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Dissertation
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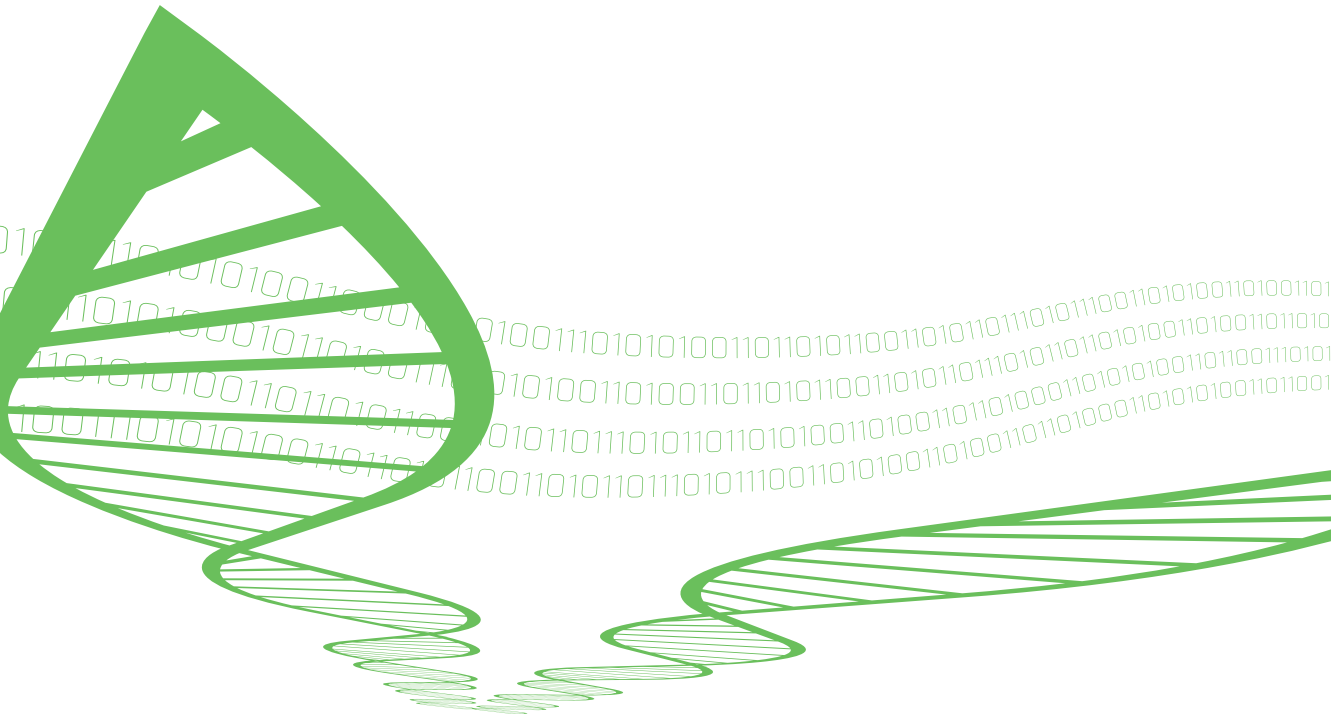
Enzymatic hydrolysis of cellulose in aqueous ionic liquids

Ronny Wahlström



VTT SCIENCE 52

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VTT Technical Research Centre of Finland

Thesis for the degree of Doctor of Technology to be presented with due permission of the School of Chemical Technology for public examination and criticism in Auditorium KE2 (Komppa Auditorium) at Aalto University School of Chemical Technology (Espoo, Finland) on the 21st of February, 2014, at 12:00.

ISBN 978-951-38-8115-3 (Soft back ed.)
ISBN 978-951-38-8116-0 (URL: http://www.vtt.fi/publications/index.jsp)

VTT Science 52

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)

JULKAISIJA – UTGIVARE – PUBLISHER

VTT
PL 1000 (Tekniikantie 4 A, Espoo)
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Kopijyvä Oy, Kuopio 2014

Enzymatic hydrolysis of cellulose in aqueous ionic liquids

Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor.
Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä. **Ronny Wahlström.**
Espoo 2014. VTT Science 52. 102 p. + app. 57 p.

Abstract

Total enzymatic hydrolysis of the polysaccharides in lignocellulosic biomass to monosaccharides is currently a focus research area. The monosaccharides obtained from lignocellulose hydrolysis can be used for the production of platform chemicals and biofuels, most notably ethanol. One major challenge in the commercialization of lignocellulosic ethanol production is the recalcitrance of lignocellulosics towards enzymatic hydrolysis, necessitating efficient pretreatment of the lignocellulosic feedstock. Certain ionic liquids (ILs, salts with melting points below 100 °C) dissolve cellulose and even lignocellulosic biomass and are as such interesting candidates for pretreatment technology. However, cellulose-dissolving ILs have been found to severely inactivate the hydrolytic enzymes (cellulases) employed in cellulose hydrolysis. This work focuses on elucidating how certain ILs affect the action of cellulases in cellulose hydrolysis. The main emphasis was on the action of purified monocomponent *Trichoderma reesei* cellulases, but some commercial cellulase preparations were also studied in IL matrices.

Hydrolysis experiments were made in solutions containing up to 90% of the two cellulose-dissolving ILs 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP). The presence of increasing amounts of IL led to decreasing yields of solubilised saccharides in enzymatic hydrolysis. Depending on the IL and cellulase, no soluble saccharides were released in hydrolysis matrices containing over 40–50% IL. There were clear differences in the severity of the effects of different cellulose-dissolving ILs on cellulase action. [EMIM]AcO was generally more harmful for cellulase action than [DMIM]DMP. Pure [EMIM]AcO completely inactivated *T. reesei* endoglucanase in 4 h in residual activity measurements, whereas pure [DMIM]DMP supported considerable cellulase activity for at least three days. These results were confirmed by time curves of microcrystalline cellulose (MCC) hydrolysis in matrices containing the two ILs. Cellulose-dissolving ILs based on carboxylate salts of the organic superbases 1,1,3,3-tetramethylguanidine (TMG) and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) have recently become available. These compounds are distillable under relatively mild conditions and are thus recyclable. However, these ILs were found to be at least as harmful for cellulase action as the studied imidazolium-based ILs and did thus not offer any benefits in terms of enzyme compatibility. *T. reesei* endoglucanases were unable to reduce the molecular weight of MCC in buffer or in any aqueous matrix containing IL, except in 90% (v/v) [DMIM]DMP in which the MCC was partially dissolved.

Cellulose-dissolving ILs were found to be basic in aqueous solution. According to the results in this work, the pH increase caused by IL basicity was not the main reason for the observed cellulase inactivation. Cellulases with confirmed activity at high pH did not perform better than acidic or neutral cellulases in IL solutions. Some indications were however obtained that cellulase thermostability may be associated with better activity in cellulose-dissolving ILs.

The studied ILs were found to have very detrimental effects on saccharide analytics. A capillary electrophoresis (CE) method was developed for the analysis of mono- and oligosaccharides in matrices containing ILs. With this CE method, the yields and product distribution of cello-oligomers produced in the hydrolysis experiments could be determined. It was found that the presence of ILs shifted the product distribution to larger cello-oligomers for some cellulases. The CE method was also used to monitor the hydrolysis of cello-oligomers with *Aspergillus niger* β-glucosidase in IL matrices. This β-glucosidase was found to be very IL sensitive.

ILs were found to affect the cellulose binding of *T. reesei* cellulases. The cellulase binding to MCC in solutions with [DMIM]DMP and [EMIM]AcO was studied with radiolabeled *T. reesei* Cel5A (endoglucanase II) and Cel7A (cellobiohydrolase I) and their respective core domains. Cel7A was able to bind to MCC with its core domain, whereas it was shown that Cel5A was very dependent on its CBM for efficient substrate binding. High cellulose binding affinity was not necessary for all the cellulases in order for them to be hydrolytically active. [EMIM]AcO interfered more with cellulase substrate binding than [DMIM]DMP. The binding ability of the *T. reesei* carbohydrate-binding modules (CBMs) was very IL sensitive.

Keywords Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor

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Sammanfattning

Enzymatisk totalhydrolys av lignocellulosans polysackarider till monosackarider är för tillfället ett mycket aktivt forskningsområde. De sålunda producerade monosackariderna kan användas som råvara vid tillverkningen av plattformkemikalier och biobränslen, av vilka särskilt kan nämnas etanol. En av de största utmaningarna i kommersialiseringen av etanoltillverkning från lignocellulosa är lignocellulosans motståndskraft mot enzymatisk hydrolys. Därför behövs effektiva förbehandlingsmetoder då lignocellulosan används som råvara. Vissa jonvätskor (definierade som salt med smältpunkt under 100 °C) löser cellulosa och till och med fullständig lignocellulosa. Jonvätskorna utgör sålunda ett intressant alternativ som förbehandlingsteknologi för lignocellulosa. Jonvätskorna har emellertid i hög grad konstaterats inaktivera de hydrolytiska enzymer, cellulaser, som används i cellulosahydrolys. Detta arbete har haft som målsättning att klargöra hur cellulosalösande jonvätskor påverkar cellulasernas funktion i cellulosahydrolys. I första hand undersöktes hur funktionen hos cellulaser renade till enkomponentpreparat från *Trichoderma reesei*, men också hos kommersiella cellulaspreparat, påverkades i vissa jonvätskelösningar.

Hydrolysexperimenten utfördes i lösningar med upp till 90 % jonvätska (1-etyl-3-metylimidazolium acetat ([EMIM]AcO) eller 1,3-dimetylimidazolium dimetylfosfat ([DMIM]DMP)). En ökande mängd jonvätska ledde till avtagande hydrolysutbyten i form av lösliga sackarider i enzymatisk hydrolys. Beroende på kombinationen av jonvätska och cellulas observerades ingen tillkomst av lösliga sackarider när jonvätskekoncentrationen steg över 40–50 %. De olika jonvätskorna var i olika utsträckning skadliga för cellulasernas funktion. [EMIM]AcO var i allmänhet mer skadlig än [DMIM]DMP för cellulasernas funktion. Ren [EMIM]AcO inaktiverade *T. reesei* endoglukanas fullständigt på mindre än 4 h, medan betydande restaktiviteter mättes efter inkubation i [DMIM]DMP under åtminstone tre dygn. Detta resultat understöddes av hydrolyskurvorna när mikrokristallin cellulosa (microcrystalline cellulose, MCC) hydrolyserades i lösningar med dessa två jonvätskor. Cellulosalösande jonvätskor som består av karboxylater av de organiska superbaserna 1,1,3,3-tetrametylguanidin (TMG) och 1,5-diazabicyklo[4.3.0]non-5-en (DBN) har nyligen blivit tillgängliga. Dessa jonvätskor är speciellt intressanta eftersom de är destillerbara under relativt milda förhållanden och sålunda är återvinningsbara. I hydrolysexperimenten konstaterades dessa jonvätskor dock vara åtminstone lika skadliga för cellulasernas funktion som de imidazoliumbaserade jonvätskorna, så dessa jonvätskor medförde ingen nytta i form av ökad enzymkompatibilitet. *T. reesei*s

endoglukanaser kunde inte reducera MCC:s molmassa i buffertlösning eller i någon jonvätskelösning, förutom i 90 % (v/v) [DMIM]DMP, vari MCC partiellt löste sig.

Jonvätskor som löser cellulosa befanns vara basiska i vattenlösning. Enligt resultaten i detta arbete skulle det stigande pH-värdet, som förosakades av de cellulosalösande jonvätskornas basiskhet, inte vara en av huvudorsakerna för den observerade inaktiveringen hos cellulaserna. Cellulaser med aktivitet i höga pH-värden presterade inte bättre i jonvätskelösningar än sura eller neutrala cellulaser. Däremot observerades det att cellulaser med ökande termostabilitet verkade bevara sin förmåga att katalysera cellulosahydrolys i jonvätskelösningar bättre, än cellulaser som är temperaturkänsliga.

De studerade jonvätskorna konstaterades vara mycket skadliga för många av de vanliga metoderna som används i kolhydratanalytik. En kapillärelektroforesmetod utvecklades för att analysera mono- och oligosackarider i jonvätskelösningar. Med den här analysmetoden kunde både hydrolysutbytena och produktdistributionen av lösliga cello-oligomerer bestämmas i jonvätskelösningar. För endel cellulaser ledde närvaron av jonvätska under hydrolysen till att produktdistributionen skiftades mot längre oligomerer, jämfört med situationen i optimumförhållanden. Kapillärelektroforesmetoden användes också för att följa med hur *Aspergillus niger*s β-glukosidas hydrolyserade cello-oligomerer i jonvätskelösningar. Detta β-glukosidas konstaterades vara mycket känsligt för närvaron av jonvätska.

Jonvätskor konstaterades påverka cellulosabindandet hos *T. reesei*s cellulaser. *T. reesei* Cel5A (endoglukanas II), Cel7A (cellobiohydrolas I) och deras respektive katalytiska domäner märktes med radioaktivt tritium och dessa cellulasers förmåga att binda till MCC studerades i lösningar innehållande [DMIM]DMP och [EMIM]AcO. Cel7A kunde binda sig till MCC direkt via sin katalytiska domän, medan det kunde påvisas att Cel5A var ytterst beroende av sin kolhydratbindande modul för att binda till cellulosa. En hög grad av bindning till cellulosa var inte nödvändig för Cel5A för att hydrolys skulle äga rum. [EMIM]AcO konstaterades påverka cellulasernas bindingsgrad till MCC mer än [DMIM]DMP. Bindningsförmågan hos *T. reesei*s kolhydratbindande moduler konstaterades vara synnerligen känslig för de studerade jonvätskorna.

Nyckelord Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä

Enzymatic hydrolysis of cellulose in aqueous ionic liquids.
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Tiivistelmä

Lignoselluloosan entsymaattista totaalihydrolyysiä tutkitaan nykyisin hyvin aktiivisesti. Lignoselluloosassa olevien polysakkaridien hydrolyysistä syntyviä monosakkarideja voidaan käyttää raaka-aineina kemikaalien, polymeerien ja biopolttoaineiden, erityisesti etanolin, tuotannossa. Iso haaste lignoselluloosapohjaisen etanolituotannon kaupallistamisessa on lignoselluloosan monimutkainen rakenne, joka vaikeuttaa entsymaattista hydrolyysiä. Tehokkaiden, lignoselluloosaa avaavien esikäsittelymenetelmien kehittäminen on siis tärkeää. Tietyt ioninesteet, jotka määritellään suoloiksi, joiden sulamispiste on alle 100 °C, liuottavat selluloosaa ja jopa lignoselluloosaa. Ne ovatkin hyvin mielenkiintoisia käytettäviksi lignoselluloosan esikäsittelyssä. Selluloosaa liuottavien ioninesteiden on kuitenkin todettu inaktivoivan hydrolyyttisiä entsyymejä, sellulaaseja, joita käytetään selluloosan totaalihydrolyysissä. Tässä työssä selvitettiin, miten tietyt ioninesteet vaikuttavat sellulaasien toimintaan selluloosan hydrolyysissä. Työssä tutkittiin pääasiassa *Trichoderma reesei* -homeen tuottamien ja puhdistettujen sellulaasien sekä myös joidenkin kaupallisesti saatavien sellulaasituotteiden toimintaa vesipitoisissa ioninesteliuoksissa.

Hydrolyysikokeita tehtiin selluloosalla vesiliuoksissa, joiden ioninestepitoisuus vaihteli; suurimmillaan se oli 90 % (joko 1-etyyli-3-metyylimidatsoliumi asetaatti ([EMIM]AcO) tai 1,3-dimetyylimidatsoliumi dimetyylifosfaatti ([DMIM]DMP)). Kasvavat ioninestepitoisuudet aiheuttivat hydrolyysisaannon pienenemisen selluloosan entsyymaattisessa hydrolyysissä. Riippuen sellulaasin ja ioninesteen yhdistelmästä liukenevia mono- ja oligosakkarideja ei syntynyt lainkaan hydrolyyseissä, joissa oli enemmän kuin 40–50 % ioninestettä. Selluloosaa liuottavien ioninesteiden vaikutuksessa sellulaasien toimintaan oli selviä eroja. [EMIM]AcO haittasi enemmän sellulaasien toimintaa kuin [DMIM]DMP. Puhtaassa [EMIM]AcO:ssa *T. reesei*n endoglukanaasi inaktivoitui täysin neljän tunnin käsittelyssä jäännösaktiivisuusmittauksen perusteella, kun taas aktiivisuus aleni hyvin vähän ja hitaasti [DMIM]DMP:ssa kolmen vuorokauden aikana. Nämä tulokset vastasivat hyvin samojen ioninesteiden vesiliuoksissa tehtyjen mikrokiteisen selluloosan (microcrystalline cellulose, MCC) entsymaattisten hydrolyysien tuloksia. Äskettäin on kehitetty selluloosaa liuottavia ioninesteitä, jotka perustuvat orgaanisten superemästen 1,1,3,3-tetrametyyliguanidiinin (TMG) ja 1,5-diatsabisyklo[4.3.0]non-5-eenin (DBN) karboksylaattisuoloihin. Nämä ioninesteet ovat tislattavia suhteellisen miedoissa olosuhteissa ja näin ollen kierrätettäviä. Hydrolyysikokeiden perusteella nämä uudet selluloosaa liuottavat ioninesteet eivät kuitenkaan olleet paremmin yhteensopivia sellulaasien kanssa kuin perinteiset imidatsoliumi-pohjaiset ioninesteet. *T. reesei*n endoglukanaasit eivät pystyneet vähentämään MCC:n molekyylipainoa puskurissa eivätkä missään muussa io-

ninestettä sisältävässä liuoksessa, paitsi 90-prosenttisessa (v/v) [DMIM]DMP:ssa, johon MCC oli osittain liuennut.

Selluloosaa liuottavien ioninesteiden todettiin olevan emäksisiä vesiliuoksessa. Tämän työn tulosten perusteella ioninesteiden aiheuttama pH-arvon nousu ei kuitenkaan ollut sellulaasien inaktivoitumisen pääsyy. Sellulaasin kyky toimia korkeissa pH-arvoissa ei tehnyt sellulaasista tehokkaampaa selluloosan hydrolyysissä ioninestematriiseissa. Sen sijaan sellulaasien termostabiilisuus vaikutti johtavan kasvavaan ioninestetoleranssiin.

Tutkittujen ioninesteiden havaittiin olevan hyvin haitallisia hiilihydraattianalytiikkamenetelmille. Työssä kehitettiin kapillarielektroforeesimenetelmä mono- ja oligosakkaridien analyysiin ioninestepitoisissa matriiseissa. Hydrolyysien saannot ja liuenneiden oligosakkaridien tuotejakaumat määriteltiin tällä menetelmällä ioninestepitoisista hydrolysaateista. Analyysien perusteella havaittiin, että ioninesteiden läsnäolo entsymaattisessa selluloosan hydrolyysissä sai tuotejakauman siirtymään pitempiin oligosakkarideihin joillakin sellulaaseilla. Elektroforeesimenetelmää käytettiin myös sello-oligomeerien hydrolyysin seuraamiseen *Aspergillus niger*in β-glukosidaasilla ioninestematriiseissa. Tämä β-glukosidaasi havaittiin hyvin ioninesteherkäksi.

Ioninesteiden havaittiin vaikuttavan *T. reesei*n sellulaasien selluloosaan sitoutumiseen. Sellulaasien sitoutumista MCC:aan tutkittiin radioleimatuilla *T. reesei* Cel5A:lla (endoglukanaasi II), Cel7A:lla (cellobiohydrolaasi I) ja niiden katalyyttisillä domeeneillä puskuriliuoksissa [DMIM]DMP:n ja [EMIM]AcO:n läsnä ollessa. Cel7A pystyi sitoutumaan MCC:aan pelkällä katalyyttisellä domeenillaan, kun taas Cel5A oli hyvin riippuvainen hiilihydraatteja sitovasta modulistaan sitoutuakseen tehokkaasti selluloosaan. Korkea sitoutumisaste ei kuitenkaan ollut tarpeellinen Cel5A:lle, jotta se olisi toiminut selluloosan hydrolyysissä. [EMIM]AcO vaikutti [DMIM]DMP:a voimakkaammin sellulaasien selluloosaan sitoutumiseen. Yleisesti tutkittujen ioninesteiden todettiin vaikuttavan herkästi *T. reesei*n hiilihydraatteja sitovien moduulien toimintaan.

Avainsanat Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase

Preface

The work in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2010–2013. As VTT has a very long experience in cellulase research, it has been a true privelege to work together with so many top scientists in this field. This work was funded by the Finnish Bioeconomy Cluster's (FiBiC) Future Biorefinery (FuBio) programme and by VTT Graduate School. In addition, the Academy of Finland Graduate School for Biorefining (BIOREGS) has provided funding for participating in conferences and financed many interesting courses. COST action FP0901 "Analytical tools for biorefineries" provided the funding to carry out a short term scientific mission to the University of Natural Resources and Life Sciences (BOKU) in Vienna in 2010. I am deeply grateful to all my funders for providing the possibility to have such a rich time in learning, networking, conferencing, studying new topics and developing as a scientist during my PhD studies.

At VTT I wish to thank Vice President, Dr. Anu Kaukovirta-Norja, my current and former Technology Managers Dr. Raija Lantto and Dr. Niklas von Weymarn, for providing me with good working facilities. I am forever thankful to my thesis supervisor at VTT, Dr. Anna Suurnäkki, for all the time she has given me even though her time schedules have been extremely tight during the last years. I greatly appreciate our regular meetings during which Anna has not only given me guidance for my scientific work but also helped me understand how our research environment and the scientific world works: in brief, being not only a supervisor, but also a mentor. In addition, I am grateful for the scientific guidance given to me by my thesis advisory board with Prof. Reija Jokela, Research Prof. Kristiina Kruus, Dr. Jarmo Ropponen and Hannu Mikkonen, who sadly passed away during the time of my thesis work. I am greatly thankful to Prof. Reija Jokela for being my supervising professor at Aalto University; I am really happy for the smooth cooperation we have had in this work. Also the Planning Officer for doctoral affairs at Aalto University, Sirje Liukko is thanked for guiding me through the university bureaucracy and Prof. Matti Leisola is thanked for supervising my minor studies. Dr. Kristiina Poppius-Levlin, the coordinator of VTT Graduate School, is thanked for her support and positive attitude in guiding us graduate school students towards the ultimate goal.

I wish to thank the pre-examiners of my thesis, Prof. Jack Saddler at University of British Columbia, Canada, and Deputy Director, Dr. Blake Simmons from Sandia

National Laboratories, US, for their extremely precious comments on the thesis manuscript. Based on their comments we had many new great ideas on how to study this topic further. I deeply believe my thesis was much improved based on the feedback I had during the preliminary examination process. Päivi Vahala and VTT publication services are thanked for helping with technical editing and Michael Bailey for carrying out linguistic corrections.

I am grateful to my team leader Dr. Terhi Hakala and my whole team at VTT for their supportive and positive attitudes towards my work. Matti Siika-aho has been a never-ending source of good advice for the practical laboratory experiments, and I hope my office mate Stina Grönqvist has enjoyed our fruitful and usually also diverting discussions as much as I have. Of the technical staff I want to thank especially Mariitta Svanberg, Riitta Alander, Jenni Lehtonen, Pirkko Saarelainen, Ulla Vornamo, Eila Turunen and Nina Vihersola for technical assistance in carrying out many of the experiments. I also want to acknowledge Dr. Tarja Tamminen for challenging a quite, at that time, reluctant young man to start conducting PhD studies in the first place. The so called “lunch group” with PhD students and young scientists has been very important for me as a forum for exchanging ideas, opinions and experiences. Jenni Rahikainen, Katariina Kemppainen, Katariina Rommi, Heini Virtanen, Piritta Niemi, Outi Santala and Kirsi Kiiveri can be counted among the most active members in this group.

I want to thank all the participants, both academic researchers and industrial tutors, in the WP2 group of the FuBio JR2 project, for giving me a forum where to discuss the ionic liquid aspect of my work. Without our co-operation this work would not have been possible to finish in its current form. Especially Prof. Ilkka Kilpeläinen and Dr. Alistair King at the University of Helsinki are thanked for their constant support. I am extremely happy for twice having had the opportunity to visit the group of Prof. Antje Potthast at BOKU University in Vienna. My visits to BOKU have been among the best parts of my PhD, both in terms of science, networking and getting new acquantancies. In addition to Prof. Potthast, I also want to thank Dr. Ute Henniges and Dr. Anna Bogolitsyna, who I had the pleasure to work with during my visits at BOKU. I also want to thank all the co-authors to my articles Dr. Stella Rovio, Dr. Anna Suurnäkki, Dr. Alistair King, Arno Parviainen, Research Prof. Kristiina Kruus, Dr. Jenni Rahikainen, Dr. Gerald Ebner, Philipp Vejdovszky, Dr. Michael Schrems, Prof. Paul Kosma, Prof. Thomas Rosenau and Prof. Antje Potthast.

Finally, I want to thank my family, my relatives and all my friends for always being supportive to me in my pursuit of the doctoral degree.

Espoo, December 2013

Ronny Wahlström

Academic dissertation

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	Professor John Saddler Department of Wood Science, University of British Columbia, Vancouver, British Columbia, Canada
Opponent	Professor Antje Spiess Enzyme Process Technology, RWTH-Aachen University Aachen, Germany

List of publications

This thesis is based on the following original publications which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers. Additional unpublished data is also presented.

I Wahlström R, Rovio S, Suurnäkki A. 2012. Partial enzymatic hydrolysis of microcrystalline cellulose in ionic liquids by *Trichoderma reesei* endoglucanases. RSC Adv 2:4472–4480.

II Wahlström R, Rovio S, Suurnäkki A. 2013. Analysis of mono- and oligosaccharides in ionic liquid containing matrices. Carbohydr Res 373:42–51.

III Wahlström R, King A, Parviainen A, Kruus K, Suurnäkki A. 2013. Cellulose hydrolysis with thermo- and alkali-tolerant cellulases in cellulose-dissolving superbase ionic liquids. RSC Adv 3:20001–20009.

IV Wahlström R, Rahikainen J, Kruus K, Suurnäkki A. 2013. Cellulose hydrolysis and binding with *Trichoderma reesei* Cel5A and Cel7A and their core domains in ionic liquid solutions. Biotech Bioeng, in Press.

