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Ionic Liquid-Assisted Enzymatic Depolymerisation of Cellulose from Biomass

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ABSTRACT

The recalcitrance of lignocellulose poses a major challenge for its sustainable utilization as source for chemicals, materials and fuels. The capability of some ionic liquids (IL) to dissolve lignocellulose and gain a precipitated amorphous material is exploited within the Cluster of Excellence "Tailor-made fuels from biomass" (www.fuelcenter.rwth-aachen.de) as an alternative pre-treatment for further (bio)-chemical conversion to fuel components. Based on scattered light intensity measurements (BioLector®, Germany) of cellulose suspended in IL, a number of IL capable of dissolving cellulose could be identified. After precipitation from the IL, enzymatic hydrolysis rates and yields are significantly enhanced (~ 20-fold rate and + 10% yield). These results can be partially transferred to wooden biomass. When retaining e.g. 10% (v/v) ionic liquid content in an aqueous system however, the enzymatic activity of commercial cellulase preparations (Celluclast®, Novozyme, Denmark) is significantly reduced to between 20 and 30% of its activity in aqueous solution. Ionic strength and viscosity of the IL have been identified as important contributing factors. Interestingly, the enzyme stability was fully maintained. However, the IL interacts differentially for endo- and exo-acting cellulases. The interpretation of these data is facilitated using mathematical models, e.g. those based on population balances (Predici), that allow incorporating both the polymeric nature of the substrate and in case of precipitated cellulose also the particle characteristics of the substrate, i.e. size, crystallinity, and porosity. As a result of the experimental and theoretical studies, improvements for IL-assisted enzymatic hydrolysis should be directed at 1) increasing enzyme activity in presence of IL and 2) tailoring the composition of the enzyme mixture to the resulting cellulosic material using the mathematical models.

Keywords: cellulase, cellulose depolymerisation, ionic liquids, lignocellulose biomass, mathematical modelling, pre-treatment
Abbreviations: **Ac**, acetate; **AMIM**, 3-allyl-1-methylimidazolium; **BMIM**, 3-butyl-1-methylimidazolium; **CMC**, carboxymethyl-cellulose; **DMP**, dimethylphosphate; **EMIM**, 3-ethyl-1-methylimidazolium; **IL**, ionic liquids; **MMIM**, 3-methyl-1-methylimidazolium, **NTP**, p-nitrophenol; **RTIL**, room temperature ionic liquids

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INTRODUCTION

Virgin biomass is naturally produced in huge amounts of more than 80 Gtons carbon year^{-1} (Petrou and Pappis 2009). Among that, lignocellulosic biomass has gained considerable interest as a renewable energy and raw material source (Ragauskas *et al*. 2006; Kunkes *et al*. 2008). In its function as the plants' structural polymer composite, lignocellulose (LC) withstands degradation (Himmel *et al*. 2007). On the other hand it contains functional groups that facilitate the further chemical conversion to intermediates (Werpy *et al*. 2004). Pre-treatment methods aiming at the separation or degradation of the polymers lignin and hemicellulose from the main compound, cellulose, typically precede the biochemical or chemical degradation of cellulose (Chandra *et al*. 2007; Agbor *et al*. 2012). Effective pretreatments improve the reactivity of cellulose by reducing crystallinity and enhancing enzyme accessibility via smaller particle size and increased porosity. They should minimise the by-product formation of both the carbohydrate and the lignin fraction, and last but not least be cost- and energy-effective (Sun and Cheng 2002). An overview on the advantages and disadvantages of various pre-treatment methods is provided in **Table 1**.

Obviously, there is yet no process ideally fulfilling all mentioned requirements for biomass pre-treatment. Therefore, the search for suitable pre-treatment methods continues. Due to their tuneable solvation properties, ionic liquids (IL) have been suggested as alternative agents in pretreatment and separation methods, extracting lignin or dissolving the carbohydrates with subsequent selective precipitation (Kilpeläinen *et al*. 2007; Zhu 2008; Chundawat *et al*. 2012), thus preparing biomass to enhance its enzymatic digestion.

IL are organic salts with a low melting point close to ambient temperature. They consist of combinations of bulky anions and cations that determine the wide variety of their physicochemical and solvation properties (Dong *et al*.

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Invited Review

Table 1 Key pre-treatment methods for ligno-cellulose degradation.

Method	Benefits	Drawbacks
Mechanical comminution	Reduction of crystallinity; reduction of particle size	Power consumption
Steam explosion	Low energy demand; cost effective for hardwoods	Some degradation of xylans to inhibitory compounds; incomplete lignin removal; less effective for softwoods
Ammonia fiber explosion (AFEX)	No formation of inhibitory compounds	No hemicellulose separation; ammonia recycle necessary; not effective for high lignin biomass
Acid hydrolysis	Dilute acids exhibit high conversion rates for hemicellulose; strong acids increase specific surface area and decrease crystallinity	pH-neutralisation necessary; concentrated acids are corrosive and must be recycled; cost higher than explosion methods; toxic compounds are formed
Alkaline hydrolysis	Particle swelling increases porosity and decreases crystallinity; effective lignin removal for low lignin content	No hemicellulose removal
Oxidative delignification	H_2O_2 reduces lignin considerably and solubilises hemicelluloses	Cost
Organosolv	Effective reduction of lignin; hemicellulose removal with addition of acid catalyst	Solvent may be inhibitory compound; solvent recycling necessary; safety aspect in solvent handling
Enzymatic oxidation and hydrolysis	Lignin degradation by white rot fungi	Very low reaction rates requiring long residence times

