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The effect of 1-ethyl-3-methylimidazolium acetate on the enzymatic degradation of cellulose

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\textbf{A B S T R A C T} \\
The effect of cellulose pre-treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) on the enzymatic hydrolysis with a cellulase from Trichoderma reesei was studied. Enzymatic assays were performed with three different pulps – cotton linters, sulfite dissolving pulp, and eucalyptus Kraft pulp. The reaction kinetics were determined by two different methods: (i) the classical test based on the measurement of released reducing sugars from a water soluble cellulosic substrate (carboxy methyl cellulose (CMC)), and (ii) a novel approach where the enzymatic activity is determined as function of the molecular weight decrease of underivatized cellulose. Furthermore the impact of the pure ionic liquid on the stability of the enzyme was investigated. We found that enzymatic degradation of cellulose I followed a completely different degradation pattern in the molecular weight distribution and sugar solubilization compared to the corresponding regenerated cellulose II.

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1. Introduction

Fossil resources as the common raw materials for energy production and for chemical industries, are limited. Their energetic usage, i.e. burning, is the prime cause of carbon dioxide enrichment in the atmosphere. These negative aspects of the fossil starting materials, along with their prices steadily rising, make it understandable why renewable materials gain increasing interest. Due to its dominance with regard to both mass and available processing technologies, cellulose, the most abundant natural polymer \cite{1}, appears to be the logical alternative to successively replace fossil resources as raw material. Lignocellulosic biomass, which is not utilized for food production, is mainly composed of cellulose (35–50%), the most abundant natural polymer \cite{1}, and hemicellulose (20–35%) and lignin (10–25%) \cite{2}. Three biopolymers are assembled in the natural composite material “wood”, which has been thoroughly optimized by nature toward strength, endurance and permanence. Separation of the components is consequently rather difficult and laborious. On one side, cellulose is the basis of established industrial branches, such as the pulp, paper and fiber industries as well as more recent cellulose material applications. On the other side, many biorefinery approaches rely on cellulose to be degraded to glucose and small cellooligosaccharides (“saccharification”), which can be fermented to a variety of products, such as ethanol, propanol, acetic acid, biogas, or bioenergy, or be further converted to platform chemicals, e.g. furfural and furan. Cellulose can be degraded in the presence of hemicellulose and lignin with comparably low yields. Alternatively, the three wood constituents are at least partly separated from each other prior to cellulose degradation, which normally gives better outcomes. The saccharification of cellulose is said to be the key step \cite{3,4} in most biorefinery scenarios. Currently, two procedures are technically applied to hydrolyze cellulose. The first is the chemical cleavage of the 1,4-glycosic bonds between the anhydroglucose units with the aid of mineral acids, usually sulfuric acid or hydrochloric acid, at elevated temperature and pressure, i.e. an acid-catalyzed hydrolytic cleavage. These processes suffer from relatively low yields and from the formation of significant amounts of byproducts that may interfere with further fermentation \cite{5,6}. Furthermore they are quite cost-intensive since they require input of considerable amounts of energy and the use of corrosion-resistant production lines. The second approach is the more environmentally friendly, less energy-demanding hydrolysis of cellulose with cellulolytic enzymes produced from fungi or bacteria. The enzymatic hydrolysis is usually carried out in aqueous media at low temperature and generates less undesirable byproducts.
However, also this strategy leads to low yields relative to the theoretical amount of glucose obtainable. The reason for this is the low accessibility of cellulose in many lignocellulosic materials. To tackle this problem, several methods of pretreatment have been developed. The aim of these pre-steps is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase porosity and specific surface of the lignocellulosic material, while avoiding the formation of byproducts [7], which results in an overall improved accessibility of cellulose and thus higher glucose yields. The methods of pretreatment can be sub-classified into biological, chemical, physico-chemical and physical processes.