V Ebner G, Vejdovszky P, Wahlström R, Suurnäkki A, Schrems M, Kosma P, Rosenau T, Potthast A. 2014. The effect of 1-ethyl-3-methylimidazolium acetate on the enzymatic degradation of cellulose. J Mol Cat B 99:121–129.

Author's contributions

I The author participated in planning the work and carried out all the laboratory work including both hydrolysis experiments and analyses. The author interpreted the results and wrote the article together with the co-authors.

II The author carried out the method development together with Dr. Stella Rovio. The laboratory work for the application examples was carried out by the author. The author interpreted the results and wrote the article together with the co-authors.

III The author planned the experiments together with the co-authors and carried out the laboratory work including both hydrolysis experiments and analyses. The author interpreted the results and wrote the article together with the co-authors.

IV The author planned the experiments together with the co-authors. The author guided the execution of the hydrolysis experiments, carried out the Tr-labeling of cellulases and the binding experiments. The author interpreted the results and wrote the article together with the co-authors.

V The author participated in the planning and execution of the cellulase inactivation experiments (endoglucanase activity measurements on soluble substrates) and the hydrolysis of beech dissolving pulp in buffer, including the analysis of the hydrolysate. The author interpreted the results from these experiments and wrote the corresponding parts of the article together with the co-authors. Results from this article have also been used in the PhD thesis of Dr. Gerald Ebner at the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria.

Contents

List of abbreviations

ABEE	4-Aminobenzoic acid ethyl ester
ABN	4-Aminobenzonitrile
AFEX	Ammonia freeze (or fibre) explosion
ANTS	8-Aminonaphthalene-1,3,6-trisulphonic acid
APTS	8-Aminopyrene-1,3,6-trisulphonic acid
ARP	Ammonia recycle percolation
AGU	Anhydroglucose unit
BGE	Background electrolyte
BASIL	Biphasic Acid Scavenging utilizing Ionic Liquids
BSA	Bovine serum albumine
CE	Capillary electrophoresis
CBM	Carbohydrate-binding domain
CMC	Carboxymethylcellullose
CBH	Cellobiohydrolase
CD	Core domain
DP	Degree of polymerization
DBN	1,5-Diazabicyclo[4.3.0]non-5-ene
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMAc	Dimethylacetamide
DMF	Dimethylformamide
DMI	1,3-Dimethyl-2-imidazolidinone
DMSO	Dimethylsulphoxide
DNS	3,5-Dinitrosalicylic acid
EG	Endoglucanase
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GPC	Gel permeation chromatography
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection

HPLC	High performance liquid chromatography
PAHBAH	*para*-Hydroxybenzoic acid hydrazide
HEC	Hydroxyethylcellulose
HMF	5-Hydroxymethylfurfural
IL	Ionic liquid
LOI	Lateral order index
LCC	Lignin-carbohydrate complex
LOQ	Limit of quantification
LSC	Liquid scintillation counting
LPMO	Lytic polysaccharide mono-oxygenase
NMMO	*N*-Methylmorpholine-*N*-oxide
4-MUC	4-Methylumbelliferyl-β-D-cellobioside
4-MUL	4-Methylumbelliferyl-β-D-lactoside
MCC	Microcrystalline cellulose
PEG	Polyethylene glycol
PHK DP	Pre-hydrolysis kraft dissolving pulp
RC	Regenerated cellulose
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
rpm	Revolutions per minute
RT	Room temperature
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDP	Sulphite dissolving pulp
TMG	1,1,3,3-Tetramethylguanidine
TCI	Total crystallinity index
VOC	Volatile organic compounds

Abbreviations of ionic liquids

[AMIM]Cl	1-Allyl-3-methylimidazolium chloride
[BzPy]Cl	Benzylpyridinium chloride
[BMIM]Ace	1-Butyl-3-methylimidazolium acesulphamate
[BMIM]DBP	1-Butyl-3-methylimidazolium dibutylphosphate
[BMIM]Cl	1-Butyl-3-methylimidazolium chloride
[BMIM]PF_6	1-Butyl-3-methylimidazolium hexafluorophosphate
[BMIM]BF_4	1-Butyl-3-methylimidazolium tetrafluoroborate
[BMPy]Cl	1-Butyl-3-methylpyridinium chloride
[DBNH]AcO	1,8-Diazabicyclo[5.4.0]undec-7-enium acetate
[DBNH]EtCOO	1,8-Diazabicyclo[5.4.0]undec-7-enium propionate
[DMIM]DEP	1,3-Dimethylimidazolium diethylphosphate
[DMIM]DMP	1,3-Dimethylimidazolium dimethylphosphate
[EMBy]DEP	1-Ethyl-3-methylbutylpyridinium diethylphosphate
[EMIM]AcO	1-Ethyl-3-methylimidazolium acetate
[EMIM]Br	1-Ethyl-3-methylimidazolium bromide
[EMIM]DBP	1-Ethyl-3-methylimidazolium dibutylphosphate
[EMIM]DEP	1-Ethyl-3-methylimidazolium diethylphosphate
[EMIM]DMP	1-Ethyl-3-methylimidazolium dimethylphosphate
[EMIM]$MeSO_4$	1-Ethyl-3-methylimidazolium methanesulphonate
[HPy]Cl	1-Hexylpyridinium chloride
[E(OH)MIM]AcO	1-Hydroxyethyl-3-methylimidazolium acetate
[DBNMe]DMP	1-Methyl-1,8-diazabicyclo[5.4.0]undec-7-enium dimethylphosphate
[P4444]OH	Tetrabutylphosphonium hydroxide
[TMGH]AcO	1,1,3,3-Tetramethylguanidinium acetate
[TMGH]*n*-PrCOO	1,1,3,3-Tetramethylguanidinium butyrate
[TMGH]COO	1,1,3,3-Tetramethylguanidinium formiate
[TMGH]EtCOO	1,1,3,3-Tetramethylguanidinium propionate
[P8881]AcO	Trioctylmethylphosphonium acetate
HEMA	Tris-(2-hydroxyethyl)methylammonium methylsulphate

1. Introduction

Cellulose is the most abundant biopolymer in the world. Different estimations of annual global cellulose production range between 9×10^{10} ton/a (Pinkert, et al. 2009) and 1.5×10^{12} ton/a (Ha, et al. 2011). Together with lignin and hemicelluloses, cellulose builds up lignocellulosic biomass into a highly complex matrix. Due to greenhouse gas emissions leading to global warming, limited supply, price stability and energy and chemical feedstock security issues, there is a need to replace fossil petroleum as raw material for fuels, chemicals and materials. Lignocellulosic biomass has been recognized as the raw material of the future, as it is renewable, carbon neutral and widely available in different forms around the world. The polysaccharides in lignocellulosic biomass can be hydrolysed to monosaccharides which can be used as raw materials for the production of a variety of commodities, such as ethanol, butanol, fatty acid ethyl esters, lactic acid and hydrogen gas (Barr, et al. 2012; Bokinsky, et al. 2011; Hofvendahl and Hahn–Hägerdal 2000; Maeda, et al. 2007).

Bioethanol production is currently the most studied process for lignocellulosic raw material conversion to value-added chemicals. Bioethanol has been used as a liquid transport fuel *e.g.* in Brazil for decades and its use is constantly growing as a liquid fuel or liquid fuel component in North America, Europe and China. However, first generation bioethanol has to a great extent relied on the use of starch as raw material source in addition to sugar cane (mainly in Brazil), and have thus directly been competing with agricultural food production. The use of food raw material for fuel production is not ethically acceptable in the long run and therefore the next generation biofuels must be produced from non-food sources, such as cellulose and hemicellulose present in lignocellulosic biomass (second generation bioethanol).

Renewable non-food sources of lignocellulosic biomass include forestry sidestreams such as logging and wood processing mill residues, removed biomass from forest management and land clearing operations, and agricultural sources such as crop residues (corn stovers, straw), perennial grasses and energy and woody crops (Perlack, et al. 2005). It has been estimated that 5–8% of the annually produced biomass would be sufficient to completely replace the consumption of fossil petroleum, other fossil resources not included (Stark 2011).

Biotechnical ethanol production from cellulosic sources consists of three distinct steps: pretreatment, total enzymatic hydrolysis and fermentation (Lozano, et al. 2012). This approach is known as separate hydrolysis and saccharification (SHF). In addition, simultaneous saccharification and fermentation (SSF), in which the hydrolysis and fermentation steps are carried out in the same vessel simultaneously, is also an increasingly studied option (Wilson 2009). The pretreatment step is needed to increase the substrate digestibility, as lignocellulosic biomass is very recalcitrant towards enzymatic hydrolysis due to various structural factors (Chandra, et al. 2007). The total hydrolysis of polysaccharides in lignocellulosic biomass is carried out by a mixture of polysaccharide-hydrolysing enzymes, cellulases and hemicellulases. Of the commercial enzymes, cellulases are the third most important group due to their diverse applications in *e.g.* cotton processing, additives in animal feed, paper recycling and detergents (Wilson 2009). When large-scale hydrolysis of lignocellulosics becomes established commercially, cellulases are expected to become the largest commercial enzyme group. Cellulases constitute the second largest operational cost factor in bioethanol production after the feedstock (Klein-Marcuschamer, et al. 2010), even though a 20-fold decrease in cellulase costs in bioethanol production has been reported for the last decade (Aden and Foust 2009). Even though some technical and economical challenges remain to be solved for economical production of lignocellulosic ethanol, several demonstration plants are currently already in operation, including in Europe *e.g.* Inbicon in Denmark and Abengoa in Spain, both of which use agricultural waste as feedstock (Larsen, et al. 2012) and many plants in North America and East Asia. Several commercial scale ethanol production facilities are under construction in different countries (Menon and Rao 2012).

1.1 Structure and enzymatic hydrolysis of cellulose

Lignocellulosic biomass consists of three major components: cellulose, hemicellulose and lignin, the ratio of which varies depending on the biomass origin. The lignocellulosic components make up a complex structural matrix which is highly recalcitrant towards hydrolysis (Figure 1). The structure and properties of cellulose, as well as cellulose hydrolysis, are described in the following sections. Hemicelluloses are heteropolymers which consist of a variety of different saccharides (Sjöström 1993). Common hemicelluloses are xylans, mannans and galactans, named after the main sugars of their backbone structure. Hemicelluloses often have branched structures. Lignin is an irregular and complex, branched polymer built up from three different phenylpropanoid monomers: 4-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, which differ from each other in the degree of methoxylation of the aromatic ring (Campbell and Sederoff 1996). Consisting of aromatic monomers, lignin has a considerable hydrophobic character, in contrast to polysaccharides. Lignin and polysaccharides have also been shown to form covalent bonds with each other, resulting in lignin-carbohydrate complexes (LCCs) (Björkman 1957).

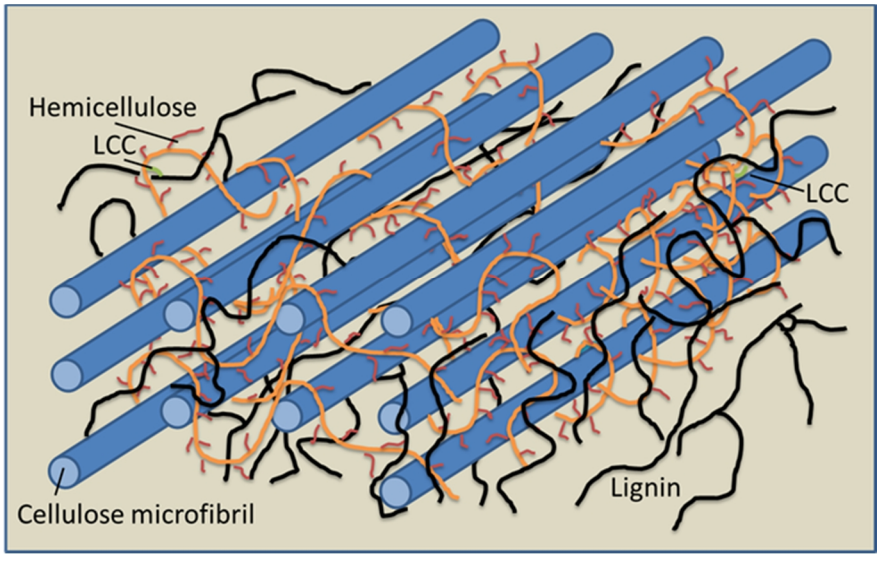

Figure 1. Schematic view of lignocellulosic biomass. The three main components, cellulose, hemicelluloses and lignin form a highly complex and entangled matrix. LCC = lignin-carbohydrate complex.

Biomass polysaccharides, cellulose and hemicelluloses, may be hydrolysed to their constituent monosaccharides either by enzymatic or mineral acid hydrolysis. Enzymatic hydrolysis offers several benefits over acid hydrolysis: 1) no need for corrosion-resistant processing equipment, 2) less acid waste and 3) less formation of undesirable by-products such as 5-hydroxymethylfurfural (HMF), which may be detrimental in downstream processing (Binder and Raines 2010; Dadi, et al. 2006). Further benefits associated with enzymatic hydrolysis includes the potential for almost complete polysaccharide conversion (Wyman, et al. 2005).

1.1.1 Structure of cellulose

Cellulose is a linear, unbranched homopolymer consisting of β-D-glucopyranose units linked by (1→4)-β-glycosidic linkages. The degree of polymerization (DP, the number of polymerized glucose units) of cellulose can vary from 20 (laboratory synthesized cellulose) up to 10 000 anhydroglucose units (AGUs) for some bacterial celluloses (Pinkert, et al. 2009). The AGUs are rotated 180° to each other and thus the (anhydro)cellobiose unit is the smallest structural element of cellulose (Figure 2).

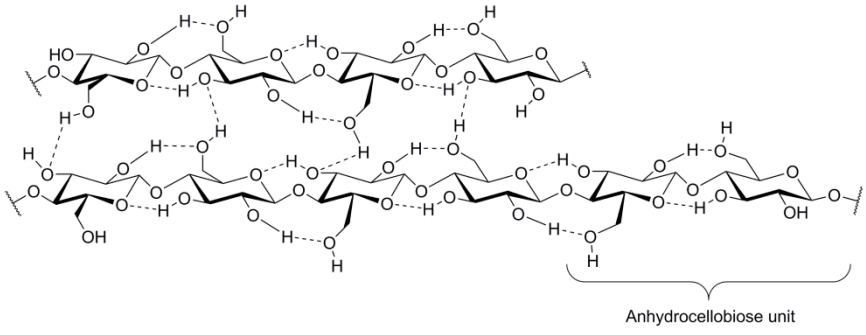

Figure 2. Schematic view of cellulose I. The anhydrocellobiose unit is the smallest repeating structure of the cellulose chain. Each anhydroglucose unit (AGU) forms two intramolecular hydrogen bonds and one intermolecular hydrogen bond.

The cellulose chains form both intra- and intermolecular hydrogen bonds, which makes the cellulose crystallites extremely rigid (Figure 2). Cellulose chains aggregate with each other to form elementary fibrils with a width of 10–20 nm, which contain both highly ordered, crystalline regions and less ordered, *i.e.* amorphous regions (Sjöström 1993). These elementary fibrils build up larger fibrils with which hemicelluloses and lignin finally form lignocellulosic fibers. Cellulose in higher plants is synthesized by cellulose synthase complexes (“rosettes”), which form 36 cellulose chains in parallel; these 36 chains have been proposed to aggregate together soon after synthesis to form the elementary microfibrils (Ding and Himmel 2006). The strong hydrogen bonding in cellulose renders it insoluble in water with increasing DP. As discussed by Zhang and Lynd (2004), cello-oligomers with DP 2–6 are water soluble and DP 7–12 are partially soluble in hot water.

As reviewed by O’Sullivan (1997), cellulose has at least six polymorphs, commonly designated as cellulose I, II, III_I, III_{II} and IV_I and IV_{II}. In addition, cellulose I has been found to exist as two polymorphs, cellulose Iα and Iβ. Cellulose I is the native form of cellulose, whereas cellulose II can be obtained from cellulose I by regeneration or mercerization. Cellulose II is thermodynamically the most stable form of cellulose. The cellulose chains in cellulose I have a parallel chain direction, whereas the chain direction in cellulose II is antiparallel. The two cellulose I polymorphs, Iα and Iβ, have the same conformation in their general skeleton, but have different hydrogen bonding patterns. The non-crystalline part of cellulose is usually termed amorphous cellulose. Microcrystalline cellulose (MCC or Avicel) is an often employed crystalline cellulose model substrate. It is prepared by partial acid hydrolysis of wood pulp followed by spray-drying (Krassig 1993). Although the amorphous regions should be removed during the acid treatment, MCC has been found to contain a significant fraction (30–50%) of amorphous cellulose (Krassig 1993; Zhang and Lynd 2004) and also some residual hemicelluloses, which may have a significant influence on the substrate properties of MCC in enzymatic hydrolysis (Várnai, et al. 2010).

1.1.2 Enzymatic hydrolysis of cellulose

Cellulose is a very stable molecule and it has been calculated that the uncatalyzed half-life of cellulose through spontaneous hydrolysis would be 5 million years (Wolfenden, et al. 1998). Thus, efficient cellulose-degrading systems are needed for industrial cellulose hydrolysis and in nature to sustain the global carbon cycle. Elwyn Reese, a pioneer in studying the systems of cellulolytic enzymes, proposed a two-step mechanism for cellulose hydrolysis in 1950 (Reese, et al. 1950). In this model two different enzyme activities, C_1 and C_x, were suggested to act stepwise on cellulose. The model has been heavily revised since 1950 and cellulose hydrolysis is currently believed to take place in three simultaneous steps: 1) physical and chemical changes to the yet unhydrolysed solid substrate, 2) primary hydrolysis, in which soluble cello-oligomers are released from the solid cellulose to the hydrolysate, and 3) secondary hydrolysis, in which the dissolved oligomers are hydrolysed to glucose (Zhang and Lynd 2004). The enzymatic hydrolysis of cellulose is performed in synergy by different cellulolytic enzymes, known as endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. In addition, it has recently been discovered that oxidative enzymes (lytic polysaccharide monooxygenases, LPMOs, some of which are classified as glycosyl hydrolase family GH61 cellulases) act on cellulose as auxiliaries to the hydrolytic cellulases, and cellulase dosage for total hydrolysis may be significantly reduced by addition of these oxidoreductase enzymes (Harris, et al. 2010; Langston, et al. 2011; Quinlan, et al. 2011; Vaaje-Kolstad, et al. 2010). The role and mechanism of LPMOs as auxiliaries in cellulose hydrolysis are still not very well known, but these enzymes appear to catalyze the oxidative cleavage of cellulose chains, in the process oxidizing the C1, C4 or C6 positions in the AGUs. Thus new cellulose chain ends are made available for the cellobiohydrolases. Other non-hydrolytic proteins may also play a role in enhancing cellulose hydrolysis, such as swollenins and expansins (Georgelis, et al. 2013; Gourlay, et al. 2013). The current system of naming and classifying carbohydrate active enzymes (including cellulases and hemicellulases) based on their structure family was introduced by Henrissat et al. (1998) and recently the classifications system for carbohydrate active enzymes has been enlarged with several new groups of auxiliary enzymes (Levasseur, et al. 2013). According to the enlargement of the classification system of the carbohydrate active enzymes, the LPMOs formerly known as GH61 enzymes should now be termed family AA9 enzymes, where AA denotes Auxiliary Activities (for plant cell wall degradation).

Cellulose-degrading enzymes are secreted by a large number of different microorganisms, such as fungi and different bacteria (Enari 1983). The most studied and efficient organism, in terms of cellulase secretion, is the filamentous fungus *Trichoderma reesei* (named in honour of Elwyn Reese). *T. reesei* is known to produce at least two cellobiohydrolases, Cel6A and Cel7A, and five endoglucanases (Cel5A, Cel7B, Cel12A, Cel45A and Cel61A) (Karlsson, et al. 2002) (Table 1). The main enzyme components of the *T. reesei* cellulase system are the

cellobiohydrolases Cel6A and Cel7A, corresponding to 20 and 60% of the total secreted cellulolytic protein, whereas Cel5A which constitutes ~12% of the secreted cellulases, is the main endoglucanase (Teeri 1997; Zhang and Lynd 2004). All *T. reesei* cellulases except Cel12A, are modular enzymes, *i.e.* they consist of a core domain (CD) connected to a carbohydrate-binding domain (CBM) through an *O*-glycosylated peptide linker.

Table 1. Cellulases produced by *Trichoderma reesei*.

Cellulase	Synonym	Modular	Ref.
Cel5A	Endoglucanase II	Yes	Saloheimo, et al. 1988
Cel6A	Cellobiohydrolase II	Yes	Teeri, et al. 1987
Cel7A	Cellobiohydrolase I	Yes	Shoemaker, et al. 1983a, b Teeri, et al. 1983
Cel7B	Endoglucanase I	Yes	Penttilä, et al. 1986
Cel12A	Endoglucanase III	No	Okada, et al. 1998
Cel45A	Endoglucanase V	Yes	Saloheimo, et al. 1994
Cel61A	Endoglucanase IV*	Yes	Saloheimo, et al. 1997

* Recently assigned as lytic polysaccharide mono-oxygenase (LPMO)

The *T. reesei* cellobiohydrolases Cel6A and Cel7A hydrolyse the cellulose chains exclusively from either the non-reducing or reducing chain end, respectively (Teeri 1997). Cellobiohydrolases have their active site in a tunnel through the CD, whereas the active sites of the endoglucanases are located in a cleft on the CD surface. The tunnel-shaped active site of *T. reesei* Cel7A was elucidated by Divne et al. (1994) and based on the tunnel structure, the enzyme was proposed to hydrolyse cellulose in a processive manner, *i.e.* without desorption of the cellulase from the cellulose between the catalytic events. In later studies the tunnel has been found to contain ten specific sites for cellulose binding (Divne, et al. 1998). Cel6A hydrolyses the cellulose from its non-reducing end (Chanzy and Henrissat 1985), but its action is not as processive as it is for Cel7A (Igarashi, et al. 2009). As discussed by Srisodsuk et al. (1998), some cellobiohydrolases have also been suggested to exhibit endoactivity, but it has been much under debate whether the observed endoactivity really is a property of the cellobiohydrolase or whether the studied enzyme preparations have contained endoglucanase as minor impurities. The catalytic mechanism can be either retaining or inverting, depending on whether the C1 anomeric centre at the hydrolysed AGU has its stereochemistry retained or inverted in the process. The hydrolytic cleavage of the glycosidic bond takes place *via* an acid catalysis mechanism in which two carboxylic acid groups act together, one activating the glycosyl bond while the other assists in the nucleophilic attack of water (Withers 2001).

The *T. reesei* endoglucanases are believed to work in a non-processive manner, probably through cycles of adsorption and desorption between the catalytic events (Linder and Teeri 1996). Endoglucanases can act on both unsubstituted

and substituted celluloses, *e.g.* on carboxymethylcellulose (CMC), hydroxyethylcellulose (HEC), cellodextrins and phosphoric acid swollen cellulose and are as such not very specific (Enari 1983). Endoglucanases are generally believed to attack only the amorphous regions of the cellulose. β-Glucosidases hydrolyse cellobiose and cello-oligosaccharides to glucose. Cellobiose causes strong end-product inhibition of the cellobiohydrolases, which is why cellobiose needs to be continuosly hydrolysed to glucose. A schematic overview of enzymatic hydrolysis of cellulose with the *T. reesei* cellulase system is presented in Figure 3.

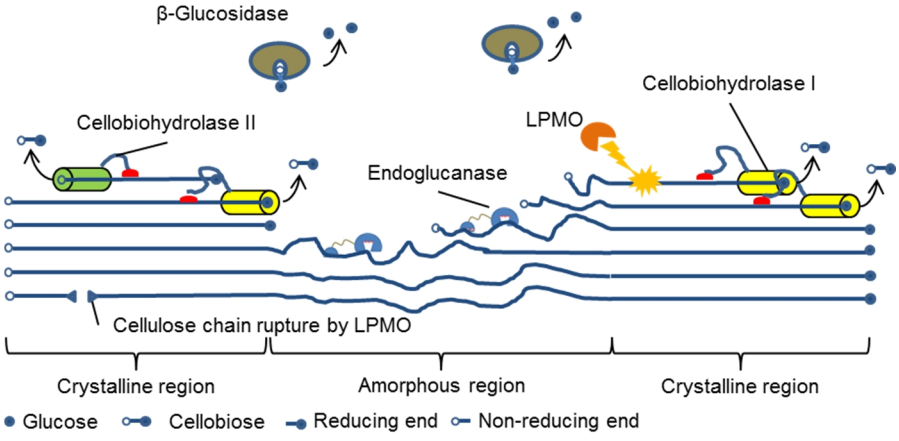

Figure 3. Schematic view of cellulose hydrolysis with the *Trichoderma reesei* cellulase system. LPMO = lytic polysaccharide mono-oxygenase enzyme.

T. reesei cellulases act synergistically, meaning that combining two or more cellulases produces more hydrolytic events together than the cumulative sum of the independent components. Most commonly synergism is exemplified by the endo/exo synergism, in which the endoactive cellulases hydrolyse intercrystal amorphous regions, thus providing the exoactive cellulases with new cellulose chain ends to work on (Srisodsuk, et al. 1998). In all, at least seven different modes of cellulase synergy have been described (Zhang and Lynd 2004).

The structure and composition of cellulosic substrates highly affect their enzymatic digestibility. Amorphous cellulose is typically hydrolysed much faster than crystalline cellulose by fungal cellulases. The high crystallinity of native cellulose hinders specifically the action of endoglucanases, whereas in regenerated cellulose (RC) more glycosidic bonds are available for scission (Dadi, et al. 2006). As reviewed by Zhang and Lynd (2004), amorphous cellulose may be hydrolysed with up to 30 times greater hydrolysis rates than crystalline cellulose, suggesting a cellulose hydrolysis model in which the amorphous regions are hydrolysed preferably before the crystalline regions. However, several studies have shown that crystallinity of the cellulosic substrate does not change during enzymatic hydrolysis (Kent, et al. 2010; Penttilä, et al. 2010; Puls and Wood 1991). Similarly, crystallite

size appears to remain constant during enzymatic cellulose hydrolysis (Penttilä, et al. 2010).

