Screening of glycoside hydrolases and ionic liquids for fibre modification

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Abstract

BACKGROUND: This study elaborates the possibility to apply combined ionic liquid and enzyme treatments for pulp fibre modification. The approach involves swelling of fibre surfaces with ionic liquid (IL) followed by enzymatic modification of the disrupted fibre surface using carbohydrate active enzymes.

RESULTS: The capacity of seven cellulose-dissolving or cellulose-swelling ionic liquids to swell pulp fibres was compared. In addition, thirteen cellulases and five xylanases were screened for their IL tolerance, which determines their applicability in combined or sequential IL-enzyme treatments of fibres. Among the studied ionic liquids, 1-ethyl-3-methylimidazolium dimethylphopshate ([EMIM]DMP) and 1,3-dimethylimidazolium dimethylphosphate ([DMIM]DMP) had the strongest effect on fibre swelling. These solvents were also found to be the least inactivating for the studied enzymes.

CONCLUSION: Enzyme compatibility and cellulose-dissolving capability are not two conflicting properties of an ionic liquid.

Introduction

Replacement of non-renewable materials such as oil-based plastics with renewable biomass-based alternatives is a global trend. Utilization of cellulose pulp fibres in new and unconventional material applications is appealing both for the forest industry, looking for new business opportunities, and for the end product manufacturers looking for industrially available, well performing and sustainable raw materials. New methods for fibre modification are required to broaden the properties of cellulosic fibres to meet the requirements of various material applications.

High accessibility and reactivity of polysaccharides in fibres is required in applications such as cellulose-based composites or man-made cellulose fibres produced via dissolution and

regeneration stages. Cellulose in wood fibres is densely packed at all of its structural levels, from elementary fibrils to the lamellar fibre structure. Different mechanical, chemical and enzymatic approaches may be used to loosen the dense packing. Pulp reactivity characterized by fibre dissolution in cellulose solvents is reported to increase with mechanical and enzymatic treatments. [1,2] On the other hand, cellulose solvents, such as ionic liquids (ILs) can be used to swell and disrupt fibre surfaces. [3]

One of the most interesting developments in cellulose chemistry during the last decades is the discovery of cellulose-dissolving ionic liquids (ILs). [4] ILs are salts with low melting points (< 100 °C). The combination of organic and ionic character makes ILs excellent solvents in various applications. The negligible vapour pressure of ILs reduces emissions of volatile organic compounds and reduces explosion hazards related to many organic solvents. Thus, ILs possess clear benefits as cellulose solvents from an industrial point of view. Currently, research is ongoing to study fibre spinning from IL solutions for textile applications [5], which might yield a new technology alternative to the Lyocell process, in which cellulose fibres are spun from N-methylmorpholine N-oxide (NMMO) solution. In addition to complete dissolution, fibre swelling in ILs is an interesting alternative for fibre processing. [6]

Endoglucanases (EC 3.2.1.4) are a group of cellulose-degrading enzymes able to internally cleave the β-1,4-glycosidic bonds in cellulose chains. As a side activity, many endoglucanases are also able to degrade hemicellulosic plant polymers such as xylan and glucomannan. [7] Endoglucanases from different structural families differ in their mode of action [8] and surface modification of pulps with enzymes from different families has been studied. [9,10] For instance, the beatability and viscosity of chemical pulp was affected differently by different endoglucanases from *Trichoderma reesei.* [10] Endoglucanase treatments have been reported to improve the alkaline solubility of dissolving pulp by carefully controlled hydrolysis of the cellulose chains. [11]

Xylanases (EC 3.2.1.8) are hydrolytic enzymes that catalyse the hydrolysis of *β*-1,4-glycosidic linkage connecting the anhydroxylose units in xylan. In fibre treatment xylanases are not only involved in xylan removal, but also assist in fibre modification. Hardwood pulp fibres treated with xylanases showed external fibrillation that could contribute to increase the interfibre bonds [12], improved resistance to pressing, higher tendency of fibre hornification and collapsibility during drying. [13] In addition, fibre porosity can be increased with a xylanase treatment. [14]

Enzymatic catalysis of various reactions has been successfully carried out in IL solutions. [15] In recent years the use of enzymes together with cellulose-dissolving ILs has been studied intensively, although difficult compatibility issues have been reported for the combination of ILs and glycoside hydrolases (GHs). [16] Discoveries of more IL-tolerant enzymes have been reported and correlation of IL-tolerance with enzyme thermostability [17,18], salt-tolerance [19] and alkali-tolerance [20] have been suggested.

In this study, our aim was to use activating or swelling ILs in sequence with enzymatic treatments for modification of fibre surfaces (Fig. 1). In this article, we report the first part of this study that is, screening of 13 endoglucanases and 5 xylanases in aqueous solutions of 7 different cellulose-swelling or dissolving ILs. Enzymes were chosen from different glycoside hydrolase (GH) families to explore whether GH family was a key enzyme characteristic for IL tolerance. Thermostable enzymes were favoured based on previous literature suggestions. [17] The swelling of pulp fibres was studied in the same ILs and the results were combined with the enzymatic screening to identify the most potential IL-enzyme combinations for both swelling pulp fibres and supporting sufficient enzymatic activity for efficiently modifying the fibre surfaces in a one-pot system.

Materials and Methods

Ionic liquids, pulps and enzymes

Seven ILs, known to dissolve or swell cellulose, were synthesized with the exception of [DMIM]DMP, which was purchased from IoLiTec GmbH (Heidelberg, Germany). Structures and full names of the studied ILs are presented in Table 1. The ^{1}H NMR spectra of [BMIM]DBP, [Chol]AcO, [BMIM]DMP, [TMGH]n-PrCOO and [EMIM]DMP are shown in the supplementary material (Fig. S1-S5).