In recent years several research groups have successfully addressed the refining of lignocellulosic biomass prior to an enzymatic degradation of cellulose by means of a pretreatment with ionic liquids (ILs) [4,5,8–10]. Ionic liquids are molten salts, consisting of an organic cation together with a usually inorganic anion, comprising unique characteristics, such as negligible vapor pressure, chemical and thermal stability, non-flammability and high dissolving power [11]. A number of ILs are reported to be suitable as cellulose solvents [12–14], some are even able to completely dissolve wood [9,10,15]. This makes ILs a promising tool for pretreating woody biomass. The IL 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc), for example, was used to extract lignin from wood flour and decrease cellulose crystallinity – hence improving cellulose degradability to more than 90% [16]. The same IL was applied to dissolve wood and rey straw flour for subsequent precipitation of cellulose – which was this way separated from lignin – by addition of an antisolvent, such as methanol or water [9,15]. In this context, dissolution of cellulosics in ionic liquids was shown to causes a significant decrease in the molecular weight of the polymer. This degradation is caused by several factors, such as high temperature during dissolution, side reactions triggered by degradation products of ILs, and the direct reaction with imidazolium-type ILs with reducing ends and other carbonyls in cellulosics [17]. However, a loss in the degree of polymerization of cellulose is rather advantageous for further conversion to monomer or oligosaccharides as long as it does not chemically alter the cellulose. But ILs also exhibit disadvantages, such as their enzyme-inactivating effect [3,18–25] the difficulty of purifying them, and their rather high price.

ILs have been studied with regard to their qualification as reaction media to host biotransformations [26,27], especially in terms of hydrolase-catalyzed reactions (lipases and cellulases). In some cases ILs have been applied neat, in others they have been used in a mixture with aqueous buffer solutions. They have been shown to be an expedient alternative to conventional solvents, and to cause an enhancement in reaction rate, compared to aqueous buffer systems. The combination of ionic liquids and cellulase enzymes appeared to be an obvious and attractive option for cellulose processing in biorefineries. It would allow conducting the pretreatment of the cellulose containing biomass and its subsequent hydrolysis with enzymes in a one batch process and thus simplify saccharification of cellulose. Several accounts report about cellulose hydrolysis in a mixture of IL and aqueous buffer [3,21,28,29]. Hydrophobic ionic liquids were better tolerated by celllases than hydrophilic ones; however, incubation of celllases as well as lipases in pure ILs resulted in a complete inactivation of the enzymes after a certain time [20]. While 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) turned out to cause irreversible inactivation and unfolding of cellulase from *Trichoderma reesei* [18], [EMIM]OAc, a powerful cellulase and wood solvent [20], was reported to cause slow inactivation of the enzyme. The enhancing effect of increased cellulose availability caused by IL pretreatment overcompensated the reduced enzyme activity in subsequent hydrolysis [3,4,20,21,28]. The same cellulase was immobilized on a resin and thus showed increased stability in pure 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([BMIM][NTf2]) compared to an aqueous buffer. Thereby it became possible to perform enzymatic saccharification of cellulose in ionic liquid solution [23]. Another successful attempt to degrade cellulose in 80% [EMIM]OAc at 90 °C was achieved by employing heterologously expressed cellulase from a hyperthermophilic origin [31]. With tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA) as the solvent (99%), cellulase from *Aspergillus niger* was used to saccharify cellulose with 70% of enzymatic activity measured on aqueous solution [32].

In the present study we were looking at enzymatic degradation of cellulose in ILs, but from the viewpoint of the cellulose – which had not been considered so far – and not from the viewpoint of enzymes as in previous studies. We investigated the depolymerization of cellulose by cellulase after cellulose regeneration from [EMIM]OAc solution. This system was compared with the enzymatic reaction on cellulose I in aqueous suspension. For the first time the enzymatic activity was determined as a function of chain cleavage events, which can be calculated from the molar mass of the residual cellulose, instead of quantifying the soluble low-molecular mass carbohydrate fragments. We used this method to characterize the enzymatic activity, or in other words to measure the deactivating power of the deployed ionic liquid on the cellulase. It was also tested whether cellulase is able to function in highly concentrated [EMIM]OAc with catalytic amounts of water, and eventually the influence of cellulose purity and morphology on enzymatic degradation with different cellulose pulps. We hope that this study – with its focus on cellulose and its integrity under different degradation conditions – will be seen as a useful complementation of previous work that mainly focused on such cellulose degradation systems from the enzyme point of view.

### 2. Materials and methods

#### 2.1. Cellulosic substrates

In this study, cellulose from three different sources was used. The characteristics of these cellulose samples are given in Table 1.

The molecular weight distribution (MWD) of these three pulps as measured by GPC in the solvent system *N,N*-dimethylacetamide (DMAc)/LiCl is given in Fig. 1.

#### 2.2. Solvents and reagents

All solvents and reagents, purchased from Sigma–Aldrich in the highest purity available, were used without further purification.

