CHAPTER 5

IONIC LIQUID PRETREATMENT OF BIOMASS FOR SUGARS PRODUCTION: DRIVING FACTORS WITH A PLAUSIBLE MECHANISM FOR HIGHER ENZYMATIC DIGESTIBILITY

In lignocellulosic biomass, the cellulose is embedded in the highly complex carbohydrate-lignin complex, which makes it recalcitrant for biological conversion using cellulases. Therefore, pretreatment is a crucial step to deconstruct the lignocellulosic biomass to release the fermentable sugars for biofuel conversion. Ionic liquids (ILs) are now being used for biomass deconstruction due to their unique tunable solvatochromic properties, i.e. hydrogen bond acidity, basicity, polarizability etc. This study will be useful to understand the driving factors responsible for biomass solubilization in ILs and helps to understand the correlation of properties of cation, anion size, Kamlet-Taft parameters, viscosity and surface tension with selective removal of xylan and/or lignin and sugar released after ionic liquid pretreatment. With that, this study is also helpful to have a deeper insight into the cellulose structural transformation (cellulose I to cellulose II) and explain the plausible mechanism for improved cellulose digestibility. The impact of these parameters on the digestibility can pave the way to customise the ILs to make biomass vulnerable to enzymatic attack.

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5.1 INTRODUCTION

As discussed in Chapter 4, cellulose is the most available and abundant biopolymer on earth can be used for the production of energy. Chapter 4 focused on pure cellulose (Avicel PH 101), which was used as a model substrate for ionic liquid dissolution and processing of regenerated cellulose for sugar yield.

Lignocellulosic biomass (LCB) is a natural and abundant source for production of fuels and chemicals in pursuit of renewable alternatives to fossil materials.[290-293] Cellulose in lignocellulosic biomass is embedded and associated with hemicellulose, lignin, extractives etc. via strong inter/intramolecular hydrogen bonding, covalent bonds and Van der Waals forces. These all together form a rigid and hard lignocellulosic complex mixture, which resist the accessibility of cellulose towards cellulase. Therefore, deconstruction of the LCB is an essential step to overcome the recalcitrance nature of LCB, as it breaks the carbohydrate-lignin complex. It disrupts variable the crystalline structure of cellulose[294-295], partially removes hemicellulose and lignin, hence, improving the enzymatic hydrolyzability.[10, 265, 296] However, most of the deconstruction methodologies target single components, *i.e.* either xylan or lignin.[297] For example, dilute acid pretreatment targets removal of xylan, whereas, alkali pretreatment targets the removal of lignin predominantly.[5, 114, 298] Hence, both of those methodologies are having their own advantages and disadvantages.[299] The acid treatment produces furfurals and acetic acid, which are inhibitors for enzymatic and fermenting yeast.[31, 282] Similarly, alkali treatment consumes a huge amount of alkali and may not be applicable for high solid loadings. Additionally, a lot of xylo-oligomers are formed in alkali treatment, which is highly detrimental to enzymes.[300] Biological methods are sluggish in nature and mechanical methods suffer from intensive energy and capital cost. Physicochemical methods require specialised equipment that can stand high pressures and high temperature.[301] Steam explosion is another method, which improves the surface area by removing the hemicellulose and some lignin making the biomass more accessible to the enzyme, but it also produces inhibitors.[52, 302] Moreover, the biggest disadvantages associated with all these methods are

consumption of a huge amount of enzyme resulting in, the high cost of sugars and ethanol.

Another method for biomass deconstruction is via Ionic Liquids (ILs), which act by dissolving the biomass and have demonstrated a great potential as green solvents.[183, 263, 303] IL pretreatment is receiving attention as a potential process that enables fractionation of LCB and produces high yields of fermentable sugars suitable for the production of renewable fuels like ethanol and butanol.[304-307] ILs are salts, comprising organic cations and organic or inorganic anions.[288, 308] They are endowed with high thermal and chemical stability, non-flammability, design ability, non-volatility, recyclability and excellent solvent properties to dissolve solutes of varying polarity.[309-310] Therefore, a deeper understanding of the driving factors and the mechanism of hemicellulose and lignin removal can help to design and improve the conventional pretreatment methods. Moreover, this process also has a good potential for commercialization.[277, 311-312] Lu et al. (2014)[313] have reported that dissolution and regeneration of microcrystalline cellulose in [C_4mim][Cl] dramatically accelerated the enzymatic saccharification. Sun et al. (2014)[268] have proposed that hydrophobic interactions between cations of ILs and cellulose (both are amphiphilic) are the predominant factors have reported that ionic liquids containing $[Lys]^-$ provide delignification (70-80% vs. 16-50%) compared to those obtained by the use of ILs containing $[OAc]^-$ and gave higher sugar yields (78-96% vs. 56-90%). Socha et al. (2014)[110] showed that [C_2mim][OAc] treated switchgrass released 90-95% of glucose and 70-75% of xylose after 72 h of cellulase hydrolysis. However, most of these studies are lacking on the evolution of factors and mechanistic pathway responsible for improved digestibility.[5, 314]

5.2 AIM OF THE STUDY

This study describes the actual lignocellulosic biomass as a feedstock for production of fermentable sugars. With that this study, we also find out the driving factors responsible for improved enzymatic digestibility of ionic liquid pretreated biomass. For this, five imidazolium-based ionic liquids, viz. [C_4mim][[BF_4],

[C_4mim][Cl], [C_2mim][Cl], [C_4mim][OAc] and [C_2mim][OAc] have been employed on mustard stalk and wheat straw as these LCBs, which are abundantly available across the globe. We have selected these two LCBs, depending upon their architectural difference. Mustard stalk belongs to stalk, more towards woody biomass, which has a different morphological, structural arrangement, while wheat straw, belongs to straws more towards relatively softer biomass. Although the chemical compositions are more or less close to each other, their response towards a particular pretreatment process may be quite different. The pretreatment of LCBs has been studied at 100 °C for 5 h and 130 °C for 2 h, as optimised for avicel in our previous work by Raj et al. (2016) as included in Chapter 4.[315] The effectiveness of pretreatment was evaluated by enzymatic hydrolysis of pretreated LCB. Cellulose deformation was analysed by the X-Ray diffractometry and FT-IR spectroscopy, whereas lignin and xylan removal was analysed by the compositional analysis. The effect of the properties of ILs, *i.e.* K-T parameters, viscosity and surface tension was evaluated to find their impact on enzymatic hydrolysis. In the present study, a comprehensive analysis of various driving factors related to the deconstruction medium and structural features of biomass responsible for improved enzymatic digestibility after pretreatment are investigated. Moreover, we have drawn a plausible mechanism of deformation of cellulose and structural factors responsible for improved enzymatic hydrolysis.

5.3 RESEARCH METHODOLOGY

Systematic steps involved in overall research methodology are shown in Figure 5.1. Imidazolium-based ionic liquids were used to pretreat the biomass at 10% solid loading at a temperature 100 and 130 °C for 2-5 h. After that, the cellulose-rich material was regenerated by addition of an antisolvent. Enzymatic saccharification of ionic liquid pretreated biomass was conducted for sugar recovery.

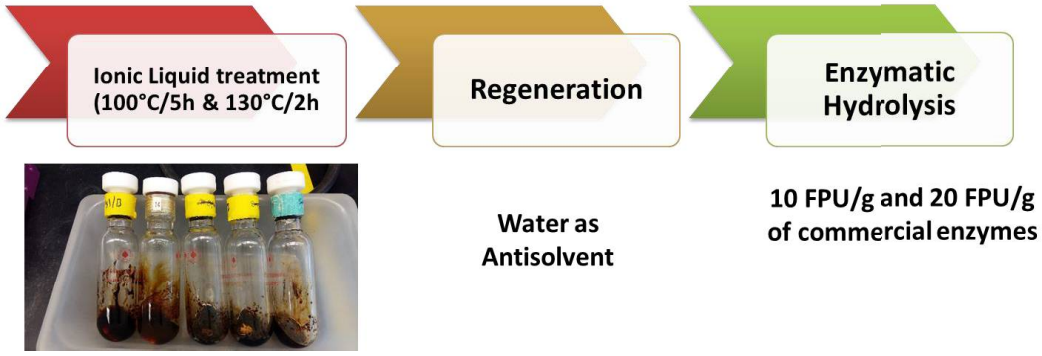


Figure 5.1 Systematic representation for biomass pretreatment and saccharification

5.4 EXPERIMENTAL

5.4.1 MATERIALS AND METHODS

All ILs and other chemical were purchased from Sigma-Aldrich, India as described in materials and methods section of Chapter 4.

Two different biomasses, i.e. Mustard (*Brassica juncea*) stalk and wheat straw (*Triticum aestivum*) was collected from the Mathura district (27.28° N 77.41° E) in Uttar Pradesh (North India) at the time of harvesting (March 2013). The biomasses were air dried, shredded to the particle size ~2 mm by knife mill and stored in airtight containers at 25 °C until further use. All the experiments were conducted using a single lot of biomass.