Cellulose crystallites in RC are usually in the form of cellulose II, which is more readily hydrolysed with enzymes than cellulose I (native cellulose) (Wada, et al. 2010). It has been suggested that van der Waals forces between the sheets of cellulose I would be stronger than in cellulose II, thus rendering cellulose I more recalcitrant towards hydrolysis (Wada, et al. 2010). Especially hydrated cellulose II has a higher hydrolysability than cellulose I. Cellulose crystallinity appears to impact more on initial hydrolysis rates than on maximum conversion (Zhu, et al. 2008). The mechanisms of enzymatic hydrolysis are partly different for regenerated substrates as compared to native lignocellulosic biomass. Especially the endoglucanase component of the cellulase cocktails has been reported to cause substantially more random hydrolysis, leading to DP reductions, on regenerated, more amorphous cellulose substrates (Engel, et al. 2012a). Recently ionic liquids (ILs) have been used to produce regenerated cellulose and lignocellulosics. For more efficient cellulase utilization, cellulase cocktails have been optimized for use on substrates regenerated from IL solution (Barr, et al. 2012; Engel, et al. 2012b).

The substrate DP is an important parameter for cellobiohydrolases, as a lower DP indicates relatively more chain ends for the cellobiohydrolases to work on (Zhang and Lynd 2004). Endoglucanase action is not known to be dependent on substrate DP. Cellulose accessibility both in terms of inner and outer surface area is important for cellulase adsorption and hydrolysis. In the total hydrolysis of lignocellulosic biomass, the accessibility of cellulose within the lignified and hemicellulose-containing matrix is especially important for efficient hydrolysis.

1.1.3 Role of the carbohydrate-binding module and cellulase substrate binding in cellulose hydrolysis

The presence and role of carbohydrate-binding modules (CBMs) in *T. reesei* cellulases were first reported for the cellobiohydrolase Cel7A (van Tilbeurgh, et al. 1986), and soon Cel6A was also reported to have a similar structure (Tomme, et al. 1988). Cleaving off the CBM from the *T. reesei* cellobiohydrolases resulted in the cellobiohydrolase core domains (CDs), which had 50–90% less hydrolytic activity on solid crystalline substrates, whereas the hydrolysis efficiency on small soluble substrates remained unchanged (Tomme, et al. 1988; van Tilbeurgh, et al. 1986). In the hydrolysis of amorphous cellulose, Cel6A CD exhibited a significant decrease in hydrolysis kinetics compared to the intact cellulase, whereas Cel7A and its CD had similar hydrolysis rates, indicating that the CBMs and CDs may have different binding and associated hydrolysis behaviour depending on the substrate morphology. At least three functions have been proposed for the CBMs: to increase the cellulase concentration close to the substrate through adsorption (Igarashi, et al. 2009; van Tilbeurgh, et al. 1986); to physically disrupt the cellulose prior to hydrolysis (amorphogenesis) (reviewed by Arantes and Saddler 2010; Din,

et al. 1994); and to target the cellulase towards specific regions on the substrate (Carrard, et al. 2000; Fox, et al. 2013).

Fungal CBMs (including those from the major cellobiohydrolases and endoglucanases of *T. reesei*) show great structural similarity and are all characterised as family 1 CBMs (Linder, et al. 1995b). The structure of these CBMs has been shown to be wedge-shaped with a rough and a flat face (Kraulis, et al. 1989). The flat face contains three aromatic amino acid residues by which the CBM interacts with the crystalline cellulose during binding. All three of these aromatic residues are tyrosines in the CBM of Cel7A, whereas one of the tyrosines is replaced by a tryptophan in the CBM of Cel5A (Linder, et al. 1995b). Replacement of a tyrosine with a tryptophan has been shown to greatly increase the cellulose binding affinity of the CBM (Linder, et al. 1995a). The spacing of the aromatic amino acid residues has been suggested to allow stacking with every second AGU of the bound cellulose chain (Tormo, et al. 1996). The flat face also contains some charged amino acid residues which are believed to participate in cellulose binding by forming hydrogen bonds (Linder, et al. 1995b).

Both the CBM and CD of *T. reesei* Cel7A bind to cellulose, but the binding of CBM is much tighter (Ståhlberg, et al. 1991). The presence of CBM does not appear to have any impact on the catalytic turnover number of the cellulases, which is linked to the sliding speed on the substrate. For both intact *T. reesei* Cel7A and its CD, this parameter was shown by high speed atomic force microscopy to be the same: 3.5 nm/s on cellulose chains (Igarashi, et al. 2009). Thus, the CBM of Cel7A has been concluded not to detach the cellulose chains from the crystalline regions and feed the chains into the catalytic tunnel, but that its role is mainly to increase the enzyme concentration close to the substrate. The hydrolysis rate per adsorbed cellulase unit has been observed to be the same on filter paper for both Cel7A and its CD, indicating that the CBM does not endow the intact enzyme with any extra catalytic properties (Nidetzky, et al. 1994). The role and structure of CBMs have been reviewed by *e.g.* Boraston et al. (2004) and Shoseyov et al. (2006).

The CBMs of *T. reesei* Cel6A and Cel7A are similar in structure and binding affinity, but whereas Cel7A CBM binds completely reversibly to cellulose (Linder and Teeri 1996), the CBM from Cel6A binds partially irreversibly (Carrard and Linder 1999). Addition of organic solvents, such as ethanol or dimethylsulphoxide (DMSO), results in decreased binding of both Cel6A and Cel7A CBMs, as is expected because substrate binding takes place mostly *via* hydrophobic interactions (Carrard and Linder 1999). The cellulose binding of *T. reesei* Cel6A and Cel7A CBMs is not pH sensitive, varying by less than 10% over a pH range of 2.5–11 (Carrard and Linder 1999). When the effect of temperature, ionic strength and pH on the binding of *T. reesei* cellulases was studied, temperature was observed to affect the binding significantly, with higher temperatures leading to decreased binding affinity, whereas the effects of ionic strength and pH varied between different enzymes (Kyriacou, et al. 1988).

A sequence comparison of known cellulases showed that the majority of the known putative cellulase genes actually do not contain a CBM (Várnai, et al. 2013). The CBM has been shown to be useful for *T. reesei* cellulases at low substrate

consistencies, whereas at high substrate consistency (20%), the CD and intact cellulases show very similar performance in cellulose hydrolysis. Screening for the most efficient cellulase candidates in diluted systems has apparently led to favouring the isolation of cellulases with CBM.

1.2 Ionic liquids in cellulose dissolution and lignocellulose pretreatment

1.2.1 Ionic liquids

Research into ionic liquids (ILs) is considered to have started with the synthesis of ethylammonium nitrate in 1914 (Walden 1914). Since a NATO Advanced Research Workshop in 2000, ILs have generally been defined as salts with melting points below 100 °C (Sun, et al. 2011). Much of the early work on ILs was carried out at the US Air Force Academy, where experiments were carried out with chloroaluminates for use in thermal batteries (Wilkes, et al. 2008). Soon, these were combined with imidazolium and pyridinium cations. The use of these ionic liquids was limited for a long time due to their reactivity with water. In the early 1990s water stable dialkylimidazolium ILs were introduced and IL chemistry as it is known today was born. Very few scientific articles dealing with ILs had appeared before 2000, whereafter the number of papers started growing rapidly (Seddon 2008). The number of potential ILs has been estimated to at least a million compounds, which means that the synthetic diversity is also great. The synthesis, purification and characterization of ILs have been reviewed by Clare et al. (2009).

ILs have some highly desirable properties. They are often called green solvents due to their very low vapour pressures, thus eliminating any emissions of volatile organic compounds (VOCs) (van Rantwijk and Sheldon 2007; Yang and Pan 2005). In addition, ILs are generally considered to be thermally and chemically stable, their properties are tunable (*e.g.* polarity, hydrophobicity) by making the correct choice of ions, and they have good solvent properties, making them good solvents for a wide range of different compounds. Due to their complex structures, ILs are able to interact with solutes through a great variety of different interactions, including dispersive, π - π, n - π, hydrogen bonding, dipolar and ionic/charge-charge interactions (Anderson, et al. 2002). ILs have low melting points, mainly due to their large and asymmetric cations, which give the salts low lattice energies for crystallisation (Huddleston, et al. 2001).

Depending on their ionic composition, ILs may be acidic, neutral or basic (MacFarlane, et al. 2006). The acid-base behaviour of different compounds may differ in ILs from those under aqueous conditions. Relatively weak acids may be very strong acids in ILs, as even weakly basic anions will shift the dissociation equilibrium strongly to the right for acid solutes because of the anion's high concentration. Acid-base conjugate ILs may be distillable if there is an equilibrium between the IL form and the unconjugated acid and base, *e.g.* in an IL with a weak basic anion and a cation with an exchangeable proton (MacFarlane, et al. 2006).

ILs have been distilled as intact ions or aggregates thereof (Earle, et al. 2006) or as anion-cation pairs (Leal, et al. 2007) at very low pressures. The distillation of 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and similar ILs has been patented (Massonne, et al. 2009). Acid-base conjugate ILs based on 1,1,3,3-tetramethylguanidium (TMG) or 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) are distillable under rather mild conditions (100–200 °C at ~1 mbar) as their unconjugated components (King, et al. 2011; Parviainen, et al. 2013).

IL polarity is not a simple concept and several different methods have been employed to determine the polarity of diverse ILs, usually giving rise to partially contradictory results (van Rantwijk and Sheldon 2007). ILs could be expected to be highly polar due to their ionic nature, but results have indicated that ILs have polarities similar to those of short chain alcohols and formaldehyde (Chen, et al. 2006c). IL polarity has little to do with the concept of hydrophobicity and hydrofilicity, and therefore predicting IL water miscibility is not straight forward (van Rantwijk and Sheldon 2007). ILs, even those labeled as hydrophobic, are at the same time hygroscopic and usually absorb some water if allowed to equilibrate with environmental moisture (Seddon, et al. 2000), which may be troublesome when carrying out reactions requiring dry conditions. Water and other solvents greatly decrease IL viscosity (Seddon, et al. 2000) and have a greater impact on the physical properties of hydrophilic ILs than on those of hydrophobic ILs (Huddleston, et al. 2001). The high viscosity of ILs may cause mass transfer problems and also causes problems in IL handling and unit operations.

ILs have a reputation of being thermostable, but studies give very different decomposition temperatures for different ILs (Huddleston, et al. 2001; Kosmulski, et al. 2004). Although ILs do not typically produce VOC emissions, their generical “greenness” has been much aquestioned. Some common IL ions are hydrolytically unstable, especially the fluorinated BF_4^- and PF_6^- anions, which may be hydrolysed to highly toxic HF (Swatloski, et al. 2003). ILs are not always inert solvents, as the C2 proton of the imidazolium ring may be deprotonated by a base to generate a highly nucleophilic carbene, which will react with most electrophiles (Aggarwal, et al. 2002). Many dialkylimidazolium ILs exhibit high ecotoxicity, which appears to increase with alkyl chain length (Docherty, et al. 2005). The biodegradability of many imidazolium-based ILs also appears to be negligible (Gathergood, et al. 2004). Other types of ILs, such as ILs based on cholinium or amino acids, are expected to be greener (van Rantwijk and Sheldon 2007). When registered for the European REACH (Registration, Evaluation, Authorisation and restriction of CHemicals) legislation, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) was labeled toxic whereas [EMIM]AcO was labeled non-toxic (Sun, et al. 2011).

Depending on their properties and solvent miscibility, ILs may be used in many different modes of operation: as pure solvent, as co-solvent or in biphasic (and even triphasic) systems (Kragl, et al. 2002). Several examples of industrial applications of ILs already exist (Maase 2008). The most well-known example is the BASIL™ (Biphasic Acid Scavenging utilizing Ionic Liquids) process introduced in 2002 by BASF, in which an IL precursor (methylimidazole) is used as acid scavenger, forming an IL upon neutralization of an acid produced in the process. A

productivity increase of $8x10^4$ compared to the earlier process was achieved, demonstrating the potential of IL technology. PetroChina has introduced an industrial scale process for isobutane alkylation, in which an IL is used as catalyst (Liu, et al. 2006). Several other industrial applications of ILs have been proposed and some of the following examples have been tested in pilot scale: use of IL as entrainer in reactive distillation, use of ILs in chlorination, ether cleavage, olefin oligomerization, hydrosilylation and fluorination reactions (Maase 2008). Further applications include electroplating processes, additives to cleaning fuels, gas storage liquids etc. ILs have been considered to be expensive, but are expected to become cheaper with increasing use. It is predicted that on mid-term time scale many ILs will become available in multi-ton quantities for 25–50 $/kg (Gordon and Muldoon 2008); more optimistic price estimations go as far down as 2.2 $/kg (Reddy 2006).

1.2.2 Cellulose dissolution in ionic liquids

Native cellulose is not easily soluble in conventional solvents. Cellulose has traditionally been dissolved in both derivatizing and non-derivatizing solvents, which may be either aqueous or non-aqueous media (reviewed by Heinze and Liebert in 2001). Dissolution of cellulose was first reported by Swatloski *et al.* (2002) in the IL [BMIM]Cl. Soon afterwards, a new type of allyl-functionalized imidazolium-based IL, 1-allyl-3-methylimidazolium chloride ([AMIM]Cl), was introduced as a powerful cellulose solvent (Wu, et al. 2004; Zhang, et al. 2005). The patent of Graenacher from 1934 has been considered to be the first account of dissolving cellulose in an IL type of solution (Graenecher 1934), but the used solvent, water-free benzylpyridinium chloride ([BzPy]Cl), contained 1–2% dry pyridine and was as such not a pure salt, neither does the pure [BzPy]Cl fit the generally accepted IL definition of having a melting point below 100 °C. Some ILs have also been found to be good solvents for native lignocellulosic biomass and even the dissolution of wood has been reported (Kilpeläinen, et al. 2007). During the last decade, cellulose and wood dissolution in ILs and the underlying mechanisms have been extensively studied and reviewed (Mäki-Arvela, et al. 2010; Pinkert, et al. 2009; Zakrzewska, et al. 2010; Zhu, et al. 2006). Homogeneous functionalization and derivatization of cellulose in IL solution has been reviewed by Barthel and Heinze (2006) and by Liebert and Heinze (2008).

According to the reviews cellulose-dissolving ILs are based on imidazolium, pyridinium, pyrrolidinium, cholinium, tetrabutylammonium, tetrabutylphosphonium and alkylalkyloxyammonium cations as well as on protonated/alkylated polycyclic amidine bases, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and DBN (Figure 4). Reported anions are *e.g.* halogens, carboxylates, amides, imides, thiocyanates, phosphates, sulphates, sulphonates and dichloroaluminates. Cellulose solubility in an IL is clearly dependent on the type of both the cation and the anion. The cellulose dissolution is very sensitive to the presence of water (Swatloski, et al. 2002). Water is thus also an efficient anti-solvent for precipitating cellulose.

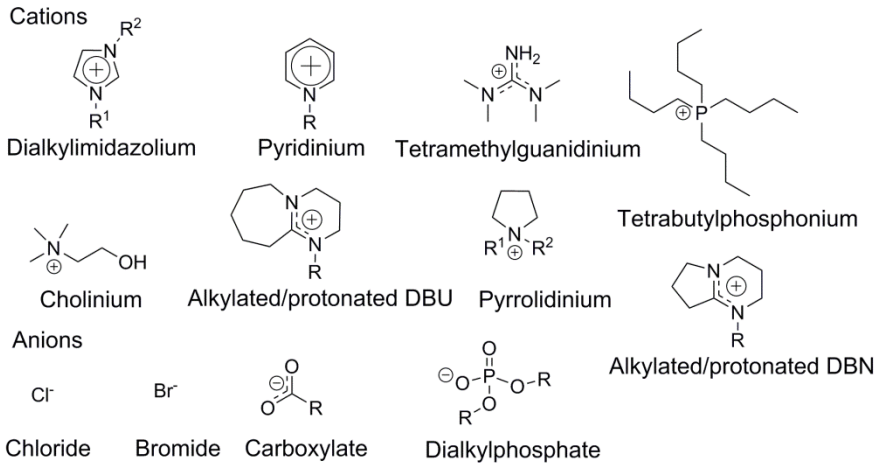

Figure 4. Different ions encountered in cellulose-dissolving ILs.

Halide-free dialkylimidazolium ILs are often encountered as cellulose solvents: dialkylphosphates (*e.g.* 1,3-dimethylimidazolium dimethylphosphate, [DMIM]DMP, reported as cellulose solvent by Mazza et al. in 2009) and acetates, of which [EMIM]AcO is a very powerful cellulose solvent (Zavrel, et al. 2009). The structures of the frequently used cellulose-dissolving ILs are presented in Figure 5. Recently, several new classes of ILs have been introduced as cellulose solvents in addition to the well-known imidazolium-based ILs. ILs consisting of conjugated acid-base pairs with cations based on the organic superbases TMG or DBN dissolve cellulose well and are reported to be distillable under rather mild conditions (King, et al. 2011; Parviainen, et al. 2013). Up to 20% (w/w) of cellulose could be dissolved in 5 min in an aqueous solution of 60% (w/w) tetrabutylphosphonium hydroxide ([P4444]OH) under very mild conditions (Abe, et al. 2012). Alkylalkyloxyammonium amino acid ILs were reported as cellulose solvents by Ohira et al. (2012a). 1-Hexylpyridinium chloride ([HPy]Cl) has also been shown to solubilize cellulose (Uju, et al. 2013). Very high cellulose dissolution rates have been reported in systems with cellulose-dissolving ILs diluted with aprotic co-solvents, such as 1,3-dimethyl-2-imidazolidinone (DMI), dimethylformamide (DMF) and DMSO (Ohira, et al. 2012b; Rinaldi 2011).

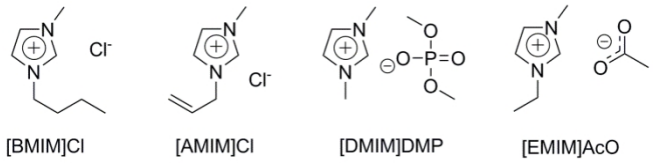

Figure 5. The most studied cellulose-dissolving imidazolium-based ILs: 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-allyl-3-methylimidazolium chloride ([AMIM]Cl), 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) and 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO).

Imidazolium-, piperidinium- and ammonium-based ILs with polyethylene glycol (PEG) subsitutents have been introduced as especially designed enzyme-compatible cellulose solvents (Figure 6) (Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009a; Zhao, et al. 2009b). The PEG substituents play a dual role in interacting with the dissolved cellulose and stabilising the enzyme through the oxygen atoms in the PEG, and the large size of the PEG-substituted cation dilutes the molar anion concentration, which has been suggested to be directly linked to enzyme inactivation (Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009b). Another cellulose-dissolving enzyme-compatible IL, 1-hydroxyethyl-3-methylimidazolium acetate ([E(OH)MIM]AcO), which has the structure of [EMIM]AcO with a terminal hydroxyl group on the ethyl side chain of the cation, was reported by Li et al. (2012).

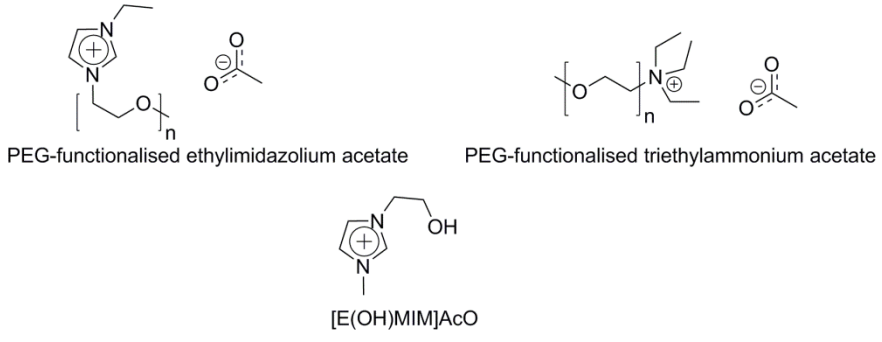

Figure 6. Tailor-made enzyme-compatible cellulose-dissolving ILs: polyethylene glycol (PEG) functionalised imidazolium and ammonium acetates and [E(OH)MIM]AcO (Li, et al. 2012; Tang, et al. 2012; Zhao, et al. 2008; Zhao, et al. 2009a; Zhao, et al. 2009b).

Cellulose-dissolving ILs are in general hydrophilic (Zhao, et al. 2008). The basicity of the anion of the ILs is generally regarded as a key property for cellulose dissolution, as the anion is believed to break up the hydrogen bond network which holds the cellulose chains together (Swatloski, et al. 2002; Tang, et al. 2012; Zhao, et al. 2009b).

Kamlet-Taft parameters can be used to predict cellulose solubillity in ILs (Doherty, et al. 2010). The Kamlet-Taft parameters are determined based on measuring solvatochromic effects (by UV-Vis) of the studied solvent on a set of dyes. Commonly used Kamlet-Taft parameters for ILs are hydrogen bond acidity (α), hydrogen bond basicity (β) and dipolarity/polarizability effects (π*) (Crowhurst, et al. 2003). Generally the β parameter (hydrogen bond basicity) efficiently predicts cellulose solubility (Doherty, et al. 2010), although recently the β-α parameter ("net basicity") has been proposed to predict cellulose solubility even more accurately (Hauru, et al. 2012). The presence of water in the solution directly decreases the β parameter, explaining the precipitation of cellulose in the waterbath regeneration process. The role of the IL cation in cellulose dissolution is somewhat under debate. Mostly, imidazolium-based cations have been studied. Already Swatloski et al. (2002) noticed that the structure of the cation also affects cellulose dissolution, as increasing the chain length of the alkyl substituents on the imidazolium led to sharply decreasing cellulose solubility. The imidazolium cation has been suggested to have hydrophobic interactions with the hydrophobic side of the cellulose (Liu, et al. 2010; Youngs, et al. 2007). The C2 proton on the imidazolium has also been proposed to interact as a weak hydrogen bond donor to the hydroxyl group oxygens in cellulose during dissolution (Youngs, et al. 2007). Recent results also suggest the cation acidity to be an important parameter for predicting cellulose solubility (Parviainen, et al. 2013). Cellulose solubility and dissolution rates are greatly affected by the IL's viscosity. Heating reduces the solution viscosity and increases cellulose solubility (Mazza, et al. 2009). Microwave irradiation can also be used to increase cellulose dissolution rates in ILs (Swatloski, et al. 2002).

1.2.3 Ionic liquid pretreatments of lignocellulosics for total enzymatic hydrolysis

Native lignocellulosic biomass is very recalcitrant towards enzymatic hydrolysis, which necessitates excessive substrate pretreatments before hydrolysis. The pretreatment process has been identified as a very expensive processing step in biorefineries and it is also decisive for designing down-stream processing (Wyman 2007). As reviewed by Chandra et al. (2007), substrate-related factors affecting the efficiency of enzymatic hydrolysis of lignocellulose are the presence and form of lignin and hemicelluloses, cellulose crystallinity, cellulose DP, and the accessible surface area of the substrate. Hemicelluloses and lignin impede cellulose hydrolysis by forming a physical barrier around the cellulose (Grethlein 1985; Mooney, et al. 1998) (Figure 1), and enzymes can also bind non-specifically to lignin (Sutcliffe and Saddler 1986). A good pretreatment process should have low capital and operating costs and be effective on a variety of different substrates, the generated side streams (hemicelluloses, lignin) should be easy to recover for further use and the formation of by-products, such as furfurals should be avoided, as these are known in many cases to inhibit down-stream processing (Chandra, et al. 2007). Furthermore, substrates which can be efficiently hydrolysed by enzymes at high substrate

loadings, with short residence times and with low enzyme concentrations should be produced (Shill, et al. 2011).

Conventional pretreatment processes are in general divided into physical, chemical and biological processes, or combinations thereof (Chandra, et al. 2007). Biological processes typically use wood degrading fungi, but the long treatment times (10–14 days) and large space requirements render this pretreatment option rather unattractive. Physical pretreatment methods involve different types of milling, reducing particle size and cellulose crystallinity while the surface area is increased. Benefits include good compatiblity with different substrate types, but high energy costs are a distinct drawback and lignin is not removed. Chemical pretreatment processes are based on both acid and alkali treatments, which primarily remove hemicelluloses and lignin. Organosolv treatments have similar lignin-removing effects. Cellulose crystallinity can be modified by treatments with cellulose swelling or dissolving solvents and reagents. Alkali- and acid-based pretreatments have the drawback of needing neutralization of the processing liquids, which in large scale can be problematical. Physicochemical pretreatment processes include steam explosion, ammonia freeze (or fibre) explosion (AFEX) and ammonia recycle percolation (ARP). In the current demonstration scale plants (*e.g.* Inbicon in Kalundborg, Denmark, SEKAB in Örnsköldsvik, Sweden and Abengoa in Salamanca, Spain) producing lignocellulosic ethanol steam/hydrothermal pretreatment is applied, in some cases combined with H_2SO_4 or SO_2 catalysis (Larsen, et al. 2012).

Certain ILs are promising in biomass pretreatment due to their unique ability to dissolve lignocellulosic biomass. The cellulosic component can be precipitated from IL solution by the addition of an anti-solvent, such as water or alcohol, and the precipitated cellulose fraction is very susceptible to subsequent enzymatic total hydrolysis (Dadi, et al. 2006). Kilpeläinen et al. (2007) were the first to demonstrate the complete dissolution of softwood in IL ([BMIM]Cl and [AMIM]Cl). In addition to softwood, hardwood has also been dissolved in ILs ([AMIM]Cl and [EMIM]AcO being the most efficient) (Zavrel, et al. 2009). Complete dissolution and regeneration of lignocellulosics is a moisture-sensitive process, as the presence of only 5–10% of water in the cellulose-dissolving imidazolium-ILs has been reported to lead to significantly decreased pretreatment efficiency (Doherty, et al. 2010). Cellulose regeneration from ILs for total hydrolysis necessitates a thorough washing of the regenerated substrate, as even residual amounts of many cellulose-dissolving ILs in the substrate have been shown to cause severe inactivation of the cellulases and inhibit down-stream microbial conversions (Hong, et al. 2012; Zhao, et al. 2009a). Whereas IL pretreatment prior to enzymatic total hydrolysis of lignocellulosics is a much studied topic, several groups have also been active in studying acid hydrolysis of plant cell wall polysaccharides dissolved in ILs (Binder and Raines, 2010; Sievers, et al. 2009; Sun, et al. 2013a). Fair hydrolysis yields have been reported for acid hydrolysis in ILs, but the by-product formation of furfurals, which are detrimental for down-stream microbial processes, could not fully be avoided in these systems.