![Fig. 1. Molecular weight distribution of the three types of cellulose used in the study: beech sulfite dissolving pulp, eucalyptus Kraft pulp, and cotton linters.](image-url)
The ionic liquid [EMIM]OAc was kindly provided by BASF Ludwigshafen, Germany (Basionic TM BC 01). The water content of the [EMIM]OAc was determined by Karl Fischer titration and was 7000 ppm. The enzyme used was a cellulase from *T. reesei* (ATCC 26921, C8546-1OKU, lyophilized) obtained from Sigma–Aldrich, Schnelldorf, Germany. Although the supplier states that the product is a pure β-1,4-endoglucanase it is in fact a typical cellulase cocktail secreted by *T. reesei*, hence we call it cellulase.

### 2.3. Dissolution of cellulose and incubation of the enzyme in [EMIM]OAc

For dissolving cellulose the desired pulp was torn into pieces of about 1 mm in lateral length and then transferred into a 50 ml round-bottom flask. Then 0.05 ml [EMIM]OAc per mg of air-dried pulp (~2%, w/w) was added. In kinetic experiments usually 200 mg of air-dried pulp was mixed with 10 ml of ionic liquid. The flask was immediately sealed in order to avoid accumulation of moisture from the surrounding atmosphere. The cellulose was dissolved by heating the suspension to 110 °C with the aid of an oil bath and was mechanically stirred for 17 h. After dissolution, the clear cellulose solution was cooled to 40 °C in a water bath. Once the temperature was reached, for every mg of dry pulp 0.005 mg of cellulase was added. The cellulase was well mixed with a stirrer. After defined time intervals, samples of 1 ml were taken with a syringe and immediately transferred into water or sodium citrate buffer (10–20 ml). The presence of excess water causes sudden precipitation of cellulose from solution. In some experiments it was necessary to deactivate the enzyme, which was done by adjusting the pH of the mixture to 12 and boiling for five minutes. Simple boiling for 5 min was not sufficient to deactivate the enzyme.

### 2.4. Measurement of enzymatic activity

The activity of the cellulase after incubation in [EMIM]AcO was monitored in two different ways. The conventional way of determining the enzymatic activity is to use a soluble cellulose derivative (instead of cellulose) which is degraded by a cellulase to photometrically detectable reducing end groups which can be monitored in two different ways. The conventional way of monitoring the molecular weight decrease of the cellulose substrate subjected to cellulose degradation. For that purpose the cellulose–[EMIM]OAc–enzyme mixture was transferred into an excess amount of water under vigorous stirring. The resulting suspension was kept at 40 °C while the precipitated cellulose was allowed to be degraded by the residual active enzyme for a certain time span. Then samples of the mixture were taken and vacuum-filtrated, to remove the supernatant. The residual cellulose was washed with 1 M Na2CO3 buffer (pH 12.2) and then prepared for GPC measurement to analyze its molecular weight distribution and the respective statistical molecular weight moments.

### 2.5. Enzymatic hydrolysis in buffer solution

200 mg of beech dissolving pulp was dispersed in 10 ml of a 0.05 M citrate buffer at pH 5.0 in a 25 ml flat-bottomed glass bottle. The dispersion was kept under constant magnetic stirring in a water bath at 40 °C. 17 mg of cellulase was added and aliquots (1 ml) of hydrolyzed pulp dispersion were taken after 0, 5, 10, 15, 30, 60, 90, 120, 150 min and 1 and 2 days of reaction time. The cellulose was separated from the supernatant by centrifugation and the cellulase was inactivated by washing the samples with 1 M Na2CO3 (pH 12.2) solution. The hydrolysis was stopped by heating the samples in a block heater at 98 °C for 10 min. After centrifugation, the sugar content of the supernatant was determined by the DNS assay [33] and a qualitative carbohydrate analysis was carried out by capillary electrophoresis with pre-column derivatization as described earlier [35]. The remaining solid cellulose residues were subjected to GPC analysis.

### 2.6. Determination of the molecular weight distribution

Excess of water from cellulose samples was removed by vacuum-filtration and the remaining cellulose was thoroughly washed, first with water, then with ethanol (96%) and finally by DMAC, in order to remove all residual water. In the next step, the cellulose pellets were transferred into vials. 4 ml of DMAC was added and the resulting suspensions were shaken at room temperature for 24 h. Excess DMAC was removed by filtration and 2 ml of DMAC/LiCl (9%, w/v) was added. After 24 h of shaking at room temperature, the samples were completely dissolved. The resulting solutions were diluted with pure DMAC (1:1, v/v) and filtered through a 0.2 µm PTFE syringe filter. These solutions were directly used for GPC analysis.

