

Research Article

Effect of Ionic Liquids on Dissolution and Identification of Wood Polysaccharides

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Abstract

To study the polysaccharide composition of wood, we previously developed a new method suitable to analyse a large number of samples needing low amounts of biomass. The method relies on wood dissolution in ionic liquids; the resulting solutions are then immuno-labelled with monoclonal antibodies (mAbs) against plant cell wall polysaccharides epitopes. In the present work, we synthesized and tested several imidazolium and 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) based ILs for their ability to solubilize Douglas-fir wood. The couple times-temperatures has been tested for its ability to produce a dissolution of wood in IL. Polysaccharide solubilization has been then analyzed thanks to ELISA technique with a set of mAbs against hemicellulose, pectin and cell wall protein. We deduced that a wood treatment at 80°C with the 1-ethyl-3-methylimidazolium bromide allows a good polysaccharides release, especially for mannans and xylans tightly bound to cellulose and lignin, with preserving the structure and that even for pectin's.

Keywords: Ionic liquid; Polysaccharide; Cell wall; Wood

Introduction

Wood biomass is a complex interacting network composed of 3 major components present in the cell wall: cellulose, lignin's and hemicelluloses. Wood also contains in smaller quantities extractives, pectin's and proteins. The knowledge of the structure and content of each polymer is a prerequisite to understand their functions during plant development and adaptation but also to optimize their industrial applications. Whatever the objective, the purification and the analysis of cell wall compounds are complicated because their multiple molecular interactions. Methods for polysaccharides purification are based on the use of chemical, physical or enzymatic treatment or by a combination of several of them [1]. To overcome long and complex methods generally used for cell wall polysaccharides extraction and purification as to reduce polysaccharide modification during their extraction, we previously developed a new method to solubilize wood components thanks to ionic liquids [2]. An ionic liquid is an organic salt in which the ions are poorly coordinated, which results in these solvents being liquid below 100°C or even at room temperature [3]. At least one

ion has a delocalized charge and one component is organic which prevents the formation of a stable crystal lattice [4]. The combinations between anions and cation allow the synthesis of a number of potential IL equal to 1012 [5]. Because of their many attractive physicochemical properties such as high thermal stability, low vapour pressure and a good ability to dissolve polar, non-polar, organic or inorganic compounds [6,7], ILs have a wide range of applications among them catalysis [7], electrochemistry, organic synthesis or even biomass pretreatment to produce biofuels or chemicals components [8,9]. Indeed, it has been shown that some ILs, mainly imidazolium based ILs, are able to completely dissolve cellulose and more complex material such as wood [10-15]. On the basis of those information's, we have sought, in this study, to demonstrate that IL are not only suitable solution for complex biomass deconstruction, but that they are also solvent allowing us to directly identify and quantify wood polysaccharide by ELISA (Enzyme Linked Immunosorbent Assay). The aim being the improvement of our previously published method [2] by the choice of one IL among 6 (1-ethyl-3-methylimidazolium bromide ([Emim]Br), 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) 1-allyl-3-methylimidazolium bromide ([Allmim]Br), 1-allyl-3-methylimi-

dazolium chloride ([Allmim]Cl), butyl-1,8-diazabicyclo[5.4.0]undec-7-ene bromide (DBUC4Br) and butyl-1,8-diazabicyclo[5.4.0]undec-7-ene chloride (DBUC4Cl), able both to solubilize the Douglas-fir biomass while preserving the cell wall polysaccharide structure. DBU based ILs were chosen because of their good ability to dissolve lignin and imidazolium ILs for their wood dissolution efficiency [15-18]. To determine the compatibility of these ILs with immuno-detection technic, we first determined the sensitivity of 14 monoclonal Antibodies (mAbs) using commercial polysaccharides dissolved in the 6 ILs before applying this method on Douglas-fir sapwood sample.

Materials and Methods

Materials

1-methylimidazole (99%), bromoethane (98%), potassium acetate (99%), 1,8-diazabicyclo [5.4.0] undec-7-ene (99%), bromobutane (98%), allyl chloride (98%) were purchased from Alpha Aesar and stabilized allyl bromide (99%) and chlorobutane (99.5%) were purchased from Fischer Scientific. Ethyl acetate (99%) was purchased from Carlo Erba, dichloromethane (99.9%) from SDS, absolute ethanol from VWR and DMSO d₆ with 0.03% tetramethylsilane (Me₄Si, 99.8% D) from Eurisotop.

¹H spectra were recorded at 400.13 MHz with a Bruker DPX-400 spectrometer using DMSO(d₆) as solvent at δ room are expressed temperature in ppm. The chemical shifts (δ) are expressed in ppm with Me₄Si as the internal standard (δ). *J* values are given in Hz. For the analysis of the DBU-ILs, two-dimensional studies were carried out: a COZY (¹H -¹H correlated spectroscopy).