1-Butyl-3-methylimidazolium dibutylphosphate [BMIM]DBP was prepared as follows: 1-methylimidazole (20.59 g, 0.2515 mol) was weighed into a 250 ml 3-neck round bottom flask. The solution was heated up to 100^{o}C and tributyl phosphate (66.83 g, 0.2509 mol) was slowly added using a pressure-equalising dropping funnel. The system was under positive pressure of argon. The reaction was then heated to 140^{o}C and left to stir for 80 hrs. The sample was then cooled to 120^{o}C for 66 hrs. The progress of the reaction was monitored using ^{1}H NMR (Fig. S1). **Cholinium acetate [Chol]AcO** was prepared as follows: Choline bicarbonate (191.49 g, 0.91 mol, 80% (w/w) in water) was weighed into a round bottom flask and diluted to about double the total volume with distilled water. Glacial acetic acid (55.69 g, 0.91 mol) was slowly added to the mixture while stirring. The progress of the reaction was monitored using ^{1}H NMR to ensure that there was a 1:1 mol ratio of cholinium cation to acetate anion (Fig. S2). **1-Butyl-3-methylimidazolium dimethylphosphate [BMIM]DMP** was prepared as follows: 59.37 g (0.478 mol) of butylimidazole was weighed into a 250 ml 3-neck round bottom flask. The solution was slowly heated up to 100°C while slowly adding 66.83 g (0.477 mol) of tributylphosphate using addition funnel. The reaction was left to stir at 140°C for 80 hours. The progress of the reaction was followed by using ^{31}P and ^{1}H NMR (Fig. S3). **1,1,3,3-Tetramethylguanadinium butyrate [TMGH]n-PrCOO** was prepared as follows: Distilled 1,1,3,3-tetramethylguanidine (56.66 g, 0.492 mol) was

slowly mixed (over 15 min) with butanoic acid (43.34 g, 0.492 mol). The mixture was thoroughly mixed and allowed to cool, to form [TMGH]n-PrCOO. The product was characterized using ^{1}H NMR (Fig. S4). **1-Ethyl-3-methylimidazolium dimethylphosphate [EMIM]DMP** was prepared as follows: 1-Ethylimidazole (45.96 g, 0.478 mol) was weighed into a 3-neck round bottom flask. The solution was heated up to 100^{o}C and trimethyl phosphate (66.83 g, 0.477 mol) was slowly added using a pressure-equalising dropping funnel. The reaction was left to stir at 120^{o}C for 80 hours. The progress of the reaction was followed using ^{1}H NMR (Fig. S5). **Tetrabutylphosphonium acetate [P$_{4444}$]AcO** was prepared by anion metathesis, according to a previous article [21]. Briefly, anion metathesis from the commercially available tetrabutylphosphonium chloride (1 eq), using potassium acetate (1.05 eq) in isopropanol, was performed. An additional acetone precipitation step was used to precipitate excess potassium salts. Filtration through celite and evaporation yielded the pure ionic liquid. Purity was followed by ^{1}H NMR (DMSO-d_6) to determine acetate to cation ratio and using Karl-Fischer titration to ensure the water content was below 1% (w/w).

Bleached softwood kraft pulp and hardwood dissolving pulp were obtained from two separate Finnish pulp mills as dry pulp sheets.

The studied endoglucanases and xylanases are listed in Table 2 with the available characterization data. *Trichoderma reesei* endoglucanases (TrGH5 and TrGH7) were purified from *T. reesei* culture filtrates. [10] The *P. horikoshii* enzyme (PhGH5) was heterologously expressed in *E. coli* and partially purified using heat treatment. [22] The TfXYN10A construct was obtained from ROAL Oy and produced in *E.coli*. TmXYN10A [23] was kindly provided as a lyophilized culture supernatant from Hairong Xiong (South-central University for Nationalities, College of Life Science, Wuhan, China) and DtXYN10B [24] was

produced in *E.coli* at Aalto University. The other enzymes were obtained from commercial sources or as pre-commercial preparates from ROAL Ltd. as indicated in Table 2.

Enzyme screening

Screening of endoglucanase and xylanase activities in the presence of ILs was done using miniaturized assays in 500 µL 96-well polypropylene microplates (Eppendorf GmbH, Germany). Carboxymethyl cellulose (CMC) and birchwood xylan were used as substrates for endoglucanases and xylanases, respectively. Enzymes were dosed to reactions based on their activity. Activity necessary to hydrolyse 1-2% of the substrate polymer in the absence of IL was determined and this dosing was used in all reactions. Reactions were performed in 200 µL volume followed by an assay of reducing sugars. A miniaturized version of the dinitrosalicylic acid (DNS) method 25 was used except with one ionic liquid, [P4444]AcO. This IL was found to be more compatible with the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay. [26] Glucose or xylose was used as standard for the reducing sugar assay and standard solutions were always prepared to the same IL solution that was used in the enzyme assay.

Endoglucanases were assayed using 1% (w/v) carboxymethylcellulose (Sigma) as substrate in sodium citrate buffer (pH 5) containing 0%, 15% or 40% (w/v) of ionic liquid. Buffer strengths 50 mM and 200 mM were used with 15% and 40% (w/v) IL concentrations, respectively. ILs were found to increase the pH of the solution (Table 1) and 50 mM citric acid was used to adjust pH to the range 5-6. Among the studied ILs [Chol]AcO, [TMGH]n-PrCOO and [P4444]AcO were the most alkaline ones and pH adjustment with citric acid was unsuccessful when 40% of these ionic liquids was present. Therefore, endoglucanase activities in these conditions were measured without pH adjustment and pH in these reactions was in the range 7-8.

Xylanase activities were assayed using 2% birchwood xylan as substrate (Sisco Research Laboratories Ltd., India) in 50 mM phosphate buffer (pH 7) containing 0,15 or 40% (w/v) of ionic liquid. To check the activity of the enzyme with different concentrations of ILs, the pH was adjusted to 7 after the addition of IL, using 50 mM phosphoric acid.