5.4.2 IONIC LIQUID PRETREATMENT

A 10% (w/w) biomass solution was carefully prepared by combining 1 g of biomass (on dry basis) with 9 g of particular IL in 50 mL two necked round bottom flasks at room temperature (25 °C). The flask was placed in the silicon oil bath preheated to the desired temperature (90-130 °C) and equipped with Teflon overhead magnetic stirring (300 rpm). The pretreatment reaction was allowed to proceed under sealed conditions for desired time periods (2 and 5 h) following which 35 mL of hot deionized water (50-60 °C) was slowly added to the sample

with stirring to precipitate the dissolved biomass, as hot water reduces the viscosity of ionic liquid and reduce the formation of gel-like phase during precipitation of cellulose-rich material (Ungurean et al., 2014). Then, the mixture of IL, water and regenerated biomass was cooled to room temperature and centrifuged at 10,000g for 10 min to separate the solid (recovered biomass) and liquid (IL and water) phases. The recovered biomass was washed five times with hot water (5×80 mL) to remove excess IL. The absence of IL in the recovered water washing was verified by measuring the absorbance at 240 nm using Shimadzu UV-Vis 2401 spectrophotometer.[316] The recovered pretreated biomass was analysed for a solid recovery and chemical composition. Solid recovery was determined by drying the biomass using a Sartorius moisture analyser at 105 °C and refers to the mass percentage of biomass (dry weight) recovered from the original biomass load and was calculated using Eq. 5.1.

$$Solid\ recovery\ \% = \frac{W_{Pret.}}{W_{Untreat.}} \times 100 \qquad Eq.\ 5.1$$

Where, $W_{pret.}$ is the mass of recovered pretreated biomass and $W_{untreat.}$ is the mass of untreated biomass.

5.4.3 COMPOSITIONAL ANALYSIS OF BIOMASS

All ILs pretreated and native biomass samples were analysed for glucan, xylan, arabinan, lignin and ash contents by two-step acid hydrolysis method developed by NREL LAP protocols no. TP-510-42618, 42619 and 42622.[317-318,319] 300 mg of dried biomass in 3 mL of 72% H_2SO_4 were incubated at 30°C while shaking at 300 rpm for 1 h. The material was diluted to 4% H_2SO_4 with distilled water and autoclaved at 121 °C for 1 h. The reaction was quenched by placing samples into an ice bath before neutralisation. The hydrolysate (20 mL) was neutralized using lime to pH 5.0, clarified through 0.45µm filter and subjected to sugar analysis using HPLC (Waters, Switzerland) fitted with Bio-Rad Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) coupled with refractive index

detector using Milli-Q water as mobile phase (0.6 mL min^{-1}, column temperature 75 °C) and refractive index detector. Furfural and hydroxymethyl furfural (HMF) were analysed with Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) column at 50 °C using a UV detector with 0.05M H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. Acid insoluble lignin (AIL) was calculated gravimetrically after ash correction as a dry weight percentage of the samples. The content of acid-soluble lignin was measured spectrophotometrically at 205 nm using an extinction coefficient of 110 $g^{-1}cm^{-1}$. The glucan and xylan content were calculated from the concentration of the corresponding monomeric sugars using an anhydro correction of 0.88 (or 132/150) for C-5 sugars and a correction of 0.90 (or162/180) for C-6 sugars. The removal of major components X (xylan or lignin) was calculated based on Eq. 5.2:

$$X\ Removal\ \% = 1 - \frac{W_{Pret.} \times C_{Pret.X}}{W_{Untreat} \times C_{Untreat.X}} \times 100\ \% \qquad Eq.5.2$$

Where, $W_{pret.}$ is the mass of pretreated biomass, $W_{untreat.}$ is the mass of untreated biomass, $C_{pret.x}$ is the composition (%) of X (xylan or lignin) in pretreated biomass and $C_{untreated.x}$ is the composition (%) of X in untreated biomass.

5.4.4 ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis of biomass samples was conducted using commercial cellulase preparation SacchariSEBC6 following NREL LAP protocol (LAP TP-510-43629).[317] All reactions were conducted at 10 % (w/v) biomass loading by placing 100 mg of biomass (dry weight) in 15 mL glass vials. The pH of the mixture was adjusted 5.0 using 0.05 M sodium citrate buffer (pH 5.0); supplemented with 0.02% sodium azide to prevent microbial contamination. The hydrolysis was performed at an enzyme dose of 10 and 20 FPU/g of pretreated biomass at 50 °C for 72 h in an incubator shaker at 150 rpm. Samples with a volume of 50 µl were withdrawn at 0, 2, 4, 6, 24, 48 and 72 h. Each sample was sealed and incubated for 5 min in a boiling water bath to denature the cellulase enzyme. The sample was

then centrifuged at 10,000 g for 5 min. 20 µl of the sample was diluted to 50 times by the addition of deionized water. The diluted supernatant was then filtered through a 0.45 µm nylon syringe filter. Sugar analysis was performed on Waters HPLC (Switzerland) using Aminex HPX-87P column coupled with the refractive index detector. Milli-Q water was used as mobile phase at a flow rate of 0.6 mL/min, with a column temperature of 75 °C. All the enzymatic saccharification experiments were conducted in triplicate. Glucose/xylose yield (%) of enzymatic hydrolysis was calculated based on total glucan/xylan present in pretreated mustard stalk/wheat straw using Eq. 5.3 and 5.4.

$$Glucose\ yield\ \% = \frac{Glucose\ released\ in\ enzymatic\ hydrolysis\ \times\ 0.9}{Total\ glucan\ present\ in\ preated\ biomass} \times 100 \quad \text{Eq. 5.3}$$

$$Xylose\ yield\ \% = \frac{Xylose\ released\ in\ enzymatic\ hydrolysis\ \times 0.88}{Total\ xylan\ present\ in\ preated\ biomass} \times 100 \quad \text{Eq. 5.4}$$

5.4.5 IL PROPERTIES AND BIOMASS CHARACTERISATION

ILs solvatochromic properties such as Kamlet-Taft parameters, viscosity, surface tension was determined as described in Material and methods section in Chapter 4.

Chemical and physicochemical analysis, i.e. Powder X-ray diffractometry, FT-IR analysis were conducted as described in Material and methods section in Chapter 4.

Statistical analysis was performed by one-way ANOVA followed Tukey's HSD post hoc tests for multiple comparisons using JMP software (SAS, US) and statistical significance were determined at 0.05 levels ($p \leq 0.05$).

5.5 RESULTS AND DISCUSSION

The objective of this study was to investigate the factors responsible for improved enzymatic hydrolysis of LCB after ILs pretreatment and to propose a

plausible mechanism for this improvement. In order to get a fair comparison between the effectiveness of ILs, two different reaction severities, *i.e.* pretreatment temperature as 100 and 130 °C and reaction time as 5 and 2 h respectively were chosen. All the experiments of enzymatic hydrolysis were performed at enzyme doses of 10 and 20 FPU/g for the pretreated mustard stalk. It was observed that high enzyme dose enhanced the initial rate of enzymatic hydrolysis. However, this enhancement reduced during the later phase of hydrolysis. For example, at 2, 4, 6, 8 and 24 h of enzymatic hydrolysis, high enzyme dose increased the glucose yield from 30.9, 45.0, 52.1, 79.6 and 94.6% to 42.1, 59.3, 68.4, 83.2 and 90.3% respectively for [C_2mim][OAc] treated biomass (at 130 °C/2 h) (Figure 5.2). Since, the glucose yield by 10 FPU dosage almost approached that by 20 FPU in 24 h, and considering the enzyme cost as a limitation for overall process economics, 10 FPU/g was chosen as the optimum dose of enzyme and discussed herein (Figure 5.3 and 5.4). To support the findings regarding the choice of IL for higher glucose yield, wheat straw, another highly abundant biomass with different structural architecture than mustard stalk has been also studied. Table 5.1 summarizes the solid recovery (% obtained on dry mass of regenerated biomass) across different pretreatment conditions, which varied in a wide range between 73.7 and 97.2% following the order: [C_4mim][BF_4]> [C_4mim][Cl]> [C_2mim][Cl]> [C_4mim][OAc]> [C_2mim][OAc]. [C_4mim][BF_4] resulted in the highest solid recovery (97.2%) followed by $[Cl]^-$ and $[OAc]^-$ based ILs.

Reduction in solid recovery is likely due to the removal/degradation of biomass components, viz. xylan, lignin and/or glucan during pretreatment. Solid recovery was assumed to be the consequence of solvation of ILs towards biomass. Higher total solids signify the lower solvation capability, *i.e.* a high solid recovery 97.2% by [C_4mim][BF_4] indicates its lowest biomass solvation capacity rendering the biomass almost in its native state. $[OAc]^-$ anion manifested the best solvation power with $[C_2mim]^+$ at 130 °C giving 73.7%, followed by $[C_4mim]^+$ leading to 78.3% solid recovery.