Dadi et al. were the first to report that dissolution of MCC in IL ([BMIM]Cl or [AMIM]Cl) followed by regeneration dramatically accelerated the enzymatic hy-

drolysis rate (Dadi, et al. 2006; Dadi, et al. 2007). The increased digestibility of the regenerated cellulose (RC) was apparently due to decreased cellulose crystallinity. Thereafter, the number of articles about IL pretreatment has increased rapidly. Table 2 gives an overview of selected IL pretreatment articles published during recent years. The total number of articles in this field is over 400 (in mid-2013) and rapidly increasing. The table illustrates the variety of lignocellulosic substrates and ILs studied, as well as the main effects of the IL pretreatment on the substrate.

The most frequently used ILs in biomass pretreatment are [EMIM]AcO, [BMIM]Cl and [AMIM]Cl (Table 2). Dialkylphosphates, most notably [DMIM]DMP, and alkyloxyalkylsubstituted imidazolium ILs are also used. Recently cholinium-based ILs, as a completely new IL class, have also been of interest for pretreatment. MCC, as a highly crystalline cellulosic model compound, is the most studied hydrolysis substrate for pretreatment. During recent years, IL pretreatment has also been studied with more industrially relevant substrates, such as corn stover, rice and wheat straw, switchgrass and wood meal. The impact of the pretreatment depends on both the type of substrate (native lignocellulosic biomass *vs.* pure cellulose) and the type of IL. The IL pretreatments increase the enzymatic digestibility of the substrates through three main effects: 1) decrystallization or crystallinity transformation from cellulose I to cellulose II, 2) removal of hemicelluloses and lignin and 3) partial depolymerization of the cellulose. Reported crystallinity changes during pretreatment involve both decreasing total crystallinity and transitions from cellulose I to cellulose II (Bian, et al. 2014; Cheng, et al. 2011; Dadi, et al. 2006; Dadi, et al. 2007). Hemicellulose removal can be achieved by mildly treating the lignocellulose with diluted solutions of certain ILs (20 or 50%) (Hou, et al. 2012; Hou, et al. 2013a; Hou, et al. 2013b). IL pretreatment also appears to break up the LCCs (Singh, et al. 2009). Treatments leading to DP reductions are directly beneficial for enzymatic hydrolysis, as cellobiohydrolases are dependent on finding cellulose chain ends as starting points for hydrolysis (Zhang and Lynd 2004). DP decreases have been reported for IL pretreatments in several articles (Bian, et al. 2014; Uju, et al. 2013). IL pretreatments have also been carried out with very high solid loadings (Wu, et al. 2011), in sequential combination with other pretreatments (Geng and Henderson 2012) or in conjunction with microwave irradiation or sonication (Ha, et al. 2011; Li, et al. 2011; Liu and Chen 2006; Yang, et al. 2010). Research interest has partly moved from the complete dissolution of cellulose and biomass to lighter IL pretreatments, such as extracting the lignin from biomass. Pretreatments can be optimized either for increased hydrolysis kinetics or maximum digestibility of the substrate.

Table 2. Selected references of IL pretreatment of lignocellulosic biomass for total hydrolysis.
MCC = microcrystalline cellulose, LCC = lignin-carbohydrate complex, DP = degree of polymerization.

Substrate	IL(s)	Impact on substrate properties	Other comments	Ref.
MCC	[BMIM]Cl	Decrystallization	50-fold increase in hydrolysis kinetics	Dadi, et al. 2006
MCC	[BMIM]Cl, [AMIM]Cl	Decrystallization	90-fold increase in hydrolysis kinetics, optimized	Dadi, et al. 2007
MCC	Six dialkylphosphate imidazolium ILs	Decrystallization, decrease in DP	Ultrasonic treatment enhanced pretreatment efficiency	Yang, et al. 2010
MCC	[DMIM]DMP	Crystallinity changes	Decrystallization not the only effect on digestibility	Xie, et al. 2012
MCC	[BMIM]Cl	Decrystallization		Lozano, et al. 2012
MCC, filter paper, cotton	[BMIM]Cl, [AMIM]Cl, four alkyloxyalkyl substituted imidazolium and ammonium acetates	Decrystallization	Residual ILs potentially inactivated cellulases	Zhao, et al. 2009a
MCC, α-cellulose, Sigmacell	[EMIM]AcO	Increased porosity and accessibility		Engel, et al. 2012a
MCC, switchgrass, pine, eucalyptus	[EMIM]AcO	Crystallinity changes, swelling, potentially DP decreases	Different effects on crystallinity depending on original substrate	Cheng, et al. 2011
MCC, straw, willow, pledget	Large variety of different IL classes	Decrystallization		Li, et al. 2011
MCC, bagasse	[HPy]Cl, [EMIM]AcO	Delignification, crystallinity changes, DP reductions	[HPy]Cl caused significant release of cello-oligomers	Uju, et al. 2013
MCC, corn stover	[BMIM]Cl	Decrystallization	Comparison between [BMIM]Cl and H_3PO_4 treatments	Sathitsuksanoh, et al. 2012
MCC, filter paper, cotton	[EMIM]AcO, [BMIM]Cl	Drastic DP decreases, changes in crystallinity	Microwave irradiation significantly enhanced pretreatment efficiency	Ha, et al. 2011
MCC, rice straw	Cholinium glycine	Lignin and partial hemicellulose extraction		Liu, et al. 2012
Cotton	[BMIM]Cl	Decrystallization, crystallinity changes, DP decreases, increased surface area	Comparison between pretreatments with [BMIM]Cl, H_3PO_4, NaOH/urea and NMMO	Kuo and Lee 2009
Cotton waste textiles	[AMIM]Cl		Down-stream processing very sensitive to residual [AMIM]Cl	Hong, et al. 2012
Switchgrass	[EMIM]AcO	LCCs disrupted, lignin extraction		Singh, et al. 2009
Switchgrass	[BMIM]Cl	Decrease in DP due to added solid acid catalyst	Prehydrolysis with acid during IL pretreatment decreased enzyme requirement with 99%	Groff, et al. 2013

Table 2. (continued)

Substrate	IL(s)	Impact on substrate properties	Other comments	Ref.
Switchgrass, poplar	[EMIM]AcO	Decrystallization		Barr, et al. 2012
Corn stover	[BMIM]Cl	Lignin removal (by alkali extraction), decrystallization by IL treatment	Combined sequential treatment: alkali + IL	Geng and Henderson 2012
Corn stover	[EMIM]AcO	Decrystallization and lignin extraction	Very high (up to 50%) solid content in IL pretreatment step	Wu, et al. 2011
Corn cob	Nine imidazolium-based ILs	Decrystallization, increase in substrate surface area	Dialkylphosphate anions combine biomass solubility and enzyme compatibility	Li, et al. 2010
Wheat straw	[EMIM]DEP, [BMIM]Cl, [EMIM]AcO, [EMIM]DBP, [EMBy]DEP	Decrystallization ([EMIM]DEP studied in detail)		Li, et al. 2009
Wheat straw	[BMIM]Cl	Decrystallization, decrease in DP, increased substrate accessiblity		Liu and Chen 2006
Rice straw	Cholinium amino acids	Lignin removal		Hou, et al. 2012
Rice straw	Aqueous cholinium amino acids	Lignin removal	50% IL sufficient for delignification, reduced solution viscosity	Hou, et al. 2013a
Rice straw	20% cholinium lysine in water	Lignin removal	Process optimized for delignification without hemicellulose removal	Hou, et al. 2013b
Energy cane bagasse	[EMIM]AcO	Decrystallization, delignification		Qiu and Aita 2013
Cellulose from sugar cane bagasse	[EMIM]AcO	50% DP decrease and crystallinity change of cellulose, increased surface area		Bian, et al. 2014
Alkali extracted sugar cane bagasse	[EMIM]DEP	Disruption of cellulose structure	Low IL concentrations in pretreatment (0–20%)	Su, et al. 2012
Milled poplar wood	[EMIM]AcO	Crystallinity changes	Comparison with acid pretreatment included	Goshadrou, et al. 2013
Maple wood flour	[EMIM]AcO	Decrystallization, lignin extraction	Potential for lignin recovery for further use	Lee, et al. 2009
Alkaline treated eucalyptus	[AMIM]Cl, [BMIM]Ace, [BMIM]Cl, [EMIM]AcO	Various effects depending on IL	$MgCl_2$ or H_2SO_4 added as catalysts in pretreatment	Sun, et al. 2013b
Fibre sludge	[AMIM]Cl, [BMIM]Cl	Increased cellulose accessibility		Holm, et al. 2012
Poplar seed floss	[BMIM]Cl, [EMIM]Cl, [BMPy]Cl	Decrystallization		Bodirlau, et al. 2010
Kenaf core fibre	Cholinium mono- and dicarboxylates	Extraction of hemicelluloses and lignin	Cholinium carboxylates were found to have low toxicity compared to [EMIM]AcO	Ninomiya, et al. 2013

The current high price of especially imidazolium-based ILs sets high recovery targets for ILs in biorefinery applications. The extensive washing of regenerated substrates to remove trace amounts of IL prior to enzymatic hydrolysis leads to extremely diluted IL solutions. For recycling, it is very expensive to separate the water from the dilute IL solutions through evaporation or reverse osmosis, due to the high energy requirements (Park, et al. 2012). For IL recovery, the simplest suggested procedure has been to evaporate the anti-solvents from the ILs and directly re-use the ILs (Lee, et al. 2009; Li, et al. 2009; Lozano, et al. 2012; Qiu and Aita 2013; Wu, et al. 2011). Another alternative for IL recovery is the creation of aqueous-IL biphasic systems by mixing the IL phase with concentrated salt solutions (Gutowski, et al. 2003; He, et al. 2005; Li, et al. 2005). However, serious problems can be expected with lignin and other components accumulating into the IL (Shill, et al. 2011; Sun, et al. 2013a). A few techno-economic studies have been carried out regarding glucose production with IL pretreatments. Abels et al. (2013) reported the IL cost to be approximately 33% of the glucose price when IL was used for pretreatment and the polysaccharides were hydrolysed enzymatically. Sen et al. (2012) made a similar study on a process in which acid hydrolysis of corn stover was carried out in IL solution. In both cases, the IL was the dominant factor in the price of the glucose product, even if high IL recovery rates (99.75% and 98%) and an IL price of only 10 €/kg or $/kg were assumed. In these studies, the production price of glucose was evaluated to be at least five times higher than the current market price.

1.3 Enzyme action in ionic liquids

1.3.1 Enzymatic reactions in ionic liquids

The first study of using enzymes in (aqueous) IL was by Magnuson et al. (1984), who studied the stability of alkaline phosphatase in ethylammonium nitrate. Several reviews on both the fundamental science and applications of biocatalysis in ILs have been published (Kragl, et al. 2002; Moniruzzaman, et al. 2010a; Naushad, et al. 2012; van Rantwijk and Sheldon 2007; Yang and Pan 2005). ILs have been recognized as desirable media in enzyme-catalysed organic synthesis mainly due to their low volatility, thus mitigating VOC emissions, and their excellent solvent properties (van Rantwijk and Sheldon 2007). Most enzymes reported to be active in ILs are lipases, which work at water-oil interfaces in their natural role (Kragl, et al. 2002). In many cases, increased enzymatic activity, stability and selectivity have been observed in IL solutions (Yang and Pan 2005). Several interesting reports have been published concerning enzymatic polysaccharide acylations in IL solutions (Chen, et al. 2006a; Chen, et al. 2013; Zhao, et al. 2008).

Three different medium types should be considered when dealing with enzyme stability and action in ILs: anhydrous hydrophobic ILs, anhydrous hydrophilic ILs, and aqueous solutions of hydrophilic ILs. As a general rule hydrophilic ILs have been regarded as being destabilizing for enzymes, whereas hydrophobic ILs are

stabilizing (Zhao, et al. 2006b). Although hydrophobic ILs stabilize suspended enzymes, they do not dissolve carbohydrates to an appreciable extent (Zhao, et al. 2009b), and are thus of marginal interest for homogeneous polysaccharide modification. Enzyme-dissolving ILs usually also inactivate enzymes, with the exception of a few ILs such as cholinium $H_2PO_4^-$ (Fujita, et al. 2005). The inactivating effect of both hydrophilic organic solvents as well as ILs has been suggested to be caused by dehydration of essential water molecules from proteins (van Rantwijk and Sheldon 2007). Especially hydrophilic ILs effectively strip essential water from enzymes (Moniruzzaman, et al. 2010b). Protein denaturation is generally a two-step process, with unfolding as a first, reversible step and aggregation of the unfolded protein as the second, irreversible step (Constatinescu, et al. 2010). Both stabilizing and destabilizing ILs appear to prevent protein aggregation. ILs are not always inactivating, but may also promote the refolding of denaturated protein, as was demonstrated with hen egg white lysozome in ethylammonium nitrate (Summers and Flowers 2000). In the early studies of enzymes in ILs, diverse problems were encountered regarding IL purity, unexpected pH shifts and precipitation of buffer salts (Kragl, et al. 2002). Even low concentrations of chloride impurities may drastically decrease enzyme activity (Lee, et al. 2006), and IL purification has been shown to confer considerable benefits in retaining enzyme activity in these systems (Park and Kazlauskas 2001).

Enzyme stability in hydrophilic solvents may be predicted by the solution's Hildebrandt solubility parameter (δ), dielectric constant (ε), dipole moments (μ) or octanol-water partition coefficient, logP (Kaar, et al. 2003). According to the log P values, many common imidazolium-based ILs, including hydrophobic ILs with PF_6^- anions, should not support enzyme activity, although 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM]PF_6 has been shown not to inactivate enzymes to a significant degree. Thus, many usually applied measures for solvent compatibility with enzymes should be applied with great care in IL systems.

Pure IL media usually have high viscosities. In enzyme-mediated reactions, the high viscosity also decreases substrate diffusion rates and thereby reaction kinetics (Zhao, et al. 2006a). Dilution with low-viscosity organic solvents has in some cases been demonstrated to be advantageous (Chen, et al. 2006b). ILs are not generally neutral in pH, but have been shown to have even considerable effects on matrix pH values in buffer solutions (Engel, et al. 2010; Li, et al. 2012). Furthermore, ILs also increase the ionic strength of the matrix, which affects enzyme activity and stability. The effects of viscosity, ionic strength and pH cannot completely explain the enzyme inactivation in many ILs, suggesting that the IL-induced enzyme inactivation is also caused by other mechanisms (Engel, et al. 2010).

Cations and anions are characterized as kosmotropes or chaotropes, depending on their ability to promote or destroy water structure, respectively (Constantinescu, et al. 2007). Generally, kosmotropic anions stabilize proteins, whereas chaotropic cations have a destabilizing effect. In Zhao et al. (2006b), enzymes were found to be stabilized by kosmotropic anions and chaotropic cations, but destabilized by chaotropic anions and kosmotropic cations, partially contradicting the previously cited rule of thumb. The enzyme inactivation rates and inactivation reversibility or

irreversibility have been proposed to be mostly anion dependent (Kaar, et al. 2003). Nucleophilic anions are possibly able to coordinate with positively charged surface residues on the enzyme and cause conformational changes (Sheldon, et al. 2002), whereas large anions spread out their charge and thus have a weaker hydrogen bond basicity to interact with the enzymes and cause less disruption of the protein structure. Carbohydrate-dissolving ILs have anions with a high hydrogen bond basicity and thus also exert a denaturing effect on enzymes (Zhao 2010). In aqueous salt solutions, the protein stability can be related to the Hofmeister series for ions (originally introduced by Hofmeister in 1888). Several studies have indicated that the impact of ILs on enzyme action in aqueous IL solutions can generally also be predicted using the Hofmeister series (Constantinescu, et al. 2007; Lai, et al. 2011). In pure ILs the ions can be expected to affect the enzymes in a much more complex manner than in aqueous IL solutions. In fact, the Hofmeister series is applicable in IL environments only when the (hydrophilic) IL is sufficiently diluted for the ions to be dissociated from each other (Zhao, et al. 2006b). The destabilizing effect of imidazolium cations has been ordered to $[DMIM]^+ < [EMIM]^+ < [BMIM]^+$ (Lai, et al. 2011). Increasing cation hydrophobicity has been found to decrease enzyme stability (Constantinescu, et al. 2007).

1.3.2 Cellulase stability and activity in ionic liquids

The first study on *Trichoderma reesei* cellulase stability in a cellulose-dissolving IL ([BMIM]Cl) was published in 2003 (Turner, et al. 2003), indicating this IL to be very inactivating for the cellulases. The Cl^- ion was proposed to be responsible for the observed enzymatic inactivation. Water dilution of the IL matrix showed the unfolding to be reversible in [BMIM]Cl for the studied enzyme. In another early article on cellulase inactivation in IL matrices, *Humicola insolens* cellulase was found to be stable in $[BMIM]PF_6$ and 1-butyl-3-methylimidazolium tetrafluoroborate ($[BMIM]BF_4$), which do not dissolve cellulose, but the cellulase was inactivated in [BMIM]Cl (Paljevac, et al. 2006).

Many studies have been carried out with mesophilic cellulases from the well-characterized *T. reesei* and *Aspergillus niger* cellulolytic systems, either with the cellulases in a monocomponent form or as cellulase cocktails (Table 3). Most of the monocomponent cellulases studied in ILs have been endoglucanases. The IL tolerance of a cellobiohydrolase has only been reported in one study (Engel, et al. 2012b) and of monocomponent β-glucosidases in three studies (Engel, et al. 2012b; Thomas, et al. 2011; Wolski, et al. 2011). Recently, cellulases tolerant to cellulose-dissolving ILs have been screened from extremophilic sources, whereas much of the early work in this field was done with mesophilic cellulases. Carboxymethylcellulose (CMC) is the dominant substrate, especially in activity measurements, but the hydrolysis of different solid substrates has also been studied.

Table 3. Published studies on cellulase activity, stability and enzymatic cellulose hydrolysis in IL-containing matrices. RC = regenerated cellulose, CMC = carboxymethylcellulose, 4-MUC = 4-methylumbelliferyl-β-D-cellobioside, (A) = activity measurement, (H) = hydrolysis experiment.

Substrate	Cellulase	IL(s)	Comments	Ref
CMC	*Aspergillus niger* cellulase	[BMIM]Cl	High pressure during hydrolysis enhanced cellulase activity in the presence of IL (A)	Salvador, et al. 2010
CMC	*Humicola insolens* cellulase	[BMIM]Cl, [BMIM]BF_4, [BMIM]PF_6	Great excess of cellulase (H)	Paljevac, et al. 2006
CMC	Bacterial cellulases	6 different ILs	Cellulose-binding module (CBM) pivotal in cellulase IL tolerance (A)	Pottkämper, et al. 2009
CMC	Bacterial cellulases	6 different ILs	IL tolerance linked to thermo- and halophilicity (A)	Ilmberger, et al. 2012
CMC	*Thermoanaerobacter tengcongensis* endoglucanase	[BMIM]Cl, [AMIM]Cl	Thermophilic endoglucanases exhibited good IL tolerance (A)	Liang, et al. 2011
CMC	*Bacillus aquimaris* cellulase	[EMIM]$MeSO_4$, [EMIM]Br	Solvent- and alkali-tolerant cellulase exhibit good IL tolerance (A)	Trivedi, et al. 2011
CMC	*Halorhabdus utahensis* cellulase	[AMIM]Cl, [EMIM]AcO, [EMIM]Cl, [BMIM]Cl	Large number of charged surface groups on protein surface endows salt tolerance (A)	Zhang, et al. 2011
RC	*Trichoderma reesei* cellulase	[EMIM]DEP	*In situ* cellulose hydrolysis in IL introduced (H)	Kamiya, et al. 2008
RC	*T. reesei* cellulase, β-glucosidase	[DMIM]DMP, [EMIM]AcO, [EMIM]lactate	Green fluorescent protein screening for enzyme inactivation in IL (A&H)	Wolski, et al. 2011
Cellulose azure	*T. reesei* cellulase cocktail	8 different ILs	HEMA showed significant cellulase stabilization (H)	Bose, et al. 2010
Cellulose azure	*A. niger* endoglucanase	3 imidazolium ILs and HEMA	(A&H)	Bose, et al. 2012
Cellulose azure	*T. reesei* cellulase	[BMIM]Cl, [BMIM]BF_4	Comparison with LiCl/DMAc, NaCl and urea solutions (A)	Turner, et al. 2003
α-Cellulose, CMC, *para*-nitrophenyl-β-cellobioside	Celluclast® 1.5 L	[DMIM]DMP, [EMIM]AcO, [BMIM]Cl, [AMIM]Cl	Evaluation of IL matrix viscosity, ionic strength and pH cellulase activity (A&H)	Engel, et al. 2010
RC, cellobiose	*T. reesei* Cel7A and Cel7B, *A. niger* β-glucosidase	[DMIM]DMP	Optimization of cellulase cocktail for RC hydrolysis in the presence of IL (A&H)	Engel, et al. 2012b

Table 3. (continued)

Substrate	Cellulase	IL(s)	Comments	Ref
RC, CMC, regenerated yellow poplar	Celluclast® 1.5L and *A. niger* β-glucosidase	[EMIM]AcO	(A&H)	Wang, et al. 2011
Filter paper (regenerated from IL)	Liquid cellulase from Imperial Jade Bio-technology	[DMIM]DMP, [EMIM]DEP, [BMIM]DBP	(A&H)	Zhi, et al. 2012
Filter paper, CMC, xylan	*Penicillium janthinellum* mutant glycosyl hydrolases	[BMPy]Cl, [BMIM]Cl	(A&H)	Adsul, et al. 2009
CMC, pretreated corn stover, MCC	*Thermotoga maritima* and *Pyrococcus horikoshii* endoglucanases	[EMIM]AcO	Thermophilic endoglucanases exhibited good IL tolerance (A)	Datta, et al. 2010
IL pretreated switchgrass	Supernatants from thermophilic bacterial consortia	[EMIM]AcO	Thermophilic bacterial consortia adapted to switchgrass at 60 °C (A)	Gladden, et al. 2011
Azo-CMC, 4-MUC, MCC, tobacco cell wall polysaccharides	*Sulfolobus solfataricus* endoglucanase	[DMIM]DMP, [EMIM]AcO	Thermostable endoglucanase, shows high activity in 80% IL at 90 °C (A&H)	Klose, et al. 2012
IL pretreated switchgrass	Thermophilic cellulases	[EMIM]AcO	Cellulase cocktail optimized based on thermophilic cellulases (A&H)	Park, et al. 2012
CMC, RC, cotton linters, algal biomass	*Pseudoalteromonas* sp. cellulase	6 different ILs	Thermo-, halo- and alkali-tolerant cellulase exhibits good IL tolerance (A)	Trivedi, et al. 2013
MCC, RC	*T. reesei* cellulase	[EMIM]DEP	Immobilization of cellulase by glutaraldehyde cross-linking, low IL content (2%) (H)	Jones and Vasudevan 2010
CMC, MCC, straw, cotton, filter paper	Cellulase powder	[E(OH)MIM]AcO	Specially designed enzyme compatible and cellulose-dissolving IL (A&H)	Li, et al. 2012
Soluble *para*-nitrophenyl glycosides	β-glucosidases, xylanase, arabinofuranosidase	[DMIM]DMP, [EMIM]DMP, [EMIM]DEP [EMIM]AcO	(A)	Thomas, et al. 2011
Cellulose powder	*Trichoderma viride* cellulase	[BMIM]Cl	Cellulase stabilized in liposomes (H)	Yoshimoto, et al. 2013

Out of eight different ILs studied by Bose et al. (2010), tris-(2-hydroxyethyl) methylammonium methylsulphate (HEMA) was found to stabilize a *T. reesei* cellulase cocktail in temperatures up to 115 °C. Although HEMA is a promising IL in view of its cellulase compatibility, cellulose solubility in this ammonium-based IL is only ~1% which limits its applicability. In Zhi et al. (2012), cellulase stability was also studied in a series of dialkylphosphate ILs with increasing alkyl substituent size ([DMIM]DMP, 1-ethyl-3-methylimidazolium diethylphosphate [EMIM]DEP and 1-butyl-3-methylimidazolium dibutylphosphate [BMIM]DBP). [DMIM]DMP, with the smallest alkyl substituents, was found to be the least inactivating. In a comparison of the action of two β-glucosidases, a xylanase and two arabinofuranosidases in the presence of three dialkylphosphate ILs ([DMIM]DMP, 1-ethyl-3-methylimidazolium dimethylphosphate [EMIM]DMP and [EMIM]DEP) and [EMIM]AcO, [EMIM]DEP was found to be the most inactivating IL (Thomas, et al. 2011). *T. reesei* Cel7A (cellobiohydrolase) and Cel7B (endoglucanase) have been found to respond similarly to the presence of [DMIM]DMP, showing some residual activity in up to 30% (v/v) IL, whereas *A. niger* β-glucosidase was more IL sensitive and lost its activity already in 15% (v/v) of [DMIM]DMP (Engel, et al. 2012b). When Engel et al. (2010) compared the compatibility of the most common cellulose-dissolving ILs ([DMIM]DMP, [BMIM]Cl, [EMIM]AcO and [AMIM]Cl) with a commercial *T. reesei* cellulase cocktail, a general decrease of 70–85% in cellulase activity was observed in the presence of 10% (v/v) IL. Comparing the same IL from different manufacturers in some cases resulted in greater differences in relative cellulase activity than between the different IL types. However, [DMIM]DMP was concluded to be the least cellulase-inactivating of the studied ILs. The storage stability was examined in 10% (v/v) [DMIM]DMP, in which a rapid loss of activity (10–40% residual activity) was measured after an incubation time of one day, whereafter no further inactivation occured. The cellulase inactivation in aqueous [DMIM]DMP was shown to be reversible.