Assay temperatures 50˚C and 60˚C were used for endoglucanases and xylanases, respectively. For *P. horikoshii* endoglucanase, *T. maritima* xylanase and *D. thermophilum* xylanases 70˚C assay temperature was used due to drastically decreasing activity in lower temperatures. Bovine serum albumin (BSA, 0.1 mg/mL) was added to each reaction to prevent unspecific enzyme binding to plate wells. Two hours incubation time with no agitation was applied for all sample plates. All reactions were carried out in triplicates.

Pulp swelling in aqueous ionic liquid solutions

Dry pulp sheets were homogenized by soaking them in water (1 g pulp/ 66 g of water) for 4 hours. The wet pulps were disintegrating using an electric kitchen mixer, followed by dewatering by vacuum filtration on a 60 µm wire cloth. After dewatering the homogenized pulps had dry contents of ca. 50%.

For swelling/dissolution experiments, 10-20 mg (dry weight) of homogenized pulp was weighed into a glass test tube. Water and IL were added, taking into account the water present in the pulp, to form a pulp dispersion in 90% (w/w) IL. The pulp dispersion in aqueous IL was heated to 80°C and subjected to magnetic stirring for 3 h in sealed test tubes in a temperature-controlled water bath. Samples were taken for light microscopy examination after 180 min. Light microscopy was performed with an Olympus BX61 microscope (Olympus Corp., Japan) and digital image recording was performed with the Soft Imaging Systems analySIS1 3.2 software.

Results and discussion

Screening aqueous ionic liquids for pulp swelling

The effect of IL treatment on pulp swelling was evaluated with seven ionic liquids from light microscopy images (Table 1). Representative microscopy images after each treatment are shown in the supplementary material (Fig. S6 and Fig. S7). The imidazolium and phosphate-based ILs were found to be the most effective in generating visually detectable changes in pulp morphology in the following order [EMIM]DMP>[DMIM]DMP>[BMIM]DMP. Thus in the imidazolium-based ILs, longer alkyl chains in the IL structure correlated with lower fibre swelling capacity. Previously, longer carboxylate chain lengths in IL anions have been found to prevent cellulose dissolution. [27] Minor changes were detected after pulp treatment with [TMGH]n-PrCOO and [P4444]AcO, whereas no visual changes were detected after treatment with [BMIM]DBP and [Chol]AcO. Pulp swelling was found to occur via two distinctive mechanisms as shown in Fig. 2. Fig. 2 A shows ballooning-type fibre swelling which typically occurred in the middle of fibres and Fig. 2 B shows unwinding which typically occurred at fibre ends. Unwinding was the most prominent mechanism observed with birch dissolving pulp whereas ballooning was more prominent with softwood kraft pulp. Ballooning-type of swelling has been previously described to occur for cotton and wood fibres in ionic liquid solutions. [6] In ballooning, S2 layer of the secondary cell wall is suggested to swell faster compared to the S1 layer, which causes the lower layer to burst out and form balloon structures to the fibre. [28] Recalcitrance of individual fibres to changes caused by the IL treatments was found to vary significantly (Fig. 2). Although only a minority of the fibres showed visible changes after IL treatment, these changes can be expected to change the bulk properties of the pulp.

Screening of endoglucanases and xylanases for ionic liquid tolerance/compatibility

The hydrolytic performance of 13 endoglucanases and 5 xylanases was studied in the presence of seven cellulose-dissolving or swelling ILs. Enzymes were selected to represent different glycoside hydrolase (GH) families to ensure a broad variation in the enzyme structures. For endoglucanases, GH families 5,7,12, and 45, and for xylanases, GH families 10 and 11 were represented (Table 2). Enzymes of bacterial and fungal origin were involved and special attention was paid to involve enzymes with notable thermostability due to the previously suggested correlation between thermostability and IL-tolerance. [17,29] A miniaturized screening assay was developed to allow high throughput operation and to minimize the use of ILs and enzymes in the screening. A long assay time (2h) was chosen to reflect factors related to both enzyme activity and stability in the aqueous IL solution.

Screening of endoglucanases

Hydrolysis of carboxymethyl cellulose (CMC) with 13 endoglucanases was studied in the presence of 15% (w/v) of ionic liquids (Fig. 3). Six enzymes from different GH families with promising IL-tolerance were chosen for an additional screening round with 40% (w/v) ILs (Fig. 4). Results are shown as relative activities (%), the 100% level representing enzyme activity in a pH 5 buffer solution. An averaged value of activity in the presence of different ILs was calculated for each enzyme aiming to describe the overall IL-tolerance of the enzyme (Fig. 3 and Fig. 4).

Enzyme activity in the presence of 15 and 40% (w/v) ILs varied widely between endoglucanases, ranging from complete enzyme inactivation (0% relative activity) to an activating effect (>100% relative activity) (Fig. 3 and Fig. 4). In most cases, ILs reduced the activity of endoglucanases. Interestingly, some enzymes were found to be activated in the presence of [DMIM]DMP, [BMIM]DMP and [TMGH]n-PrCOO.

An averaged value of relative activity was calculated for each enzyme which represents the overall stability of the enzyme in the presence of all studied ILs (last rows in Fig. 3 and 4). According to the averaged activity values, PhGH5 and 1_GH45+CBM were the most IL-tolerant enzymes in the presence of 15% (w/v) and 40% (w/v) of ILs. Both enzymes, PhGH5 and 1_GH45+CBM, originate from thermophilic organisms, i.e. from the extremophile bacterium *P. horikoshii* and from a thermotolerant fungus, respectively. For PhGH5, good IL tolerance has been reported previously. [17] In all cases, good thermostability alone did not result in good IL tolerance. The commercial endoglucanases CtGHX, TmGH5 and DtGHX from thermophilic bacteria *Clostridium thermocellum*, *Thermotoga maritima* and *Dictyoglomus turgidum*, respectively, had the poorest IL tolerance among the studied enzymes. Less than 20% of relative activity (averaged value) was retained in the presence of 15% (w/v) of the ILs. Endoglucanases TrGH7 and TrGH5 from the mesophilic and industrially important organism *Trichoderma reesei* retained 57% and 44% of activity (averaged values) in the presence of 15% (w/v) IL, respectively. TrGH7 (former EGI) was found to be generally more IL tolerant compared to the other endoglucanase TrGH5 (former EGII) from *T. reesei*, although TrGH7 structure is more labile at elevated temperatures. [30] In this study, the most IL-tolerant endoglucanases represented GH families 5 and 45.