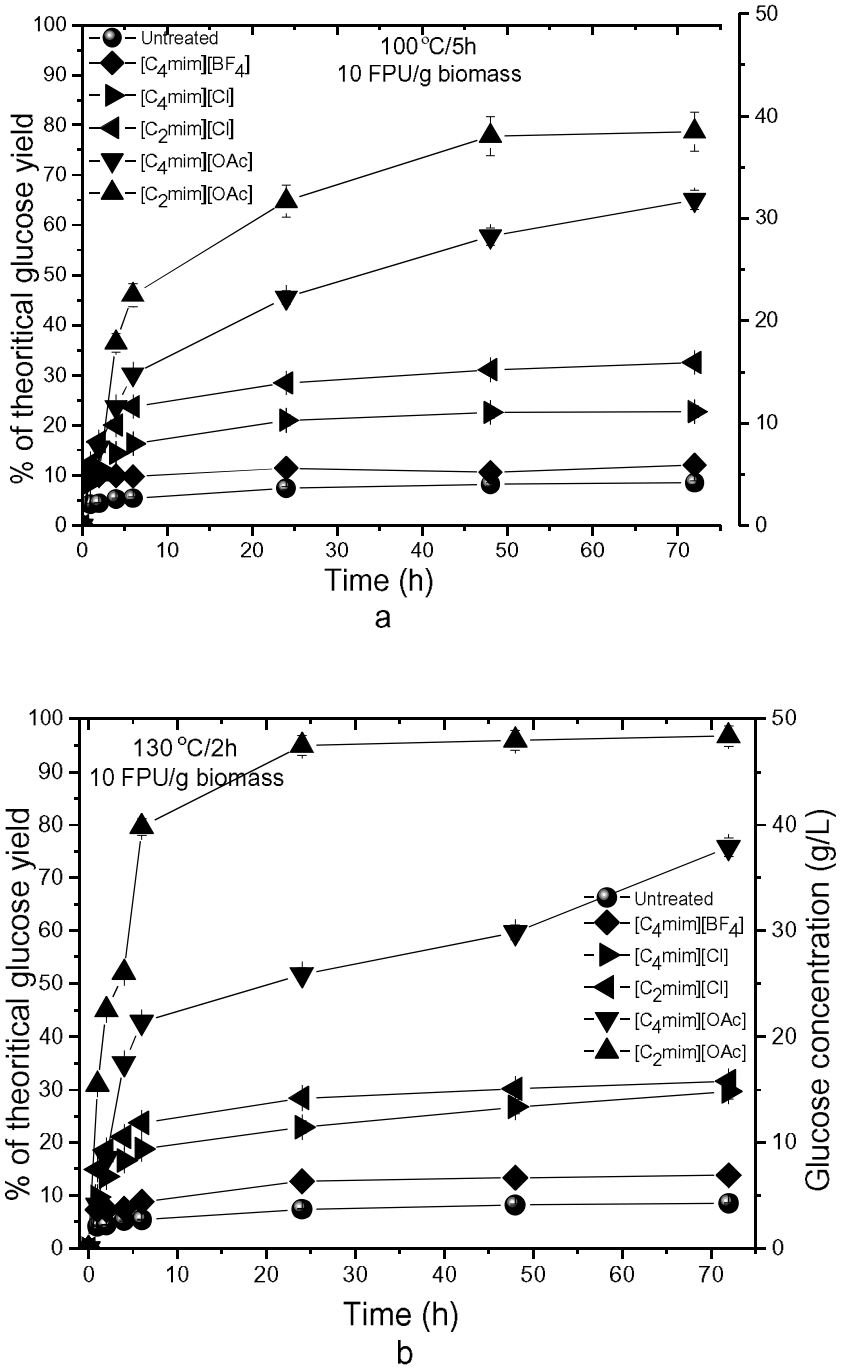


Figure 5.2 Glucan digestibility of the pretreated mustard stalk with two different pretreatment conditions: 100 mg untreated/pretreated biomass, 0.8 mL of sodium citrate buffer, 0.05M, 10 FPU/g of pretreated biomass of SacchriSEBC6, 50 °C, 150 rpm, time, 72 h). **(a)** 100 °C/5 h; **(b)** 130 °C/2 h

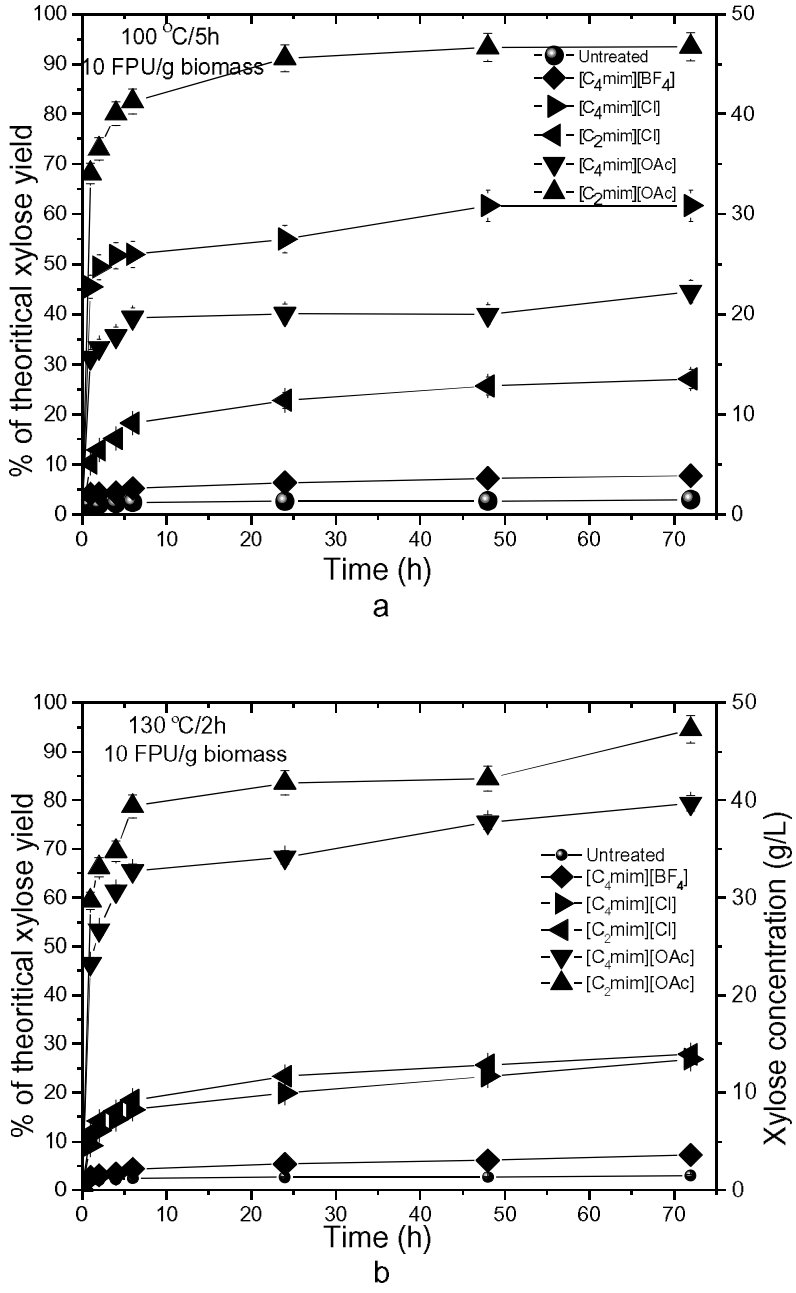


Figure 5.3 Xylan digestibility of the pretreated mustard stalk with two different pretreatment conditions: 100 mg untreated/pretreated biomass, 0.8 mL of sodium citrate buffer, 0.05M, 10 FPU/g of pretreated biomass of SacchriSEBC6, 50 °C, 150 rpm, time, 72 h). **(a)** 100 °C/5 h; **(b)** 130 °C/2 h.