Ionic Liquid Synthesis

Two groups of ionic liquid have been synthesized (i) Imidazolium-based IL cations associated with 3 different anions (Figure 1) and (ii) two 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) ionic liquids (Figure 2).

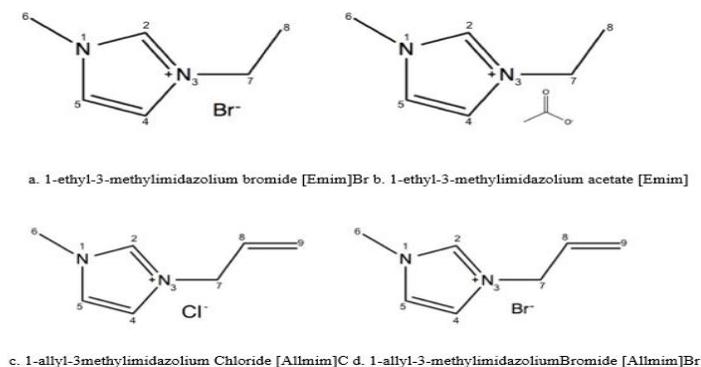


Figure 1: Imidazolium-based IL cations synthesized here differs by the length of the cation chain: a, b ethyl for [Emim] and c, d allyl for [Allmim] as well as by the counter ion: a, d Bromide; b, acetate, c, chloride.

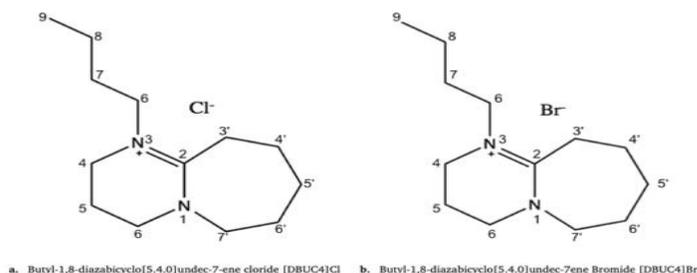


Figure 2: Butyl-1,8-diazabicyclo [5.4.0] undec-7-ene (a) chloride [DBUC4] Cl and bromide [DBUC4] Br.

1-Ethyl-3-Methylimidazolium Bromide, [Emim]Br

A mixture of 1-methylimidazole (41.06 g, 0.5 mol) and bromoethane (65.38 g, 0.6 mol) was stirred at 35°C for 20 h. The product was washed with ethyl acetate (2 × 100 ml) and dried in a vacuum. The [Emim]Br is obtained with a yield of 96% (91.72 g) and is in a form of a friable solid of light gray color (Figure 1A).

¹H NMR (DMSO d₆): δ_H 9.44 (1H, s, H-2), 7.92 (1H, t, H-5, *J*_{5,4} = 1.7), 7.81 (1H, t, *J*_{4,5} = 1.7), 4.22 (2H, q, *J* = 7.3), 3.87 (3H, s), 1.38 (3H, t, *J* = 7.3).

1-Ethyl-3-Methylimidazolium Acetate, [Emim]Ac

[Emim]Ac was synthesized through the ion exchange reaction according to Zhang L, et al. [19] adapted by Wang N, et al. [20]. Dried potassium acetate (37.75 g, 0.385 mol) was completely dissolved in 350 ml of ethanol before adding slowly [Emim]Br (70 g, 0.385 mol). The mixture is heating at 40°C for 5h. The solution is then put at -20°C during 1h before being filtered to remove the potassium bromide precipitate. The ethanol was evaporated and the product was then placed at -20°C for overnight and filtered again. The resulting pale yellow viscous liquid was obtained in 84% yield (52.1 g).

¹H NMR (DMSO d₆): δ_H 9.87 (1H, s, H-2), 7.85 (1H, t, H-5, *J*_{5,4} = 1.7), 7.76 (1H, t, H-4, *J*_{4,5} = 1.7), 4.21 (2H, q, H-7, *J*_{7,8} = 7.3), 3.87 (3H, s, H-6), 1.55 (3H, s, H-2'), and 1.41 (3H, t, H-8, *J*_{8,7} = 7.3).

1-Allyl-3-Methylimidazolium Chloride, [Allmim]Cl

This IL was synthesized according to [21]. Allyl chloride (11.65 g, 0.152 mol) was slowly added to 10 g (0.122 mol) of 1-methylimidazole (molar ratio 1.25:1) in a round-bottomed flask with a reflux condenser in an ice bath. After 12 h at 55°C, unreacted chemical reagents were removed by evaporation to obtain a yellow viscous liquid in 97% yield (16.94 g).