Two structural variants of a 1_GH45 endoglucanase were included in the study, one carrying a family 1 carbohydrate binding module (CBM) linked to a core domain via a peptide linker (1_GH45+CBM) and the other being the same enzyme without a linker and a CBM (1_GH45) (Table 2). At low substrate consistency, CBMs are known to improve cellulase enzyme action on insoluble cellulosic substrates by concentrating the enzymes on substrate surfaces and promoting the formation of enzyme-substrate complexes. The CBM-free variant 1_GH45 reached 39% relative activity (averaged value) at 40% (w/v) IL concentration, whereas the corresponding enzyme with a CBM tolerated ILs significantly better (50%). In general, CBMs do not affect enzyme activity on soluble substrates [31], which

suggests that the better performance of 1_GH45+CBM is caused by other factors. The carbohydrate binding module in the enzyme 1_GH45+CBM is connected to the catalytic core domain through a linker peptide. The linker peptide is likely highly O-glycosylated in a fungal expression host. [32] O-glycans have been shown to increase the enzyme resistance to proteolytic cleavage and increase thermostability. [33] In this study the highly O-glycosylated linker peptide may have shielded the enzyme from the inactivating effect of ILs. When endoglucanase action on solid cellulosic substrate has been studied, ionic liquids are found to interfere with CBM interaction with cellulose. [34] Accordingly, CBM or linker-bound O-glycans may bring additional structural stability to an enzyme in the presence of ILs but the functional role of CBM in targeted cellulose binding may be compromised.

Screening of xylanases

The hydrolysis efficiency of five GH10 and GH11 xylanases were tested in 15% (w/v) and 40% (w/v) IL solutions (Fig. 5 A and Fig. 5 B, respectively). Enzyme tolerance to ILs varied greatly, although the GH10 xylanases (TfXYN10A, TmXYN10B, GH10) showed generally higher IL tolerance [23,35] over the GH11 xylanases (DtXYN11B, GH11). [24] Among the studied enzymes TfXYN10A was the most IL-tolerant enzyme retaining 100% and 48% of relative activity (averaged) in the presence of 15% and 40% IL, respectively. TfXyn10A retained its full activity even in the presence of 15% [P4444]AcO which was found to be a highly inactivating IL for the other enzymes. TfXyn10A was found to outperform the other xylanases almost in all conditions. This enzyme was earlier shown to have very low competitive inhibition in the presence of [EMIM]OAc, [EMIM]DMP and [DBNH]OAc. [36] The present study showed that this enzyme tolerates a large array of hydrophilic ILs. Among GH10 xylanases, the order of IL tolerance was TfXYN10A > GH10 > TmXYN10B and it remained the same in both 15% and 40% IL concentrations. Among the two GH11 xylanases DtXyn11B was more IL-tolerant compared to the pre-commercial GH11 xylanase.

Although enzyme thermostability has been correlated with IL-tolerance [17] the results obtained with GH10 xylanases highlight also that thermostability does not inherently implicate good IL tolerance. [24] Among the studied xylanases TmXYN10B was the most thermostable enzyme, but it was not the most IL-tolerant one. However, this enzyme has demonstrated its hydrolytic efficiency in the presence of IL at higher temperatures (80-100°C). [23]

In previous studies and in this study, family GH10 xylanases were generally found to be more IL-tolerant compared to family GH11 enzymes. [35] Earlier findings with [EMIM]AcO suggest that the IL interferes with substrate binding in the active site and this competitive inhibition may be more pronounced with family GH11 xylanases. [24] The difference in activity among these enzymes in the presence of ILs appears to be based on differences in the active site and was revealed by molecular docking studies between the enzyme and the IL cation. [35,36] Probably the narrow and deep active site of GH11 xylanases allows transient binding of a large amounts of $[EMIM]^+$ cations, thus preventing the binding of a substrate. Consequently, the activity is hindered by competitive inhibition posed by binding of the IL cation. [37] Jaeger and Pfaendtner (2013) proposed a mechanism based on molecular dynamics simulation that IL cations may bind to some places in the xylanase active site. [37] Later molecular docking studies revealed a much larger number of potential binding sites in the narrow active site canyon of GH11 xylanases [35], in which, a large part or even whole active site canyon could be filled with (transiently) bound IL cations. It was also observed that in the wider active site of GH10 xylanases, the binding of $[EMIM]^+$ cations is distributed in a more restricted area than in GH11 xylanases [35], which is in line with the conclusion of lower competitive inhibition in GH10 enzymes. Nevertheless, the high thermostability probably protects against the structural unfolding effect of ILs also in xylanases.

Comparison of the effect of ionic liquids on enzyme activity and fibre-swelling capability

Among the studied ILs, [DMIM]DMP was the most enzyme-compatible IL at 40% (w/v) consistency both with endoglucanases and xylanases (Fig. 3 and Fig. 5 B, respectively). In a previous study [DMIM]DMP has been identified as least inhibitory to the studied enzymes among four different ILs. [38] In this study, the imidazolium and phosphate based ionic liquids [DMIM]DMP, [EMIM]DMP, [BMIM]DMP and [BMIM]DBP shared a similar structural basis except that the anions and cations in the series have growing alkyl side chains (Table 1). There appears to be a clear trend that longer alkyl side chains in the IL structure led to lower compatibility with enzymes. This trend has previously been observed with a series of imidazolium- and phosphate-based ILs. [39] Within the series of imidazolium- and phosphate-based ILs, [BMIM]DBP, which carries the longest alkyl side chains both in the anion and cation was also least compatible with the studied endoglucanases and xylanases. Among the studied ILs, [P4444]AcO was clearly the most inhibitory to all studied enzymes, both endoglucanases and xylanases, except that TfXyn10A was highly active in it at 15% (w/v) IL concentration. Tetrabutylphosphonium [P4444] cation carries four four-carbon alkyl chain substituents and the IL is also highly alkaline in aqueous solutions (Table 1), which may be predictive of poor enzyme compatibility. In a recently published study [Chol]AcO has been found to be reasonably cellulase-compatible as compared to [EMIM]AcO. [40] In this study [Chol]AcO was also among the least toxic of the studied ILs. [TMGH]n-PrCOO has in a previous study been found to be more inactivating for *T. reesei* Cel5A than [EMIM]AcO [18], while the results in this study place [TMGH]n-PrCOO between the imidazolium-based ILs in terms of enzyme compatibility.