Sources: Sun and Cheng 2002; Chandra *et al*. 2007, Galbe and Zacchi 2007

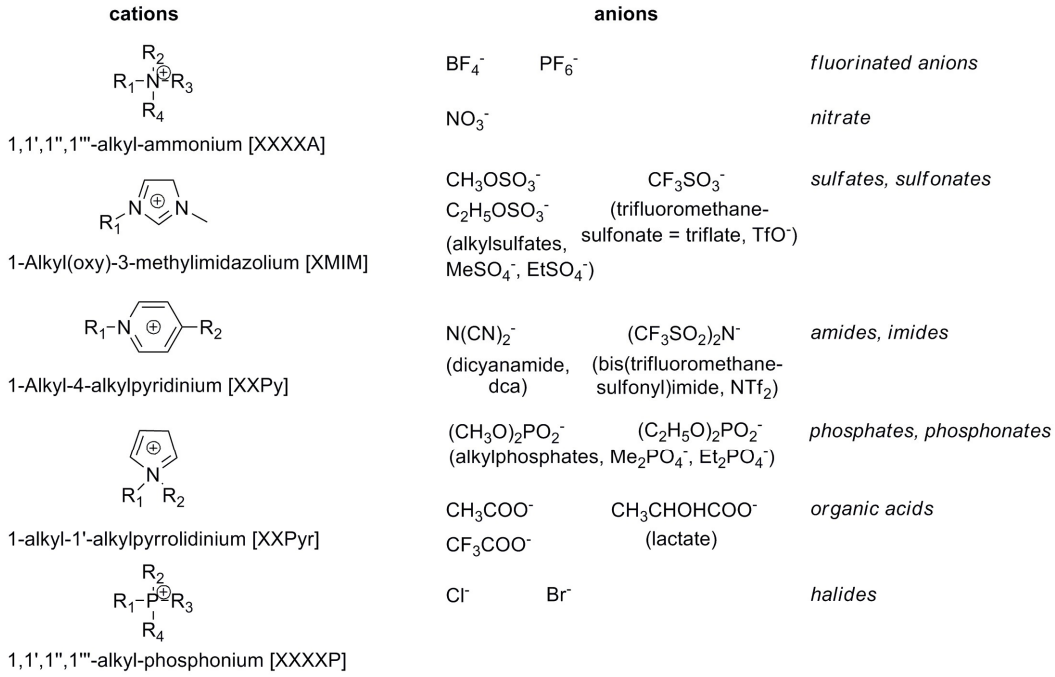

Fig. 1 Ionic liquid cations and anions often used in biocatalysis and/or for carbohydrate polymer dissolution.

2007). The huge number of possible combinations motivated their perception as tuneable or designer solvents (Seddon 2002) (**Fig. 1**). ILs with a melting temperature below 25°C are termed room-temperature ILs (RTILs). Although the first liquid salt had been synthesised already in 1914 by Walden, the intensive exploitation of the IL properties to new applications began only in the 90ies (Welton 1999). ILs have interesting properties from both the environmental and the processing perspective: they have a low volatility, thus reducing greenhouse gas emission (Earle *et al*. 2006) and are non-flammable as bulk liquids (Deetlefs and Seddon 2006). Many ILs are thermally very stable and non-toxic (Swatloski *et al*. 2003; Wells and Combe 2006), thus deserving the title green solvent (Earle and Seddon 2000). Of course, the solvation properties are central – and ILs display ionic interactions, opening new solvation properties unprecedented by organic solvents. ILs solvate 'difficult-to-dissolve' molecules such as polymers and carbohydrates in particular (Kubisa 2004; Ueki and Watanabe 2008), while maintaining the stability of (bio)-catalysts that are negatively influenced by traditional polar organic solvents (Kragl *et al*. 2002; Park and Kazlauskas 2003; van Rantwijk and Seddon 2007).

Particularly ILs with high hydrogen bond basicity dissolve polymers (Anderson *et al*. 2002), yielding polymer solutions or gels. Both for applications in biocatalysis and in the field of carbohydrate polymer dissolution, the most used IL contain imidazolium cations (Swatloski *et al*. 2002; Roosen *et al*. 2008; King *et al*. 2009; Tadesse *et al*. 2011) (**Fig. 1**). Favourable interactions with the aromatic lignin structures in wood have been reported for 1-allyl- or 1-benzyl-3-methylimidazolium (King *et al*. 2009). The application ranges of IL anions, however, differ for biocatalysis and dissolution of carbohydrates, respectively. ILs with weakly coordinating anions, such as PF_6^- and BF_4^-, are particularly compatible with biocatalysts (Cantone *et al*. 2007), but do not dissolve carbohydrates (Vitz *et al*. 2009; Zavrel *et al*. 2009), whereas those with anions of high hydrogen basicity, such as halides, and organic acid anions are successful in disrupting the strong hydrogen bonding