¹H NMR (DMSO d₆): δ_H 9.45 (1H, s, H-2), 7.83 (1H, t, H-5, *J*_{5,4} = 1.8), 7.81 (1H, t, H-4, *J*_{4,5} = 1.8), 6.06 (1H, ddt, H-8, *J*_{8,9B} = 16.9; *J*_{8,9A} = 10.3; *J*_{8,7} = 6.0), 5.35 (1H, dq, H-9A, *J*_{9A,8} = 10.3; *J*_{9A,9B} = 1.3),

5.30 (1H, dq, H-9B, $J_{9B,8} = 16.9$; $J_{9B,9A} = 1.3$), 4.90 (2H, dt, H-7, $J_{7,8} = 6.0$), 3.90 (3H, s, H-6).

1-Allyl-3-Methylimidazolium Bromide, [Allmim]Br

The allyl bromide (13.84 g, 0.114 mol) was slowly added to 7.5 g (0.092 mol) of 1-methylimidazole (molar ratio 1.25:1) in a round-bottomed flask with a reflux condenser in ice bath because of its high exothermic. After 48 h at 22°C, unreacted chemical reagents were removed using a rotary evaporator at 80°C at 1 h. The resulting brown viscous liquid was obtained in 96% yield (21.06 g).

¹H NMR (DMSO *d*₆): 9.24 (1H, s, H-2), 7.79 (1H, t, H-5, $J_{5,4} = 1.8$), 7.76 (1H, t, H-4, $J_{4,5} = 1.8$), 6.06 (1H, ddt, H-8, $J_{8,9B} = 17.0$; $J_{8,9A} = 10.3$; $J_{8,7} = 6.0$), 5.36 (1H, dq, H-9A, $J_{9A,8} = 10.3$; $J_{9A,9B} = 1.3$), 5.31 (1H, dq, H-9B, $J_{9B,8} = 17.0$; $J_{9B,9A} = 1.3$), 4.89 (2H, dt, H-7, $J_{7,8} = 6.0$), 3.89 (3H, s, H-6).

Butyl-1,8-Diazabicyclo [5.4.0] Undec-7-ene Chloride, [DBUC4] Cl

A mixture of DBU (40.8 g, 0.268 mol) and chlorobutane (29.77 g, 0.32 mol) was stirred at 70°C for 72 h followed by 20 h at 90°C. The resulting orange gum was washed with dichloromethane (2 × 100 ml) and dried in a vacuum. Yield: 96% (63.07 g).

¹H NMR (DMSO *d*₆): 3.62 (2H, m, H-2), 3.50 (2H, t, H-12, $J_{12,13} = 7.5$), 3.47 (2H, t, H-9, $J_{9,10} = 5.7$), 3.44 (2H, t, H-11, $J_{11,10} = 5.7$), 2.86 (2H, m, H-6H), 1.96 (2H, qn, H-10, $J = 5.7$), 1.66 (2H, m, H-4), 1.62 (4H, m, H-3 and H-5), 1.52 (2H, qn, H-13, $J = 7.5$), 1.30 (2H, sx, H-14, $J = 7.5$), 0.90 (3H, t, H-15, $J_{15,14} = 7.5$).

Butyl-1,8-Diazabicyclo [5.4.0] Undec-7-ene Bromide [DBUC4] Br

DBU (8.31 g, 0.055 mol) was introduced in a round flask

placed in an ice bath. 1.2 equivalent of bromobutane (0.066 mol, 8.98 g) was slowly added under stirring. The flask was then fitted with a reflux condenser and put in an oil bath. The oil bath temperature was slowly increased until 80°C for 2 h and 90°C for 6 h more. Unreacted chemical reagents were removed by evaporation at 80°C at 1 h to give a brown solid in 96% yield (15.11 g).

¹H NMR (DMSO *d*₆): 3.63 (2H, m, H-2), 3.50 (2H, t, H-12, $J_{12,13} = 7.4$), 3.48 (2H, t, H-9, $J_{9,10} = 5.8$), 3.45 (2H, t, H-11, $J_{11,10} = 5.8$), 2.86 (2H, m, H-6), 1.97 (2H, qn, H-10, $J = 5.8$), 1.68 (2H, m, H-4), 1.63 (4H, m, H-3 and H-5), 1.52 (2H, qn, H-13, $J = 7.4$), 1.30 (2H, sx, H-14, $J = 7.4$), 0.90 (3H, t, H-15, $J_{15,14} = 7.4$).

Wood Sampling and Dissolution

Wood samples (1-2 cm thick disks) were collected at 1.3 m from the trunk of a 20 year-old Douglas-fir tree (*Pseudotsuga menziesii*) in October at Gimel, France (45°18'N, 1°50'E). Once the bark removed, the softwood part of the disks was grounded into a fine powder (<250 μm) with a Danguomeau steel ball mill. Wood powder were then homogenized and stored in dry atmosphere until analysis.