Interestingly, the fibre swelling capacity of the studied ILs appeared to follow the same trend as was the case for enzyme compatibility (Table 1, Fig. 3 and Fig. 4). Especially in the case of the imidazolium-based ILs, longer alkyl substituents on either the IL cation, anion or on both was detrimental to their capability to make visible changes to the fibre morphology,

meaning that the most enzyme-compatible ILs were also the most potential ones for fibre swelling. This can be seen as an encouraging result for making the IL and enzymatic treatment in a one-pot process. The observed effect of alkyl chain length in imidazolium ILs on cellulose-dissolving capacity is in good accordance with results published by Swatloski et al (2002) on a different set of cellulose-dissolving imidazolium ILs. [4]

Conclusions

The aim of this study was to find ionic liquid (IL) and enzyme combinations that may be used simultaneously or sequentially to modify pulp fibres. At 40% (w/v) IL concentration several IL-enzyme combinations retained more than 50% of activity. Endoglucanase from hyperthermophilic organism *P. horikoshii* was found to be highly stable in the presence of ILs together with a family GH45 endoglucanase (1_GH45+CBM) of fungal origin. Among the studied xylanases, GH10 *T. flexuosa* xylanase (TfXYN10A) was found to be the most IL-tolerant xylanase. In general, family GH10 xylanases were more IL-tolerant compared to family GH11 xylanases.

ILs with imidazolium- and phosphate-based ions were found to be most effective in fibre swelling. [EMIM]DMP and [DMIM]DMP with the shortest alkyl side-chains were found to be more effective compare to [BMIM]DMP and [BMIM]DBP with longer alkyl side-chains. ILs based on imidazolium cation and phosphate anion were also found to be enzyme-compatible if short-chain alkyl substituents were attached to the core molecules (such as in [DMIM]DMP). Longer alkyl substituents resulted in increased enzyme inactivation and lower fibre-swelling capacity. Most detrimental to enzyme activity were the ILs [BMIM]DBP and [P4444]AcO both rich in long chain alkyl substituents. As groups, the cellulases and xylanases responded partially differently to the presence of different ILs, and it was also noticed that thermostability alone was not indicative of good IL tolerance. For endoglucanases the most IL-tolerant enzymes were from families 5 and 45 whereas

xylanases from GH family 10 were the most IL-tolerant. For the integrated IL and enzymatic treatment of fibres, it can be considered a promising result that the most suitable fibre-swelling ILs also were the least inactivating ones.

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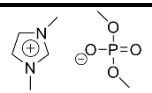
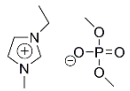
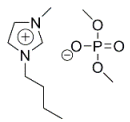
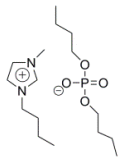
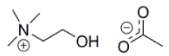
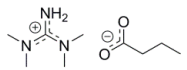
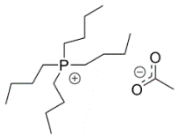
Table 1 Ionic liquids (ILs) and their effect on fibre swelling

Abbreviation	Full name	Chemical structure	Effect on fibre swelling[b]	pH in assay conditions[a]
[DMIM]DMP	1,3-Dimethylimidazolium dimethylphosphate		+++	6
[EMIM]DMP	1-Ethyl-3-methylimidazolium dimethylphosphate		++++	6.5
[BMIM]DMP	1-Butyl-3-methylimidazolium dimethylphosphate		++	5.5-6
[BMIM]DBP	1-Butyl-3-methylimidazolium dibutylphosphate		0	6-7
[Chol]AcO	Cholinium acetate		0	8
[TMGH] n-PrCOO	1,1,3,3-Tetramethylguanidinium butyrate		+	7.5-8

[P4444]AcO	Tetrabutylphosphonium acetate		+	9

[a] pH value was measured from a mixture of 40% IL in 50 mM sodium citrate buffer (pH 5) using pH indicator paper

[b] no effect (0), minor effect (+), major effect (++++), effect on dissolving pulp fibres was evaluated from light microscopy images. Examples of representative light microscopy images are available in the supplementary material (Fig. S6 and S7).

Table 2 Endoglucanases and xylanases used in ionic liquid compatibility screening.

Source organism	Activity	GH family	Short name	Source of enzyme[a]	Thermal stability
Trichoderma reesei	endoglucanase	5	TrGH5	*T. reesei*	T_{opt} ~55˚C, T_m 75˚C [30]
Trichoderma reesei	endoglucanase	7	TrGH7	*T. reesei*	T_{opt} ~55˚C, T_m 65˚C [30]
Pyrococcus horikoshii	endoglucanase	5	PhGH5	*E. coli*	T_{opt} ~100˚C [22]
Aspergillus niger	endoglucanase	12	AnGH12	Megazyme	T_{opt} 60˚C [b]
Bacillus amyloliquefaciens	endoglucanase	5	BaGH5	Megazyme	T_{opt} 60˚C [b]
Talaromyces emersonii	endoglucanase	5	TeGH5	Megazyme	T_{opt} 70˚C [b]
Thermotoga maritima	endoglucanase	5	TmGH5	Megazyme	T_{opt} 80 ˚C [b]
Clostridium thermocellum	endoglucanase	n.a.	CtGHX	Sigma	T_{opt} 70˚C [c]
Dictuoglomus turgidum	endoglucanase	n.a.	DtGHX	Sigma	T_{opt} 70˚C [c]