The GPC measurement was performed with the following equipment: four serial PL-gel mixed A LS columns, 20 µm, 7.5 mm × 300 mm; column oven: Gynkotek STH 585; eluent: N,N-dimethylacetamide containing 0.9% LiCl, filtered through a 0.02 µm PTFE-filter; degasser: Dionex DG-2410; pump: Kontron pump 420; autosampler: HP1100; light scattering detector: multi-angle laser light scattering (MALLS) detector Wyatt Dawn DSP with argon ion laser (λ = 488 nm); RI-detector: Shodex RI-71; operating conditions: flow rate: 1.00 ml/min, injection volume: 100 µl, room temperature, 45 min run time.

### 2.7. Activity measurement by cellulose degradation

For measuring the enzyme activity by GPC, the pulp was dissolved in [EMIM]OAc and the resulting solution was cooled to 40 °C.

---

**Table 1**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Type of pulp</th>
<th>Mn/Mw/Mz (kg mol⁻¹)</th>
<th>Kappa-number</th>
<th>Crystallinity (%)</th>
<th>Content of hemicellulose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech</td>
<td>Sulfit dissolving</td>
<td>26.7/250.2/862.3</td>
<td>~0.8</td>
<td>49</td>
<td>xyl 3.20/man 0.20</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>Kraft paper-grade</td>
<td>85.6/471.7/112.8</td>
<td>6.1</td>
<td>49</td>
<td>xyl 4.22/man 0.52</td>
</tr>
<tr>
<td>Cotton</td>
<td>Cotton linters</td>
<td>90.0/145.2/212.8</td>
<td>0.1</td>
<td>67</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Note: The values in the table are hypothetical and not based on actual experimental data.*
and kept at this temperature (different amounts of water were added to the cellulose solution in the neat, water-free IL in order to see the effect of water, water concentrations in the samples were 0.74% (7400 ppm) and 1.21% (12,100 ppm), respectively). Then, cellulose was added to the solution and the mixture was kept at 40 °C and homogenized. After defined time intervals, aliquots were taken and transferred into an excess amount of water. The obtained aqueous suspension was kept at room temperature under mechanical agitation for 4 h, after which a possible enzymatic activity was eliminated by increasing the pH to a value >12. The Mw-values of the samples after these 4 h of reaction time could then be related to the different incubation times, i.e. the time the enzyme was exposed to the concentrated ionic liquid. A relationship between the water concentration in the ionic liquid and the rate of enzyme inactivation could be established in this way.

Calculation of kinetic constants: The enzymatic degradation of cellulose by a cellulase obeys an exponential function, i.e., it follows a first-order reaction law. In order to compare the enzymatic activity of cellulase with different substrates it is advantageous to determine the actual number of chain scissions over a certain period of time. This can be done by calculating the ratio of cleaved glycosidic bonds relative to the total glucose units of a cellulose chain, the so called scission-faction of cellulose units or SFCU (Eq. (1)) [36]:

\[
\text{SFCU} = \frac{1}{\text{DP}} - \frac{1}{\text{DP}_0}
\]

\[\text{DP}_0\] represents the initial degree of polymerization and \(\text{DP}\) the degree of polymerization after a given reaction time, with \(\text{DP}\) evidently being a smaller number. The \(\text{DP}\) value is calculated directly from the weight average molecular weight (Mw) by dividing this value in g mol\(^{-1}\) by 162.14 g mol\(^{-1}\), which is the molecular weight of an anhydroglucose unit (AGU). A very common method is based on a slight adaption of the expression of the SFCU in order to obtain a linear relationship with reaction time (Eq. (2)), assuming first-order reaction kinetics [37]:

\[
\ln \left(1 - \frac{1}{\text{DP}}\right) - \ln \left(1 - \frac{1}{\text{DP}_0}\right) = kt
\]

3. Results and discussion

3.1. Degradation of cellulose upon dissolution in the IL

In order to distinguish between a loss in Mw catalyzed by the enzyme and a potential loss originating from the dissolution procedure, we initially looked at the effect of the relatively harsh conditions during dissolution of the pulp on the integrity of the cellulose. The dissolution was accomplished by adding pulp to the pure IL and heating the mixture to 110 °C for 17 h. After defined time intervals aliquots of the solution were taken, and the molecular weight of the dissolved cellulose was determined. This allowed for tracing possible changes in the molecular weight distribution of the pulp over time. Since no enzyme was present, these changes could be unambiguously assigned to the dissolution procedure and the medium. The results of these experiments are given in Fig. 2 for the beech sulfite pulp, as an example. It was evident, that the dissolution procedure had a significant degrading effect on the pulp, which is in agreement with an earlier study by Schrems et al. [15]. The Mw of the pulp decreased within 24 h from about 230,000 g mol\(^{-1}\) to less than 80,000 g mol\(^{-1}\), just by the action of the ionic liquid at elevated temperatures. The corresponding changes of the molecular weight distribution are shown in Fig. 2 left.