Douglas-fir sapwood was dissolved at 10 mg per 5.23 mmol in each IL previously synthesized ([Emim]Ac, [Emim]Br, [Allmim]Br, [Allmim]Cl, [DBUC4] Br and [DBUC4] Cl) (5.23 mmol [Emim]Br correspond to 1 g [Emim]Br). Wood dissolution has been performed at 80°C and 100°C until complete solubilization of wood shucks. Dissolution advancement was followed by optical microscopy.

Polysaccharide Immunodetection

The polysaccharide identification is made thanks to an ELISA test with a set of 14 monoclonal antibodies (mAbs) as described (Table 1).

Antibody	Polymers recognition	Animal	Supplier	Reference
LM21	Mannan, glucomannan, galactomannan	Rat	PlantProbes	[22]
LM10	Un-substituted xylan, low substituted arabinoxylan	Rat	PlantProbes	[23]
LM11	Un-substituted xylan, low substituted arabinoxylan	Rat	PlantProbes	[24]
LM15	Xyloglucan XXXG	Rat	PlantProbes	[25]
LM25	Xyloglucan XLLG, XXLG, XXXG	Rat	PlantProbes	[26]
CCRC-M14	Rhamnogalacturonan I	Mouse	CarboSource	[27]
CCRC-M31	Rhamnogalacturonanane I	Mouse	CarboSource	[27]
LM5	Galactan	Rat	PlantProbes	[28]
LM6	Arabinan	Rat	PlantProbes	[29]
LM19	Un-esterified Homogalacturonan	Rat	PlantProbes	[30]
CCRC-M38	Un-esterified Homogalacturonan	Mouse	CarboSource	[27]

Table 1: Monoclonal list of antibodies (mAbs). Antibody, name code of the antibody; Polymer recognition, epitope or polysaccharide recognized by the antibody; Animal, immune animal use for antibody production; supplier manufacture or laboratory; reference paper.

Commercial polysaccharides (guar from *Cyamopsis tetragonoloba* (Sigma-Aldrich), β -(1-4) mannopentaoses (Megazyme), xylans from birchwood (Sigma-Aldrich), xyloglucans from tamarind seeds (Megazyme), pectin from citrus (Sigma-Aldrich), arabic gum (Sigma-Aldrich), karaya gum (Sigma-Aldrich), arabinans from sugar beet (Megazyme)) were dissolved in the 6 pure ILs (10 mg mL⁻¹) thanks to a heating treatment at 80°C for 3 h. All dilutions of commercial and Douglas-fir solubilized polysaccharides-IL were done in deionized water to decrease the viscosity of the IL-polysaccharide solution and facilitate an accuracy sample. ELISA were conducted as described by Pattathil and co-workers [27] with some minor modifications. Diluted polysaccharides-IL, wood-IL and control-IL (solution without commercial or wood polymers) solutions were applied to 96-well microplates (Nunc, ref 269620 ThermoFischer) over a broad concentration range (0.5 ng to 5 μ g for polysaccharides or 0.05 to 100 μ g for wood solutions). Microplates were incubated overnight at 37°C, then washed with 300 μ L Tris-Buffer Saline (TBS) (50 mM Tris-HCl, NaCl 150 mM, pH 7.6) before being coated with 300 μ L of 1% (w/v) nonfat dry milk in TBS for 1 h [31]. The blocking solution was discarded from the microplate well and replaced by 50 μ L of primary mAb (1:100 v/v) in TBS, then incubated for 4 h at room temperature. The supernatant was removed and wells were washed three times with 300 μ L of 0.1% (w/v) nonfat milk in TBS (wash buffer). Then, 50 μ L of diluted (1:5000 v/v) secondary peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG antibodies (Sigma-Aldrich), depending on the previously primary antibody used, were added to each well and incubated for 1 h. Wells were washed five times with 300 μ L of wash buffers. The revelation was done in 50 μ L of 1-Step Ultra TMB (3,3',5,5'-Tetramethylbenzidine) - ELISA Substrate Solution (Life Technologies) for 20 min before being stopped by adding 50 μ L of 0.5 N sulfuric acid. Absorbance has been read at 450 nm and 655 nm with a microplate reader (BMG-Labtech, FLUOstar Omega) [32].

Results and Discussion

Ionic Liquid Synthesis

Six ILs, [Emim]Ac, [Emim]Br, [Allmim]Br, [Allmim]Cl, [DBUC4]Br and [DBUC4]Cl, were synthesized to compare their efficiency for dissolving Douglas-fir sapwood. These ILs have been chosen because of their good ability to solubilize either cellulose, lignin or even wood as described in the literature [8,10-15]. The cation forming the ionic liquid are generally synthesized by alkylation of an atom bearing a lone pair of electrons (nitrogen, phosphorus or Sulphur). Imidazolium-based IL cations diversity (Emin, Allmin) lies on the length of the alkyl chains linked in it. The modification of the anion generally occurs in the final step by exchange between the latter and an alkali metals ion or alkaline salt. All ILs synthesized have been produced with more than 96% purity during a one-step synthesis proceeding as describe in ma-

terial and methods. To reduced IL viscosity, un-fluorinated anion has been chosen [33]. Nitrogen cation have been preferred because of their lower capability of decomposition, increasing their thermal stability and reducing their volatility [14].