n.a.	endoglucanase	45	1_GH45	ROAL Oy	Enzyme from a thermotolerant fungal species
n.a.	endoglucanase	45	1_GH45+CBM	ROAL Oy	Enzyme from a thermotolerant fungal species
n.a.	endoglucanase	45	2_GH45	ROAL Oy	n.a.
n.a.	endoglucanase	5	GH5	ROAL Oy	n.a.
Thermopolyspora flexuosa	xylanase	10	Tf Xyn10	*E.coli*	T_{opt} 78°C [41]
***Thermotoga maritima* MSB8**	xylanase	10	TmXyn10	*P. pastoris*	T_{opt} 100°C [23]
Dictyoglomus thermophilum	xylanase	11	DtXyn11	*E.coli*	T_{opt} 90°C [24]
n.a.	xylanase	10	GH10	ROAL Oy	n.a.
n.a.	xylanase	11	GH11	ROAL Oy	n.a.

GH=glycoside hydrolase, n.a. = not available

[a]commercial source or expression host specified, enzymes provided by ROAL Oy are pre-commercial preparates

[b]according to manufacturer's product sheet

[c]temperature used in manufacturer's product sheet

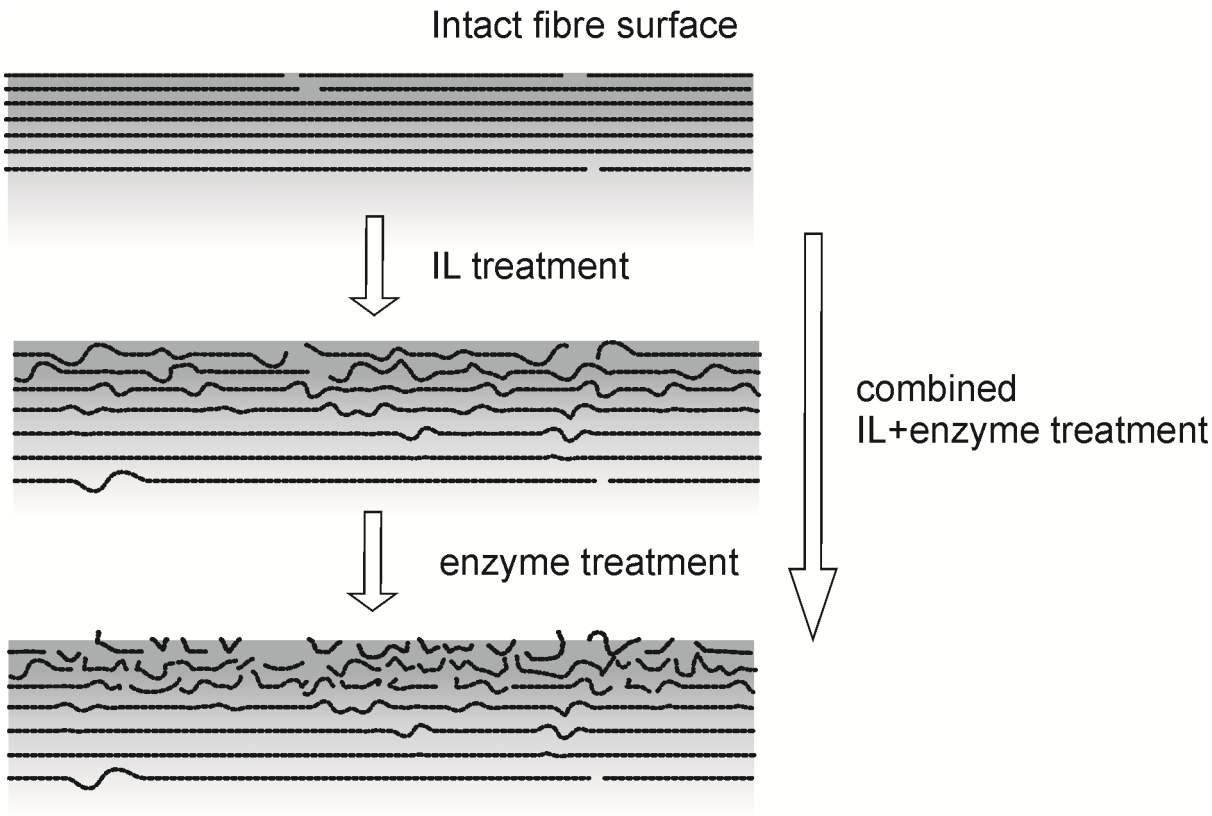


Fig. 1. Envisioned modification of cellulose pulp fibre surfaces by Ionic liquid (IL), enzyme treatment and their combinations.