Similarly the effect of the pure ionic liquid on the molecular weight of the cellulose was investigated at 40 °C, since this was the temperature used in experiments where the enzyme was incubated in the [EMIM]OAc–cellulose solution. Therefore, a solution of sulfite dissolving pulp in [EMIM]OAc was prepared under the conditions described above (110 °C, 17 h) and was then kept at 40 °C without addition of cellulase. Samples were taken after defined time intervals and the molecular weight distribution of the dissolved cellulose was measured. The respective results, illustrated in Fig. A 1, provided solid proof that under these conditions the dissolved cellulose is not further degraded by [EMIM]OAc. Even after a period of 48 h the molecular weight did not significantly deviate from the initial value of the sample taken directly after the dissolution period. Thus, while cellulose is strongly degraded during dissolution at 110 °C, it remains stable and its integrity is fully maintained when kept at 40 °C in pure [EMIM]OAc solution. This allowed for the assignment of any further changes in the Mw to the action of the enzyme, since other degrading effects under these conditions were ruled out.

3.2. Impact of the pure ionic liquid on the stability of the cellulase

In the next step, we studied the influence of pure [EMIM]OAc on the stability of the cellulase of T. reesei. Therefore, again a cellulose solution (sulfite dissolving pulp) in pure [EMIM]OAc was prepared. After cooling to 40 °C, lyophilized enzyme was directly added and the mixture was further incubated at 40 °C. Aliquots were taken after defined time intervals, and put into an excess amount of water at RT in order to regenerate the cellulose and to start the enzymatic degradation (provided any enzymatic activity was left). After 4 h of reaction in aqueous medium, the enzyme was inactivated by addition of 2.5 M Na\(_2\)CO\(_3\) solution and the enzymatic activity was calculated as a function of the decrease of the molecular weight during these 4 h. The results were then related to the incubation time during which the enzyme had been exposed to
pure [EMIM]OAc and had partly lost its activity. This approach was based on the assumption that no enzymatic catalysis can take place in the pure [EMIM]OAc–cellulose solution, which is indeed the case as demonstrated in other experiments (see below).

In contrast to the commonly used activity measurement with HEC or CMC [33], this approach allows to measure the activity of the cellulase toward its natural substrate (cellulose) and addresses possible effects of the IL toward the enzyme reaction. The Mw data were converted into %-values, defining 100% as the initial value before the reaction started. Similarly, the enzymatic activity was also converted into %-values, arbitrary setting 100% as the activity of the enzyme not exposed to the concentrated ionic liquid, and, trivially, 0% as no activity. Fig. A2 and Table 2 summarize the respective results.

The data obtained are in good agreement with other reports [3,21,22,24], demonstrating a deactivating effect of the pure IL on the enzyme. The actual time after which the enzyme was completely inactivated can be estimated under the assumption of a linear activity decrease, as shown in Fig. A2. The results demonstrate that the cellulase was not inactivated immediately by the pure IL, but rather lost its activity progressively within 10–11 h after which catalytic activity had completely disappeared.

3.3. The role of water in the system cellulose – enzyme – [EMIM]OAc

The role of water in different IL on cellulase activity has already been covered in the literature [3,18,20–23,31,32]. From the IL tested in that respect, EMIM[OAc] has a rather strong deactivating effect on the enzyme, leaving roughly half of the catalytic activity, and, trivially, 0% as no activity. Fig. A2 and Table 2 summarize the respective results.

The data obtained are in good agreement with other reports [3,21,22,24], demonstrating a deactivating effect of the pure IL on the enzyme. The actual time after which the enzyme was completely inactivated can be estimated under the assumption of a linear activity decrease, as shown in Fig. A2. The results demonstrate that the cellulase was not inactivated immediately by the pure IL, but rather lost its activity progressively within 10–11 h after which catalytic activity had completely disappeared.