Wolski et al. (2011) developed a screening method in which green fluorescent protein is employed to determine protein stability in ILs, based on fluorescence measurements. With this method, [DMIM]DMP and [EMIM]lactate were identified as potentially enzyme-compatible ILs for *in situ* cellulose hydrolysis. In validating the screening results, *T. reesei* cellulases retained their activity in up to 40% [DMIM]DMP or [EMIM]lactate and *A. niger* β-glucosidase in up to 60% [DMIM]DMP. [DMIM]DMP was found to be more enzyme compatible than [EMIM]lactate. Inactivation of *A. niger* cellulase in [BMIM]Cl appears to correlate linearly with the water activity (Salvador, et al. 2010). Based on activity measurements on CMC after incubation in 10% [BMIM]Cl the cellulase regains activity upon dilution, supporting the earlier observations of Turner et al. (2003) concerning the reversibility of cellulase inactivation in aqueous [BMIM]Cl. Cellulase inactivation is temperature dependent, as a *T. reesei* cellulase mixture with *A. niger* β-glucosidase showed only minor activity losses at 4 °C during an incubation time of 1.5 h in up to 30% [EMIM]AcO, whereas inactivation proceeded much faster at 50 °C (Wang, et al. 2011).

Enzyme thermo- and halotolerance have been linked to IL tolerance, and IL-tolerant cellulases have been screened from different extremophilic and halophilic sources for better enzyme performance in the hydrophilic ILs used for cellulose

dissolution (Datta, et al. 2010; Gladden, et al. 2011; Ilmberger, et al. 2012; Klose, et al. 2012; Liang, et al. 2011). Increased IL tolerance has also been reported for enzymes active at high pH originating from solvent-tolerant bacteria (Trivedi, et al. 2011; Trivedi, et al. 2013). A cellulase from the haloalkaliphilic *Halorhabdus utahensis* has also been shown to have good IL tolerance (Zhang, et al. 2011), which was suggested to be due to the presence of a great number of negatively charged amino acid residues on the protein surface, low content of hydrophobic amino acids and a compact packing of the protein structure. The negative charge on the protein surface is anticipated to interact well with both water and high ion concentrations, as in an IL. Similar conclusions regarding the relationship between protein structure and IL tolerance were drawn by Karbalaei-Heidari et al. (2013).

Screening metagenomic libraries and mutation experiments have yielded encouraging results in finding cellulases with increased IL tolerance. Adsul et al. (2009) found several glycosyl hydrolases with improved ionic liquid tolerance from *Penicillium janthinellum* mutants. Pottkämper et al. (2009) screened the IL tolerance of 24 bacterial cellulases derived from metagenomic libraries in a number of different ILs. Most of the screened cellulases had very low IL tolerance and the most IL tolerant cellulases also displayed remarkably high halotolerance, which again would suggest that halotolerance and IL tolerance are correlated. Mutations in the CBM of the cellulases led to increased activity in the presence of ILs in some cases, which suggested that the CBM would play an important role in how cellulases are affected by IL.

Currently marketed commercial cellulose-degrading enzyme cocktails display low tolerance towards biomass-dissolving ILs, and some new cellulase mixtures have therefore been especially optimized for IL matrices. With an optimized cellulase cocktail based on thermophilic enzymes, over 50% of the original activity in aqueous conditions was reported to be retained in 20 (w/v) % [EMIM]AcO at 70 °C (Park, et al. 2012). Engel et al. (2012b) optimized a cellulase cocktail for the hydrolysis of RC in the presence of 10% (v/v) [DMIM]DMP based on inactivation data obtained for different cellulase components (*T. reesei* Cel7A and Cel7B, *A. niger* β-glucosidase) in this IL.

Some efforts have been made in designing cellulase-compatible ILs (Section 1.2.2 and Figure 6). Li et al. (2012) demonstrated the successful hydrolysis of straw, cotton and filter paper with cellulase powder in 15% (w/v) of a specially designed IL, [E(OH)MIM]AcO (see Section 1.2.2). The cellulase displayed good residual activities (over 50% of original activity) in up to 25% of this IL after a one day incubation and the unfolding temperature of the cellulase was found to increase in the presence of [E(OH)MIM]AcO. The presence of this IL caused the pH of the hydrolysis medium to increase which was suggested to be a major factor causing loss of cellulase activity in the studied system.

Different stabilization techniques have been applied to obtain better cellulase performance in ILs. Jones and Vasudevan (2010) reported cellulase cross-linking with glutaraldehyde and the use of this catalyst in media containing low concentrations (2% v/v) of the IL [EMIM]DEP. Cellulases have effectively been stabilised for better perfromance in ILs by either lyophilizing the enzyme together with PEG

(Turner, et al. 2003) or by covalently attaching PEG chains to the N-terminal end of the enzyme (Li, et al. 2013). The PEG chain is suggested to form a protective, hydrophilic region around the cellulase, which protects the cellulase from the IL and may also increase interactions between the modified cellulase and cellulose. Lozano et al. (2011) immobilized a commercial cellulase on Amberlite XAD4, a polymeric support, and coated the particles with a hydrophobic IL, which was observed to stabilize the cellulase against thermal inactivation. When coated by stabilizing IL, the immobilized enzyme showed a better stability in the very inactivating IL [BMIM]Cl. Stabilisation of *Trichoderma viride* cellulase as liposomes has also been reported to be a successful strategy for increasing the IL tolerance of enzymes (Yoshimoto, et al. 2013). Interestingly, cellulases have been found to show increased thermal stability in the presence of RC, indicating that inactivation kinetics measured in IL solutions without substrate may not give the complete picture of cellulase inactivation (Zhao, et al. 2009a). Enzyme stabilization in ILs has been reviewed by Moniruzzaman et al. (2010b) and by Zhao (2010).

1.3.3 Enzymatic hydrolysis of cellulose in cellulose-dissolving ionic liquids

The fact that cellulose is soluble in certain ILs opens up interesting possibilities for enzymatic hydrolysis of dissolved or regenerated cellulose. Regenerating lignocellulosic biomass from IL solution is known to be an efficient pretreatment prior to enzymatic hydrolysis (Section 1.2.3), but the high dilution ratio of the IL caused by the regeneration and subsequent washing presents challenges to economical IL recycling (Park, et al. 2012). Some residual IL is trapped inside the regenerated substrate, potentially causing enzyme inactivation during the subsequent hydrolysis (Hong, et al. 2012; Zhao, et al. 2009a). Therefore, it is of considerable interest to study direct hydrolysis of the regenerated substrate without removing the IL in a separate step between pretreatment and hydrolysis. It is however known, that cellulose-dissolving ILs have anions with a high tendency to form hydrogen bonds and inactivate enzymes (Zhao 2010). A great variety of substrates, enzyme preparations, ILs and methods have been used to assess IL effects on cellulase stability, activity on soluble substrates and yields from the hydrolysis of solid cellulosic substrates (Table 3).

Kamiya et al. (2008) introduced the term *in situ* saccharification, which means a combined one-pot procedure in which the substrate is first dissolved in a cellulose-dissolving IL, whereafter the regeneration is done by adding buffer and the enzymatic hydrolysis is carried out in the same vessel without removing the IL. Some other interesting concepts for biomass hydrolysis in IL matrices have also been proposed. A hyperthermophilic endoglucanase gene has been added *in planta* (Klose, et al. 2012); the enzyme is inactive during plant growth conditions, but in pretreatment conditions at high temperatures (90 °C) it becomes active and degrades the plant cell wall from inside the plant. Furthermore this cellulase showed an extraordinary tolerance to [EMIM]AcO and [DMIM]DMP, which means that cellulose-dissolving ILs can be used in the pretreatment process. Nakashima et al.

(2011) proposed the combination of IL pretreatment, enzymatic hydrolysis and fermentation to ethanol in a one-pot procedure. The used yeast displayed cellulases on its cell surface and hydrolysis could be efficiently carried out when additional free cellulases were added. The yeast was shown to be capable of fermenting the liberated sugars to ethanol, but the yeast tolerated a maximum IL concentration of only 200 mM. Also other studies have indicated microbial fermentation to be sensitive to inhibition by low IL residuals in the hydrolysates (Ninomiya, et al. 2013; Hong, et al. 2012).

An almost complete conversion of RC has been achieved with a cellulase cocktail of *T. reesei* cellulases and β-glucosidase from *A. niger* in the presence of 15% (v/v) [EMIM]AcO (Wang, et al. 2011), whereas IL-pretreated yellow poplar had rather low hydrolysis conversion (33%) in the same conditions. Regenerated filter paper was enzymatically hydrolysed in the presence of [DMIM]DMP, which doubled the hydrolysis yield compared to untreated filter paper (Zhi, et al. 2012). Engel et al. (2010) compared the enzymatic hydrolysis of α-cellulose both untreated and regenerated from [DMIM]DMP in buffer and in 10, 20 and 30% (v/v) [DMIM]DMP. The initial hydrolysis rates were greater for RC even in 30% (v/v) [DMIM]DMP than for untreated α-cellulose in buffer. However, the increasing presence of this IL lowered the hydrolysis yields in prolonged hydrolyses. Wolski et al. (2011) compared the enzymatic hydrolysis of IL-preatreated *Miscanthus* with a commercial *T. reesei* cellulase cocktail in aqueous [DMIM]DMP and [EMIM]lactate and found [DMIM]DMP to be a significanlty more enzyme-compatible IL than [EMIM]lactate, as hydrolysis still took place in 50% (w/w) IL. The most IL-tolerant cellulase to date was reported by Klose et al. (2012): a hyperthermophilic and halophilic GH12 endoglucanase lacking CBM from *Sulfolobus solfataricus* hydrolysed dissolved or regenerated MCC well in 80% (v/v) [DMIM]DMP and [EMIM]AcO at 90 °C. Based on these results, efficient pretreatment and saccharification should be possible with this enzyme in a single stage.

Separation of the hydrolysis products from the IL-containing hydrolysates is a challenge. Boronate-saccharide complexation with subsequent extraction (Brennan, et al. 2010) and different large-scale chromatographic procedures have been proposed for this task (Binder and Raines 2010; Feng, et al. 2011). The one-pot or *in situ* hydrolysis is a relatively new concept and currently suffers from problems with enzyme performance in high concentrations of biomass-dissolving ILs, but in the light of recently reported highly IL-tolerant cellulases this concept can indeed be seen as a future alternative to the earlier proposed regeneration pathway.

1.4 Carbohydrate analysis in solutions containing ionic liquids

Carbohydrate analytics form an important part of the research both for understanding fundamental mechanisms of enzymatic hydrolysis and for optimizing total hydrolysis procedures. Reports of IL effects on carbohydrate analytics are scarce, but it has been reported in several references that ILs have disturbing effects on different analytical methods (Hyvärinen, et al. 2011; Klembt, et al. 2008). Typically,

spectrophotometric methods such as the 3,5-dinitrosalicylic acid (DNS) (Sumner 1924) or *para*-hydroxybenzoic acid hydrazide (PAHBAH) (Lever 1973) assays are used for quantifying the total amount of reducing sugars, whereas high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE) techniques are used for analysing the identity of different mono- and oligosaccharides. Some references contain indications of ILs or impurities in ILs interfering with photometric assays or HPLC methods, typically used for enzyme kinetic and activity measurements (Klembt, et al. 2008). Analysis in IL matrices has been reported to be very challenging with HPLC and GC, as the columns tolerate only low amounts of salts (Hyvärinen, et al. 2011). In Hyvärinen et al. (2011) the chromatograms showed broad IL peaks which in some cases overlapped with the saccharide peaks, and the retention times of the IL peaks varied depending on the sample composition. The HPLC system was compatible with a maximum amount of 1.8% of IL in the sample.

ILs have been used as auxiliary substances in both chromatographic and electrophoretic methods (Stalcup and Cabovska 2005). In analytical applications ILs have been used as *e.g.* mobile phase additives, capillary wall coating agents, for covalent attachment to column and capillary walls etc. However, the usefullness of ILs in these applications cannot be compared to samples containing undefined amounts of different ILs, as in the case of typical hydrolysates from *in situ* saccharification. Vaher et al. (2011) used ILs as components in the background electrolyte (BGE) in capillary electrophoresis (CE). Many ILs are UV active and may as such be used as chromophores for indirect UV detection. Some ILs are suggested to act selectively with saccharides, which facilitates their analytic separation. The IL concentration must be kept low (below 20–30 mM) in these applications, as high contents of IL have been found to lead to baseline fluctuations.

Saccharide standard curves in the DNS spectrophotometric assay for samples containing 0–20% (v/v) [EMIM]AcO have been reported not to show any interference of the IL with the analysis (Wang, et al. 2011). DNS results were confirmed by HPLC for the hydrolysates, but no mention was made of IL effects on the chromatographic analysis.

Sugar derivatization with aromatic amines for saccharide analysis with HPLC in IL-containing hydrolysates has been reported in several studies. In Kamiya et al. (2008), glucose and cellobiose were analysed as 4-aminobenzoic acid ethyl ester (ABEE) derivatives in hydrolysates containing [EMIM]DEP by HPLC on a C18 column. However, no information was provided on the dilution factors or disturbing effects of the IL presence. Liquors containing ILs have also been analysed for carbohydrates with similar procedures in other studies, but nothing is generally commented about the final IL concentration in the samples or about the effect of IL on the analysis results (Sun, et al. 2013a; Uju, et al. 2013).

In addition to general harmful effects that ILs may have on analytical methods, they may also react with the saccharide analytes. Ebner et al. (2008) demonstrated that imidazolium-based ILs ([BMIM]Cl in this study) form carbenes at their C2 position in the imidazolium, which further react with the reducing ends of saccharides. Du and Qian (2011) further confirmed this reaction for other ILs, most notably

[EMIM]AcO, by quantum mechanical calculations. Currently, it is not known to what extent the formation of saccharide-imidazolium adducts affects saccharide analytics in IL matrices and whether this adduct formation is reversible during the preparation of samples for analysis.

2. Aims of the work

The overall goal of this work was to study the enzymatic hydrolysis of cellulose in aqueous ionic liquids (ILs). Mainly, cellulases from the mesophilic fungus *Trichoderma reesei* and some commercial cellulases were used. Monocomponent cellulase preparations were used to study how different enzymes respond to the presence of cellulose-dissolving ILs. Principally, the model substrates microcrystalline cellulose (MCC) and carboxymethylcellulose (CMC) were employed.

More specifically, the aims of this work were:

- To study the effect of both known imidazolium-based and newly introduced cellulose-dissolving IL classes on the hydrolysis of cellulose by *T. reesei* cellulases and commercial cellulases (hydrolysis yield, product distribution in the hydrolysates, molecular mass changes in the cellulose) (I–IV)
- To study *T. reesei* cellulase inactivation rates and the reversibility/ irreversibility of cellulase inactivation in the presence of cellulose-dissolving ILs, as well as reasons for IL-induced cellulase inactivation (I–V)
- To study the effect of ILs on *T. reesei* cellulase binding to cellulose with intact modular cellulases and their corresponding core domains (IV)
- To develop analytical methods allowing the necessary mono- and oligosaccharide analyses in the presence of ILs (II)

3. Materials and methods

This section presents the main experimental techniques and materials used in this work. More detailed information can be found in the original papers I–V.

3.1 Cellulosic substrates

The cellulosic substrates used in this work are listed in Table 4. Carboxymethylcellulose (CMC) was used as dissolved substrate in the endoglucanase activity assays; the other substrates were used in suspension in the hydrolysis experiments. Regenerated cellulose (RC) was prepared by dissolving microcrystalline cellulose (MCC) in [EMIM]AcO overnight at 80 °C and precipitating the dissolved cellulose by adding water. The RC was washed with water until the washing liquid was colourless and then dried at 13 mbar at room temperature (RT) overnight to yield a brownish brittle solid. The weight average molecular mass (M_w) did not decrease due to the regeneration procedure (I, Table 3).

Table 4. Cellulosic substrates used in this work. MCC = microcrystalline cellulose, PHK DP = pre-hydrolysis kraft dissolving pulp, SDP = sulphite dissolving pulp, CMC = carboxymethylcellulose.

Substrate	Supplier	Specifications	Used in
MCC	Serva Gmbh	Particle size 0.020 mm	I, II, III, IV
Eucalyptus PHK DP	Bahia Specialty Cellulose	2.5% xylan	III
Beech SDP	Lenzing AG	3.2% xylan, 0.2% mannan	V
CMC	Sigma	Low viscosity CMC	I, II, III, IV, V

3.2 Enzymes and enzyme assays

Pure monocomponent preparations of *Trichoderma reesei* cellulases were mainly used in this work. In addition, some commercial endoglucanase preparations were used. Table 5 presents the enzyme preparations used, together with their supplier or production procedure if produced at VTT, and additional comments about the enzymes and the original article in which they were used.

Table 5. Cellulase preparations used in this work. *T. reesei* = *Trichoderma reesei*, *T. maritima* = *Thermotoga maritima*, *A. niger* = *Aspergillus niger*, EG = endoglucanase, CBH = cellobiohydrolase, CD = core domain.

Enzyme	Supplier / production procedure	Comments	Used in
T. reesei Cel5A	Suurnäkki, et al. 2000	Formerly EGII	I, II, III, IV
T. reesei Cel5A CD	Suurnäkki, et al. 2000	Formerly EGII CD	I, IV
T. reesei Cel7A	Rahikainen, et al. 2013	Formerly CBHI	IV
T. reesei Cel7A CD	Suurnäkki, et al. 2000	Formerly CBHI CD	IV
T. reesei Cel7B	Suurnäkki, et al. 2000	Formerly EGI	I
T. reesei cellulase	Sigma-Aldrich	Commercial cellulase cocktail	V
T. maritima Cel5A	Megazyme International	Thermophilic endoglucanase preparation	III
Puradax HA® 1200E	DuPont Industrial Biosciences	Endoglucanase preparation used in washing liquids	III
IndiAge® ONE	DuPont Industrial Biosciences	Endoglucanase preparation used in denim finishing	III
A. niger β-glucosidase	Novozymes	Crude β-glucosidase preparation	II

Endoglucanase activity was determined using a 1% (w/v) CMC solution as substrate for 10 min at 50 °C and quantifying the amount of reducing chain ends produced by the DNS assay (Section 3.6, assay conducted according to Ghose (1987) with the DNS reagent solution prepared as in Sumner (1924). Cellobiohydrolase activity for Cel7A and Cel7A core domain (CD) was determined on 4-methylumbelliferyl-β-D-lactoside (4-MUL) with an assay described in (van Tilbeurgh, et al. 1988). β-Glucosidase activity was determined on 4-nitrophenyl-β-D-glucopyranoside as described in (Bailey and Linko 1990) and xylanase activity on birch glucuronoxylan as described in (Bailey, et al. 1992). All activities were expressed as nanokatals (nkat), with 1 nkat defined as the number of catalysed reactions (mol) per time unit (s) (Dykbaer 2001).

The protein content of the enzyme preparations was detemined (in I, II, III) with a commercial kit based on the Lowry method (Lowry, et al. 1951), with bovine serum albumine (BSA) as protein standard. The molar protein concentrations of pure *T. reesei* monocomponent cellulase preparations were determined on the

basis of their absorption at 280 nm in IV. Protein purity was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), in which protein visualization is based on a UV-light driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin, et al. 2002) with a Criterion stain-free imaging system.

For determining residual activities after different treatments, endoglucanase samples were incubated in matrices containing IL for different time periods in hydrolysis conditions (V). Thereafter, samples were withdrawn and their residual endoglucanase activity was determined on CMC. Inactivation time curves were determined on the basis of the residual endoglucanase activities. Endoglucanase samples were also boiled for 10 or 20 min after incubation in buffer and IL matrices under hydrolysis conditions in order to determine the efficiency of boiling for enzyme inactivation and the protecting effect of the ILs towards boiling (I and unpublished results).

3.3 Ionic liquids

The ILs employed in this study are presented in Table 6 and their structures in Figure 7. The nomenclature for the tetra-alkylphosphonium ILs is based on their alkyl substituents: the cations have four alkyl subsituents and the four numbers after the P denotes the length of the individual alkyl chains.

Table 6. Ionic liquids used in this work, their supplier/method of preparation and aggregation state at room temperature (RT) or melting point (mp).

IL	Supplier / preparation	Melting point	Used in
[DMIM]DMP	IoLiTec	Liquid at RT	IV
	Bradaric, et al. 2003		I, II
[EMIM]AcO	IoLiTec	Liquid at RT	I, II, III, IV
	BASF (Basionic TM BC 01)		V
[TMGH]COO	King, et al. 2011	78–83 °C	III
[TMGH]AcO	King, et al. 2011	90–97 °C	III
[TMGH]EtCOO	King, et al. 2011	62 °C	III
[TMGH]*n*-PrCOO	King, et al. 2011	67 °C	III
[DBNMe]DMP	Parviainen, et al. 2013	Liquid at RT	III
[DBNH]AcO	Parviainen, et al. 2013	Solid at RT, mp < 80 °C	III
[DBNH]EtCOO	Parviainen, et al. 2013	Liquid at RT	III
[P4444]OH	ABCR	Provided in 40% aqueous solution	Unpublished
[P8881]AcO	King, et al. 2013	Liquid at RT	Unpublished

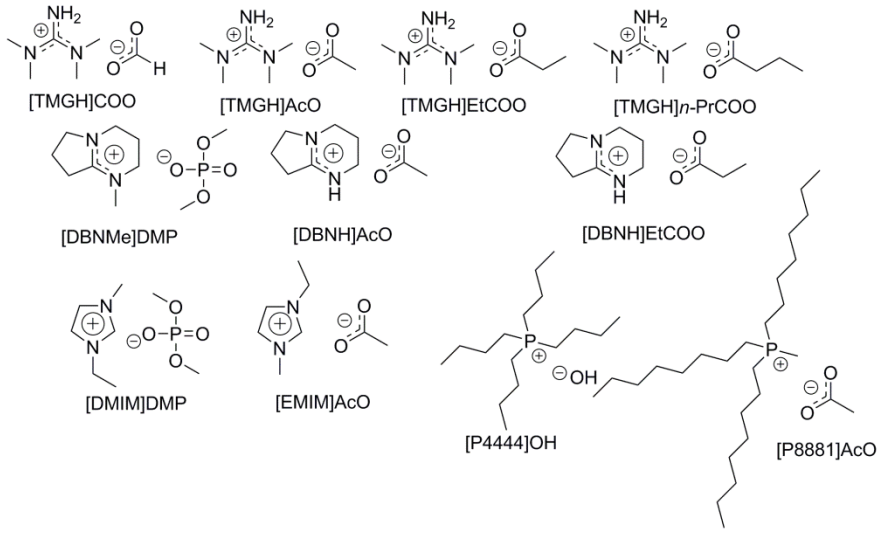

Figure 7. Structures of ILs employed in this work.

3.4 Hydrolysis of solid polymeric substrates in aqueous IL solutions

Enzymatic hydrolysis of solid cellulose was carried out with IL dosages of 0, 20, 40, 60, 80 and 90% (v/v in I, II or w/w in III, IV) in buffer (0.050 M citrate, pH 5.0 or 0.100 M phosphate, pH 6.0). 30 mg (dry weight) of substrate was weighed into a test tube, the defined amount of buffer was added and the mixture was stirred to homogeneity (Figure 8). The defined volume of IL was added to the mixture, and the mixture was temperated to the hydrolysis temperature (45 °C). Those ILs which were not liquid at RT were melted in a heated oil bath before addition to the hydrolysis mixture. The enzyme preparation was added with the cellulase dosage corresponding to an endoglucanase activity (on CMC) of 2000 nkat/g cellulose in I and II, or to a protein content of 1 mg/g of substrate (III). In IV, the enzyme dosage was based on cellulase concentration, which was 400 nM in the final hydrolysis mixture. The total hydrolysis sample volume was 3 mL (in I, II) or had a total weight of 3 g (in III, IV). The hydrolysis was carried out at 45 °C in closed test tubes in a water bath with continuous magnetic stirring for the specified hydrolysis time.

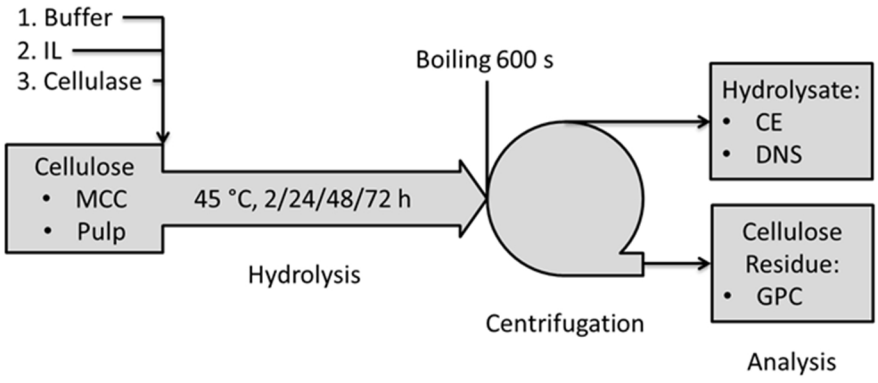

Figure 8. Flow chart for the hydrolysis experiment with product separation and analysis. CE = capillary electrophoresis, DNS = 3,5-dinitrosalicylic acid assay, GPC = gel permeation chromatography.

The hydrolysis was terminated by boiling the sample for 600 s. After cooling to RT, the reaction tube was centrifuged at 3000 rpm for 10 min and the clear supernatant was separated from the solid cellulose residue. In the high-IL hydrolysis samples the cellulose was (partly) dissolved and thus needed to be regenerated. This was done by adding 3 mL or g of distilled water after boiling, followed by vigorous mixing before centrifugation. When using RC as substrate (I), the procedure as described above was followed with the exception that the substrate was stirred and swollen in buffer overnight before the addition of enzyme. All experiments were carried out in triplicate. Reference samples were treated in the corresponding conditions without addition of enzyme.

3.5 Hydrolysis of cello-oligomers with β-glucosidases in IL solutions

Enzymatic hydrolysis of cello-oligomers (in the range of cellobiose to cellohexaose) to glucose with *A. niger* β-glucosidase was carried out in buffer (0.050 M citrate, pH 5.0 or 0.100 M phosphate, pH 6.0) in the presence of [DMIM]DMP and [EMIM]AcO, as described in II. The hydrolysis temperature was 45 °C and the hydrolysis time 20 h. The hydrolysate cello-oligomer composition was analysed by CE with pre-column derivatization as described in Section 3.6 and in II.