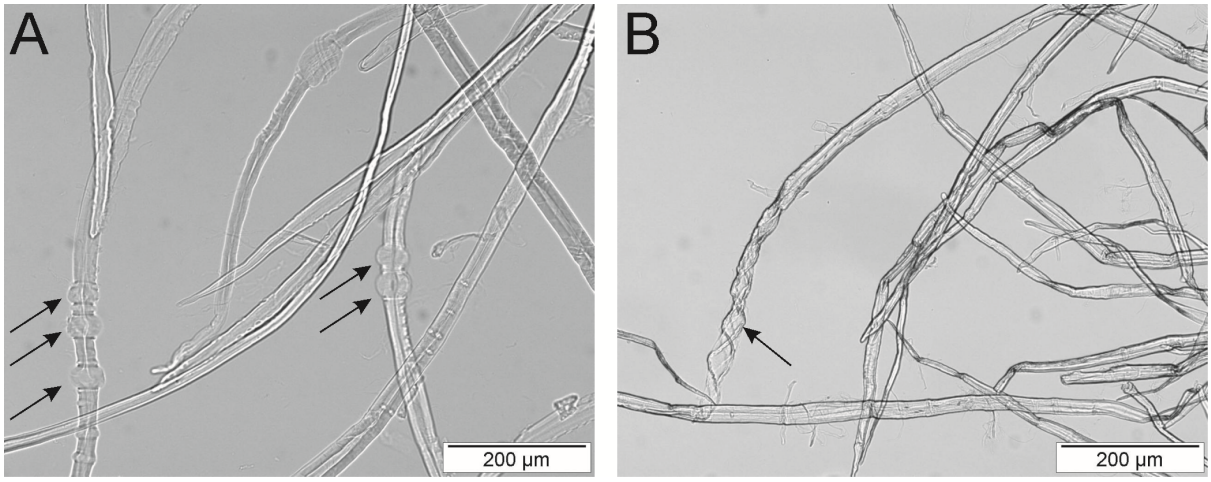


Fig. 2. Swelling of pulp fibres by ionic liquids. Light microscopy images of typical changes in pulp fibres after treatment with ionic liquid. A) Bleached softwood kraft fibre treated with 90% (w/w) [EMIM]DMP (3h, 80˚C), B) Dissolving pulp treated with 90% (w/w) [TMGH]n-PrCOO (3h, 80˚C). Fibre swelling with ballooning (red arrows in panel A) and unwinding (red arrow in panel B) mechanisms are shown.

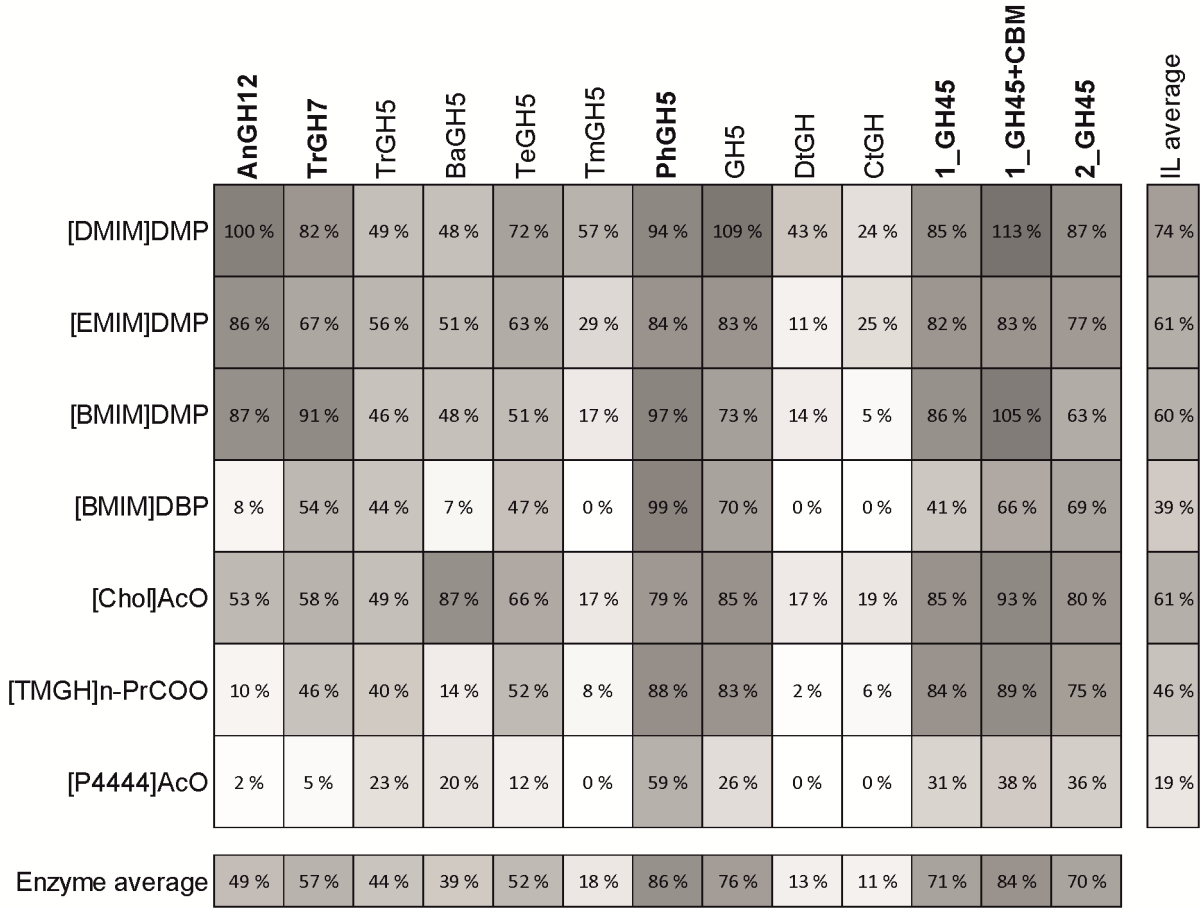


Fig. 3. Action of endoglucanases in 15% ionic liquid. Relative endoglucanase activities in 15% (w/v) ionic liquid after a 2 h assay. Reaction without added IL represents the 100% level. The darker the grey colour, the higher the enzyme activity in the IL solution. Enzymes marked with boldface were selected for subsequent screening round with 40% (w/v) ionic liquid.