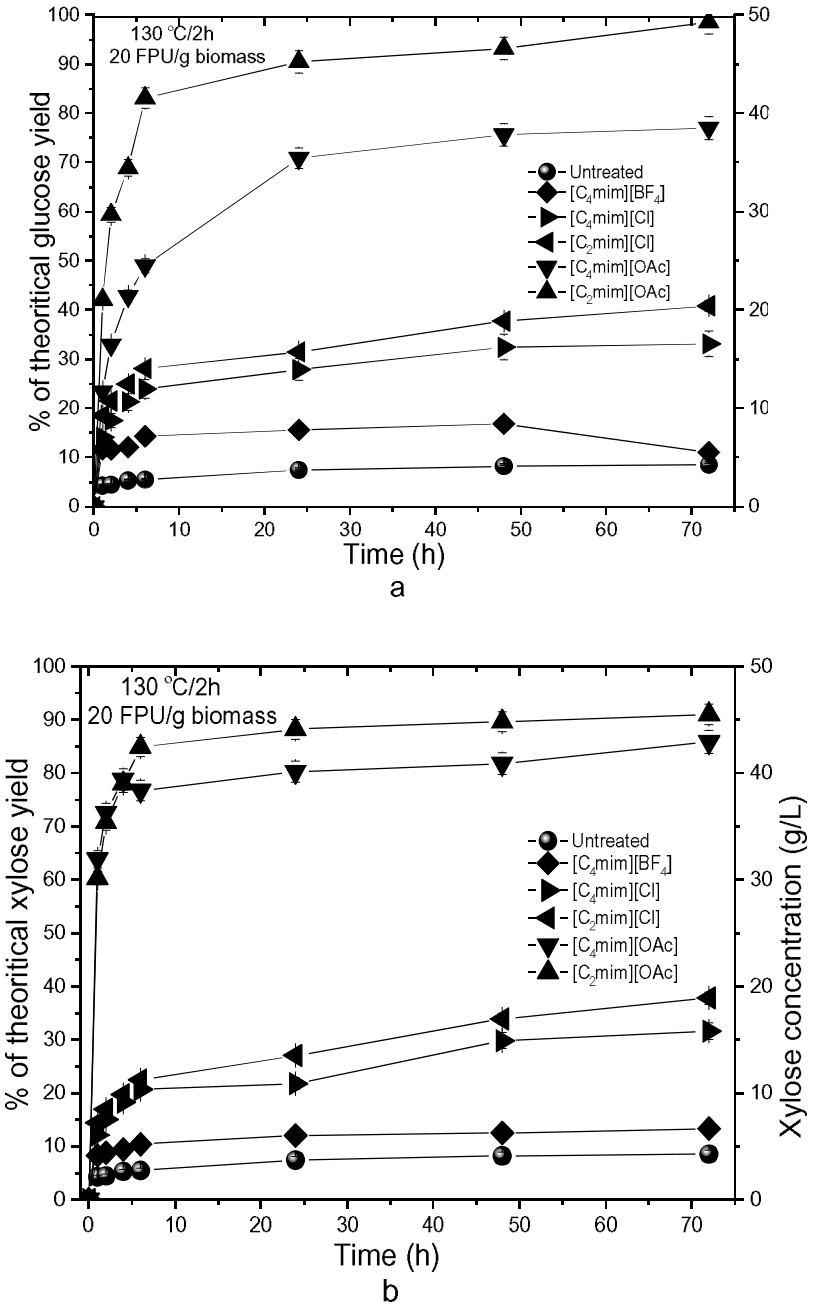


Figure 5.4 Glucan and Xylan digestibility of the pretreated mustard stalk with two different pretreatment conditions: 100 mg untreated/pretreated biomass, 0.8 mL of sodium citrate buffer, 0.05M, 20 FPU/g of pretreated biomass of SacchriSEBC6, 50 °C, 150 rpm, time, 72 h); 130 °C/2 h.

Table 5.1 Compositional analysis untreated and ILs pretreated biomass

Pretreatment			Composition of pretreated biomass (%)					Removal after pretreatment (%)[b]	
ILs	*T/t*	Solid Recovery (%)[a]	Glucan	Xylan	Lignin	Ash	Acetate[c]	Xylan	Lignin
Mustard Stalk	**N/A**	**NA**	**39.94±0.3**	**22.3±0.5**	**20.8±0.5**	**1.3±0.1**	**2.9±0.4**	**N/A**	**N/A**
[C_4mim][BF_4]	100/5	97.2±0.2^{A}	38.7±0.5^{A}	20.9±0.4^{A}	20.9±0.4^{A}	0.05±0.0	2.8±0.4	4.4±0.1^{A}	2.1±0.5^{A}
	130/2	96.2±0.2^{A*}	39.2±0.2^{A*}	21.9±0.4^{A*}	19.9±0.2^{A*}	0.05±0.01	2.7±0.6	9.1±0.3^{A*}	6.8±0.2^{A*}
[C_4mim][Cl]	100/5	80.2±0.5^{B}	38.9±1.0^{A}	20.3±0.9^{A}	21.7±0.2^{A}	1.3±0.2	0.4±0.9	27.4±0.5^{B}	1.2±0.1^{A}
	130/2	78.7±0.6^{B*}	39.5±0.8^{A*}	19.3±0.6^{B*}	20.0±0.3^{A*}	0.2±0.4	0.5±0.01	32.2±0.2^{B*}	8.2±0.4^{A*}
[C_2mim][Cl]	100/5	83.4±0.2^{C}	40.5±0.7^{A}	21.8±0.8^{A}	22.6±0.5^{A}	1.3±0.2	0.6±0.01	19.2±0.2^{C}	9.6±0.5^{B}
	130/2	81.9±0.9^{C*}	40.5±0.2^{A*}	20.7±0.2^{B*}	20.4±0.5^{A*}	0.1±0.04	0.6±0.02	24.5±0.5^{C*}	19.8±0.3^{B*}
[C_4mim]OAc]	100/5	81.3±0.4^{D}	45.4±0.8^{B}	11.2±0.3^{B}	23.8±0.4^{B}	0.2±0.01	0.7±0.01	64.0±0.5^{D}	7.2±0.5^{C}
	130/2	78.3±1.0^{B*}	48.8±0.2^{B*}	9.4±0.8^{C*}	20.4±0.5^{A*}	0.3±0.01	0.6±0.01	75.4±0.6^{D*}	23.1±0.6^{C*}
[C_2mim]OAc]	100/5	80.8±0.1^{D}	46.7±0.5^{B}	8.5±0.5^{B}	24.5±0.6^{C}	0.1±0.001	0.6±0.01	69.5±0.6^{E}	5.1±0.4^{D}
	130/2	73.7±0.4^{D}	50.1±0.7^{B*}	8.7±0.5^{C*}	22.5±0.4^{B*}	0.05±0.01	0.5±0.02	71.4±0.6^{E*}	20.4±0.9^{B*}
Wheat Straw	**N/A**	**NA**	**34.6±1.1**	**22.5±1.9**	**17.2±1.0**	**10.5±0.9**	**3.3±0.8**	**N/A±**	**N/A**
[C_4mim][Cl]	100/5	89.2±0.4^{P}	34.9±0.2^{P}	18.1±0.5^{P}	17.4±0.2^{P}	4.1±0.5^{P}	2.7±0.5	28.3±0.2^{P}	9.9±0.8^{P}
	130/2	85.0±0.5^{P*}	35.1±0.9^{P*}	17.3±0.5^{P*}	17.2±0.3^{P*}	3.7±0.8^{P*}	2.5±0.8	34.7±0.6^{P*}	14.9±0.5^{P*}
[C_2mim]OAc]	100/5	76.7±0.4^{Q}	41.0±0.8^{Q}	25.9±0.5^{Q}	15.5±0.3^{Q}	3.6±0.9^{P}	1.9±0.1	11.3±0.8^{Q}	42.9±0.4^{Q}
	130/2	62.0±0.3^{Q*}	42.1±0.8^{Q*}	14.9±0.2^{Q*}	13.7±1.2^{Q*}	3.6±0.3^{P*}	0.7±0.02	58.9±0.2^{Q*}	50.6±0.4^{Q*}

[a]Solid recovery (%) = (Mass of regenerated biomass on oven dry basis)/(Mass of initial biomass on oven dry basis)×100.
[b]Xylan/lignin removal is the percentage of xylan/lignin in pretreated biomass (calculated based on xylan/lignin present in untreated biomass. [c]Acetate (%) was calculated using 0.983 conversion factor (acetic acid to acetate on extractive free basis). The sum of the total is less than 100; another component may non-sugars, water soluble and acetate (<2%).
All experiments were conducted in triplicate and the mean is reported with ±S.D. Values in the same column for same pretreatment temperature with different superscripts letter indicate the significance difference at $P \leq 0.05$. Letter with a star (*) superscript show statistical significance at 130 °C.