Wood Dissolution

Two heating treatments (80°C, 100°C) were tested to dissolve Douglas-fir sapwood in the different ILs. After 72 h at 100°C, the wood biomass was totally dissolved by all them with the exception of the [DBUC4]Br and in a lesser extent by [DBUC4]Cl (Figure 3, Table 2).

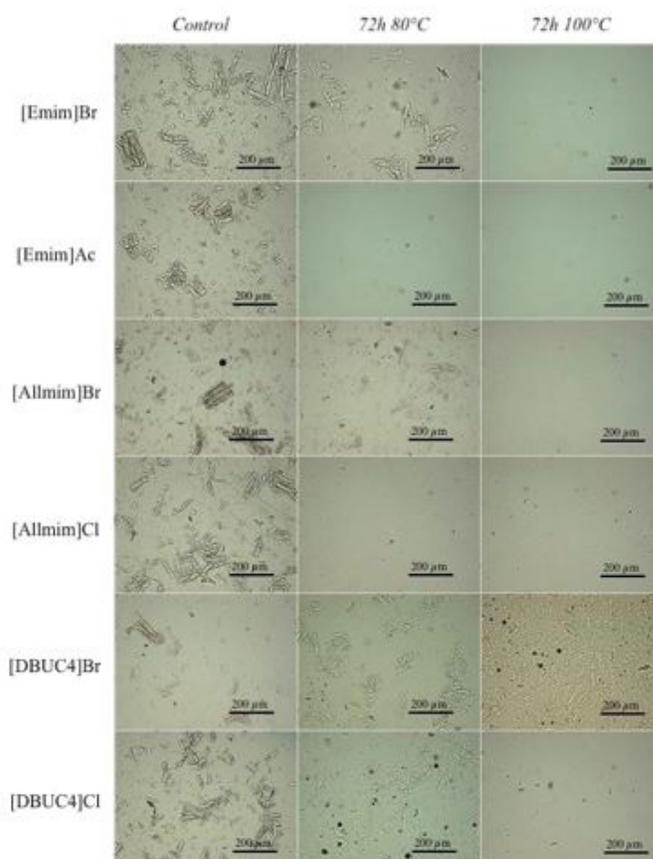


Figure 3: Optic microscopy of Douglas-fir wood chip after 72 h heating treatment (80°C, 100°C) [Emim]Ac, [Emim]Br, [Allmim]Br, [Allmim]Cl, [DBUC4]Br and [DBUC4]Cl. Control, picture take at t_0 before treatment.

As we can see, the temperature affects the dissolution rate probably through a decrease of the viscosity of the IL [34]. Moreover, high temperatures result also in the destabilization of hydrogen bonds of the cellulose. This explains that the higher is the temperature, the faster is the wood dissolution [35]. Comparing the efficiency of the cation (for [Allmim]Br, [Emim]Br and [DBUC4] Br), we demonstrate that the cation [Allmim] is more efficient to dissolve Douglas-fir sapwood than [Emim] and [DBUC4].

Indeed, the imidazolium cation with its relocate aromatic electron may interact with cellulose hydroxyl oxygen atoms, preventing cross linking of the cellulose molecules [11,36,37]. The interactions between the cation and the aromatic rings of lignin could also occur. Unlike [DBUC4] and [Emim], [Allmim] cation is the only one to present an electron displacement not only on the imidazole ring but also on its side chain, which may explain its superior efficiency to dissolve wood [35]. In terms of efficiency related to the anion, we found that acetate and chloride were more efficient than the bromide whatever the cation. Several polarity scales have been used to explain and predict solubility of biopolymers in solvents such as the Kamlet-Taft polarity parameters. These parameters are determined by measuring the UV-Vis spectra of dyes. In this system, is the parameter describing the hydrogen-bond basicity, is a measure of the solvents hydrogen-bond acidity and * of its interactions through bipolarity and polarizability [38]. In ionic liquid, is influenced by the anion [39]. For cellulose solubility, it was shown that ionic liquid that dissolve cellulose is characterized by high values for parameters describing solvent hydrogen-bond basicity [14], and that chloride anion has a higher hydrogen-bond basicity (0.97) than bromide anions (0.90) [40].

Ionic liquid	80°C	100°C
[Emim]Br	144	75
[Emim]Ac	54	28
[Allmim]Br	96	72
[Allmim]Cl	54	14
[DBUC4]Br	144	80
[DBUC4]Cl	96	75

Table 2: Time course (h) for a full Douglas-fir dissolution in [Emim]Ac, [Emim]Br, [Allmim]Br, [Allmim]Cl, [DBUC4]Br and [DBUC4]Cl of softwood chip after 2 heating treatments (80°C, 100°C).