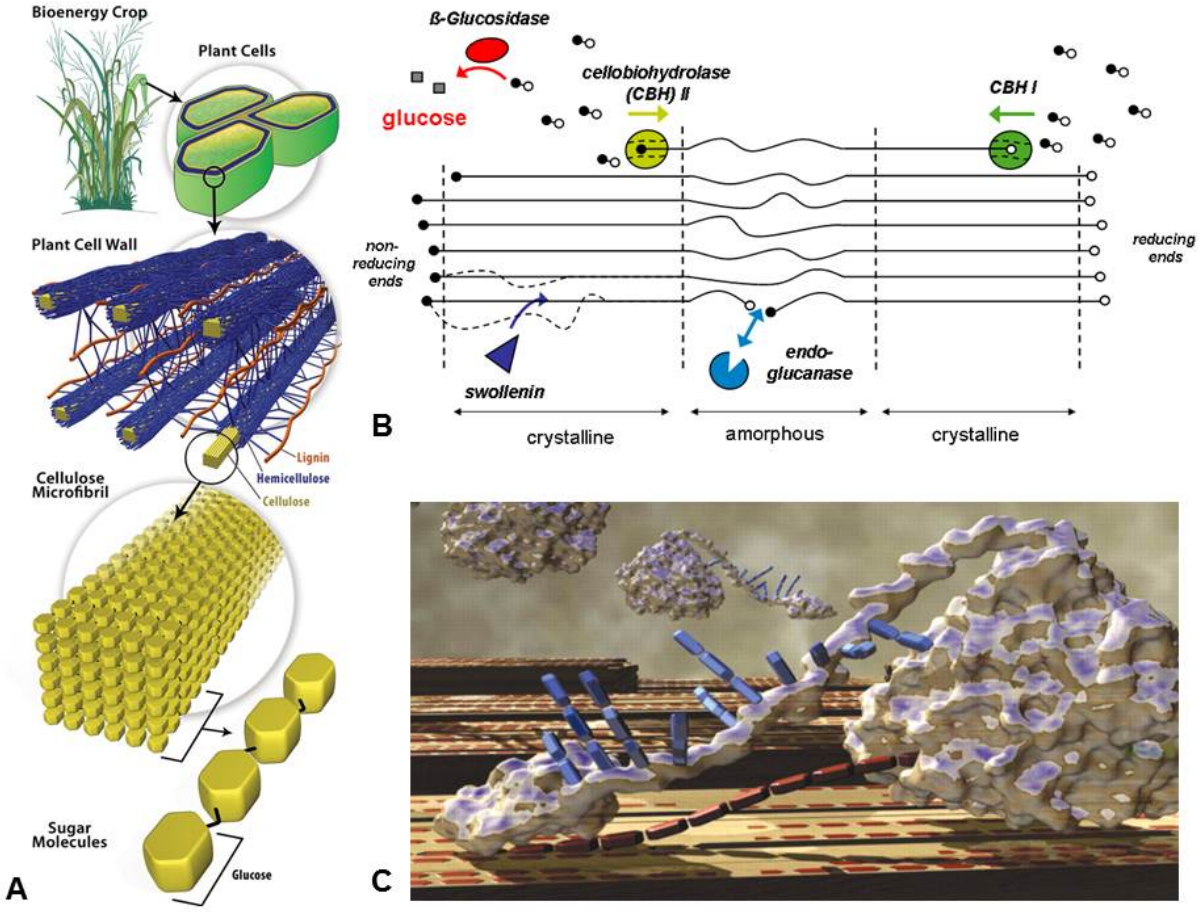

Fig. 2 Structural and enzymatic aspects of lignocellulose degradation. (**A**) Lignocellulose structure (U.S. Department of Energy Genomic Science Program, genomicscience.energy.gov). (**B**) Concerted enzymatic attack of the cellulose biopolymer at amorphous and crystalline regions from (non)-reducing ends, modified from Teeri (1997). (**C**) Artistic concept of CBH action during cellulose hydrolysis, where the cellulose binding domain adsorbs to the cellulose surface and guides the catalytic domain along the fibre (U.S. Department of Energy Genomic Science Program, genomicscience.energy.gov).

network of cellulose (Anderson *et al*. 2002; Ohno and Fukaya 2009), but typically destabilise biocatalysts (Cantone *et al*. 2007). ILs with sulphate or sulfonate anions and particularly alkylbenzenesulfonate additionally extract lignin from lignocellulose (Pu *et al*. 2007; Tan *et al*. 2009). Thus, ILs have a significant potential for biomass pretreatment by extracting lignin and dissolving carbohydrates.

In nature, the enzymatic degradation of lignocellulose in plant cell walls is accomplished by a large number of synergistically acting oxidative (Levasseur *et al*. 2008) and hydrolytic enzymes (Cantarel *et al*. 2009) with different specificities for degrading the lignin sheets and cleaving the hemicellulose network surrounding the cellulose fibrils, and finally, degrading cellulose itself (**Fig. 2A**). Lignin and hemicellulose cleavage require many different enzyme functions, but the accessibility of lignin- and hemicellulose-degrading enzymes poses no major problem. In contrast, cellulose structure necessitates the concerted action of a well-balanced mixture of enzymatic activities for its degradation (**Fig. 2B**). Unlike other polysaccharides, *e.g*. amylose and amylopectin, cellulose fibres arrange regularly. The β-1,4-linked cellobiose repeat units favour hydrogen bonding both in the plane of the pyranose rings and between several layers, resulting in a densely stacked layer of cellulose fibres. To attack this intricately bound structure, a whole family of glucanases is required. Endo- and exo-glucanases degrade cellulose chains in the interior of the chain, and from the end, respectively. β-Glucosidase then cleaves the resulting cellobiose units, reducing end-product inhibition of the exoglucanases (**Fig. 2B**).

Using ILs for the pre-treatment of biomass for improved degradability will obviously disrupt cellulose structure (Dadi *et al*. 2006; Ohno and Fukuya 2009), but also impact the biocatalysts' activity. We need experimental information on the interaction of the three key components in the reaction system, ILs, cellulose, and the enzyme preparation. Thus, light is shed onto the development of online measurement methods for gaining integral information on cellulose particle dissolution or degradation. Then, preliminary approaches to future (ligno)-cellulose degradation processes combining chemo- and biocatalysis are shown, demonstrating the potential of IL pre-treatment of biomass. To further rationalise these efforts, an enzyme kinetic modelling approach based on population balances is finally presented.

ANALYSIS METHODS FOR CELLULOSE DISSOLUTION AND HYDROLYSIS

Having identified ILs as potential solvents for cellulose (Swatloski *et al*. 2002), the obvious next question is which solvent to choose. Both the high number and cost of IL render high-throughput screening on a small scale desirable (Holbrey and Seddon 1999; Joglekar *et al*. 2007). The choice of the ideal cellulase preparation for particular (ligno)-cellulose particles likewise requires screening (Himmel *et al*. 2007; Jing *et al*. 2007; Decker *et al*. 2009) which also requires solids handling and in addition, product quantification (Navarro *et al*. 2010; Santoro *et al*. 2010; Bharadway *et al*. 2011). Spectroscopic techniques identify and quantify many compounds non-invasively and in online mode (Workman *et al*. 2003). However, heterogeneous systems with particles are difficult to investigate spectrometrically due to the particle light scattering. If the particle characteristic is changing over time as in case of the dissolution or degradation of cellulose particles, light scattering and also absorption may be used as online monitoring signal.