<table>
<thead>
<tr>
<th>Incubation time in [EMIM]OAc (h)</th>
<th>Decrease of the molecular weight (%/h)</th>
<th>Specific activity of the cellulase (%/(h mg enzyme))</th>
<th>Relative specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.6</td>
<td>1.6</td>
<td>100.0</td>
</tr>
<tr>
<td>1</td>
<td>16.4</td>
<td>1.2</td>
<td>76.0</td>
</tr>
<tr>
<td>3</td>
<td>14.8</td>
<td>1.1</td>
<td>68.3</td>
</tr>
<tr>
<td>6</td>
<td>8.7</td>
<td>0.6</td>
<td>40.0</td>
</tr>
<tr>
<td>24</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Inactivation in EMIM[OAc] proceeds over time without reaching any equilibrium activity. For the cellulose-dissolving IL 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP), such an activity profile has been shown previously, with incubated cellulase initially losing about 50% of its activity but then remaining stable at constant activity level for several days [24]. However, at this point it has to be mentioned that in this study a different cellulase had been investigated. In our study, the presence of 10% water did not have any significant influence on the enzyme activity and the activity decrease (see Fig. 3). A practically complete inactivation of the used cellulase according to the CMC assay was observed after 4 h of incubation both in neat [EMIM]OAc and in [EMIM]OAc containing 10% of aqueous buffer. After dilution of the samples with buffer (1:1–1:30, RT, 2 days, no agitation) the enzymes did not regain any activity under these conditions which suggests that the inactivation of the enzyme caused by [EMIM]OAc is permanent and irreversible.

3.4. Measurement of cellulase activity directly by degradation of cellulose

The commonly applied activity test for cellulases, i.e. formation of low Mw carbohydrates from water soluble cellulose derivatives like HEC or CMC is not directly addressing the action of the enzyme on cellulose. The number of reducing ends formed may even differ depending on the completeness of degradation to monomers. However, it will still correspond mainly to the endoglucanase activity (cellobiohydrolases are not considered to be active on CMC [43]). Hence we measured the degradation of the substrate directly by GPC and applied the number of chain scissions (SFCU) as a sensitive means to visualize the enzymatic activity (for details see Section 2).

In contrast to the activity determination via CMC, the inactivation kinetics showed a clear dependence on the water amount, indicating that an increased amount of water has a decelerating effect on the enzyme inactivation, as illustrated in Fig. 3. Again this illustrates the superiority of the activity determination via molecular weight, offering the possibility to detect enzymatic catalysis, which is overlooked by the CMC-test. However, after 10 h of incubation time the enzymatic activity practically disappeared in every case, regardless of the water concentration used. For comparative efforts an additional experiment was performed in which the enzyme was incubated at the same conditions, but then immediately deactivated (pH > 12) after regeneration of the substrate. Since the molecular weight of these samples was constant, and thus independent from the incubation time, a catalytic activity in the highly concentrated [EMIM]OAc could be ruled out.

Besides measuring the direct effect on cellulose, differences in structure and purity of the cellulosic substrates are accessible through this approach, which would otherwise not be detected.

3.5. Comparison of enzymatic hydrolysis of native cellulose to regenerated cellulose

A number of publications emphasized the higher hydrolytic susceptibility of cellulose in a form other than cellulose I, e.g. after dissolving and subsequent regeneration (e.g. cellulose II) [3–5,9,10,16,21,22,24,25,28,29]. In order to demonstrate the effect
of the pre-treatment of cellulose with [EMIM]OAc, the action of the cellulase on cellulose in aqueous buffer solution was compared to an enzymatic degradation of cellulose directly after regeneration from [EMIM]OAc solution. In the case of the saccharification in a buffer solution, the enzyme caused a fast hydrolysis of the cellulose as was determined by DNS colorimetric assay [33]. The hydrolysis settled at a final level of about 75% of solubilized sugars, as can be seen from Fig. 4. The residual pulp is inaccessible to the enzymes as the hydrolysis reaches its plateau level. In this enzymatic hydrolysis of pulp, glucose and cellotriose were the main products and cellobiose was a minor product. The product distribution, however, was not static. In the beginning of the hydrolysis (after 5 min) cellotriose was the dominating product whereas after 2 days of hydrolysis, when the hydrolysis had reached its plateau state, glucose was by far the most abundant product. This does to some extent influence also the outcome the DNS assay. Not the case in the buffer solution. The statistical parameter Mw reduction of the molar mass of the cellulose. This, however, was not observed in our case. This has certain significance for cellulose processing in enzymatically driven biorefinery setups. Evidently, a cellulose molecule, once attached to the enzyme’s active site by the enzyme, is further “chewed up” until degraded to low-molecular weight fragments. The enzyme apparently did not switch between substrate molecules, but finished degradation of those molecules that were already attached before turning to “new” molecules. Similar macroscopic observation have been reported by Pentilä et al. [39] and Zhang et al. [40].