5.5.1 IL PROPERTIES VIS-A-VIS ENZYMATIC HYDROLYSIS

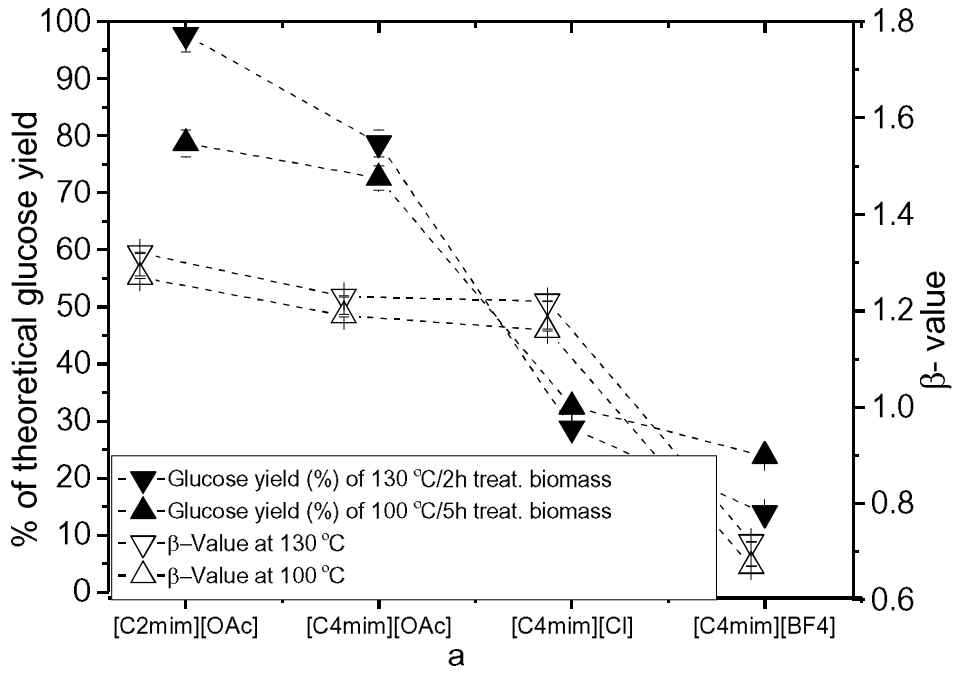
5.5.1.1 KAMLET–TAFT PARAMETER

ILs are known to solubilize cellulose by disrupting the crystal lattice as a result of hydrogen bond formation with the donor-OH groups within the cellulose.[313] K-T parameter of ILs was determined to find out its impact on the overall process. There are three properties under K-T parameters: β-value (hydrogen bond basicity), α-value (hydrogen bond acidity) and π^*(polarizability of ILs).[286] The β and α-value, respectively, indicate the solvent's ability to donate electron density to form the hydrogen bond and hydrogen bond acceptor ability of a given solvent, while the π^* value measures the residual polarity of the solvent after the hydrogen-bonding effects have been removed. As we have discussed before in Chapter 4, Table 4.3 that β-values ranged between 0.67 and 1.27 at 100 °C, with an increasing trend from $[BF_4]^-$ to $[Cl]^-$ to $[OAc]^-$ based ILs and seem to be governed primarily by the anion. For example, $[C_2mim][OAc]$ and $[C_4mim][OAc]$ showed the highest β-values (1.27 and 1.19) followed by $[C_4mim][Cl]$ as 1.16 and $[C_4mim][BF_4]$ as 0.67 at 100 °C. At 130 °C, a slight increase of β-values with similar trends was observed. From β-values shown in Table 4.3 in Chapter 4, it is observed that an increase in β-values was faster for $[OAc]^-$ containing ILs with temperature. Since, $[C_2mim][Cl]$ IL has a high melting point (*ca.* 75 °C), its parameters could not be determined. Figure 4.8 in Chapter 4 showed that besides the nature of anion, *i.e.* $[OAc]^-$, the length of alkyl chain moiety also contributed to the β-values with $[C_2mim]^+$ having a higher value (1.27) than $[C_4mim]^+$ (1.19) at 100 °C. Similarly, at 130 °C, $[C_2mim]^+$ and $[C_4mim]^+$ with $[OAc]^-$ showed 1.32 and 1.23 β-values respectively. The reduction in H-bonding capacity (β-values) of an anion with an increase in alkyl chain length of cation may be attributed to the fact that, a longer alkyl chain leads to the larger molecular size, apparently lowering down anion concentration. Similar observations have been also reported in literature.[262]

H-bond formation between ILs and cellulose is expected to disturb the intact cellulose structure itself as well as its interaction with the hemicellulose and lignin

of the LCB. The consequence of which was observed when enzymatic hydrolysis of the pretreated mustard stalk was conducted (Fig. 5.2 and 5.3). Figure 5.3 showed the time course of enzymatic hydrolysis, which showed that the initial rate of hydrolysis is a discriminating factor. For example, sugar release with [C_2mim][OAc] treated biomass at 130/2 h and 100/5h shows a sharp rise in hydrolysis within six hours of reaction, i.e. 130/2h treated mustard stalk gave 79.6% of glucose yield as compared to 100/5h treated mustard stalk (44.3%) within six hours respectively.

A correlation between the glucose and xylose yield and the β-values of ILs is presented in Figure 5.5a and 5.5b. The biomass pretreated with ILs having higher β-values yields higher glucose after enzymatic hydrolysis, which shows higher hydrogen bond basicity coincided with the higher hydrolysis rate, although exceptions, such as $[BF_4]^-$ were apparent, since, $[BF_4]^-$ is a non-coordinating anion, which does interact poorly with cellulose.



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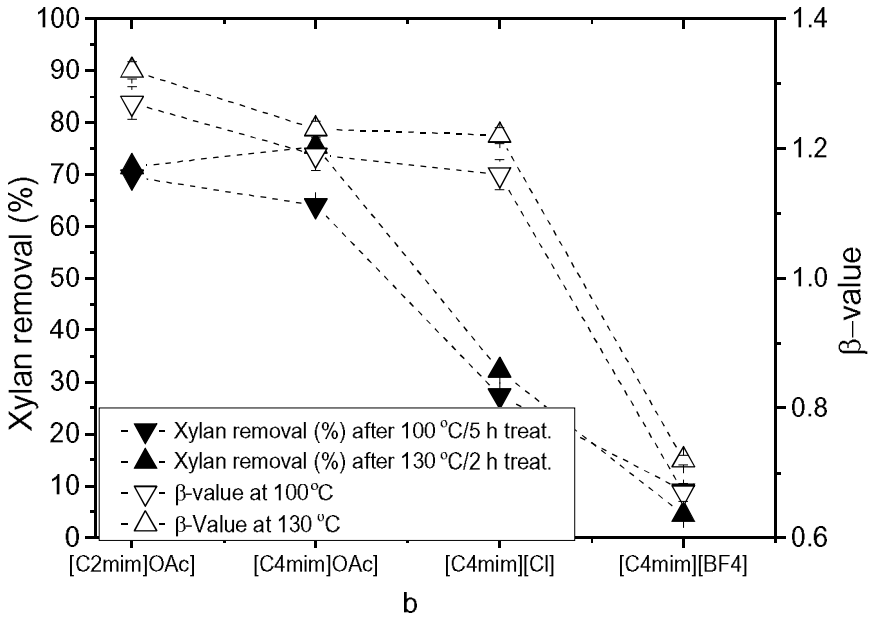


Figure 5.5 (a) Impact of β-value on glucose yield (%) after 72 h enzymatic hydrolysis with 10 FPU/g of SaccharISEBC6 enzymes; **(b)** Impact of β-value on xylan removal (%) of the pretreated mustard stalk.

Table 5.2 shows the glucan/xylan recovery for all ILs treated biomass calculated based upon glucan/xylan present in native and pretreated biomass and varied from 89.8 to 95.7 and 28.8 to 91.2% respectively. Although the glucan recovery for $[BF_4]^-$, $[Cl]^-$ and $[OAc]^-$ containing ILs appears to be the same, the glucose yield upon enzymatic saccharification is remarkably different. Similarly, glucose/xylose yields obtained after 72 h of enzymatic hydrolysis and were calculated based upon glucan/xylan present in pretreated biomass. Overall glucose/ xylose yield represents the yield considering whole process (both pretreatment and enzymatic hydrolysis). Keeping the cation moiety as $[C_2mim]^+$ and temperature as 100 °C, $[OAc]^-$ was found to be most effective in increasing the accessibility of mustard stalk to cellulases yielding 78.7% glucose, followed by $[Cl]^-$ which released 32.5% glucose. The effect of increasing the pretreatment temperature from 100 to 130 °C was most pronounced for $[C_2mim][OAc]$ both for mustard stalk and wheat straw increasing the glucose yield from 78.7 to 97.7% and 61.9 to 98.7% respectively. As shown in Table 5.2, the glucose yield of the biomass pretreated

with different ILs at 100 °C/5h, i.e. [C_2mim][OAc] (78.7%) and [C_4mim][OAc] (72.6%) are higher than [C_2mim][Cl] (32.5%) and [C_4mim][Cl] (23.8%). Similar trends were also observed for biomass pretreated at 130 °C/2h. Higher β-value of [C_2mim][OAc] (1.32), [C_4mim][OAc] (1.23) at 130 °C results in higher glucose yield, *i.e.* 97.7 and 72.8%, than the [C_2mim][Cl], [C_4mim][Cl] (1.22) treated biomass resulting in 31.5 and 28.7% yields respectively and hence, reflects that higher β-value results in higher glucose yields. Upon a critical analysis of the data presented in Table 4.3 in Chapter 4; pertaining to α and π^*-values, shows that π^*-values ranged between 0.91 and 0.99 at 100 °C. The α-values ranged between 0.59 and 0.72 at 100 °C and was observed to increase with an increase in pretreatment temperature. The α-value of [C_2mim][OAc] with smaller alkyl chains is higher (0.72), compared to [C_4mim][OAc] (0.60), [C_4mim][Cl] (0.72) and [C_4mim][BF_4] (0.59) at 100 °C. Also, [C_2mim][OAc] gave 78.7% of glucose yield, while, [C_4mim][OAc] and [C_4mim][BF_4] with longer alkyl chain and lower α-values (Table 4.3, Chapter 4), resulted only in 72.6 and 12.1% glucose yield. This also shows the effect of alkyl chain on glucose yield keeping the anion $[OAc]^-$ constant. Interestingly, [C_2mim][OAc] and [C_4mim][Cl], despite having similar α-values as 0.72, resulted in different glucose yields, which cannot be explained by their α-values. However, their respective β-values (1.27 and 1.16 respectively) support this difference. Thus, β-values in comparison to α-values have a better correlation with glucan conversion for the same cation across different anions. Conclusively, for better glucan conversion, structure and properties of both the anion and cation is important. Similar observations regarding the α-values were reported in previously.[262]

Table 5.2 shows that, xylose yield (%) at 100 °C/5 h, *i.e.* [C_2mim][OAc] (94.5%) and [C_4mim][OAc] (61.6%) are higher than [C_2mim][Cl] (27.8%) and [C_4mim][Cl] (24.9%) respectively. Similar trends were also observed for biomass pretreated at 130 °C/2 h, with a maximum xylose yield was in case of [C_2mim][OAc] (96.5%), followed by [C_4mim][OAc] (79.3%), [C_2mim][Cl] (27.8%) and [C_4mim][Cl] (26.8%).