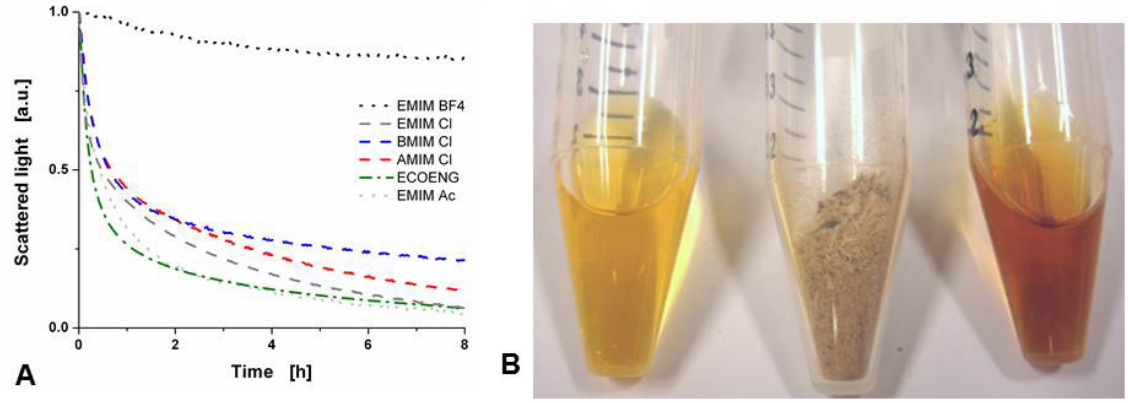

Fig. 3 Dissolution of lignocellulose. (**A**) Dissolution profiles of 4% (w/w) Avicel at 50°C observed using scattered light. (**B**) EMIM Ac and beech before and after dissolution of 5% (v/v) at 90°C for 12 h. Figure from **Zavrel M, Bross D, Funke M, Büchs J, Spiess AC** (2009) High-throughput screening for ionic liquids dissolving (ligno-)cellulose. *Bioresource Technology* **100**, 2580-2587, ©2009, with kind permission from Elsevier Ltd., Oxford, United Kingdom.

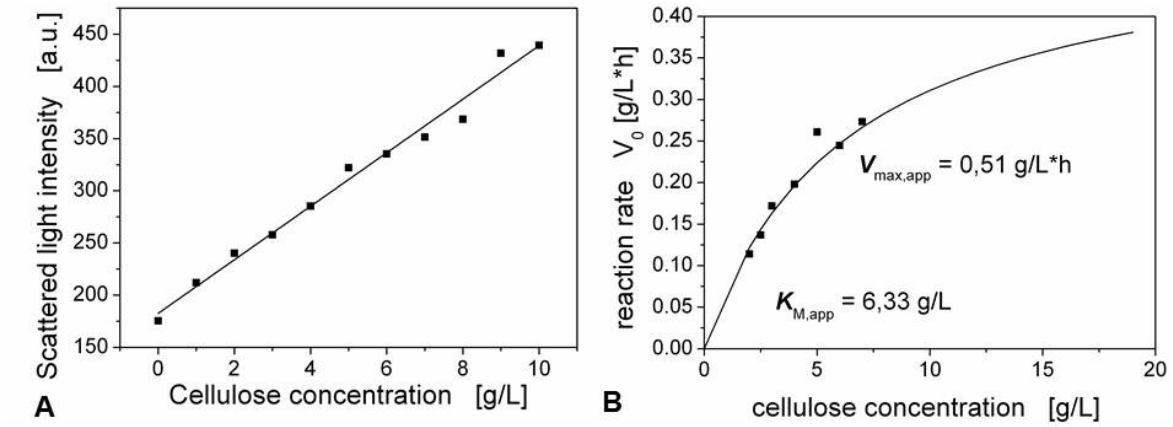

Fig. 4 Determination of apparent kinetic parameters for cellulose hydrolysis using scattered light measurement. (**A**) Calibration of initial cellulose concentration vs. scattered light intensity. (**B**) Apparent binding parameter and maximal reaction rate for cellulose conversion estimated from initial (< 10% conversion) decrease in scattered light intensity. Figure from **Wulfhorst H, Jäger G, Ellinidou E, Büchs J, Spiess AC** (2010) Charakterisierung von Cellulasepräparationen mittels Streulicht. *Chemie Ingenieur Technik* **82**, 117-120, ©2010, with kind permission from Wiley-VCH, Weinheim, Germany.

The BioLector technology has been originally developed at AVT – Biochemical Engineering for monitoring fermentation processes in shaken microtitre plates using scattered light (Samorski *et al*. 2005; Kensy *et al*. 2009), and has now been used to observe the changes of solids in a suspension.

The scattered light signal of cellulose particles suspended in IL is proportional to its mass concentration (Zavrel *et al*. 2009). The dissolution of cellulose leads to a reduced signal strength. Thus, more than 20 IL have been screened for their ability to dissolve cellulose using the BioLector technology, and a selection thereof for their ability to dissolve wood particles (**Fig. 3**). The relative scattered light intensity remains nearly unaltered in IL unable to dissolve cellulose, *e.g*. EMIM BF_4 (1-ethyl-3-methylimidazolium tetrafluoroborate), whereas it is significantly reduced in IL known for their capability to dissolve cellulose. IL can thus be ordered according to their rate or extent of dissolution (**Fig. 3A**), identifying EMIM Ac (-acetate) and MMIM DMP (1,3-dimethylimidazolium dimethylphosphate, EcoEng 1111P®) as best solvents for cellulose. The screening for cellulose solvents is merely limited by the colouration of some IL and their melting point if they are beyond the application range of the microtitre plates and their readers. At least partial dissolution of lignocellulose was demonstrated for those IL dissolving cellulose (**Fig. 3B**). Here, AMIM Cl (1-allyl-3-methylimidazolium chloride) and EMIM Ac were identified as the best solvents for the dissolution of a range of hard- and softwoods among the tested ones. Until now, more than 70 IL have been tested for their ability to dissolve cellulose, lignin and lignocellulose (Pinkert *et al*. 2009; Wang *et al*. 2012). More than 50 IL have been shown to be solvents for cellulose, more than 25 for lignin, and more than 15 for lignocellulose (Mäki-Arvela *et al*. 2010). The obtained screening results on a small scale (Zavrel *et al*. 2009) have even been confirmed to be predictive for the order of magnitude of the dissolution capacity of both cellulose and lignocellulose if information on lignin dissolution is taken into account (Mäki-Arvela *et al*. 2010). Thus, established screening methods have the potential to support the understanding of the molecular reasons for dissolution capability of (ligno)-cellulose in ILs.

A similar impact may be expected for the evaluation of cellulase preparations for the assessment of degradation of various (ligno)-cellulose substrates, which is currently achieved using sampling and chemical analysis of formed sugars (Kim *et al*. 1998; Jing *et al*. 2007; Studer *et al*. 2010). Similar to the dissolution of cellulose in IL, also the degradation of cellulose leads to reduction in number and size of the cellulose particles. Thus, the scattered light signal, which could be shown to rise linearly with increasing concentration of suspended cellulose (**Fig. 4A**), can be used to follow the hydrolysis progress (Jäger *et al*. 2010; Wulfhorst *et al*. 2010). From the initial slope of the hydrolysis curves, initial rates of cellulose degradation can be obtained that may be used to determine apparent cellulose particle degradation kinetics (**Fig. 4B**). Of course, the applied Michaelis-Menten kinetics is not capable of reproducing the mechanistic details of the heterogeneous cellulose hydrolysis, but the obtained semi-quantitative parameters may help to assemble suitable cellulase preparations on particular cellulosic substrates for biomass refining (Zhang *et al*. 2006).

