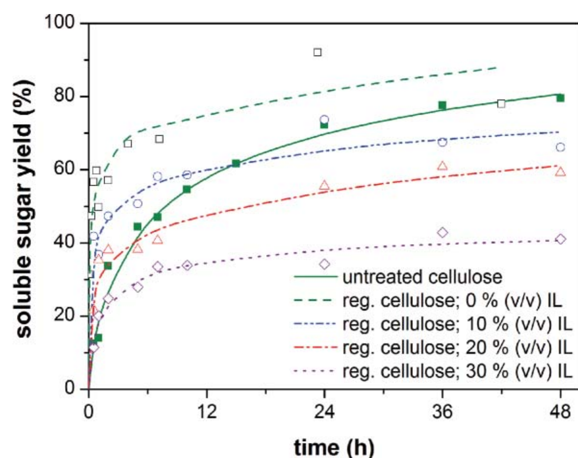

Fig. 7 Conversion curves of cellulose hydrolysis for untreated and regenerated α-cellulose at different [MMIM] [DMP] concentrations. Soluble sugars formed from 10 mg mL^{-1} substrate at 45 °C by using 1.8 mg mL^{-1} desalted cellulase were measured. Reaction conditions correspond to Fig. 6.

As seen in Fig. 7, the conversion curves illustrate that the initial reaction rates are indeed much higher for regenerated α-cellulose. After 48 h the yields did not increase further significantly for both untreated as well as regenerated cellulose. Without ionic liquid, similar maximum yields of approximately 80% were reached, but unexpectedly, with residual ionic liquid present the maximum sugar yield were only between 40% and 70%. In previous studies, the ionic liquid was removed before the enzymatic hydrolysis; similar yields of 80–90% were hereby reached within 24 h.[24,35,39] Reaction rates for regenerated cellulose were increased by only 2- to 6-fold in comparison to untreated cellulose for cellulases from thermophilic organisms.[37] The same authors also found that, in the presence of more than 10% (v/v) ionic liquid, the enzyme activity on regenerated cellulose was already lower than for untreated cellulose without any ionic liquid.

The reason for lower sugar yield using regenerated cellulose with residual ionic liquid may be the composition of the different cellulolytic enzymes in the commercial Celluclast® preparation. It is likely that the ionic liquid specifically influences each of the enzymes in the Celluclast® mixture in a different way. Since *endo*- and *exo*-glucanases have different substrate preferences such as amorphous or crystalline celluloses, the respective reactions will proceed differently on two substrates. This is supported by the results shown in Fig. 2 where the hydrolysis of the two tested soluble substrates was affected less by ionic liquids than the insoluble α-cellulose tested. The effect of ionic liquids on purified *endo*- and *exo*-glucanases as well as β-glucosidase will be investigated in future studies. These prospective results could lead to novel cellulase mixtures to optimize the hydrolysis of regenerated cellulose with needing to wash out any residual ionic liquid. Furthermore, future work has to extend our findings for α-cellulose to a true lignocellulose substrate as there will be present in a biorefinery. In particular, the changes in pH and viscosity of the hydrolysis liquor due to the hemicellulose and lignin content will have to be investigated in detail.

4. Conclusions

Sometimes the predictability of lab experiments for real processes can only be ensured by a careful control of the reaction parameters. In the case of enzymatic hydrolysis of cellulosic substrates for generation of sugars, it was found that residual ionic liquid concentrations of only 10% (v/v) exerted an enormous impact on enzymatic activity – even decreasing the cellulase activity by 70%. Even though it is common to use soluble substrates in the laboratory, this study shows this is unrealistic, since industrial applications always deal with insoluble substrates. Thus, it is expedient to apply the insoluble α-cellulose in laboratory screening experiments or regenerate it by precipitation from ionic liquid. However, here it is important to keep the concentrations of ionic liquid low to maximize sugar yields. In the future, laboratory screening experiments regarding cellulose hydrolysis should focus on using individual enzymes. Ultimately, these perspective results will lead to the production of novel cellulase mixtures to optimize the hydrolysis of regenerated cellulose without needing to wash out any residual ionic liquid.

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