3.6 Saccharide analysis in hydrolysates

The saccharides solubilized in the enzymatic hydrolysis of solid substrates were analysed by DNS assay (III, IV and V) or by a CE method (I, II, III and V), which was especially optimised for saccharide analysis in IL matrices (II). DNS assay was carried out according to the procedure described in Ghose (1987) with the

DNS reagent solution prepared as described in Sumner (1924). When hydrolysates containing IL were analysed with the DNS assay, it was important to correct the background with the reference samples. Saccharide quantification was also carried out using the PAHBAH assay as described in Lever (1973) in III. In order to have a comparable quantification of reducing saccharides in hydrolysates with different distributions of oligosaccharides, a secondary acid hydrolysis was performed to degrade all oligosaccharides to monosaccharides. For acid hydrolysis, the sample (1 mL, pH adjusted to below 7) was mixed with 0.05 mL 70% H_2SO_4, autoclaved for 1 h at 120 °C and then diluted to 2.5 mL with milli-Q water.

CE with pre-column derivatization was employed for analysing hydrolysis products (mono- and oligosaccharides) in the hydrolysates. The method development and applicability are described in Section 4.1.2 and in more detail in II. The hydrolysate saccharides with galactose added as internal standard were derivatized by 4-aminobenzonitrile (ABN, in buffer and [DMIM]DMP matrices) or 4-aminobenzoic acid ethyl ester (ABEE, in [EMIM]AcO and [TMGH]carboxylates) through reductive amination. After centrifugation, aliquots of the sample liquid were analysed by CE. Saccharide quantitation was carried out against standard curves acquired for each analyte in the different matrices. Full technical details of this method can be found in II.

3.7 Analysis of solid cellulose hydrolysis residues

The molecular mass distribution of the solid cellulose residues after enzymatic hydrolysis and regeneration was determined by gel permeation chromatography (GPC) as described in I. The samples were washed to remove any residual IL (dry samples were activated by mixing with water) and a solvent exchange procedure was carried out in which the cellulose samples were treated three times with ethanol and three times with dimethylacetamide (DMAc), before dissolution in a dry solution of 8% (w/v) LiCl in DMAc. After complete cellulose dissolution, the sample was diluted, filtered and subjected to GPC analysis on the HPLC system described in I. The molecular mass distribution was determined by comparison to pullullan standards.

Fourier transform infrared (FTIR) spectroscopy with photoacoustic detection was applied to study crystallinity changes in the solid hydrolysis residues after enzymatic treatments in IL matrices (I). Cellulose crystallinity indices can be calculated from the FTIR spectra peaks. In this work, the lateral order index (LOI) was calculated as the peak absorption ratio between the peaks located at 1437 cm^{-1} and 899 cm^{-1} (Hurtubise and Krassig 1960) and the total crystallinity index (TCI) as the ratio between the 1378 cm^{-1} and 2900 cm^{-1} peak absorptions (Nelson and O'Connor 1964).

3.8 Cellulase substrate binding experiments with ^{3}H-labeled *Trichoderma reesei* cellulases

The binding of *T. reesei* cellulases and their core domains (CDs) to MCC in the presence of selected ILs was elucidated with labeled proteins (IV). *T. reesei* Cel5A, Cel5A CD, Cel7A and Cel7A CD were labeled with ^{3}H through reductive amination of free amines (lysine residues and N-terminus) with formaldehyde as methyl group donor and [^{3}H]$NaBH_4$ as reducing agent, as described in detail in IV. The ^{3}H-labeled protein preparations were characterized by activity assays and SDS-PAGE (IV, supplementary material), according to which no protein degradation or any reduction of specific enzyme activity had occurred during the labeling reaction. Binding experiments were carried out at 4 °C in 1% (w/w) MCC dispersion in 0.050 M citrate buffer (pH 5.0) containing [DMIM]DMP or [EMIM]AcO (0, 20 and 40% w/w) and initial cellulase concentrations of 0.1–10 µM. An equilibration time of 4 h was used, which was sufficient for equilibration in all the studied matrices at 4 °C. Measurements were carried out at equilibrated binding in order to ensure that the binding results were comparable with each other and not time dependent. 4 °C was chosen as binding temperature in order to suppress enzymatic hydrolysis of the MCC. The cellulase binding was calculated on the basis of the amount of unbound cellulase in the supernatant as quantified by Liquid Scintillation Counting (LSC) by comparing samples with MCC to reference samples without MCC. The presence of IL in the samples did not cause any observable interference with the LSC analysis. Isotherms were plotted based on the calculated bound enzyme per gram of MCC against the concentration of free enzyme at equilibrium.

4. Results and discussion

4.1 Analytical considerations and development of ionic liquid-compatible analytical methods (II)

4.1.1 Effects of ionic liquids on common carbohydrate analysis methods

In this work, the DNS assay was used for quantifying the total amount of reducing sugars in the hydrolysates (III, IV, V), or as a complimentary method to CE analysis (I, II, III). In general, ILs were observed to give a background absorption in the spectrophotometric detection (II). Some of the used ILs were coloured and additional colour formation usually took place during the heating phase of the DNS assay, suggesting that the DNS reagent solution reacted with the IL or some impurity in it. Thus it was of great importance to carefully correct the DNS result for any sample with the corresponding reference samples as background. The studied imidazolium-based ILs, [DMIM]DMP and [EMIM]AcO, were in all concentrations miscible with the DNS reagent solution, whereas 1,1,3,3-tetramethylguanidinium acetate ([TMGH]AcO) caused precipitation in concentrations greater than 20% (w/w). DNS analysis could be carried out for samples containing 20% (w/w) concentrations of [TMGH]carboxylates and DBN-based ILs (unpublished results). For the phosphonium-based ILs (tetrabutylphosphonium hydroxide [P4444]OH and trioctylmethylphosphonium acetate [P8881]AcO), precipitates were formed even with low concentrations of IL in the DNS assay. The PAHBAH assay could be used for analysis of samples containing [P4444]OH, but not [P8881]AcO. It appears that the IL compatibility of the DNS and PAHBAH assays must be evaluated experimentally for each IL separately. The limit of quantification (LOQ) for the DNS assay was 0.1 mg/mL for glucose in the experimental setup used in this work (*i.e.* 1% substrate consistency in the hydrolysis experiments), corresponding to a hydrolysis degree of 1% of the dry matter (III, IV).

The application of a secondary acid hydrolysis of cellulose hydrolysates containing the ILs [DMIM]DMP and [EMIM]AcO was studied with the aim of converting cello-oligomers to glucose, to ensure that the hydrolysis yield quantification as total amount of reducing saccharides was comparable as glucose equivalents. It was observed that the higher the concentration of IL, the less of the cello-

oligomers were hydrolysed to glucose (unpublished results). In Table 7, the hydrolysis yield (72 h MCC hydrolysis with *T. reesei* Cel7A in 0.050 M citrate buffer, pH 5.0 at 45 °C), as determined by DNS assay, of the crude hydrolysate is compared to the hydrolysis yield of the same samples after secondary acid hydrolysis. An increase factor was calculated to describe how much the secondary acid hydrolysis increased the analysed yield. A falling trend in this increase factor can be seen in the hydrolysates with increasing concentrations of both [DMIM]DMP and [EMIM]AcO. It was not studied whether any acid degradation of the saccharides in the sample solutions took place, in addition to the hydrolysis of the glycosidic bonds in the cello-oligomers. The acid hydrolysis was problematic when working with low hydrolysis yields, because the procedure further diluted the samples by a factor of 2.5. At least one reason for the problems encountered appeared to be the unpredictable pH shifts caused by the presence of ILs. Acid hydrolysis was not used in this work, but possibly this method could be optimized to work for samples containing IL in the future.

Table 7. Hydrolysis yields from 72 h MCC (1% w/w) hydrolysis with *Trichoderma reesei* Cel7A (400 nM) at 45 °C in 0.050 M citrate buffer (pH 5.0). The yields were determined before and after secondary acid hydrolysis by DNS assay. The increase factor illustrates how much the apparent yield increased as a result of the acid treatment before DNS assay. LOQ = limit of quantification.

Matrix	Crude Yield	StDev	Yield AH	StDev	Increase factor
Buffer pH 5.0	9.2	0.4	14.9	0.1	1.62
10% [DMIM]DMP	5.1	0.5	8.1	0.1	1.58
20% [DMIM]DMP	5.1	0.1	7.2	0.6	1.42
30% [DMIM]DMP	4.5	0.3	5.1	0.7	1.13
40% [DMIM]DMP	2.5	0.1	2.6	0.1	1.04
50% [DMIM]DMP			< LOQ		NA
10% [EMIM]AcO	6.8	0.5	8.2	0.1	1.20
20% [EMIM]AcO	4.2	0.3	4.6	0.3	1.09
30% [EMIM]AcO			< LOQ		NA
30% [EMIM]AcO			< LOQ		NA
40% [EMIM]AcO			< LOQ		NA
50% [EMIM]AcO			< LOQ		NA

Because the distribution of soluble oligosaccharides in the hydrolysates was of interest in this work, the ability of different high performance methods to characterize the various oligosaccharides in IL matrices was tested. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described in Tenkanen and Siika-aho (2000) allows the quantitative analysis of linear cello-, manno- and xylo-oligomers up to DP 6 in aqueous solutions.

However, in the presence of IL (tested with [DMIM]DMP), the saccharide standards could not be identified at all in the chromatograms (II). CE methods with direct (Rovio, et al. 2008) and indirect (Soga and Ross 1999) detection were also assessed for saccharide analysis in [EMIM]AcO and [DMIM]DMP matrices, but the saccharide standards did not give any identifiable signals in the test runs (unpublished results).

It is known that imidazolium-based ILs may be deprotonated at their C2 position to yield highly nucleophilic carbenes, which in turn react with the reducing ends of saccharides (see Section 1.4). Currently it is not known to what extent this reaction affects the saccharide quantification analytics. It can be concluded that ILs interfere with both quantitative and qualitative methods generally used for saccharide analysis in aqueous solutions. There is thus a clear need to further develop both robust and sensitive IL-compatible saccharide analytics in the future.

4.1.2 Development of a capillary electrophoresis method for carbohydrate analysis in aqueous ionic liquid matrices

Inspired by the work of Kamiya et al. (2008), in which hydrolysate saccharides were analysed with HPLC as ABEE derivatives in matrices containing the IL [EMIM]DEP, a new CE method to analyse saccharides in the presence of ILs was developed (II). In this method, reducing saccharides were derivatized with aromatic amines through reductive amination (Figure 9) prior to CE analysis (II). The CE method developed in this work was based on analysis conditions described in Sartori et al. (2003), with saccharide derivatization carried out as in Dahlman et al. (2000). It was found that the presence of ILs did not prevent the derivatization reaction from taking place (II). Depending on the type of IL present in the sample, better detector responses were obtained by using either ABEE or ABN as derivatization reagent. Tests were also made with 6-aminoquinoline, but this derivatization reagent was found to give many interfering peaks of unknown origin in the electropherogram. Generally, the benefits of derivatization are higher detection sensitivity and selectivity.

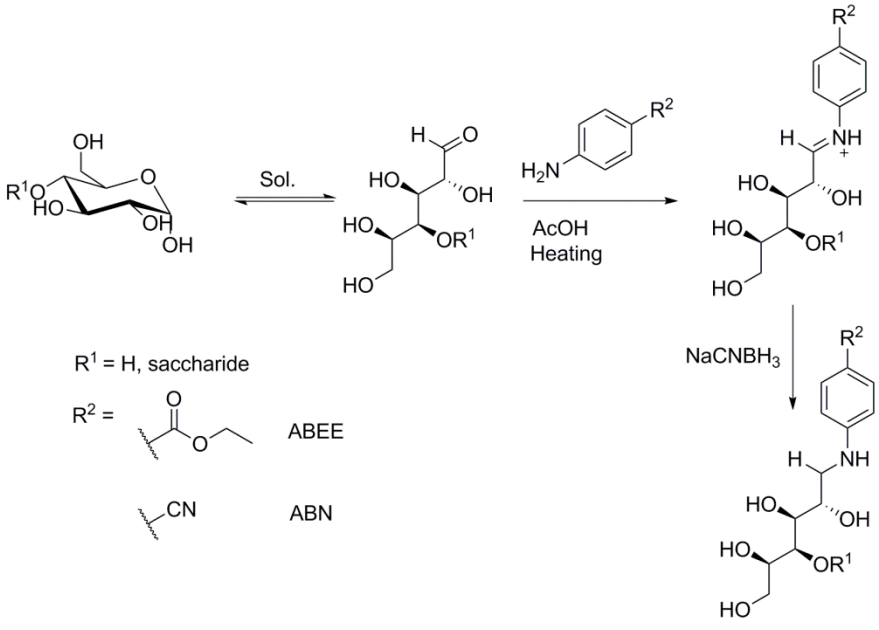

Figure 9. Saccharide labeling with aromatic amines through reductive amination.

Analysis conditions for labeled saccharides were optimized for CE (II). Increasing the analysis temperature from 15 °C (Sartori, et al. 2003) to 30 °C greatly improved the detector response and analyte peak shapes, possibly due to decreased sample viscosity. When optimizing the borate concentration of the background electrolyte (BGE), the optimum concentration was found to be the same (450 mM) in both aqueous and IL-containing samples, although the high ion content of the IL matrices was anticipated to seriously change the optimal BGE constitution. Borate ions in the BGE forms complexes with the saccharides, thus inducing charge to the otherwise neutral saccharides (Dahlman, et al. 2000; Hoffstetter-Kuhn, et al. 1991; Sjöberg, et al. 2004). The BGE composition was also optimized by leaving out the organic components used in (Sartori, et al. 2003), as these were found to offer no benefits in the presence of ILs. The electrophoresis was carried out in reverse polarity mode, which was highly beneficial because the saccharides could thus be detected before the very large peak of excess derivatization reagent, which seriously affected the baseline at higher migration times. The sample and BGE together form a very complex system, necessitating excessive capillary rinsing to minimize capillary blocking due to precipitations.

The applicability of the optimized CE method was studied with the monosaccharides galacturonic acid, glucose, mannose, arabinose and xylose, and cello-, manno- and xylo-oligomers in the range of monosaccharide to hexasaccharide. Of the monosaccharides, the mannose, glucose and arabinose peaks partially overlapped (II, Figure 2), whereas the others were well separated. Cello- and xylo-

oligomers were mainly separated, although some of the longer oligomers overlapped (II, Figure 3). Manno-oligomers could not be separated from each other with this method, as they migrated in the same peak (mannobiose to mannohexaose). Further details concerning the detection and quantification limits can be found in II.

In addition to the imidazolium-based ILs [DMIM]DMP and [EMIM]AcO, the CE method was also found to be applicable to samples containing other hydrophilic and cellulose-dissolving ILs: [TMGH]AcO and all three of the DBN-based ILs studied in this work (III and unpublished results). Depending on the type of IL, the method was compatible with 20 to 40% (v/v) of IL in the sample matrix. To further improve the method, it could be fruitful to study the use of fluorescing tags such as 8-aminopyrene-1,3,6-trisulfonic (APTS) or 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) for derivatising the analyte saccharides. Labeling the saccharides with fluorescing tags would potentially greatly improve the sensitivity of the analysis.

4.2 Dissolution of cellulosic substrates in aqueous ionic liquid solutions (I, III)

In this work it was important to know the conditions in which partial dissolution of cellulose takes place, as the dissolved fraction (either in solution or partially regenerated) can be expected to have very different substrate properties from the native cellulose in enzymatic hydrolysis (Dadi, et al. 2006; Dadi, et al. 2007; Goshadrou, et al. 2013). The dissolution of the cellulosic substrates under enzymatic hydrolysis conditions in IL solutions was followed by visual inspection, light microscopy and light transmission measurements (I). Based on previously published studies, cellulose was expected to dissolve only in IL matrices with low water content (Doherty, et al. 2010; Swatloski, et al. 2002). With the low substrate consistency of 1% (w/v) used in the hydrolysis experiments, partial or complete cellulose dissolution was observed when the IL concentration was higher than 80% (v/v). Based on the light transmission measurements, [EMIM]AcO was a much more efficient cellulose (MCC) solvent than [DMIM]DMP (I, Figure 2), with virtually full MCC dissolution in 90% (v/v) [EMIM]AcO. MCC did not completely dissolve even in 100% [DMIM]DMP under the applied mild conditions (45 °C, 1 d magnetic mixing). Partial dissolution was observed as increasing light transmission when the [DMIM]DMP content was above 90% (v/v). On the basis of visual inspection, [TMGH]AcO appeared to be between [EMIM]AcO and [DMIM]DMP in dissolving power for MCC. The dissolution of MCC under hydrolysis conditions as a function of [EMIM]AcO concentration in buffer is shown in Figure 10. In 90% [EMIM]AcO, the substrate was dissolved to give a completely transparent solution. In the experiments with eucalyptus pre-hydrolysis kraft dissolving pulp, the pulp was observed not to dissolve as well as the MCC (III). The less efficient dissolution of dissolving pulp was probably due to two factors: 1) the pulp had a higher molecular weight (M_w) of 300 000 g/mol (III, Figure 7) than MCC with a M_w of 50 000 g/mol (I, Table 3) and 2) the ground dissolving pulp was found to cluster to

some extent with concomitant mixing problems in both aqueous and IL containing hydrolysis matrices, whereas the mixing for the MCC was close to ideal due to its fine and even particle size (III).

Figure 10. MCC (1% w/v) dissolution in [EMIM]AcO/buffer (45 °C, 1 d mixing) as a function of [EMIM]AcO concentration. From the left: buffer, 20, 60, 80 and 90% (v/v) [EMIMAcO in buffer. Note that the bottles also contain a magnetic stirring bar.

The degree of dissolution of cellulose in ILs was anticipated to lead to crystallinity changes in the regenerated cellulose residues after hydrolysis, and therefore the crystallinity changes in the hydrolysis residues were assessed by FTIR spectroscopy. The total crystallinity index (TCI) and the lateral order index (LOI) were calculated from the FTIR spectra as explained in Section 3.7. TCI is a measure of the overall degree of order in the cellulose, whereas LOI is a measure of the ordered regions perpendicular to the chain direction and is greatly influenced by cellulose processing (Široky, et al. 2012). As expected, the crystallinity of dissolved and subsequently regenerated MCC samples was signicantly lower than the crystallinity of the original MCC (Table 8). The LOI values corresponded well with the expected crystallinity changes, whereas the TCI values did not correlate with the LOI values or with the expected crystallinity changes. Similar observations of the applicability of TCI and LOI were recently made by Zhao et al. (2009a). Clearly, samples treated in [EMIM]AcO had much lower crystallinity than samples treated in [DMIM]DMP, correlating with the superior dissolving power of [EMIM]AcO. The samples treated in [DMIM]DMP had a slightly lower LOI value than that of the original MCC. Interestingly, the RC did not show the lowest LOI value, but this was found in the hydrolysis samples treated in 90% [EMIM]AcO. This might be due to different regeneration conditions.

Table 8. Lateral order index (LOI) for MCC samples treated in [DMIM]DMP and [EMIM]AcO under hydrolysis conditions (I and unpublished results).

Sample	LOI
MCC, untreated	1.098
Regenerated MCC (RC)	0.415
RC, 72 h hydrolysis, Cel7B, buffer	0.458
MCC, 72 h, 90% [DMIM]DMP + buffer (no enzyme)	0.816
MCC, 72 h hydrolysis, Cel7B, 90% [DMIM]DMP + buffer	0.697
MCC, 72 h hydrolysis, Cel5A, 90% [DMIM]DMP + buffer	0.871
MCC, 48 h, 90% [EMIM]AcO + buffer (no enzyme)	0.124
MCC, 48 h hydrolysis, Cel7B, 90% [EMIM]AcO + buffer	0.015
MCC, 48 h hydrolysis, Cel5A, 90% [EMIM]AcO + buffer	0.242

4.3 Action of *Trichoderma reesei* cellulases in aqueous ionic liquid solutions (I, II, III, IV, V)

In this work, the action of the *T. reesei* endoglucanases Cel5A and Cel7B on MCC was studied in matrices containing 0–90% (v/v) [EMIM]AcO and [DMIM]DMP (I, II) and the *T. reesei* cellobiohydrolase Cel7A (IV) was studied in 0–50% (w/w) solutions of these two ILs. In addition, the action of *T. reesei* Cel5A on MCC and a eucalyptus pre-hydrolysis dissolving grade pulp was studied in matrices containing two novel classes of cellulose-dissolving ILs, based on the organic superbases TMG and DBN (III).

4.3.1 Effect of cellulose-dissolving ILs on cellulase activity (I, V)

Inactivation kinetics of the endoglucanase activity of a commercial *T. reesei* cellulase (lyophilized cellulase powder from Sigma) in 90 and 100% [EMIM]AcO (V) and 100% [DMIM]DMP (unpublished results) was studied in the absence of substrate. The cellulase was incubated in IL at 40 °C and its residual endoglucanase activity on CMC was measured at given time points (Section 3.2). The results show a distinct difference in inactivation rate (Figure 11). When incubated in [EMIM]AcO, the cellulase lost its endoglucanase activity completely (to less than 1% of the original activity) in 4 h. The inactivation rates were similar in both 90 and 100% (v/v) [EMIM]AcO. In 100% (v/v) [DMIM]DMP, on the other hand, the cellulase appeared to be rather stable and only a very slow gradual loss of endoglucanase activity was measured during a 72 h incubation period. Based on this experiment, [EMIM]AcO is a highly inactivating IL whereas [DMIM]DMP supports cellulase activity for prolonged periods. Several other recent studies also indicate

[DMIM]DMP to be comparably less inactivating for cellulases than most other cellulose-dissolving ILs (Engel, et al. 2010; Wolski, et al. 2011).

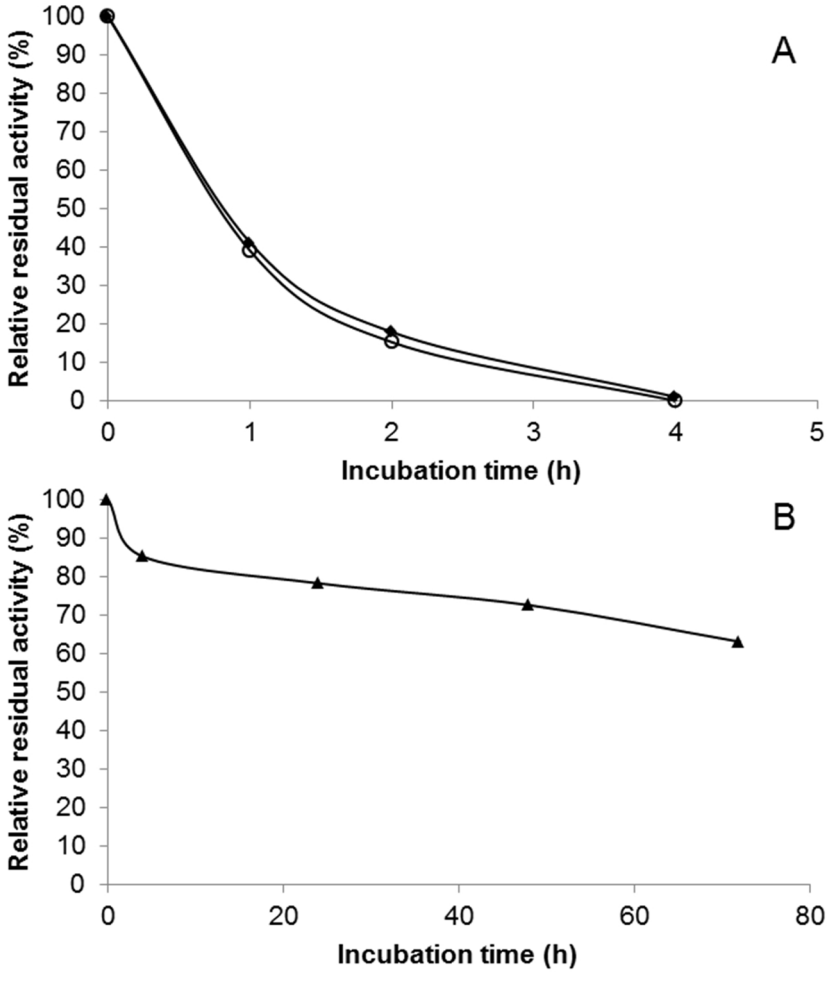

Figure 11. Inactivation of a commercial *Trichoderma reesei* cellulase powder during incubation in (A) [EMIM]AcO or (B) [DMIM]DMP. Legend: ○ 90% (v/v) [EMIM]AcO, ♦ 100% [EMIM]AcO, ▲ 100% [DMIM]DMP. The 100% activity level was measured as endoglucanase activity in 0.050 M citrate buffer (pH 5.0).

The reversibility of the inactivation of the commercial *T. reesei* cellulase was studied by diluting the samples incubated in [EMIM]AcO with buffer to very low final IL concentrations (below 4% v/v IL). No regeneration of activity was observed, suggesting that the inactivation of the enzyme in [EMIM]AcO was irreversible, or at least that the inactivated cellulase was not able to regain its activity in the pres-

ence of even as low [EMIM]AcO concentrations as 3% (w/w). In V, the residual cellulase activity after incubation in [EMIM]AcO was further studied by following the enzymatic effect on the molecular mass distribution of regenerated pulp. According to the results obtained by this alternative activity measurement method, some residual cellulase activity was left after incubation in [EMIM]AcO for even 10–11 h. These results indicate the endoglucanase activity measurement on CMC used in this work to be insufficient, and that alternative methods are needed to analyse the activity of cellulases in the presence of ILs. The studied cellulase did not regain its activity on CMC when diluting with buffer after incubation in [EMIM]AcO, but in the case in which the incubation in IL was done in the presence of pulp, the pulp may have had a protecting effect on the enzyme, possibly explaining the difference between the incubation times required for complete enzyme inactivation. Zhao et al. (2009a) reported an increase in the thermal stability of a commercial cellulase cocktail in the presence of regenerated cellulose, which was explained by a higher adsorption of the enzyme to the regenerated cellulose with high surface area. Possibly, similar effects led to the higher residual endoglucanase activity in the experiments, in which the cellulases were incubated together with pulp before activity assay.