PROCESS CONSIDERATION FOR CHEMO-ENZYMATIC HYDROLYSIS OF CELLULOSE

Before applying IL in biocatalytic processes, their effects on the biocatalyst have to be understood, both for rational decisions on the unit operations and also quantitatively for modelling purposes. The positive effects of IL pretreatment on enzymatic (ligno)-cellulose hydrolysis, which are enhanced rate and conversion, have been observed with thoroughly washed material (Dadi *et al*. 2006; Kuo and Lee 2009; Lee *et al*. 2009; Zhao *et al*. 2009; Uju *et al*. 2012). However, this implies a dilute stream of IL in recovery that may render the process economically inefficient (Li *et al*. 2010) unless the IL can be efficiently recycled (Shill *et al*. 2011). Thus, the application of enzymes with residual IL seems desirable. It was shown earlier that cellulases do not tolerate high amounts of IL, but denature (Turner *et al*. 2003). Few studies investigated residual cellulase activity and found none above 40% v/v or even less IL. This is the case for *Trichoderma reesei* cellulases in EMIM diethylphosphate (Kamiya *et al*. 2008), for *Penicillium janthinellum* cellulase variants in BMIM Cl (1-butyl-3-methylimidazolium chloride; Adsul *et al*. 2009), for cellulases from thermophilic organisms, *Thermotoga maritima* and *Pyrococcus horikoshii* in EMIM Ac (Datta *et al*. 2010) or for halophilic cellobiohydrolase I from *Halorhabdus utahensis* in (Zhang *et al*. 2011), and for cellulases screened from metagenomic sources in a range of IL (Pottkämper *et al*. 2009). To better understand this coincidence, the effects of IL on the industrial benchmark, *T. reesei* cellulases (Celluclast®, Novozymes), were investigated in more detail.

It could be shown that already 10% (v/v) IL lead to a significant enzyme activity loss, even for the same type of IL from different suppliers. A significant variation in residual activity was also found for different batches of one enzyme preparation from the same supplier with one IL. The specific influence of one particular IL, MMIM DMP is shown in **Fig. 5**. The earlier results on the effects of increasing IL volume fractions could be confirmed for cellulases from *T. reesei* and differentiated for the type of substrate. With the soluble cellulosic materials CMC and NTP-cellobioside Celluclast® exhibited activity until 40% (v/v) MMIM DMP, but only until 20% (v/v) with insoluble α-cellulose (**Fig. 5A**). The assay durations might imply that enzyme deactivation is the differentiating factor, but this could be disproved both by studies under operational and storage conditions. Celluclast® activity remained stable for more than 10 days in 10% (v/v) of various IL, after one day of adaptation to the non-conventional environment. An investigation of the concomitant change of ionic strength and viscosity with increasing IL volume fraction revealed that ionic strength and viscosity significantly reduced the cellulolytic activity on α-cellulose. This implies that part of the IL impact may be due to mass transfer effects to the surface of the cellulose particles (**Fig. 5B**). The 'conventional' cellulase from *T. reesei* was found to be IL-tolerant similar to a range of cellulases isolated from metagenomic sources or thermophilic microorganisms (Pottkämper *et al*. 2009; Datta *et al*. 2010; Zhang *et al*. 2011). Thus, further work will be required to identify cellulases with higher residual activity in IL and to find IL with lower viscosity.

The downsides of the specific degradation of cellulose to the glucose building blocks using enzyme cocktails become obvious. The key drawback is the low specific activity of cellulases on the solid particles (Zhang *et al*. 2006; Merino and Cherry 2007). This corresponds to a high protein amount and associated cost required to speed up the reaction. In batch reactors at higher conversion this leads to jamming, *i.e*. the mutual obstruction of the cellulolytic enzymes, on the solid surface, eventually slowing the reaction down to a halt and thus limiting the maximal obtainable conversion (Bommarius *et al*. 2008; Igarashi *et al*. 2011). An alternative to enzymatic catalysis for cellulose hydrolysis is the rather cheap chemical catalysis either using homogeneous, typically acid, catalysts (Li *et al*. 2008; Vanoye *et al*. 2009), or heterogeneous, such as supported metal or solid acid, catalysts (Dhepe and Fukuoka 2008; Rinaldi and Schüth 2009). The key disadvantage of chemical catalysis is the low specificity, leading to dehydration by-products reducing the yield of fermentable sugars. Obviously, chemical and biocatalysis are complementary, suggesting the combination of enzymatic conversion of chemically pre-hydrolysed cellooligomers to gain both speed and selectivity (Rinaldi *et al*. 2008; Schüth *et al*. 2010).

To this end, α-cellulose is dissolved in BMIM Cl and chemically hydrolysed using solid acid catalyst Amberlyst 15DRY, subsequently precipitated by addition of water and thoroughly washed to remove IL residues from the resulting cellooligomer hydrogel (Rinaldi *et al*. 2010). The cellooligomers are then subjected to enzymatic hydrolysis with nearly quantitative yield. The precipitation of the cellooligomers results in a hydrogel with shorter, amorphous