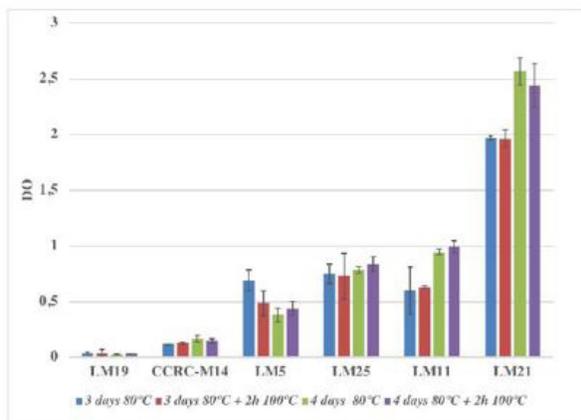
The complete dissolution of Douglas-fir wood in synthesized [Emim]Ac occur after 54 h at 80°C and 28 h at 100°C. Similar results have been published for red oak and pine wood powder (125-250 µm) after 25 and 46 h respectively, at 110°C [15]. The authors explain the time difference they observed between softwood (coniferous) and hardwood (deciduous) by the difference in composition between the two woods. Nevertheless, results with the our synthesized [Emim]Ac on Douglas-fir wood are closer to those obtained for the Red Oak, although Douglas-fir is a softwood. For [Allmim]Cl, complete dissolution of pine wood powder (8% w/w; particle size: 0.1-2 mm) occurs after 8 h at 80°C [18]. At this temperature, 54 h were needed to dissolve finer chips (<250 µm) of Douglas-fir wood (Table 2). Differences dissolution rate

between pine and Douglas-fir can be explained by the presence of water in our IL and wood biomass. Indeed, Kilpelainen and co-workers [18] worked under an inert atmosphere from freeze-dried wood and IL with a minimum water content (<1%). In our case, the wood was lyophilized but we have not sought to maintain the ILs before and during the dissolution process at their minimum water content, with the aim of simplifying, subsequently, the dissolution step of a large number of samples. Most hydrophilic ILs can absorb up to 10% of their water mass when they are exposed to the atmosphere at room temperature over 4 h [7,34]. One study showed that the presence of 3 to 5% of water completely inhibited *Miscanthus sp* solubilization in [Emim]Ac and [Emim]Cl [41]. In the case of cellulose, Swatloski and co-workers [42] observed a decrease in the solubility of this polymer in the presence of water in ILs, due to the competition between the cellulose and water to form hydrogen bonds with the IL anion.

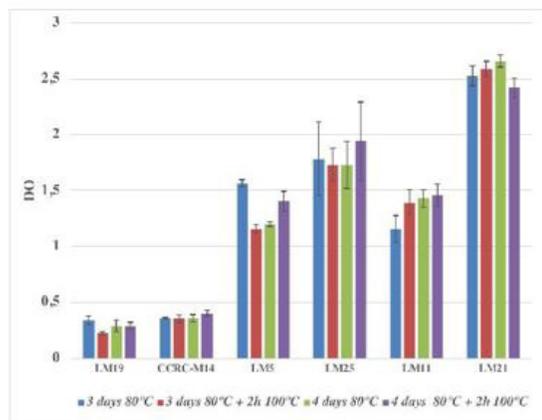
Polysaccharide Immuno-Detection

Once solubilized in the ILs, the wood cell wall polysaccharides were analyzed thanks to ELISA. About 200 monoclonal antibodies, with different specificities for the major cell wall polysaccharide families, are available. For one polymer family, a set of antibodies can each detect a specific pattern, allowing a detailed analysis of the wall features depending on the plant species [43]. In this study, 14 antibodies (Table 1) able to recognize the major hemicelluloses, pectins, and wall proteins (AGPs) epitopes have been selected. Purification or solubilization of cell wall polymers before immuno-labelling have the advantage, in theory, of making all polymers, and so their epitopes, freely accessible for antibodies [25]. Here, the ELISA method has been chosen in place of the dot-blot, that we had initially used to analyze the polysaccharide composition of walnut [2]. In fact, Douglas-fir being more colorful wood, dot-blot had some limitations such as pre-colored membrane before revelation. Before wood sample analyzing, some pure commercial polysaccharides have been first dissolved for 3 h at 80°C in each of the 6 synthesized ILs. Calibration curves established over concentration ranges from 0.5 to 2.5 µg of pure polymers show correlation coefficients always greater than 0.95. However, immune-detection of commercial polysaccharides show slight variation suggesting that the method is not absolute but IL-dependent (data not show). Polymers processing by the ILs may thus lead to degradation or chemical modification of them resulting in a reduction of their affinity to the polycarbonate matrix of the ELISA plates as to antibody recognition [44]. In the case of one of them, [Emim]Ac, the heating treatment during wood dissolution induced a slight pectin browning suggesting a Maillard reaction after β-elimination of pectin monosaccharide.