Inactivation of *T. reesei* cellulases by boiling was studied to determine whether boiling was sufficient for complete enzyme inactivation and whether ILs protected the enzymes during boiling. *T. reesei* Cel5A was preincubated for different times (0.25, 2 and 72 h) in matrices containing [DMIM]DMP and thereafter boiled for 10 or 20 min. Boiling for 10 min reduced Cel5A activity on CMC by approximately 50% when preincubation (0.25 h) was performed in buffer. When incubated in 20–90% (v/v) [DMIM]DMP and boiled for 10 and 20 min, the residual endoglucanase activity was 20–60% of the original activity (unpublished results). The 20 min boiling clearly reduced the endoglucanase activity more than 10 min boiling. The presence of [DMIM]DMP appeared to protect the *T. reesei* Cel5A from inactivation during boiling (I and unpublished results). The data obtained in this work does not give any indication on how [DMIM]DMP could protect the cellulases during boiling, but it has been described in several articles how ILs can be used to stabilize enzymes under inactivating conditions *e.g.* by coating the enzymes with suitable ILs (Lozano, et al. 2011; Monhemi, et al. 2012). Based on these experiments, significant endoglucanase activity was present in the hydrolysates after boiling and it cannot be excluded that the residual active cellulase could be able to hydrolyse the regenerated cellulose during washing or regeneration after hydrolysis. The effect of [EMIM]AcO during boiling was not studied as this IL had been found to inactivate *T. reesei* cellulases irreversibly in 4 h (Figure 11).

4.3.2 Effect of ILs on yield and products in enzymatic cellulose hydrolysis (I, II, III, IV)

The effect of several different classes of cellulose-dissolving ILs (Figure 7) on the yields in cellulose hydrolysis with *T. reesei* cellulases was studied. [EMIM]AcO

was found to be more harmful than [DMIM]DMP in MCC hydrolysis with both *T. reesei* Cel5A (I, II, IV), Cel7B (I) and Cel7A (IV) (Table 9). No clear differences were observed in the effect of ILs on the hydrolysis yields of the two endoglucanases Cel5A and Cel7B. The action of cellobiohydrolase Cel7A was clearly less affected than that of the endoglucanases by the presence of IL. Although Cel5A was more efficient in the beginning of the hydrolysis (2 h timepoint), Cel7A gave higher yields both in buffer and in IL matrices (IV, Figures 1A and 1B). No soluble saccharides were released by the endoglucanases when the IL concentrations increased above 40–50% (w/w), whereas the cellobiohydrolase Cel7A was able to produce low amounts of saccharides from MCC even in 50% (w/w) of both ILs, as determined by the DNS assay. In Kamiya et al. (2008) it was also observed that a *T. reesei* cellulase cocktail was almost completely ineffective in cellulose hydrolysis when the IL concentration in the hydrolysis matrix increased above 40% (v/v). Some differences in hydrolysis yield were also observed depending on enzyme dosage and analysis method (Table 9). No cellulose dissolution occurred in these IL concentrations (see Section 4.2).

Table 9. Hydrolysis yields (in % of substrate dry weight) in 72 h MCC hydrolysis with *T. reesei* cellulases at 45 °C. Yields were determined by CE analysis, the IL concentration was in % (v/v) and the enzyme dosage was 2000 nkat/g of MCC, with the activity measured on CMC. In the entries marked with *, the yields were determined with the DNS assay, the IL concentration was in % (w/w) and the enzyme concentration was 400 nM. The error values are based on the standard deviation of three parallel samples.

Cellulase	Buffer	[DMIM]DMP		[EMIM]AcO		Ref.
		20% IL	40% IL	20% IL	40% IL	
Cel5A	8.8 ± 1.0	2.0 ± 0.3	Traces	0.4 ± 0.0	0.0	I
Cel5A*	9.5 ± 0.7	2.7 ± 0.5	1.1 ± 0.0	2.4 ± 0.4	Traces	IV
Cel7A*	9.2 ± 0.4	5.1 ± 0.1	2.5 ± 0.1	4.2 ± 0.3	Traces	IV
Cel7B	5.6 ± 0.5	1.0 ± 0.3	Traces	Traces	0.0	I

A distinct difference in the hydrolysis yields of *T. reesei* Cel5A as a function of time was observed in [DMIM]DMP and [EMIM]AcO matrices (II, Figure 5). The presence of [DMIM]DMP (20 and 40% v/v) slowed down the enzymatic hydrolysis rather than completely preventing it and the maximum hydrolysis level was not attained in 72 h. In 20% (v/v) of [EMIM]AcO the hydrolysis yield was very low with Cel5A and did not increase after the first hours of hydrolysis, suggesting that the cellulase was no longer able to hydrolyse MCC. This observation of the different effects of these two ILs on the hydrolysis time curves correlates well with the inactivation measurement data presented in Section 4.3.1. Taking into account how differently Cel5A is inactivated in [DMIM]DMP and [EMIM]AcO, the differences in 72 h hydrolysis yields are surprisingly small, suggesting that direct inactivation by

the ILs may not be the only reason for the observed low hydrolysis yields in IL matrices. There is a certain discrepancy between the hydrolysis yields obtained in [EMIM]AcO matrices in the different experimental series, the reason of which is unknown (I, II, III and IV). To some extent the differences might be explained by the use of two different analysis methods, CE with pre-column derivatization and DNS assay. With other ILs, of which the most studied was [DMIM]DMP, the results were more consistent.

T. reesei Cel5A was used as reference cellulase to compare the effects of [EMIM]AcO, [DMIM]DMP, tetra-alkylphosphonium-, TMG- and DBN-based ILs on the enzymatic hydrolysis of MCC (III and unpublished results). Four cellulose-dissolving [TMGH]carboxylates and three DBN-based ILs were included in the study: 1,1,3,3-tetramethylguanidinium formiate ([TMGH]COO), [TMGH]AcO, 1,1,3,3-tetramethylguanidinium propionate ([TMGH]EtCOO), 1,1,3,3-tetramethylguanidinium butyrate ([TMGH]*n*-PrCOO), 1,5-diazabicyclo[4.3.0]non-5-enium acetate ([DBNH]AcO), 1,5-diazabicyclo[4.3.0]non-5-enium propionate ([DBNH]EtCOO) and 1-methyl-1,5-diazabicyclo[4.3.0]non-5-enium dimethylphoshate ([DBNMe]DMP) (Figure 7), as well as [P4444]OH and [P8881]AcO. The compatibility screening was made at 20% (w/w) IL concentration for 72 h at 45 °C. In buffer solution (pH 5.0) the hydrolysis yield was 7.7%, whereas the hydrolysis yield was decreased by 70% or more in all the IL matrices. Thus, it was clear that all of the studied ILs were very harmful for the action of *T. reesei* Cel5A (Figure 12). In most cases, the difference between the effects of ILs were rather small and even within the error limits. The DBN-based ILs were the most detrimental to Cel5A action. The DBN-based cation is probably not very cellulase-compatible, as the other used acetates and dimethylphosphates were less inhibiting for the cellulase. Of the TMG-based ILs, the acetate was the most cellulase-compatible. Samples containing [P8881]AcO could not be analysed in this work because this IL precipitated with both the DNS and PAHBAH reagent solutions. The [P4444]OH samples were analysed with the PAHBAH assay and this IL was clearly very harmful to the action of *T. reesei* Cel5A. Based on this experiment it is clear that the new cellulose-dissolving IL classes studied here are even less cellulase-compatible than the imidazolium-based ILs, even though the new ILs have other desirable properties, such as distillability and a low price of starting materials.

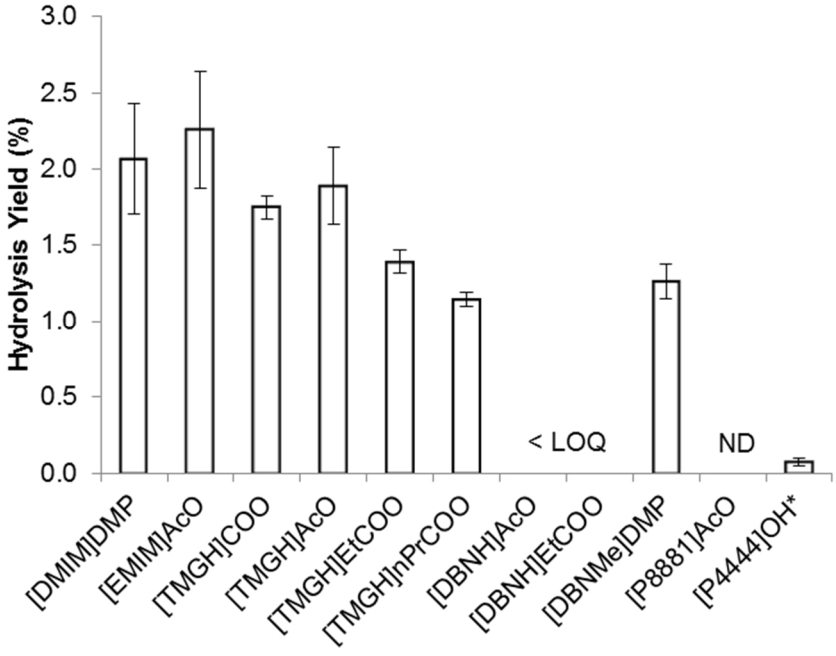

Figure 12. Comparison of the effect of 20% (w/w) of different cellulose-dissolving ILs on 72 h MCC hydrolysis yields (% of dry weight) with *Trichoderma reesei* Cel5A. The enzyme dosage was 2000 nkat/g MCC based on activity on CMC. Hydrolysis yields were determined by the DNS assay, except for samples containing [P4444]OH (marked with *), for which the yield was determined by the PAHBAH assay. The limit of quantification (LOQ) with the DNS assay was 1% yield, for PAHBAH assay the LOQ was below 0.1%. ND denotes that neither DNS nor PAHBAH assays were applicable. Error bars are based on the standard deviation for three replicate samples. The hydrolysis yield of the buffer reference was 7.7%.

In addition to the hydrolysis yields, the cello-oligomer distribution of the saccharides released from MCC by *T. reesei* Cel5A and Cel7B in the different IL matrices (I, III and V) were also determined using the CE method described in II and in Section 4.1.2. *T. reesei* Cel5A has been reported to produce glucose, cellobiose and cellotriose in MCC hydrolysis in buffer at pH 5.0, whereas the main products of Cel7B are glucose and cellobiose (Karlsson, et al. 2002). The same product distribution was observed in buffer (pH 5.0) in this work (I, Tables 1 and 2). The presence of IL in the hydrolysis matrix shifted the cello-oligomer distribution from glucose towards cellobiose and cellotriose for both Cel5A and Cel7B. No longer cello-oligomers were observed. The same shift in the cello-oligomer distribution was observed when the hydrolysis was carried out in phosphate buffer at pH 7.0 instead of in citrate buffer at pH 5.0. Thus, it cannot be ruled out that the shift to longer cello-oligomers in the product distribution could be a pH effect, as the IL did

cause the hydrolysis matrix pH to shift to more alkaline values (I, Figure 3). In (III) a similar shift was also observed in the product distributions of *T. reesei* Cel5A and IndiAge® ONE, but not in the product distribution of Puradax® HA 1200E, when these enzymes were studied in [EMIM]AcO and [TMGH]AcO (III, Table 2). Obviously, the shift to longer cello-oligomers is not only dependent on the IL, its concentration and the pH of the matrix, but also on the studied cellulase. The shift towards longer cello-oligomers in hydrolysates containing IL could also have an effect on any potential end-product inhibition, but in this work such an effect was considered negligible due to the very low concentrations of cello-oligomers in the final hydrolysates and the shift in the product distribution being rather modest.

4.3.3 Effect of IL on the molecular mass distribution of cellulose in enzymatic hydrolysis (I, III)

The solid cellulose residues from the partial hydrolysis of MCC with the *T. reesei* endoglucanases Cel5A and Cel7B in the presence of ILs were analyzed by GPC in order to study the changes in the molecular mass distribution. The ILs did not cause any complications in this analysis, as they could be washed off from the residual cellulose before cellulose dissolution for GPC analysis.

The weight average molecular mass (M_w) of MCC was not affected by the IL or endoglucanase treatments in buffer or in IL-containing matrices in which cellulose dissolution did not occur (Section 4.2 and I, Table 3). A significant reduction of the M_w from 50 000 g/mol to 35 000–36 000 g/mol was observed for MCC treated with Cel5A or Cel7B in 90% (v/v) [DMIM]DMP. The same was not observed in any of the [EMIM]AcO matrices, including those in which MCC had clearly decreased crystallinity after complete dissolution in aqueous [EMIM]AcO (Section 4.2 and Table 8). The M_w reduction appears to be limited by the substrate properties rather than by the used cellulase, as the M_w reduction as a function of hydrolysis time was almost identical for Cel5A and Cel7B and doubling the cellulase dosage did not lead to increased M_w reduction (I, Table 3). On the other hand, with the core domain (CD) of *T. reesei* Cel5A (lacking the carbohydrate-binding module, CBM), the M_w reduction did not reach the same level as with the intact Cel5A. The M_w reduction was not accompanied with any detected formation of soluble saccharides in MCC hydrolysis with *T. reesei* Cel5A and Cel7B (I), suggesting a different mode of endoglucanase action on the partially dissolved cellulose. The endoglucanases may have a preference for random chain scission of the dissolved cellulose molecules instead of producing soluble cello-oligomers, as during the hydrolysis of undissolved MCC. This mode of action is possibly coupled with low cellulase adsorption to the substrate in high IL concentrations (see Section 4.4.2). Based on the M_w reductions there appears to be a fundamental difference between how the studied endoglucanases were able to act in concentrated [DMIM]DMP and [EMIM]AcO solutions, correlating with the residual activity measurements after incubation in these two ILs (Section 4.3.1).

Two alternatives are possible for the observed molecular mass reductions in the residual cellulose: 1) the *T. reesei* endoglucanases Cel5A and Cel7B hydrolysed the dissolved cellulose chains in the aqueous 90% (v/v) [DMIM]DMP solution or 2) the endoglucanases were inactive in 90% (v/v) [DMIM]DMP throughout the hydrolysis time but regained their activity during regeneration and hydrolysed the amorphous regions of the partly regenerated MCC in the diluted IL. The M_w reduction was clearly dependent on the hydrolysis time for both Cel5A and Cel7B (I, Table 3), which would support alternative 1. Alternative 2 is supported by the observation that endoglucanases may retain significant residual activity during prolonged incubation times in [DMIM]DMP (Section 4.3.1 and Figure 11). As discussed in Section 4.3.1, boiling was not sufficient to completely inactivate the used endoglucanases, which means that significant endoglucanase activities were still present in the hydrolysis mixtures after the hydrolysis had been terminated. Furthermore, [DMIM]DMP appears to inactivate cellulase reversibly (Engel, et al. 2010), making it possible that the cellulases inactivated in highly concentrated IL could regain their activity when the IL matrix was diluted with water *e.g.* during regeneration of dissolved MCC or during washing of the MCC. In future work, other inactivation methods (*e.g.* chemical inhibition) should be considered to avoid having residual endoglucanase activities in the regeneration mixtures.

In order to determine whether post-hydrolysis (alternative 2) of residual cellulose can take place during the after-treatment of the hydrolysis mixtures, a 30 min hydrolysis of cellulose regenerated from partial dissolution in 90% (v/v) [DMIM]DMP for 72 h was carried out either in buffer or in 45% (v/v) [DMIM]DMP with fresh *T. reesei* endoglucanase using a dosage corresponding to the endoglucanase activity measured in the hydrolysates after boiling for 10 min (unpublished results). 45% (v/v) [DMIM]DMP corresponds to the hydrolysis matrix in the potential post-hydrolysis, as regeneration after hydrolysis was always carried out by adding one part water to one part of hydrolysis mixture. The residual cellulose samples treated in buffer with cellulase showed a clear decrease in M_w corresponding to the M_w reduction observed for samples treated with endoglucanases in 90% (v/v) [DMIM]DMP. However, the samples treated in 45% (v/v) [DMIM]DMP showed no decrease in M_w, indicating that the *T. reesei* endoglucanases were unable to hydrolyse the solid, partially regenerated residual cellulose under the conditions of the hydrolysis mixture after-treatment in this high content of IL. Based on this experiment, alternative 1 would be supported, *i.e.* the dissolved fraction of the cellulose was hydrolysed in 90% (v/v) [DMIM]DMP, in which the endoglucanases also exhibited a certain degree of activity. At the same time it also became clear that the partially regenerated cellulose could easily and rapidly be degraded by the endoglucanases in buffer. This experiment confirmed that no cellulose degradation due to adsorbed cellulases being released to the washing liquor occurred during washing of the cellulose residues. Endoglucanase denaturation and the possible regeneration of activity after incubation in highly concentrated IL is a topic which clearly requires further investigation.

Whereas the studied *T. reesei* endoglucanases were not able to reduce the M_w of MCC in buffer, even when 8.8% or 5.6% of the cellulose was solubilised by

Cel5A and Cel7B, respectively (I, Tables 1 and 2), the M_w of RC was considerably reduced during hydrolysis (I, Table 3). The reduction of M_w of RC was clearly different for the two cellulases. Cel5A reduced the M_w from 47 000 g/mol to 12 000 g/mol, whereas with Cel7B the M_w decreased from 47 000 g/mol to 32 000 g/mol. The M_w reductions were paired with approximately doubled yields of solubilized cello-oligomers (I, Tables 1 and 2) when RC was compared to untreated MCC as substrate. These results support the previous findings that endoglucanases play a more significant role in the hydrolysis of regenerated, more amorphous cellulose than in the hydrolysis of highly crystalline substrates (Engel, et al. 2012a). Furthermore, there appear to be great differences between the actions of different *T. reesei* endoglucanases on regenerated substrates.

4.4 Ionic liquid effects on hydrolysis and cellulose binding of *T. reesei* cellulases and their core domains (IV)

4.4.1 MCC hydrolysis with *T. reesei* Cel5A and Cel7A in ionic liquid matrices: comparison between intact and core domain cellulases

In this work, the role of the carbohydrate-binding module (CBM) in enzymatic cellulose hydrolysis and cellulase binding to MCC in IL matrices was studied systematically for the first time. In (I) the action of the *T. reesei* endoglucanase Cel5A and its core domain (CD) on MCC was compared in buffer and in 20% (v/v) [DMIM]DMP. In buffer, the intact Cel5A was manyfold more effective in MCC hydrolysis than its CD (I, Table 2), as was expected from previous studies (Tomme, et al. 1988). When 20% of [DMIM]DMP was present in the matrix, the intact Cel5A showed a 52% decrease in the 72 h hydrolysis yield, whereas surprisingly the hydrolysis yield of Cel5A CD was not affected by the presence of IL. In (IV), similar results were obtained when the hydrolysis of MCC with the *T. reesei* endoglucanase Cel5A, cellobiohydrolase Cel7A and their CDs was further studied at 45 °C in buffer and 10–50% (w/w) of [DMIM]DMP and [EMIM]AcO (IV, Figures 1A and 1B). Increasing IL concentrations affected the hydrolysis yields of all four enzymes negatively. From the hydrolysis results, it is clear that [EMIM]AcO was more harmful to the cellulase performance than [DMIM]DMP, as was observed thoughout this work.

Some interesting differences were observed in how the hydrolysis yields of MCC with intact and CD cellulases changed with the introduction of IL. When adding [DMIM]DMP (10% w/w) to the MCC hydrolysis matrices, the hydrolysis yields of the intact cellulases decreased to approximately the level of their corresponding CDs, suggesting that the CBM was not able to promote cellulose hydrolysis in [DMIM]DMP matrices. With [EMIM]AcO the same was observed for Cel5A but not for Cel7A, suggesting this effect to be potentially IL- and enzyme-dependent. After the drastic initial decrease in the hydrolysis yields of the intact cellulases caused by introducing IL into the hydrolysis matrix, both the intact and CD cellulases showed evenly decreasing yields with increasing IL concentrations.

4.4.2 Substrate binding of *T. reesei* Cel5A, Cel7A and their core domains in the presence of ionic liquids

Binding of ^{3}H-labeled *T. reesei* Cel5A, Cel7A and their respective CDs to MCC was studied in buffer and in the presence of 20 and 40% (w/w) of [DMIM]DMP and [EMIM]AcO. The low binding temperature (4 °C) was chosen in order to suppress cellulose hydrolysis, which would make comparing the binding results difficult.

In buffer, *T. reesei* Cel5A and Cel7A bound to MCC with similar binding isotherms (IV, Figure 2). Cel7A CD also bound relatively well to MCC, probably by using the substrate binding zone in its active site tunnel, as has been suggested in previous studies (Kotiranta, et al. 1999; Linder and Teeri 1996). Cel5A CD, on the other hand, exhibited extremely low binding to MCC in buffer, indicating that the CBM had an important role in the substrate binding of the intact *T. reesei* Cel5A. The difference in the binding ability of Cel5A CD and Cel7A CD is apparently due to the different structures of the substrate binding zones close to the catalytic site, which in cellobiohydrolases is a tunnel and in endoglucanases a cleft on the protein surface (Divne, et al. 1994; Teeri 1997).

The substrate binding of the studied cellulases decreased with increasing IL concentration (IV, Figures 3A, 3B and 3C). [EMIM]AcO had a stronger negative influence on substrate binding than [DMIM]DMP, similarly to its effect on the hydrolysis yields (IV, Figures 1A and 1B). However, the binding was more affected by ILs for *T. reesei* Cel5A than for Cel7A and its CD. In 40% (w/w) [DMIM]DMP the intact *T. reesei* Cel5A showed some binding, whereas no binding was observed in 40% (w/w) [EMIM]AcO. Cel7A could still bind to the substrate to some extent even in 40% (w/w) of the two ILs (IV, Figure 3B). The binding of Cel7A CD was the least affected by the presence of IL (IV, Figure 3C). The binding of Cel5A and Cel5A CD could not be compared in IL matrices, as Cel5A CD displayed extremely low binding in all of the studied matrices.

The negative influence of the ILs on the substrate binding of the studied cellulases could be due to different reasons. One possibility is that the IL causes conformational changes to the structure of the CBM, decreasing or completely annihilating its binding affinity, similarly to what has been proposed for the inactivation of intact enzymes in ILs (Sheldon, et al. 2002). In previous studies, the addition of organic solvents has also been observed to be detrimental to cellulase substrate binding (Carrard and Linder 1999). Solvents have been proposed to interfere with the hydrophobic interactions which play a major role in the cellulase binding to cellulose. As the ILs employed in this study have a significant organic character, similar interference with the hydrophobic interactions dominating substrate binding cannot be ruled out. Further studies are needed to understand the precise manner in which ILs affect the substrate binding of cellulases.

The effects of IL on the hydrolytic action and cellulose binding of the studied cellulases are compared in Table 10. On the basis of the results, there is not always a clear correlation between substrate binding and hydrolysis and the effects of ILs on these parameters. Cellulase substrate binding is known to be tempera-

ture-sensitive, with higher temperatures usually leading to decreased substrate binding (Carrard and Linder 1999; Kyriacou, et al. 1988; Linder and Teeri 1996). Therefore, it could be expected that enzyme binding in the presence of ILs is even more affected at hydrolysis temperatures than at 4 °C, which was used in these binding experiments. Significant substrate binding is apparently not needed in all cases for hydrolysis to take place, as was demonstrated with Cel5A CD in buffer, so not all of the negative IL effects on enzymatic hydrolysis can be attributed only to interference with binding.

Table 10. Comparison of the impact of [DMIM]DMP and [EMIM]AcO on the hydrolytic action and cellulose binding of *Trichoderma reesei* Cel5A, Cel7A and their core domains. The "+" sign indicates the degree to which the cellulase hydrolysis or binding was reduced by IL.

Cellulase	Reduction of hydrolysis yield vs buffer		Reduction of binding to MCC	
	[DMIM]DMP	[EMIM]AcO[1]	[DMIM]DMP	[EMIM]AcO
Cel5A	+++	+++	++	+++
Cel5A CD	+	+	ND	ND
Cel7A	++	+	+	++
Cel7A CD	+	+	+	+

[1]In high IL concentrations (>20% w/w), [EMIM]AcO was clearly more harmful than [DMIM]DMP in hydrolysis for all cellulases.

4.5 Study of the effect of ILs on commercial cellulases

4.5.1 IL effects on the action of commercial alkali- and thermostable cellulases (III)

The link between cellulase alkali- and thermostability and IL tolerance was studied in this work using three commercial thermo- or alkali-stable cellulase preparations: a thermophilic cellulase (Cel5A) from *Thermotoga maritima* and Puradax® HA 1200E and IndiAge® ONE (III). Puradax® HA 1200E is produced for detergent applications and IndiAge® ONE for denim finishing of textiles. *T. reesei* Cel5A was included in the study for comparison. The commercial cellulase preparations were characterized by SDS-PAGE, which showed them to be rather pure monocomponent preparations (III, Figure 1). Both MCC and eucalyptus pre-hydrolysis kraft dissolving pulp were used as substrates in the hydrolysis experiments. Two ILs were employed: [EMIM]AcO, and [TMGH]AcO, which, based on the IL screening described in Section 4.3.2, was chosen as a representative of the new cellulose-dissolving superbase ILs.

IndiAge® ONE was the most efficient cellulase in the hydrolysis of solid cellulose both in buffer and in the presence of IL (III, Figure 5), although Puradax® HA

1200E showed much higher specific activity on dissolved CMC (III, Figure 2). In the presence of ILs, the enzymatic hydrolysis yields were on the same level for MCC and dissolving pulp (III, Figures 5A and 5B). IndiAge® ONE was the most IL-tolerant cellulase, with some hydrolytic action even in 40% (w/w) IL, and its hydrolysis yields decreased linearly with the IL concentration. Puradax® HA 1200E was affected differently by ILs, as the hydrolysis yield was almost the same in 20% (w/w) IL as in buffer, but then rapidly decreased in higher IL concentrations. By comparison, *T. reesei* Cel5A was much more IL-sensitive than the commercial cellulase preparations. The differences between the impact of [EMIM]AcO and [TMGH]AcO on the hydrolysis yields were rather small. *T. maritima* Cel5A was found to give very poor hydrolysis yields on MCC both at 45 °C and at its optimum temperature (80 °C) in both buffer and IL matrices, so its IL tolerance could not be determined (unpublished results). The presence of ILs was observed to shift the product distribution to longer cello-oligomers for IndiAge® ONE but not for Puradax® HA 1200E (III, Table 2). The same effect was also noticed for *T. reesei* Cel5A and Cel7B (I, Tables 1 and 2) and appears to depend on the specific cellulases rather than on the IL. The cellulases were able to reduce the molecular mass of pulp but not of MCC in buffer and in some IL matrices (III, Figure 7).