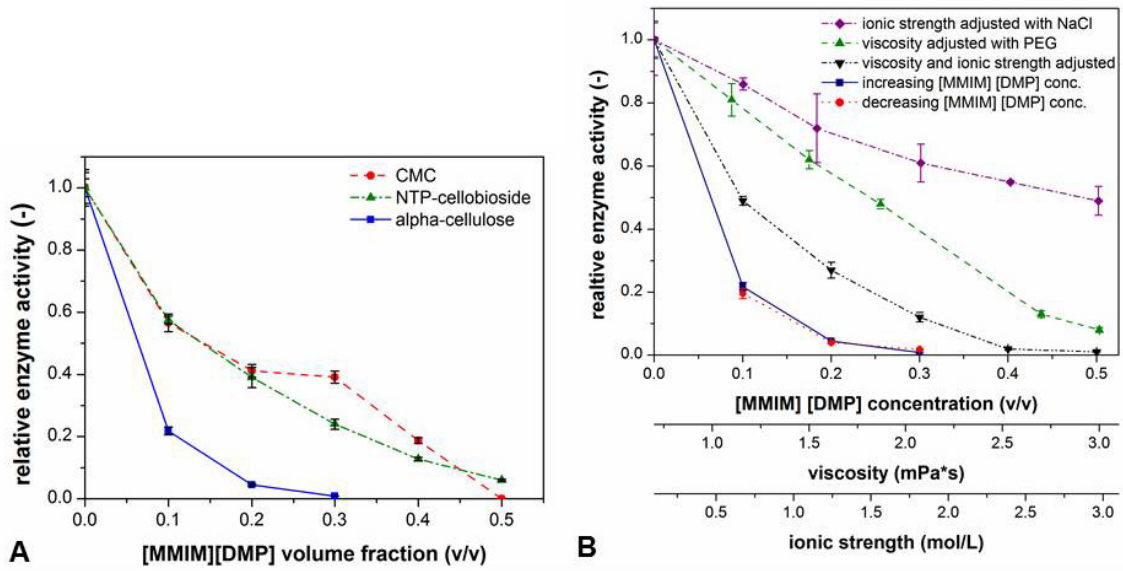

Fig. 5 Effect of ionic liquid MMIM DMP on the cellulolytic activity of Celluclast®. (**A**) Activity assays using carboxymethylcellulose (CMC) and α-cellulose were performed analysing DNS reducing sugar formation after 10 and 30 min, respectively, whereas hydrolysis of p-nitrophenol-(NTP)-cellobioside was followed spectrophotometrically. (**B**) Activity based on α-cellulose affected by viscosity and ionic strength corresponding to the respective MMIM DMP volume fraction. Figure from **Engel P, Mladenov R, Wulfhorst H, Jäger G, Spiess AC** (2010) Point by point analysis: how ionic liquid affects the enzymatic hydrolysis of native and modified cellulose. *Green Chemistry* **12**, 1959-1966, ©2010, with kind permission from RSC Publishing, Cambridge, UK.

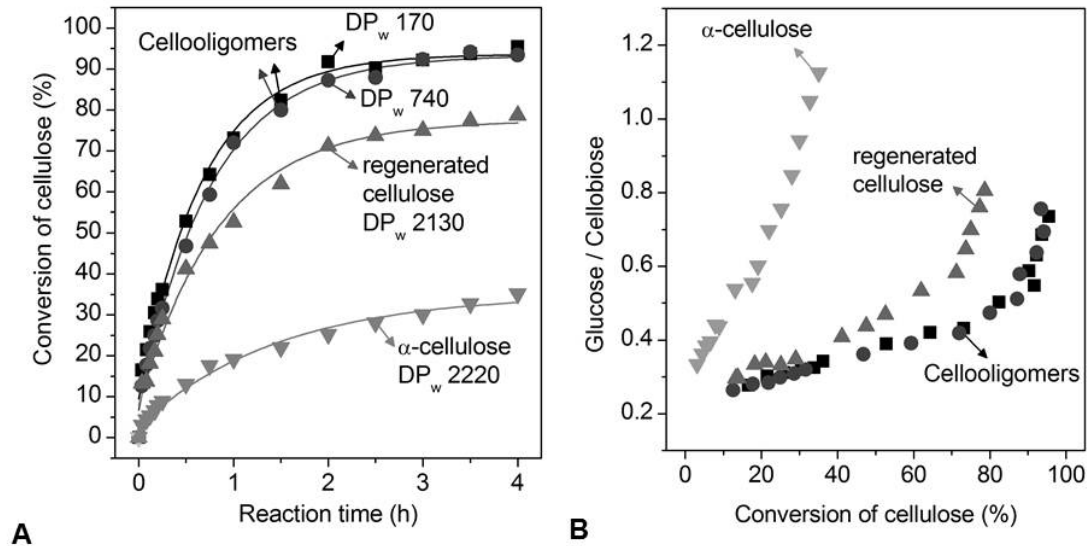

Fig. 6 Enzymatic hydrolysis of pre-processed cellulose. Reaction conditions: Cellulose 10% (w/w), Celluclast® 0.5% (v/v), pH 4.5, 45°C. (**A**) Time course of cellulose degradation of cellooligomers. (**B**) Glucose-to-cellobiose yield ratio. Figure from **Rinaldi R, Engel P, Büchs J, Spiess AC, Schüth F** (2010) An integrated catalytic route to fermentable sugars from cellulose. *ChemSusChem* **3**, 1151-1153, ©2010, with kind permission from Wiley-VCH, Weinheim, Germany.

Formation of Michaelis-Menten complex	$E + C_s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}C_s$		BG: s = 2, EG, CBH: s > 2
Formation of higher order complex	$E + E\text{-}C_s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}E\text{-}C_s$		EG, CBH: s > 7
Cellulose degradation	$E\text{-}C_s \xrightarrow{k_2}$	$E + C_{s\text{-}r} + C_r$	BG: s = 2, r = 1 EG: r < s CBH: r = 2
Processive cellulose degradation	$E\text{-}C_s \xrightarrow{k_2}$	$E\text{-}C_{s\text{-}2} + C_2$	CBH: s > 2
Competitive inhibition	$E + C_{1/2} \underset{k_{-3/5}}{\overset{k_{3/5}}{\rightleftharpoons}} E\text{-}C_{1/2}$		BG: only C_1
Un-competitive inhibition	$E\text{-}C_s + C_{1/2} \underset{k_{-4/-6}}{\overset{k_{4/6}}{\rightleftharpoons}} C_{1/2}\text{-}E\text{-}C_s$		BG: only C_1

Fig. 7 Overview on relevant elementary reactions for the glycosyl hydrolases involved.

cellulose chain exposed to a large surface, thus providing better accessibility to the cellulases (Dadi *et al*. 2006; Uju *et al*. 2012). A nearly quantitative conversion of the cello-oligomers is achieved in short process times compared to α-cellulose directly precipitated from a BMIM Cl solution and 'native' α-cellulose (**Fig. 6**). IL pretreatment raises the cellulose conversion from 35% to *ca*. 80% after 4 h. The chemical pre-hydrolysis to oligomers results in a further increase to nearly 95% conversion after 4 h, which is unprecedented (**Fig. 6A**). The reaction products are devoid of dehydration by-products. Interestingly, the relative proportions of mono- and disugars formed change depending on the extent of the pretreatment, with higher amounts of cellobiose formed for the cellooligomers, indicating a domination of cellulose hydrolysis by cellobiohydolases (**Fig. 6B**), pointing at the need of tailoring cellulase composition to the specific substrate applied, best based on a quantitative understanding of the cellulase hydrolysis process.