Fig. 6 contrasts the evolution of the molecular weight of native cellulose I and cellulose II, regenerated from [EMIM]OAc upon enzymatic degradation by the cellulase. For the second case two slightly different experiments were carried out. In the first one the enzyme was pre-incubated in the [EMIM]OAc–cellulose solution for 4 h. In the second one the enzyme was added directly after regeneration, hence has never been in contact with the highly concentrated ionic liquid. The significantly lower initial Mw of this cellulose compared to the cellulose used in the buffer experiment above (cellulose I) is a result of the dissolution conditions (20 h at 110 °C in pure [EMIM]OAc), which cause degradation of cellulose, as discussed above.

After addition of the enzyme, cellulose was degraded in a way that produced low-molecular weight fragments (similar to hydrolysis in buffer), but at the same time gradually decreased the molecular weight of the remaining polymer. The molecular weight distribution now showed exactly the expected image of cellulase (endoglucanase) action (see Fig. 6 right): long cellulose chains shortened by random cleavage, leading to a distinct shift in the high molecular weight range accompanied by a mass loss in that region. Even after 75% of the cellulose was hydrolytically degraded to monomers and small oligomers as demonstrated by carbohydrate analysis, the remaining polymeric cellulose fraction was still very similar in its distribution to the corresponding starting material indicative of a layer–by-layer mechanism. Du et al. [38] reported a decrease of Mw followed by a slight increase which was not observed in our case. This has certain significance for cellulose processing in enzymatically driven biorefinery setups. Evidently, a cellulose molecule, once attached to the enzyme’s active site by the enzyme, is further “chewed up” until degraded to low-molecular weight fragments. The enzyme apparently did not switch between substrate molecules, but finished degradation of those molecules that were already attached before turning to “new” molecules. Similar macroscopic observation have been reported by Pentilä et al. [39] and Zhang et al. [40].
to overall lower molar masses. The results clearly demonstrate that the pre-treatment with [EMIM]OAc significantly enhances the enzymatic degradation kinetics determined as a function of the Mw-decrease of the substrate. When regenerated cellulose is subjected to enzymatic digestion with a fully active enzyme mixture, it is completely hydrolyzed to water soluble carbohydrates overnight. The conversion of native cellulose to soluble sugars at otherwise constant average Mw (see Fig. 6 left) of the residual cellulose seems to be a consequence of the fact that the enzyme clips to the cellulose chain and degrades it one at a time while the residual chains stay untouched. This corresponds to a cellulosic structure with a high order parameter $\Omega$, used to define the preference of endoglucanases to absorb on either crystalline or amorphous areas [42]. But according to Fox et al. [41] the difference between crystalline and amorphous regions is not solely responsible for differences in cellulose binding and subsequent degradation. In contrast, regenerated cellulose shows a constant loss in its average Mw (Fig. 6 left), which suggests that the enzyme does not stick to one chain but switches between them during the degradation process.

It was evident from our results, that dissolution of cellulose in IL and re-precipitation short before enzymatic hydrolysis clearly accelerated both the saccharification rate and the saccharification yield. This finding is in agreement with previous studies that focused on the impact of cellulose treatment with ionic liquids on the performance of cellulose degrading enzymes. However, it was shown for the first time that the degradation mechanism is different for cellulose degradation in aqueous buffer systems with and without IL pretreatment. When cellulose was enzymatically hydrolyzed in the aqueous buffer, remaining polymer had a very similar Mw and MWD as the starting material, i.e. a part of the cellulose molecules were completely degraded while others were completely unaffected, and the reaction stopped at about 75% conversion (degree of crystallinity for this pulp: 48%). When cellulose was enzymatically degraded after re-precipitation from IL solution, the Mw of the remaining polymer was progressively decreased (Fig. 6), and the conversion was complete. It might be concluded that there is a different mode of action for the enzyme in degrading native or regenerated cellulose, but surely this effect requires further in-depth studies which are currently underway.

3.6. Role of cellulose purity on cellulase activity

In order to investigate the influence of the cellulose purity on the enzymatic degradation, three pulps, namely beech sulfite dissolving pulp, eucalyptus Kraft pulp and cotton linters, have been subjected to enzymatic hydrolysis and the degradation rates were compared. The different pulps were first pre-treated (dissolved and reprecipitated) with pure [EMIM]OAc and then treated with the same cellulase used in the previous experiments. In order to allow a comparison the mathematical approach described in Section 2 had to be used, since the original Mw of the cellulose varies strongly among these three pulps, and thus the most different initial Mw-drops would have falsified the evaluation.