From the above discussion, it may be concluded that higher β-value resulted in higher glucose/xylose yields and since the β-value of any ionic liquid is dependent of the cation and anion, therefore, an optimum combination of these two can result in higher glucose yield. Similar results were also observed in the case of xylose yields while using a wheat straw with ILs (Table 5.2).

5.5.1.2 IMPACT OF VISCOSITY AND SURFACE TENSION OF ILS ON ENZYMATIC HYDROLYSIS

Kinematic viscosity (η) of all the five ILs was analysed at 100 and 130 °C and the data are presented in Table 4.4 as described in Chapter 4. The surface tension of all the ILs was measured at 25, 50 and 75 °C and extrapolated by the linear fit to get the values for 100 and 130 °C. Table 4.4, Chapter 4 showed an increase in temperature results in a significant decrease in viscosity. Viscosity of all ILs at 100 °C and 130 °C was following the order as: [C_2mim][OAc] <[C_4mim][BF_4] <[C_4mim][OAc] <[C_2mim][Cl] <[C_4mim][Cl]. In order to find the correlation between the viscosity of ILs and the glucose yields, Figure 5.6a and 5.6b were drawn, which shows that the glucose yield was inversely proportional to the viscosity and surface tension, except the case of [C_4mim][BF_4]. Highest glucose yield (78.7 and 97.7%) was achieved with [C_2mim][OAc] pretreated mustard stalk with low viscosity (8.3, 4.4 cSt/sec) and surface tension, i.e. 32.5 and 30.2 mN/m at 100 and 130 °C respectively. However, [C_4mim][BF_4], despite having a low viscosity (8.2 and 4.5 cSt/sec) and high surface tension (41.7 and 40.7mN/m) at 100 and 130 °C does not improve the enzymatic hydrolysis, and gave poor glucose yield (12.1 and 13.8%) respectively. It may be attributed to the low hydrogen bond basicity, i.e. (β=0.72) of $[BF_4]^-$ and being a non-coordinating anion, it does not interrupt the hydrogen bonding in cellulose, hence, an insignificant improvement on enzymatic hydrolysis.[158]

Table 5.2 Overall glucose/xylose yield (%) after 72 h of enzymatic hydrolysis

ILs	*T/t*	SacchariSEBC6 enzyme (10FPU/g of pretreated biomass) Glucan recovery[b]	Glucose Yield (%)[a]	Overall Glucose yield[s]	Xylan recovery[b]	Xylose yield (%)[a]	Overall xylose yield[c]	PXRD[d] CrI %
Mustard Stalk	N/A	N/A	11.3±1.1	N/A	N/A	7. 2±0.9	N/A	62.5
[C_2mim][BF_4]	100/5h	94.3±0.4^{A}	12.1±0.4^{A}	11.4±0.3^{A}	91.2±0.6^{A}	8.7±0.8^{A}	7.9±0.2^{A}	61.0
	130/2h	95.5±0.4^{A*}	13.8±0.8^{A*}	13.2±0.6^{A*}	95.9±0.5^{A*}	11.7±0.9^{A*}	11.2±0.3^{A*}	60.1
[C_4mim][Cl]	100/5h	90.3±0.5^{B}	23.8±0.4^{B}	21.5±0.8^{B}	72.6±0.5^{B}	24.9±0.5^{B}	18.08±0.2^{B}	59.7
	130/2h	89.8±0.3^{B*}	28.7±0.1^{B*}	25.8±0.3^{B*}	67.8±0.5^{B*}	26.8±0.5^{B*}	18.2±0.5^{B*}	55.6
[C_2mim][Cl]	100/5h	93.8±0.3^{A}	32.5±0.0^{C}	30.5±1.0^{C}	81.6±0.4^{C}	27.8±0.4^{C}	22.7±0.4^{C}	54.1
	130/2h	92.2±0.2^{C*}	31.5±0.5^{C*}	29.0±0.9^{C*}	76.3±0.6^{C*}	27.8±0.1^{C*}	21.2±0.6^{C*}	47.4
[C_4mim][OAc]	100/5h	92.6±0.5^{C}	72.6±0.9^{D}	67.2±1.1^{D}	36.3±0.6^{D}	61.6±0.3^{D}	22.5±0.5^{D}	37.7
	130/2h	95.7±0.2^{A*}	72.8±0.5^{D*}	69.7±0.4^{D*}	24.9±0.5^{D*}	79.3±0.1^{D*}	19.8±0.1^{D*}	46.9
[C_2mim][OAc]	100/5h	94.6±0.1^{A}	78.7±0.4^{E}	74.5±0.8^{E}	30.7±0.9^{E}	94.5±0.9^{E}	29.0±0.3^{E}	32.3
	130/2h	92.5±0.9^{C*}	97.7±3.0^{E*}	90.4±0.6^{E*}	28.8±0.2^{E*}	96.5±0.6^{E*}	27.8±0.9^{E*}	42.0
Wheat straw	NA	N/A	13.4±0.2	N/A	N/A	8. 2±0.2	N/A	62.1
[C_4mim][Cl]	100/5h	89.9±0.6^{P}	41.9±0.9^{P}	37.3±1.0^{P}	71.6±0.1^{P}	25.4±0.5^{P}	18.2±0.5^{P}	58.3
	130/2h	86.2±0.4^{P*}	43.8±0.3^{P*}	37.8±0.5^{P*}	65.3±1.0^{P*}	22.9±0.4^{P*}	14.9±0.5^{P*}	54.9
[C_2mim][OAc]	100/5h	78.8±0.8^{Q}	61.9±0.6^{Q}	48.8±0.6^{Q}	89.0±0.9^{Q}	79.6±0.8^{Q}	70.8±0.5^{Q}	36.4
	130/2h	75.4±0.2^{Q*}	98.7±0.7^{Q*}	74.4±0.4^{Q*}	41.5±0.8^{Q*}	42.2±0.5^{Q*}	17.5±0.5^{Q*}	40.3

[a]Glucose/xylose yield (%) is yield after 72 h of enzymatic hydrolysis (calculated based on total glucan/xylan present in pretreated mustard stalk). Glucan/xylan recovery is the percentage of recovered glucan/xylan after pretreatment (calculated based on glucan/xylan present in the untreated mustard stalk. [b]Overall glucose/xylose yield represents the overall glucose/xylose yield considering the whole process (both pretreatment and enzymatic hydrolysis).

[d]PXRD is crystallinity index calculated based on the equation, *i.e.* $CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$

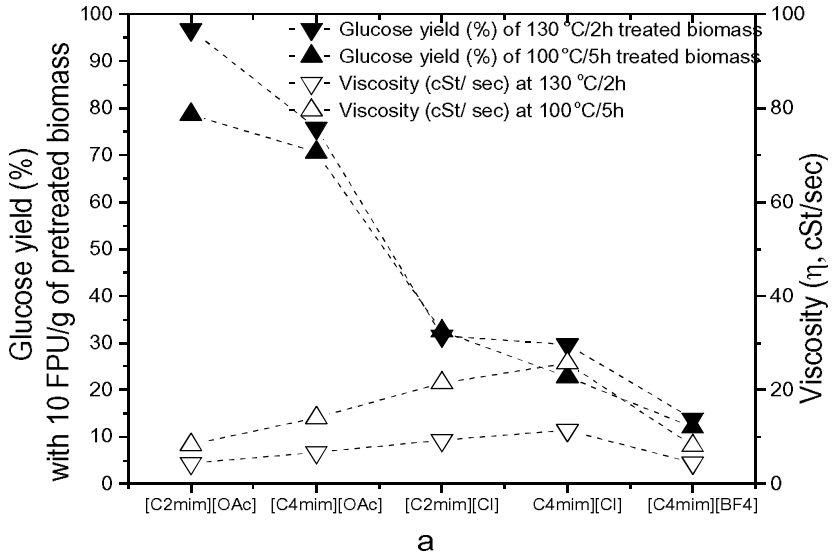
[b]Calculated using (overall yield = glucan recovery × glucose yield/100). All experiments were done in triplicate and the mean is reported with ±S.D. Values in the same column for same pretreatment temperature with different superscripts letter indicate the significance difference at P≤ 0.05. Letter with a star (*) superscript show statistical significance at 130 °C.

It was mentioned in Table 4.4; Chapter 4, that the viscosity/surface tension decreases with an increase in temperature. ILs with same anion and cation of different alkyl chain length shows an increase in surface tension with increasing alkyl chain length. Coming to the effect of the anion, comparing the surface tension of ILs with butyl substitution on cation the order is $[OAc]^-$ >$[Cl]^-$>$[BF_4]^-$. It has been observed that the effect of viscosity, surface tension and β-value on glucose yields, β-value was more preferred than the viscosity/surface tension when comparing the ILs treatment efficacy. From the above discussion, it is also observed that ILs having $\beta \geq 0.72$, with low viscosity/surface tension results in higher glucose yields. From the above data, it also can be noticed that the viscosity and surface tension have a significant impact on glucose yield if the variation is induced either in anion or cation. And a possible explanation for the efficacy of ILs with lower viscosity/surface tension that is liquids with lower surface tension have low surface forces and could easily penetrate into the material and provide better mass transfer resulting in more interaction with cellulose hydroxyls. This argument further supports a steep rise in enzymatic hydrolysis at 130 °C than 100 °C with [C_2mim][OAc].