To ensure the thermo-stability of the polymers in IL, [Emim] Br has been chosen to make a comparative ELISA experiment with 6 of the 14 antibodies used for this study (Figures 4a and 4b).



a. 9th early wood ring (Douglas-fir sapwood)



b. 13th latewood ring (Douglas-fir heartwood)

Figure 4: Immuno-labelling intensity (OD) measurement for a set of 6 antibodies in function of time and temperature after Douglas-fir wood polysaccharide dissolution in [Emim]Br. Quantities of wood dissolved: 10 µg for CCRC-M14, LM19, LM25, 5 µg for LM5 and 0.5 µg for LM11 and LM21. Dissolution and ELISA were done in triplicate.

In [Emim]Br, 6 days at 80°C were necessary to completely dissolve the wood powder obtained with the Danguomeau steel ball mill (Table 2). In contrast, only 3 days in this IL were enough to completely dissolve sapwood and heartwood powder ground into liquid nitrogen in the ball-mill Retsch. This can be explained by a greater fineness of the ground powder in liquid nitrogen. After wood dissolution at 80°C or at 80°C followed or not by 2 h at 100°C, soluble polysaccharides could be well identified whatever the treatment and the antibodies use (Figures 4a and 4b). The standard deviation of repeated measurements is rather low (Figures 4a and 4b), even if some standard deviations are higher for some samples (for example LM11, 3 days at 80°C (Figure 4a); LM25 for all treatment (Figure 4b). Whatever, the method stays reproducible for all the antibodies. Regarding the results for the early wood (Figure 4a), immuno-labelling is comparable regardless of the dissolution treatment for CCRC-M14, LM25, LM19, LM11 and LM21 antibodies. The LM5 immuno-labelling is more intense after the 3-day treatment at 80°C in early wood (Figure 4a) as in latewood (Figure 4b) than the other treatments. For the sapwood, immuno-detection difference is also observed for LM21 and LM11 for which labelling are more intense after 4 days at 80°C followed or not by 2 hours at 100°C. However, the labelling differences between treatments are low, that allow us to suggest that time as temperature have a slight impact on Douglas-fir dissolution and polysaccharide immuno-detection.

The douglas-fir wood samples were then analyses after full dissolution in each of the ILs ([Emim]Ac, [Emim]Br, [Allmim]

Br, [Allmim]Cl, [DBUC4]Br and [DBUC4]Cl). In the case of [DBUC4]Br, low labelling was observed for all polymers (Figures 5a and 5b). In addition, some pectin and xyloglucan labelling disappear with a higher temperature dissolution (100°C against 80°C). The [DBUC4]Cl is one of our IL that has the best labelling rate for all polymers, including pectin. Despite these results, the [DBUC4]Cl and the [DBUC4]Br were not conserved for future studies because of the too high viscosity of them. More than solution manipulation, strong viscosity level reduced the efficiency of polysaccharide dissolution by decreasing the mobility of IL inside the wall. Moreover, wood cell wall polysaccharide liberation induced simultaneously an increase of viscosity in the final solution. Low labelling was observed for pectin after wood dissolution in [Allmim]Cl and [Emim]Ac at 80 and 100°C (Figures 5a and 5b). Wood dissolved in [Emim]Br and [Allmim]Br at 80°C present interesting labelling levels for all polymers. At a dissolution temperature of 100°C, some pectin labelling disappear in the case of [Emim]Br. Usually, for all the ILs, the labelling obtained for xylans and mannans are higher when the wood is dissolved at 100°C than at 80°C (Figures 5a and 5b). Mannan and xylan epitopes remain inaccessible to antibodies at lower temperature than 100°C (Figures 5a and 5b). Thus, a higher heating temperature allow a better dissociation of these hemicelluloses from other cell wall components. In softwood, (galacto)glucomannans are oriented parallel to the cellulose micro fibrils with which they exhibit strong interactions [45]. (Arabino)glucuronoxylans and (glucurono)arabinoxylans are linked to lignin via ester bonds at the uronic acids of their side chains [46,47].