The activity in high pH, thermostability, activity on CMC and hydrolysis yields on solid substrates in buffer and in the presence of ILs of the studied cellulases are presented in Table 11. As can be seen be seen from this table, enzyme activity in high pH did not give any direct benefits in cellulose hydrolysis in the presence of ILs. Activity on CMC correlated poorly with the hydrolysis of solid cellulose, suggesting that activity on soluble substrates is a poor indicator of action on solid substrates in hydrolysis experiments. Thermostability appeared to correlate better with IL tolerance, as was exemplified by IndiAge® ONE, which was more thermostable than Puradax® HA 1200E and *T. reesei* Cel5A (III, Figures 6A, 6B and 6C) and was also the most IL-tolerant cellulase. In order to obtain conclusive correlations between enzyme properties such as thermostability and IL tolerance a larger library of enzymes with various properties should be screened in selected ILs in the future.

Table 11. Comparison of the hydrolytic performance on solid and soluble substrates in IL matrices with alkali- and thermostability of cellulases (III). The “+” sign indicates the degree to which the specific property is assigned to the cellulase.

Cellulase	Activity in high pH	Thermostability	Activity on CMC	Solid hydrolysis in buffer	Solid hydrolysis in ILs
T. maritima Cel5A	+	+++[1]	+	-	-
Puradax® HA 1200E	+++	-	+++	+	++
IndiAge® ONE	+	++	+	+++	+++
T. reesei Cel5A	ND	+	+++[2]	++	+

[1] Based on manufacturers note
[2] Measured in I

4.5.2 Cello-oligomer hydrolysis with β-glucosidases in aqueous ionic liquid solutions (II)

The action of commercial *Aspergillus niger* β-glucosidase (Novozyme 188) on soluble cello-oligomers was studied in the presence of 20 and 40% (w/w) of [DMIM]DMP and [EMIM]AcO (II). Cello-oligomers in the range of cellobiose to cellohexaose were all completely hydrolysed to glucose in optimum conditions (buffer, pH 5.0), whereas increasing the pH to 6.0 partially impeded the hydrolysis of cellopentaose (II, Table 2), indicating the β-glucosidase to be pH sensitive. The presence of [DMIM]DMP and [EMIM]AcO led to lower hydrolysis degrees for both cellobiose and cellopentaose and the buffer pH also appeared to play a role in limiting the degree of hydrolysis. The β-glucosidase partially hydrolysed cello-oligomers even in 40% (w/w) [DMIM]DMP, whereas hydrolysis degrees were very low in the matrices containing [EMIM]AcO. This difference cannot be explained by pH effects. The pH value was the same (6.6) in 20% (w/w) [DMIM]DMP and 20% (w/w) [EMIM]AcO (with phosphate buffer in both), nevertheless the difference in hydrolysis degree was great. [EMIM]AcO is clearly more harmful for β-glucosidase action than [DMIM]DMP.

In this work, the crude β-glucosidase preparation was also found to contain some xylanase and endoglucanase activities which may have had minor effects on the cello-oligomer hydrolysis (II). Engel et al. (2012b) have previously reported that the β-glucosidase (Novozyme 188) did not exhibit any activity on cellobiose in matrices containing more than 15% (v/v) of [DMIM]DMP. Long hydrolysis experiments may clearly lead to different conclusions regarding IL-induced cellulase inactivation, as the β-glucosidase in this work was found still to hydrolyse cello-oligomers partially in up to 40% (w/w) [DMIM]DMP.

4.6 pH Effects on cellulase action in ionic liquid solutions (I, II, III)

Throughout this work, the pH values of the solution matrices in the hydrolysis experiments were monitored (I, II, III and unpublished results). The measured pH values of different IL matrices have been summarized in Table 12. Generally, all of the cellulose-dissolving ILs used in this work were basic in aqueous solution. This is not surprising, as the cellulose dissolution capacity is believed to be mainly dependent on the anion's capacity to accept H-bonds (*i.e.* basicity) (Doherty, et al. 2010; Tang, et al. 2012). However, pH values measured in high concentrations of IL should be regarded with a certain degree of caution, as the pH scale is defined for diluted water solutions. The basic IL shift could not be compensated by increasing the concentration of the acid component in the buffer (Table 12, rows 3 and 4). Based on the pH data in Table 12, not only the anion but also the cation plays a distinct role in the pH of aqueous IL matrices. In most cases, the added buffer (citrate at pH 5.0 or phosphate at pH 6.0) had only a minor impact on the matrix pH.

Table 12. pH of IL-containing hydrolysis matrices. C = 0.050 M citrate buffer (nominal pH 5.0), C* = 0.500 M citrate buffer (nominal pH 5.0); P = 0.100 M phosphate buffer (nominal pH 6.0). pH values in bold are from matrices in which IL and buffer were mixed on a volume basis (v/v); other pH values were measured from matrices in which IL and buffer were mixed on a weight basis (w/w).

		pH as function of IL concentration (%)						
IL	**Buffer**	**0**	**20**	**40**	**60**	**80**	**90**	**Ref**
[DMIM]DMP	C	**5.0**	**5.4**	**6.3**	**7.7**	**9.0**	**9.7**	I
[DMIM]DMP	P	6.0	6.6	7.2				II
[EMIM]AcO	C	**5.0**	**6.3**	**7.3**	**8.9**	**11.0**	**12.7**	I
[EMIM]AcO	C*	**5.0**	**6.2**	**7.2**	**8.8**	**11.1**	**12.3**	Unpublished results
[EMIM]AcO	P	6.0	6.5	7.4	8.8			II, III
[TMGH]COO	C	5.0	6.5					Unpublished results
[TMGH]AcO	C	5.0	5.6					Unpublished results
[TMGH]AcO	P	6.0	5.6	6.1	7.1			III
[TMGH]EtCOO	C	5.0	6.5					Unpublished results
[TMGH]*n*-PrCOO	C	5.0	6.4					Unpublished results
[DBNMe]DMP	P	6.0	6.8	8.1	10.5	12.6	13.6	III + unpublished results
[DBNH]AcO	P	6.0	7.1	7.9	9.7	11.2		Unpublished results
[DBNH]EtCOO	P	6.0	7.0	7.9	9.3	11.1	11.9	Unpublished results

In some recently published studies, the basicity of IL matrices has been attributed as a partial reason (Engel, et al. 2010) or even a dominating factor (Li, et al. 2012) for cellulase inactivation. The impact of IL basicity on enzyme action is probably very different for different enzymes depending on their pH activity curves. The β-glucosidase from *A. niger* studied in II was pH sensitive, but even in that case [EMIM]AcO was more harmful to the β-glucosidase action than [DMIM]DMP in matrices with practically the same pH, indicating that the ILs have other detrimental effects on the enzymes than basicity.

Comparison of the steeply decreasing hydrolysis yields of the *T. reesei* and commercial endoglucanases in increasing IL concentrations (Sections 4.3.2 and 4.5.1) with the IL matrix pH values displayed in Table 12 does not support the hypothesis that the poor cellulase performance in IL solutions would be explained only by a high matrix pH. At the same it was shown in hydrolysis experiments and activity measurements at different pH values in buffer that the studied enzymes

have considerable hydrolytic activity at pH 7 (I, Tables 1 and 2: MCC hydrolysis at pH 7.0 and III, Figure 2). In Section 4.5.1 it was clearly shown with the example of Puradax® HA 1200E that having activity in high pH does not necessarily lead to good performance on solid substrates in basic IL matrices. To conclude this discussion, the increase in pH value of the hydrolysis matrix is not the major reason for poor cellulase performance observed in this work in IL solutions. ILs probably have other harmful effects on the cellulases, such as structural changes in the protein structure leading to inactive protein conformations (Sheldon, et al. 2002). These effects need to be further studied for specific IL and enzyme combinations in the future.

5. Conclusions and future prospects

Two distinct process alternatives are available for the total enzymatic hydrolysis of lignocellulosics using ionic liquid pretreatment technologies: separate pretreatment with IL followed by washing off the IL before enzymatic hydrolysis (regeneration procedure), and the one-pot procedure in which the enzymatic hydrolysis is carried out in the same vessel as the pretreatment with the IL still present. The one-pot procedure offers several technical advantages such as simplified processes with less washing, separation and evaporation steps and easier IL recycling. However, the presence of high concentrations of cellulose-dissolving ILs effectively hinders the action of enzymes in hydrolysis. In this work, the effects of ILs on enzymatic hydrolysis of cellulose were elucidated. Some of the phenomena related to IL-induced cellulase inactivation and the effects of ILs on both the cellulases and the substrate in hydrolysis conditions were studied. Working with cellulose hydrolysis in IL solutions is challenging, as the ILs interfere with the commonly used analytical methods in carbohydrate chemistry. A CE method compatible with moderate amounts of ILs was successfully developed for the analysis of hydrolysates containing ILs. However, more work should be carried out in the future on developing saccharide analytics applicable in high salt concentrations for IL matrices.

ILs were confirmed to have a negative impact on the cellulose hydrolysis with mesophilic *Trichoderma reesei* cellulases, of which Cel5A, Cel7A and Cel7B were studied in detail. However, clear differences were observed regarding the inactivation rates and mechanisms of cellulases in ILs. [DMIM]DMP supported cellulase activity during prolonged incubation times, whereas enzyme inactivation in [EMIM]AcO was rapid and irreversible. Cellulase activity in ILs is apparently not the only factor affecting cellulose hydrolysis and possibly other factors, such as IL coating of the substrate, limit enzymatic hydrolysis. More studies are needed to elucidate the discrepancy between low hydrolysis yields and well-retained cellulase activity in ILs such as [DMIM]DMP. *T. reesei* endoglucanases were observed to cause reductions in the molecular weight of microcrystalline cellulose in 90% (v/v) of [DMIM]DMP, in which cellulose was partially dissolved. This observation was interesting as it suggested that cellulases may retain their activity even in very high concentrations of certain cellulose-dissolving ILs. The endoglucanases appeared to reduce the molecular weight without any detectable formation of cello-

oligomers, which is a different mode of action in comparison to the hydrolysis of undissolved MCC.

The results obtained in this work using commercial cellulases with increased thermo- and alkali-stability suggested that enzymes with increased IL tolerance may be found in already available enzyme products. Thermostablilty rather than activity in high pH appears to be linked to IL tolerance. In order to strengthen this hypothesis, wide screening studies with cellulases of different thermostability should be undertaken. The studied cellulose-dissolving ILs were all basic in aqueous solution to different degrees, but the basic impact was in this work generally determined not to be the main reason for the poor cellulase performance in ILs. Other IL properties affecting cellulase performance in hydrolysis, but which were not studied in this work, include high matrix viscosity, ionic strength and specific IL effects, which with some probability consist of protein unfolding and conformational changes to the active structure of the enzymes. The studies of cellulase binding and the role of the carbohydrate-binding module in ILs showed that cellulase substrate binding is probably severely hindered by the presence of ILs in the hydrolysis matrix. However, it was also shown that high substrate binding is not always a requirement for hydrolysis to take place.

If ILs are to become part of large-scale industrial biomass processing, such as polysaccharide hydrolysis and fermentation, in which cheap bulk products are produced, several challenges remain to be solved. ILs are still relatively expensive, even though their price is expected to decrease in the future. Virtually complete recyclability is needed. The distillable superbase ILs, which were also studied in this work, represent an interesting opportunity for good recyclability through distillation, and these ILs may be produced from cheaper starting materials than the conventional cellulose-dissolving imidazolium-based ILs. The biocompatibility, toxicology and biodegradability of ILs is also a topic which requires increased research efforts. This topic is not made any easier by the large number of different ILs available. Nevertheless, the large-scale use of ILs will not be possible until these issues have been sufficiently elucidated.

In the recent literature several breakthroughs have been made in designing enzyme-compatible IL systems for cellulose modification. Two main routes for tackling this problem are possible: designing cellulose-dissolving ILs which support enzyme activity or developing IL-tolerant enzymes. In the light of recently reported advances, it is reasonable to expect that the problems with enzyme inactivation in cellulose-dissolving ILs will have found a technical solution within next few years, although applying this solution to large-scale processing may not be straightforward. Cellulose hydrolysis is not the only interesting enzymatic modification of cellullose in ILs. Other potentially feasible enzymatic modifications of cellulose dissolved in IL solutions include *e.g.* oxidation, acylation and other derivatizations which could lead to new products or better production routes for existing industrial cellulose products.

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Series title and number
VTT Science 52

Title	**Enzymatic hydrolysis of cellulose in aqueous ionic liquids**
Author(s)	Ronny Wahlström
Abstract	Total enzymatic hydrolysis of the polysaccharides in lignocellulosic biomass to monosaccharides is currently a focus research area. The monosaccharides obtained from lignocellulose hydrolysis can be used for the production of platform chemicals and biofuels, most notably ethanol. One major challenge in the commercialization of lignocellulosic ethanol production is the recalcitrance of lignocellulosics towards enzymatic hydrolysis, necessitating efficient pretreatment of the lignocellulosic feedstock. Certain ionic liquids (ILs, salts with melting points below 100 °C) dissolve cellulose and even lignocellulosic biomass and are as such interesting candidates for pretreatment technology. However, cellulose-dissolving ILs have been found to severely inactivate the hydrolytic enzymes (cellulases) employed in cellulose hydrolysis. This work focuses on elucidating how certain ILs affect the action of cellulases in cellulose hydrolysis. The main emphasis was on the action of purified monocomponent *Trichoderma reesei* cellulases, but some commercial cellulase preparations were also studied in IL matrices. Hydrolysis experiments were made in solutions containing up to 90% of the two cellulose-dissolving ILs 1-ethyl-3-methylimidazolium acetate ([EMIM]AcO) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP). The presence of increasing amounts of IL led to decreasing yields of solubilised saccharides in enzymatic hydrolysis. [EMIM]AcO was generally more harmful for cellulase action than [DMIM]DMP. Pure [EMIM]AcO completely inactivated *T. reesei* endoglucanase in 4 h in residual activity measurements, whereas pure [DMIM]DMP supported considerable cellulase activity for at least three days. The cellulase compatibility of several novel classes of cellulose-dissolving ILs were studied in hydrolysis, but these ILs were found to be at least as harmful for cellulase action as the studied imidazolium-based ILs. *T. reesei* endoglucanases were unable to reduce the molecular weight of microcrystalline cellulose (MCC) in buffer or in any aqueous matrix containing IL, except in 90% (v/v) [DMIM]DMP in which the MCC was partially dissolved. The studied ILs were found to have very detrimental effects on saccharide analytics. A capillary electrophoresis (CE) method was developed for the analysis of mono- and oligosaccharides in matrices containing ILs. The cellulase binding to MCC in solutions with [DMIM]DMP and [EMIM]AcO was studied with radiolabeled *T. reesei* Cel5A (endoglucanase II)and Cel7A (cellobiohydrolase I) and their respective core domains. Cel7A was able to bind to MCC with its core domain, whereas it was shown that Cel5A was very dependent on its CBM for efficient substrate binding. [EMIM]AcO interfered more with cellulase substrate binding than [DMIM]DMP. The binding ability of the *T. reesei* carbohydrate-binding modules (CBMs) was very IL sensitive.
ISBN, ISSN	ISBN 978-951-38-8115-3 (Soft back ed.) ISBN 978-951-38-8116-0 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Date	January 2014
Language	English, abstracts in Swedish and Finnish
Pages	102 p. + app. 57 p.
Keywords	Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase
Publisher	VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland, Tel. +358 20 722 111

Seriens namn och nummer
VTT Science 52

Namn	**Enzymatisk cellulosahydrolys i vattenhaltiga jonvätskor**
Författare	Ronny Wahlström
Referat	Enzymatisk totalhydrolys av lignocellulosans polysackarider till monosackarider är för tillfället ett mycket aktivt forskningsområde. De sålunda producerade monosackariderna kan användas som råvara vid tillverkningen av plattformkemikalier och biobränslen, av vilka särskilt kan nämnas etanol. En av de största utmaningarna i kommersialiseringen av etanoltillverkning från lignocellulosa är lignocellulosans motståndskraft mot enzymatisk hydrolys, varför effektiva förbehandlingsmetoder är nödvändiga. Vissa jonvätskor (definierade som salt med smältpunkt under 100 °C) löser cellulosa och till och med fullständig lignocellulosa. Jonvätskorna utgör sålunda ett intressant alternativ som förbehandlingsteknologi för lignocellulosa. Jonvätskorna har emellertid i hög grad konstaterats inaktivera de hydrolytiska enzymer, cellulaser, som används i cellulosahydrolys. Detta arbete har haft som målsättning att klargöra hur cellulosalösande jonvätskor påverkar cellulasernas funktion i cellulosahydrolys. I första hand undersöktes hur funktionen hos cellulaser renade till enkomponentpreparat från *Trichoderma reesei*, men också hos kommersiella cellulaspreparat, påverkades i vissa jonvätskelösningar. Hydrolysexperimenten utfördes i lösningar med upp till 90 % jonvätska (1-etyl-3-metylimidazolium acetat ([EMIM]AcO) eller 1,3-dimetylimidazolium dimetylfosfat ([DMIM]DMP)). En ökande mängd jonvätska ledde till avtagande hydrolysutbyten i form av lösliga sackarider i enzymatisk hydrolys. [EMIM]AcO var i allmänhet mer skadlig än [DMIM]DMP för cellulasernas funktion. Ren [EMIM]AcO inaktiverade *T. reesei* endoglukanas fullständigt på mindre än 4 h, medan betydande restaktiviteter mättes efter inkubation i [DMIM]DMP under åtminstone tre dygn. Ett antal nya sellulosalösande jonvätskor, baserade på organiska superbaser studerades i hydrolysexperiment, men dessa jonvätskor konstaterades vara åtminstone lika skadliga för cellulasernas funktion som de imidazoliumbaserade jonvätskorna. *T. reesei*s endoglukanaser kunde inte reducera mikrokristallin cellulosas (MCC) molmassa i buffertlösning eller i någon jonvätskelösning, förutom i 90 % (v/v) [DMIM]DMP, vari MCC partiellt löste sig. De studerade jonvätskorna konstaterades vara mycket skadliga för många av de vanliga metoderna som används i kolhydratanalytik. En kapillärelektroforesmetod utvecklades för att analysera mono- och oligosackarider i jonvätskelösningar. *T. reesei* Cel5A (endoglukanas II), Cel7A (cellobiohydrolas I) och deras respektive katalytiska domäner märktes med radioaktivt tritium och dessa cellulasers förmåga att binda till MCC studerades i lösningar innehållande [DMIM]DMP och [EMIM]AcO. Cel7A kunde binda sig till MCC direkt via sin katalytiska domän, medan det kunde påvisas att Cel5A var ytterst beroende av sin kolhydratbindande modul för att binda till cellulosa. [EMIM]AcO konstaterades påverka cellulasernas bindingsgrad till MCC mer än [DMIM]DMP. Bindningsförmågan hos *T. reesei*s kolhydratbindande moduler konstaterades vara synnerligen känslig för de studerade jonvätskorna.
ISBN, ISSN	ISBN 978-951-38-8115-3 (Print) ISBN 978-951-38-8116-0 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online)
Datum	Januari 2014
Språk	Engelska, referat på svenska och finska
Sidor	102 s. + bil. 57 s.
Nyckelord	Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase
Utgivare	VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland, Tel. +358 20 722 111

Julkaisun sarja ja numero
VTT Science 52

Nimeke	**Selluloosan entsymaattinen hydrolyysi vesipitoisissa ioninesteissä**
Tekijä(t)	Ronny Wahlström
Tiivistelmä	Lignoselluloosan entsymaattista totaalihydrolyysiä tutkitaan nykyisin hyvin aktiivisesti. Lignoselluloosassa olevien polysakkaridien hydrolyysistä syntyviä monosakkarideja voidaan käyttää raaka-aineina kemikaalien, polymeerien ja biopolttoaineiden, erityisesti etanolin, tuotannossa. Iso haaste lignoselluloosapohjaisen etanolituotannon kaupallistamisessa on lignoselluloosan monimutkainen rakenne, joka vaikeuttaa entsymaattista hydrolyysiä. Tehokkaiden, lignoselluloosaa avaavien esikäsittelymenetelmien kehittäminen on siis tärkeää. Tietyt ioninesteet, jotka määritellään suoloiksi, joiden sulamispiste on alle 100 °C, liuottavat selluloosaa ja jopa lignoselluloosaa ja ovat näin ollen hyvin mielenkiintoisia käytettäviksi lignoselluloosan esikäsittelyssä. Selluloosaa liuottavien ioninesteiden on kuitenkin todettu inaktivoivan hydrolyyttisiä entsyymejä, sellulaaseja, joita käytetään selluloosan totaalihydrolyysissä. Tässä työssä tutkittiin pääasiassa *Trichoderma reesei* -homeen tuottamien ja puhdistettujen sellulaasien sekä myös joidenkin kaupallisesti saatavien sellulaasituotteiden toimintaa ja inaktivoitumista vesipitoisissa ioninesteliuoksissa. Hydrolyysikokeita tehtiin selluloosalla vesiliuoksissa, joiden ioninestepitoisuus vaihteli; suurimmillaan se oli 90 % (joko 1-etyyli-3-metyylimidatsoliumi asetaatti ([EMIM]AcO) tai 1,3-dimetyylimidatsoliumi dimetyylifosfaatti ([DMIM]DMP)). Kasvavat ioninestepitoisuudet aiheuttivat hydrolyysisaannon pienenemisen selluloosan entsyymaattisessa hydrolyysissä. [EMIM]AcO haittasi enemmän sellulaasien toimintaa kuin [DMIM]DMP. Puhtaassa [EMIM]AcO:ssa *T. reesei*n endoglukanaasi inaktivoitui täysin neljän tunnin käsittelyssä, kun taas aktiivisuus aleni hyvin vähän [DMIM]DMP:ssa kolmen vuorokauden aikana. Useamman uuden superemäspohjaisen, selluloosaa liuottavan ioninesteryhmän sellulaasiyhteensopivuus tukittiin hydrolyysikokeissa, mutta nämä ioninesteet eivät kuitenkaan olleet paremmin yhteensopivia sellulaasien kanssa kuin perinteiset imidatsoliumi-pohjaiset ioninesteet. *T. reesei*n endoglukanaasit eivät pystyneet vähentämään mikrokiteisen selluloosan (MCC) molekyylipainoa puskurissa eivätkä missään muussa ioninestettä sisältävässä liuoksessa, paitsi 90-prosenttisessa (v/v) [DMIM]DMP:ssa, johon MCC oli osittain liuennut. Tutkittujen ioninesteiden havaittiin olevan hyvin haitallisia hiilihydraattianalytiikkamenetelmille. Työssä kehitettiin kapillarielektroforeesimenetelmä mono- ja oligosakkaridien analyysiin ioninestepitoisissa matriiseissa. Sellulaasien sitoutumista MCC:aan tutkittiin radioleimatuilla *T. reesei* Cel5A:lla (endoglukanaasi II), Cel7A:lla (cellobiohydrolaasi I) ja niiden katalyyttisillä domeeneillä puskuriliuoksissa [DMIM]DMP:n ja [EMIM]AcO:n läsnä ollessa. Cel7A pystyi sitoutumaan MCC:aan pelkällä katalyyttisellä domeenillaan, kun taas Cel5A oli hyvin riippuvainen hiilihydraatteja sitovasta modulistaan sitoutuakseen tehokkaasti selluloosaan. [EMIM]AcO vaikutti [DMIM]DMP:a voimakkaammin sellulaasien selluloosaan sitoutumiseen. Yleisesti tutkittujen ioninesteiden todettiin vaikuttavan herkästi *T. reesei*n hiilihydraatteja sitovien moduulien toimintaan.
ISBN, ISSN	ISBN 978-951-38-8115-3 (nid.) ISBN 978-951-38-8116-0 (URL: http://www.vtt.fi/publications/index.jsp) ISSN-L 2242-119X ISSN 2242-119X (painettu) ISSN 2242-1203 (verkkojulkaisu)
Julkaisuaika	Tammikuu 2014
Kieli	Englanti, tiivistelmä ruotsiksi ja suomeksi
Sivumäärä	102 s. + liitt. 57 s.
Avainsanat	Ionic liquid, cellulase, hydrolysis, carbohydrate, cellulose, inactivation, carbohydrate-binding module, cellulase binding, glycoside hydrolase
Julkaisija	VTT PL 1000, 02044 VTT, puh. 020 722 111

Enzymatic hydrolysis of cellulose in aqueous ionic liquids

The rapidly growing global demand for energy and chemical resources, economic and political feedstock security issues and the concern for global warming direct us from the utilization of fossil to renewable feedstocks. Lignocellulosic biomass from different origins is a widely available and currently much underutilized feedstock for the production of e.g. liquid biofuels. Hydrolysis of lignocellulosic polysaccharides results in monosaccharides which can be further transformed into fuels and other chemical products by biotechnological means. The hydrolysis of lignocellulosics is preferably carried out using hydrolytic enzymes, cellulases and hemicellulases, as catalysts. The native lignocellulosic feedstock is, however, highly recalcitrant towards enzymatic hydrolysis and therefore costly pretreatments are needed prior to hydrolysis.

Ionic liquids are salts with low melting points (<100 °C). Some classes of ionic liquids have been noticed to dissolve cellulose, and more recently, to possess the quite unique property to dissolve even native lignocellulosic biomass. Thus, ionic liquids hold a great potential as pretreatment technology prior to enzymatic hydrolysis. It is, however, known that ionic liquids also inactivate cellulases used for cellulose hydrolysis. In this work, the performance of different cellulases in both commercial and non-commercial cellulose-dissolving ionic liquids was studied. The aim of this work was to elucidate the inactivation of cellulases in ionic liquids, study the effect of ionic liquids on the hydrolytic performance of cellulases during long hydrolysis experiments, and elucidate different ionic liquid- and enzyme-related factors affecting the hydrolytic ability of cellulases in the presence of ionic liquids.

ISBN 978-951-38-8115-3 (Soft back ed.)
ISBN 978-951-38-8116-0 (URL: http://www.vtt.fi/publications/index.jsp)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)