5.5.2 XYLAN REMOVAL VIS-A-VIS ENZYMATIC HYDROLYSIS

Xylan removal under different ILs pretreatment conditions is summarized in Table 5.1, which fell in the range: 4.4-9.1, 19.2-32.2 and 64.0-75.4% for $[BF_4]^-$, $[Cl]^-$ and $[OAc]^-$ based ILs respectively. This wide variation across different ILs signifies the critical role that anion plays in targeting hemicellulose. Removal of xylan is caused by hydrogen bond interactions between the -OH of polysaccharides and the anions of ILs resulting in disruption of inter/intramolecular H-bonding within the cellulose and hemicellulose. This process results in solubilizing both, cellulose and hemicellulose. The addition of anti-solvent (water in our case) to the soluble material resulted in precipitation of cellulose and transformation to amorphous cellulose (II) leaving the hemicellulose and lignin in the solution. Irrespective of the type of cation, $[OAc]^-$ as anion was found to be superior to $[Cl]^-$ and $[BF_4]^-$ in targeting the hemicellulose. The temperature was also observed to facilitate the H-bonding

interaction between anions and polysaccharides. While $[OAc]^-$ removed xylan most effectively, the impact of increasing the temperature (from 100 to 130°C) was not very predominant, leading to 64.0% and 75.4% for $[C_4mim][OAc]$ and 69.5 and 71.4% for $[C_2mim][OAc]$.



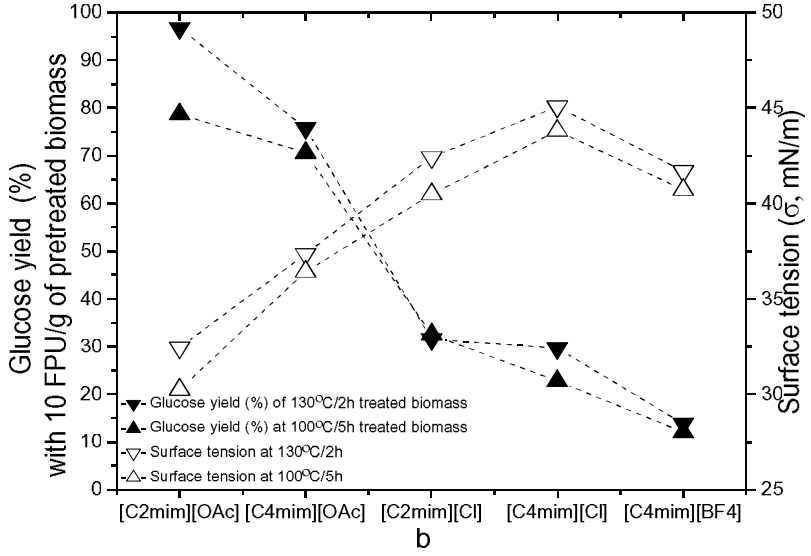


Figure 5.5 a) Correlation of kinematic viscosity vs. glucose yield (%) after 72 h of enzymatic hydrolysis with 10 FPU/g of pretreated biomass using a SacchariSEBC6 enzyme; **b**) Correlation of surface tension vs. glucose yield (%) after 72 h of enzymatic hydrolysis with 10 FPU/g of pretreated biomass using a SacchariSEBC6 enzyme.

On the other hand, $[BF_4]^-$ was least efficient in removing xylan but the impact of reaction temperature was most pronounced for its leading to almost

two-fold increase from 4.4 to 9.1% and from 27.4 to 32.2% xylan removal, when increased from 100 to 130 °C. $[Cl]^-$ also showed an increase in the xylan removal to 27.4% and 32.2% for $[C_4mim][Cl]$ and 19.2 and 24.5% for $[C_2mim][Cl]$ by increasing temperature from 100 to 130 °C. It is worth mentioning that ILs with high hydrogen bond forming capability is least affected for removing xylan by the increase in the temperature. Hence, $[OAc]^-$ reached up to that level (75.4%) with $[C_4mim]^+$ as cation at 130 °C. The overall trend of xylan removal was: $[C_4mim][BF_4]$ <$[C_2mim][Cl]$ <$[C_4mim][Cl]$ <$[C_2mim][OAc]$ <$[C_4mim][OAc]$.

Glucose yields from all the pretreated mustard stalks were correlated with xylan removal. Table 5.2 shows that glucose yields varied between 12.1-13.8% for $[BF_4]^-$, 23.8-32.5% for $[Cl]^-$ and 72.6-97.7% for $[OAc]^-$ following the order: $[OAc]^-$ >$[Cl]^-$ >$[BF_4]^-$, which shows that higher xylan removal resulted in higher glucose yields. Effect on glucose yield was most pronounced (97.7%) with $[OAc]^-$ attached to cation $[C_2mim]^+$. Although for $[C_4mim][OAc]$, xylan removal increased from 64.0 to 75.4% by raising the temperature from 100 to 130 °C, glucose yield did not show a noticeable increase (72.6 to 72.8%). However, for $[C_2mim][OAc]$, an increase in xylan removal from 69.5 to 71.4% significantly increased the glucose yield from 78.7 to 97.7%. The impact of high β-value for $[C_2mim][OAc]$ on enzymatic hydrolysis was further established, though ILs having [OAc] anion resulted in almost similar xylan removal. Hence, this may be argued that there are other parameters related to the pretreated LCB apart from the xylan removal which facilitates higher glucose yield.

5.5.3 LIGNIN REMOVAL VIS-A-VIS ENZYMATIC HYDROLYSIS

In biomass, lignin, in addition, to being linked with cellulose and hemicellulose is highly cross-linked heteropolymer in itself. Table 5.1 summarises lignin removal across different IL pretreatment and was found to be in the range: 2.1-6.8, 1.2-19.8 and 5.1-23.1% (w/w) for $[BF_4]^-$, $[Cl]^-$ and $[OAc]^-$ based ILs respectively. The significant role that the IL anion plays is evident by this wide variation in lignin removal. Figure 5.7a and 5.7b showed that keeping the cation constant as $[C_4mim]^+$, with an increase in temperature

from 100 to 130 °C, lignin removal was increased by 3.2, 6.8 and 3.2 folds for $[BF_4]^-$, $[Cl]^-$ and $[OAc]^-$ respectively. The effect of temperature was most pronounced for $[Cl]^-$ but the overall lignin removal was highest for $[C_4mim][OAc]^-$ leading to 23.1%. Lignin removal is the result of cellulose solubilization brought about by H-bond interaction between the anions of ILs and the -OH groups of celluloses (Figure 5.8), which are strong H-bond donors Depending upon the H-bond formation capacity to break down the extensive inter/intramolecular hydrogen bonding, more and more lignin gets exposed, hence, results in higher delignification. $[OAc]^-$ caused highest delignification as it has two binding sites (Figure 5.8a) for receiving H-bonds with cellulose while $[Cl]^-$ has just one.[320] Table 5.1 also shows that ILs cation plays a role in further strengthening the interaction by H-bonding between its acidic protons with the -OH of cellulose and hydrophobic interaction.

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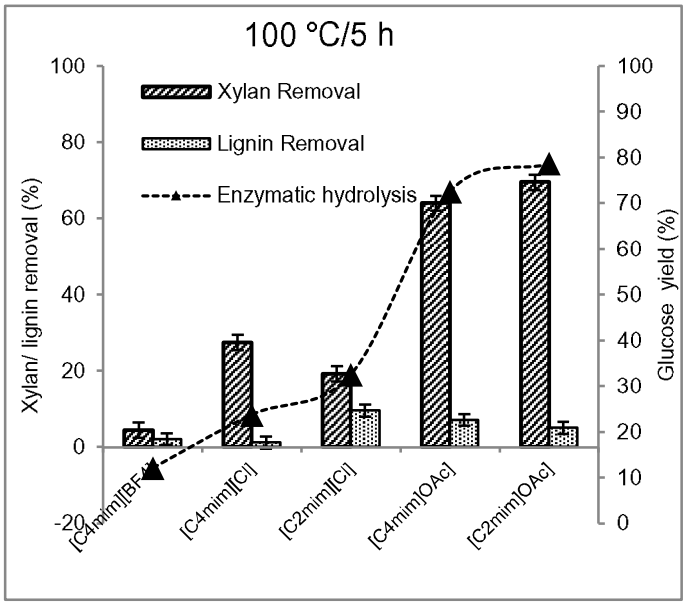


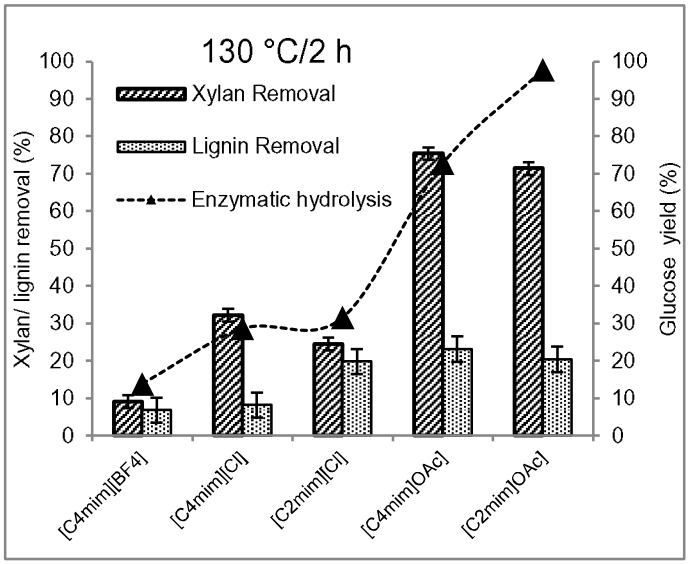


Figure 5.6 Impact of xylan/lignin removal on glucose yield (%) for 100 °C/5 h and 130 °C/2 h treatment with 10 FPU/g of the pretreaated biomass of SacchariSEBC6.