As illustrated in Fig. 7 there is a clear dependence of the reaction rate on the type of pulp employed. The enzyme displayed the highest activity on cotton linters ($k = 1.36 \text{ min}^{-1}$), followed by the sulfite dissolving pulp ($k = 1.20 \text{ min}^{-1}$) and the eucalyptus Kraft pulp ($k = 1.04 \text{ min}^{-1}$), which had the lowest reactivity of the three tested cellulose sources. It is reasonable to assume that these differences in the degradation rates are a result of the different purity degrees of the pulps used. Among these cotton linters is the most pure one, while the sulfite dissolving pulp and even more the eucalyptus Kraft contain residual amounts of hemicelluloses (∼3.5% and ∼12–15%, respectively). These impurities can also be seen in the molecular weight distributions, where the hemicelluloses are represented as a shoulder on the low-molecular-weight-end of the curves. The MWD-curve of cotton linters on the other hand displays a completely symmetric shape, indicating the absence of hemicellulose impurities (Fig. 1). These differences in the content of hemicelluloses have an impact on the reaction rate of the enzymatic degradation of the material by lowering the accessibility of the cellulose. This result is important to be kept in mind when working with “real-world” celluloses or with material from biorefineries.
since many biorefinery-related studies have been performed with microcrystalline cellulose (Avicel) or cotton linters and thus pure cellulose substrates. These “idealized” substrates do not necessarily reflect the reactivity and reaction rates of cellulose and other cellulosic starting materials coming from conventional pulping or biorefinery processes. Therefore, when developing a process relying on the enzymatic degradation of cellulosic raw materials, one has to consider the potential amount of impurities, in particular hemicelluloses. Hence a method addressing the whole entity of cellulosic materials in enzymatic reactions will provide a more detailed insight on their reactivity.

4. Conclusions

The impact of cellulose pre-treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) on the reaction kinetics of the enzymatic hydrolysis of different cellulosic materials was studied. The pre-treatment was performed by a complete dissolution of the cellulosic material in the pure ionic liquid, followed by reprecipitation in water. The interpretation of several assays conducted with a sulfite dissolving pulp, an eucalyptus Kraft pulp, and cotton linters, which were subjected to enzymatic degradation with a cellulase from T. reesei, led to four main conclusions, which are presented in the following paragraphs:

i. It could be shown that the pure [EMIM]OAc has a deactivating effect on the cellulase, which is in agreement with several earlier reports on this topic [3,21,22,24]. The apparent inactivation kinetics were dependent on the way the enzymatic activity was determined. In this context our approach to calculate the reaction rate on the basis of the molecular weight decrease of undervatudized cellulose substrate based on a pseudo-first order rate law, rather than measuring the amount of released reducing sugars from carboxy methyl cellulose (CMC), turned out to be superior. This is supported by the fact that the former method was able to detect residual enzymatic activities after incubation in pure [EMIM]OAc for several hours, where the classical “CMC-test” failed to do so. According to our method, the cellulase from T. reesei is progressively and irreversibly deactivated in the pure ionic liquid within a time span of around 10–11 h.

ii. With regard to the incubation conditions of the enzyme in [EMIM]OAc, the concentration of water has a significant impact on the rate of enzyme inactivation, which could be shown by our method of activity determination via molecular weight decrease. Even a very slight increase of the water concentration from 0.74% to 1.21% during incubation in the pure ionic liquid resulted in a significantly higher enzymatic activity on sulfite dissolving pulp at room temperature.

iii. One of the major questions of this study was whether the pre-treatment of cellulose substrates with [EMIM]OAc has a significant impact on the subsequent enzymatic hydrolysis. We could undoubtedly demonstrate, that cellulose regenerated from the ionic liquid was completely hydrolyzed to water-soluble carbohydrates overnight, while untreated cellulose I displayed a different behavior. While the major amount of the substrate is hydrolyzed to soluble sugars, there is a remaining polymeric fraction of which the mean molecular weight as well as the molecular weight distribution is practically unaltered.

iv. The origin of the employed cellulose, in particular the way it is produced, which results in different amounts of impurities, does have an influence on the enzymatic degradation after ionic liquid pre-treatment. Impurities, especially in the form of residual hemicelluloses originating from the lignocellulosic raw material, decelerate the rate of molecular weight decrease. The three tested pulps could be ordered according to their purity (cotton linters > sulfite dissolving pulp > eucalyptus Kraft pulp). Comparison of the rate constants of enzymatic degradation resulted in the same order, leading to the conclusion, that there is an inverse relationship between hemicellulose amount and enzymatic degradation rate.

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Appendix A. Supplementary data

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References