MODELLING APPROACHES FOR HOMOGENEOUS AND HETEROGENEOUS HYDROLYSIS OF CELLULOSE

The hydrolysis of insoluble cellulose takes place at the solid/liquid interface after adsorption of the dissolved cellulases to the available solid cellulose surface (Holtzapple *et al*. 1984; Zheng *et al*. 2009), resulting in a more or less dense surface coverage of the cellulose particles (Igarashi *et al*. 2009). The enzymes then act according to their substrate specificities on amorphous regions or chain ends (**Fig. 2B**). For a proper description of synergistic effects, models require a differentiation of the various hydrolytic activities (Okazaki and Mooyoung 1978; Nidetzky *et al*. 1994; Peri *et al*. 2007), including inhibition of the enzymes (Gusakov *et al*. 1985; Zheng *et al*. 2009). Good models for the description of the substrate characteristics consider particle size (Converse and Grethlein 1987), and porosity (Zhou *et al*. 2009), together resulting in specific surface area available for enzyme adsorption (Movagarnejad *et al*. 2000; Levine *et al*. 2010), degree of polymerisation (Okazaki and Mooyoung 1978; Zhang and Lynd 2006; Griggs *et al*. 2011), and finally cellulose crystallinity (Gusakov *et al*. 1985). Most of the cited models consider only selected phenomena and descriptors. Capturing the development of the chain length distribution of polymers during reaction requires specific modelling strategies. Population balances are one way to incorporate the polymer chain length distribution into a mechanistic depolymerisation model (Wulkow 2008; Hosseini and Shah 2011).

Populations summarise chemically identical species differing in one or several discrete or continuous properties. Cellulose varies *e.g*. continuously in particle size, and discretely in chain length of the cellulose fibres, which is our focus. Since the chemical species is maintained during cellulose hydrolysis, *i.e.* glucose is considered as a cellulose of degree of polymerisation DP or $s = 1$, changes to the cellulose population's concentration $c(s,t)$ occur via hydrolysis reducing s, or via changes in the reactor volume. In contrast to chemical catalysts, enzymes bind to the cellulosic substrate, resulting in further populations of enzyme-substrate-

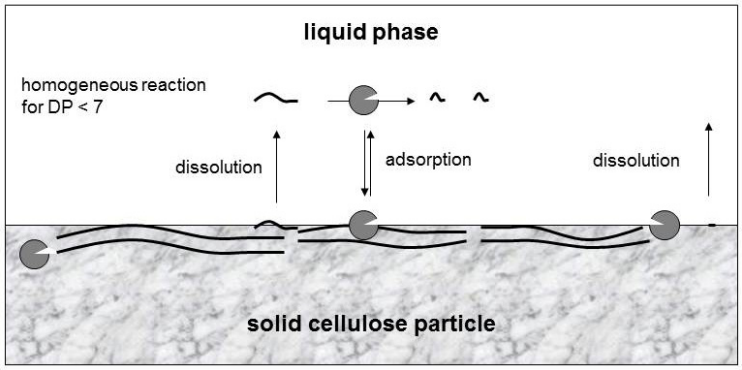

Fig. 8 Phenomena occurring in the cellulose particle and the aqueous phase during cellulose hydrolysis.

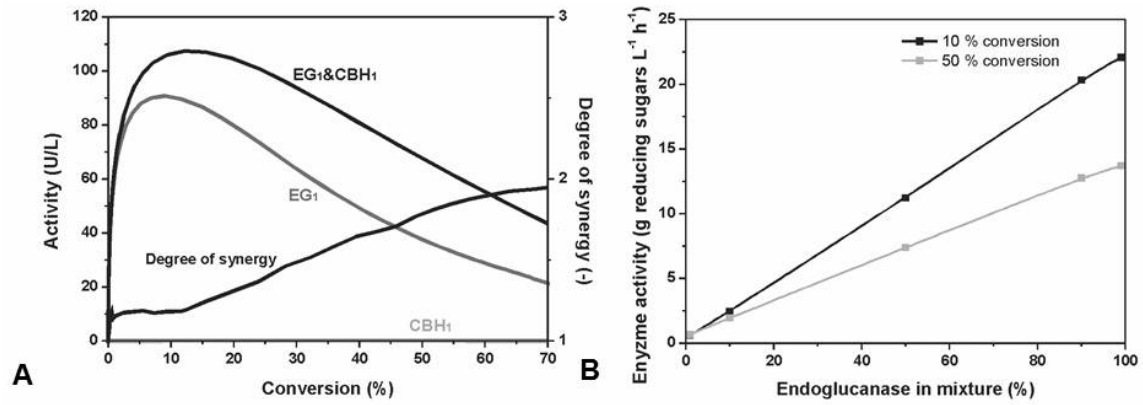

Fig. 9 Synergism of EG1 and CBH1 in homogeneous reaction. (**A**) Enzymatic activity in U L^{-1} (equals µmol min^{-1} L^{-1}) of the individual and combined cellulases and degree of synergy for combined cellulase action as a function of conversion. (**B**) Sensitivity of enzymatic activity in terms of reducing sugar formation on variation of endoglucanase content in the mixture at 10% and 50% conversion. The simulated activities correspond to the arithmetic mean of the individual activities within 1% accuracy, *i.e.* the degree of synergy equals 1.

complexes. Consequently, the calculation of the cellulose population concentrations $c(s,t)$ requires a mapping of the particular elementary reactions and their boundary conditions catalysed by the enzymes in the cellulase mixture (**Fig. 7**) to a representation of the populations in terms of the appropriate degree of polymerisation.