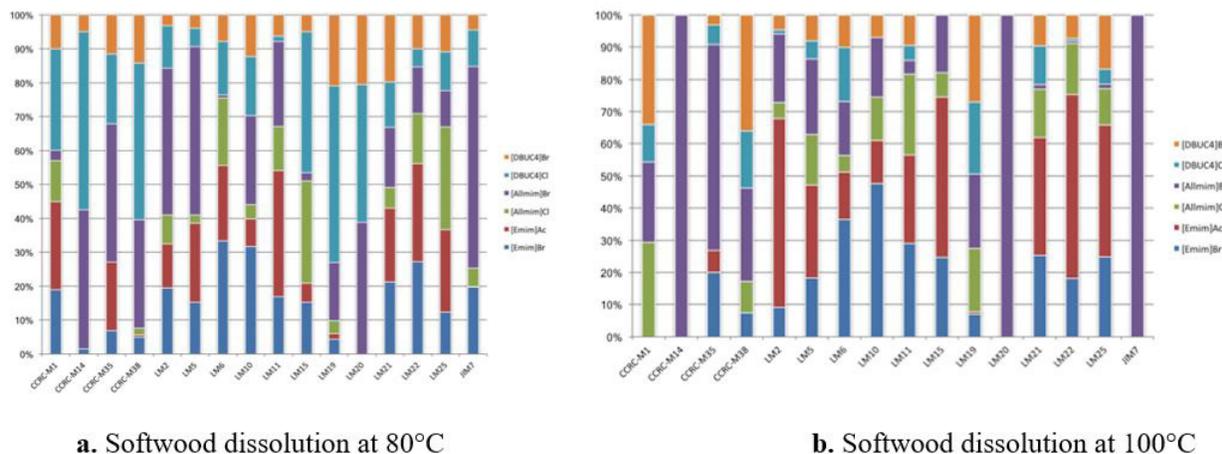


Figure 5: Combined effect of temperature (a, b) and IL ([Emim]Ac, [Emim]Br, [Allmim]Br, [Allmim]Cl, [DBUC4]Br and [DBUC4]Cl) on wood polysaccharide labelling efficiency for a set of 14 antibodies.

Hence, higher wood dissolution temperatures in ILs would better separate mannans and xylans from cellulose and lignin. However, it was found that pectin labelling decreased or disappeared by increasing the temperature from 80 to 100°C. Too high temperature treatment can lead to damage of polymers and/or of chemical epitope modifications. We noticed in fact, in the analysis by dot blot walnut wood dissolves in [Emim]Ac, a decreased labelling for Homogalacturonans (HGs) after a microwave treatment before conventional heating [48]. Socha and co-workers [48] studied immunolabeling profiles of a cereal, *Panicum virgatum*, after a sequential extraction of cell wall polymers with or without pretreatment with the [Emim]Ac (3 h at 160°C). They found that with the IL pretreatment, HG (CCRC-M38, JIM5, JIM7 among others), rhamnogalacturonan I (RGI) and some arabinogalactan epitopes disappeared. However, after a pretreatment with the [Emim]Ac, galactomannan epitopes appeared and xylan and xyloglucan epitope labelling are more intense. By solubilizing pine and oak wood, or microcrystalline cellulose in [Emim]Ac, Sun and co-workers [15] found minor degradation of the polymers after 16 h at 110°C (presence of oligosaccharides). It was also shown that the acetate anion of IL can easily be protonated with organic acids naturally present in the wood to form acetic acid [49]. Acidic conditions may cause partial hydrolyze of hemicellulose, cellulose and lignin [49] as well as HGs [50]. The acetate of [Emim]Ac can also acetylate polysaccharides and therefore hide some polymer epitopes. This has been shown for cellulose: after 20 min at 150°C, the cellulose has an acetyl degree of substitution of 0.017 [51]. A study has been conducted on the consequences of a total solubilization of bamboo powder in [Allmim]Cl after 12 h at 100°C [52]. These authors observed partial degradation of hemicelluloses and lignin. In addition, they also showed a potential cleavage of glucuronic acid substitution of 4-méthylarabinogluronoxylanes.

The Douglas-fir wood dissolved in [Allmim]Cl at 80°C has one of the lowest xylan labelling. The ILs are not the only solvents used to pretreat the lignocellulosic biomass in the optics to produce bio-ethanol. Other pre-treatments have been developed such as AFEX (ammonia fiber expansion). This pretreatment has been applied, more or less severely, on different plant biomass (including Douglas-fir wood) before sequential extraction of cell wall polymers and immunoassays [53]. More treatment severity increases the occurrence of substituted or unsubstituted of xylan epitopes, whereas the pectin epitopes (HG and RGI) disappear. These results show, as in our case, that is complicated to make accessible all wall polymers, even those strongly linked to cellulose and lignin, while not degrading those more easily solubilized such as pectin.

Conclusion

Different imidazolium or DBU based IL were synthesized and their efficiency to dissolve Douglas-fir wood powder by heating at 80 and 100°C were compared. [Allmim]Cl and [Emim]Ac are the most efficient. The dissolution process at 80°C in [Allmim]Br, [Emim]Br and [DBUC4]Cl need more time but would polymers seem to be less modified. We showed, moreover, with [Emim]Br that the IL/ELISA method is reproductive to analyses plant cell wall polysaccharide after IL dissolution.

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