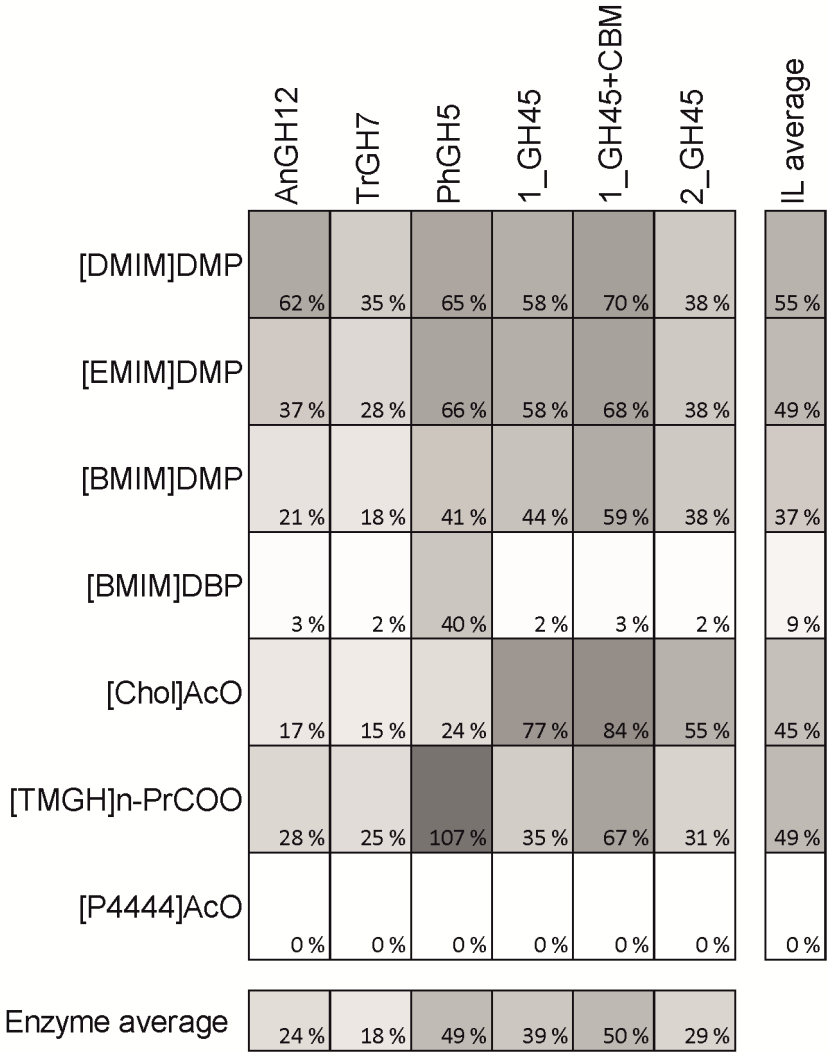


Fig. 4. Action of endoglucanases in 40% ionic liquid. Relative endoglucanase activities in 40% (w/v) ionic liquid after a 2 h assay. Reaction without added IL represents the 100% activity level. The darker the grey colour, the higher the enzyme activity in the IL solution.

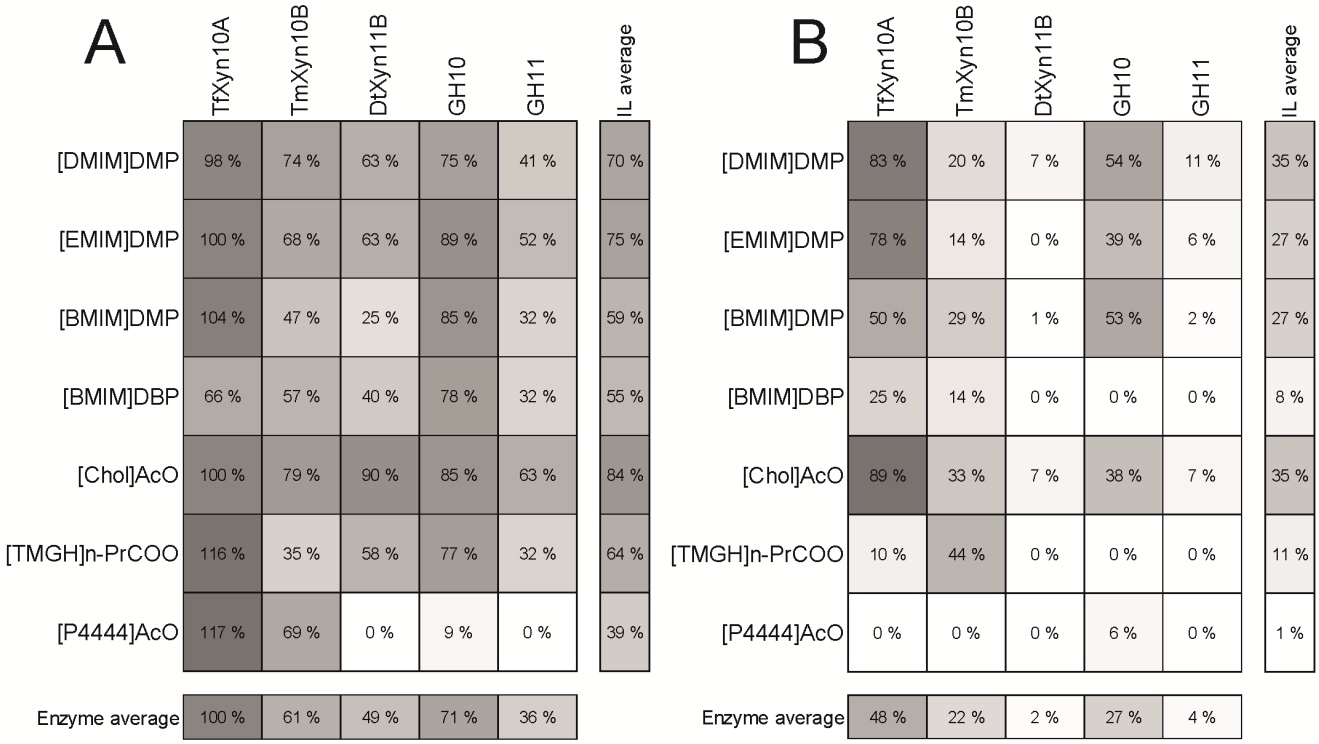


Fig. 5. Action of xylanases in 15% and 40% ionic liquid. Relative xylanase activities in A) 15% (w/v) and B) 40% (w/v) ionic liquid after a 2 h assay. Reaction without added IL represents the 100% activity level. The darker the grey colour, the higher the enzyme activity in the IL solution.