During heterogeneous cellulose hydrolysis, degradation occurs both on the surface of the solid phase and for short cellooligomers in the liquid phase (**Fig. 8**). The adsorption equilibrium of the enzymes between aqueous phase and cellulose solids is modelled via competitive Langmuir adsorption isotherm, however as a fast dynamic process. Cellulose structural parameters enter the model in terms of the particle size determining the cellulose available at the surface for degradation, as crystallinity impeding the action of endoglucanases (EG), and porosity defining the accessibility of the enzymes to the interior of the particle. Cellulose structural parameters are easily available in literature for the standardised laboratory substrates (Walker and Wilson 1991). Adsorption parameters are more difficult to determine reliably and have mostly been reported on mass basis (Zhang and Lynd 2004), which unfortunately complicates the consideration of shrinking particles.

Simulations of the hypothetical homogeneous cellulose hydrolysis using pure endoglucanase demonstrate the statistical cleavage, resulting in an apparent shift of the chain length distribution (CLD) to shorter DP. In contrast, cellobiohydrolases (CBH) apparently reduces only the relative weight of the polymer fraction in comparison to the single product, cellobiose. Reducing sugars are formed nearly at a constant and low rate. Combining EG and CBH the polymer fraction is both shifted towards shorter DP and reduced in weight in comparison to the monomers. Therefore, synergistic behaviour is found for the homogeneous reaction system, with an estimated degree of synergism of up to 2 at conversions higher than 10% (**Fig. 9**). This is in the lower range of the values reported for heterogeneous systems (Okazaki and Mooyoung 1978) and thus coincides with the amorphous character of dissolved cellulose (Zhang and Lynd 2004). If, however, simulations are performed with varying EG and CBH content, but constant total protein amount, then the enzymatic activity of the mixture corresponds to the arithmetic mean of the activity of the individual enzymes, corresponding to a degree of synergism of unity based on a more conservative definition of DS (Henrissat *et al*. 1985). Since EG has the higher specific activity of both enzymes, hydrolysis of dissolved cellulose should preferably be performed using exclusively EG (**Fig. 9B**).

The pattern of CLD change and reducing sugar formation simulated in the heterogeneous cellulose system is similar to the one of the homogeneous system. During hydrolysis the particles shrink. Thus, the cellulose mass available for enzyme adsorption and surface area for cellulose degradation is reduced, resulting in a significantly lower reaction rate at later reaction times (**Fig. 10**). Of the cellulose structural parameters, smaller particles expectedly improve the hydrolysis rate. Lower crystallinity and higher porosity also had a positive effect, but to a lesser extent. Thus, the general pre-treatment aims (Chandra *et al*. 2007; Kumar *et al*. 2009) are supported by the simulations, although quantitative prediction will require fine-tuning of the model.

Already few simulations based on parameters estimated from literature data demonstrate the potential of the novel cellulose hydrolysis model that is based on population balances (Engel *et al*. 2011). The key advantage is the fully mechanistic description of the kinetic and thermodynamic phenomena that enables the proper analysis and assignments of root causes for often observed coupled effects in enzymatic cellulose hydrolysis, such as cellulase synergism,

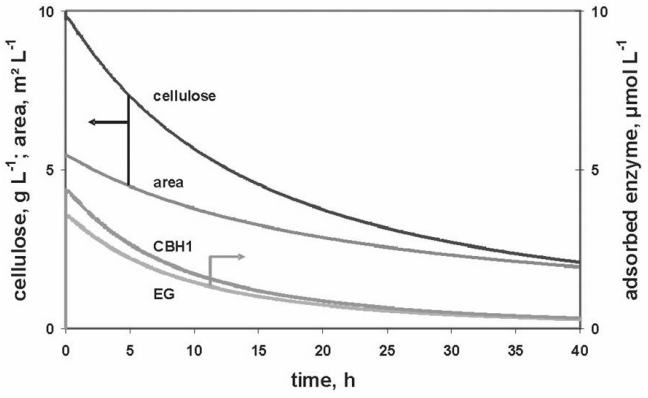

Fig. 10 Simulation results for effects of adsorption in the heterogeneous enzymatic cellulose hydrolysis. Progress of solid cellulose concentration, area and adsorbed enzyme concentration.

slow reaction, and jamming. Beyond that, the suggestion of process improvements becomes possible, such as improved enzymes or enzyme preparations (Levine *et al*. 2011; Engel *et al*. 2012).

Of course, the exploration of parameter spaces without foundation on experimental evidence is not very reliable, thus parameter estimation and model discrimination based on experimental data is the logical next step. The model will be further used to answer typical chemical engineering questions, such as the definition of optimal pre-treatment based on the substrate structural parameters, the composition of optimal enzyme mixtures for specific substrates, and optimal reaction control strategies. In the future, the mechanistic modelling concept will be extended to lignocellulosic substrates.

CONCLUSIONS

IL-assisted enzymatic hydrolysis of cellulose has the potential to establish as alternative process for the exploitation of lignocellulosic biomass, since its selectivity exceeds that of the existing processes. To complement the available analytical methods for soluble sugar formation that rely on sampling with high-throughput-capable online measurements, a scattered light screening method for cellulose particle disappearance has been adapted to compare ILs for their potential to dissolve cellulose or even wood and to compare cellulases for their degradation ability, respectively.

ILs have been exploited for enzymatic cellulose degradation mostly by dissolving and destroying cellulose structure with subsequent precipitation of the material in an amorphous form. This approach has been very successful in enhancing hydrolysis rates, however not necessarily conversion. The effect of IL on the residual activity of cellulases has been investigated in more detail and attributed partially to unavoidable mass transfer limitations due to high viscosity of the IL. Using an innovative combined process, where both chemical and biocatalyst catalyse the same reaction, a significantly enhanced hydrolysis rate with almost quantitative conversion was found. Obviously, there is a significant potential for more innovative and integrated reaction concepts involving ionic liquids and enzymes. However, the compatibility of the enzymes with ionic liquids needs to be clearly understood and improved.

Quantitative model-based evaluation may serve for further process development. It has been sketched here that a rigorously mechanistically derived population balance model has the potential to integrate interpretation experimental data ranging from biochemical to process studies. Examples are the successful simulations of coupled phenomena occurring in cellulose degradation, such as apparent cellulase synergisms, and the effects of competitive enzyme adsorption. The model-based experimental analysis of homogeneous and heterogeneous cellulose degradation should be able to contribute to lower the experimental burden of the complex substrates and catalyst mixtures.

In conclusion, even if ILs may yet appear too expensive for large-scale processes, their selectivity and processing advantages may render them economically attractive in future. Novel analytical concepts and modelling strategies as collected here will support that technology conversion.

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