It is interesting to note that for $[Cl]^-$, there is significant cation effect observed for lignin removal when $[C_2mim]^+$ and $[C_4mim]^+$ are compared. Lignin removal increases from 1.2 to 8.2% (~8 folds) for $[C_2mim]^+$ and from 9.6 to 19.8% (~2 folds) for $[C_4mim]^+$ at 100 and 130 °C respectively. [320] explained the possible transitions between different intermediate states during H-bond formation with cellulose. Depending upon the nature of anion moiety, the time span of an intermediate state may or may not depend upon the cation chain length of the ionic liquids. This can be explained by Figure 5.8a, which shows that for $[Cl]^-$, there occurs a transition from state S_1 to S_2. The likelihood of occurring in state S_1 reduces drastically and the next most favourable state S_2 is populated where a single [Cl] anion can form H-bond with two hydroxyls of cellulose. The possibility of populating the state S_2 becomes twice when $[Cl]^-$ is associated with C_4 alkyl chain than that with C_2 (0.25 to 0.45 fraction of the total time).[320] This means that the residence time of S_2 state with C_2 chain almost doubles than that with C_4 chain. There is a gain in a number of H bonds, which has a direct effect on lignin removal increasing it from 1.2 to 8.2%. However, for the transition between S_{01} and B_{11} state (next favourable state for $[OAc]^-$), there is no change in the time span with increasing the alkyl chain length from C_2 to C_4 (it remains 0.35 fractions of total time). Therefore, even though the

number of H-bonds increased in the B_{11} state, there is an almost similar lignin removal and hence, alkyl chain does not have any significant impact. Same theory may also be applicable for xylan removal as [C_4mim][OAc] results in slightly higher xylan removal than [C_2mim][OAc].

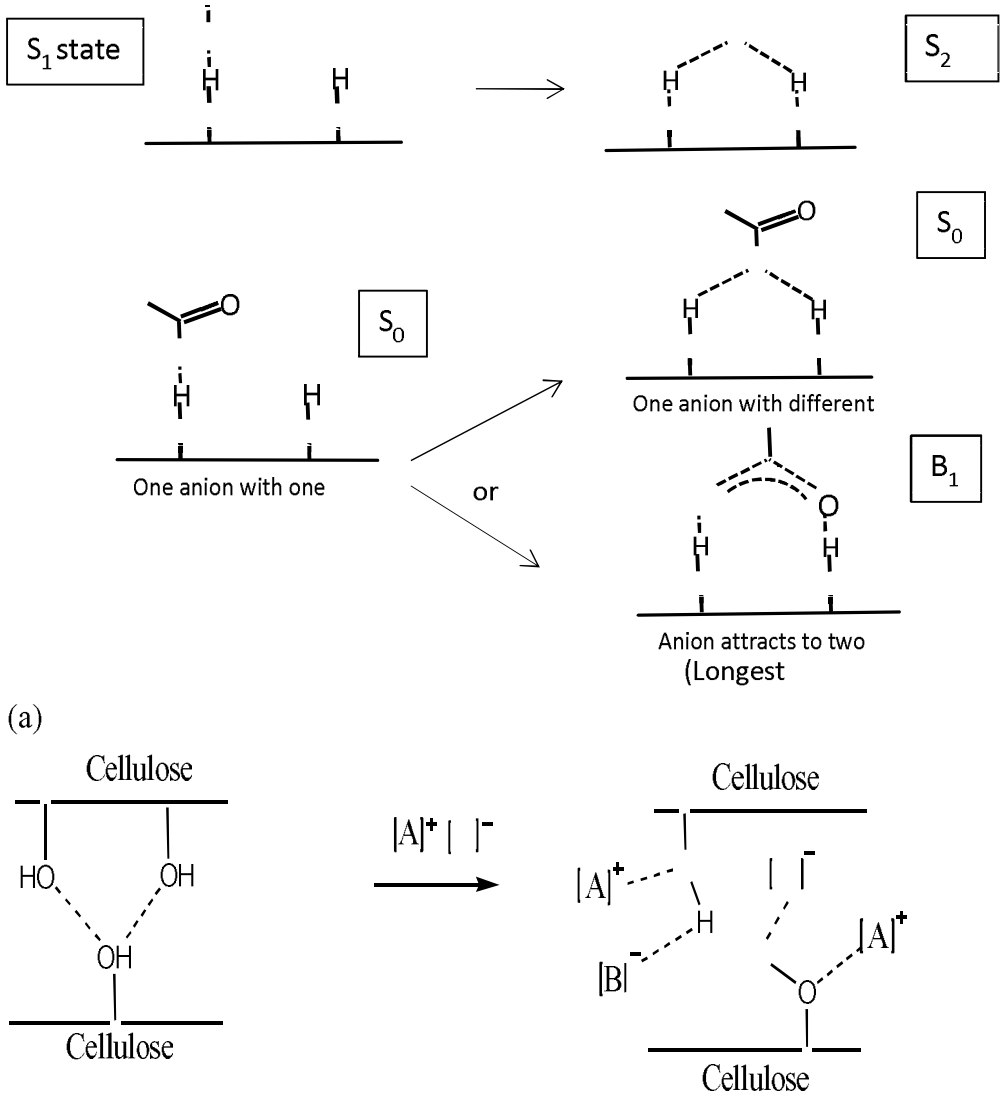


Figure 5.7 (a) Promotion of an acetate ion from the S_{01} state to the B_{11} state (a) directly and (b) via the S_{02} transition state; **(b)** Dissolution of cellulose by ionic liquid $[A]^+[B]^-$.

Lignin removal from the biomass is anticipated to be favourable for enzymatic hydrolysis. Table 5.2 shows that glucose yields varied between 12.1-13.8% for $[BF_4]^-$, 23.8-32.5% for $[Cl]^-$ and 72.5-97.7% for $[OAc]^-$ following the order: $[OAc]^- > [Cl]^- > [BF_4]^-$. It shows that effect on glucose yield was most

pronounced with $[OAc]^-$ attached to cation $[C_2mim]^+$. Although, for $[C_4mim][OAc]$ and $[C_2mim][OAc]$, the lignin removal shows a marginal reduction from 23.1 to 20.4% at 130 °C glucose yield substantially increased up to 97.7% in comparison to 72.8%.

The efficiency of pretreatment was evaluated by the glucose yield obtained after enzymatic hydrolysis of the IL pretreated residues. Keeping the cation moiety as $[C_2mim]^+$ and temperature as 100 °C, $[OAc]^-$ was found to be most effective in increasing the accessibility of mustard stalk to cellulases yielding 78.7% glucose. It was followed by $[Cl]^-$, which released 32.5% of glucose. $[BF_4]^-$ pretreated biomass was least hydrolyzed yielding only 12.1% glucose. The effect of increasing the pretreatment temperature from 100 to 130 °C was most pronounced for $[C_2mim][OAc]$ both for mustard stalk and wheat straw (Table 5.2) increasing the glucose yields from 78.7 to 97.7% and 61.9 to 98.7% respectively. It is reported that ILs work as an organic solvent for delignification and partly to xylan removal. Contrary to it, we have found in all the pretreatment experiments, primarily ILs solubilizes xylan and thereafter it works for partial delignification. For example, the highest enzymatic hydrolysis was obtained (97.7%), where the xylan and lignin removal is 71.4 and 20.4%. Therefore, it may be argued that the ILs preferentially removes xylan and consequently improve enzymatic hydrolysis. It may further be argued that xylan is relatively higher recalcitrant than the lignin. However, removal of lignin helps to reduce the enzyme dose per unit of biomass as the enzyme doses were used on the basis of pretreated LCB. Higher enzyme dose, 20 FPU/g results in higher enzymatic hydrolysis are given in Figure 5.4a and 5.4b. From the above discussion, it is evident that at 130 °C, $[C_4mim]$ [OAc] is more effective in lignin and xylan removal than the $[C_2mim][OAc]$. However, higher enzymatic hydrolysis using $[C_2mim][OAc]$ could not be explained on the basis of lignin and xylan removal.

5.5.4 EFFECT OF BIOMASS STRUCTURAL FEATURES ON ENZYMATIC HYDROLYSIS

Apart from the removal of xylan and the lignin, there could be several structural features of cellulose, which could influence the enzymatic hydrolysis,

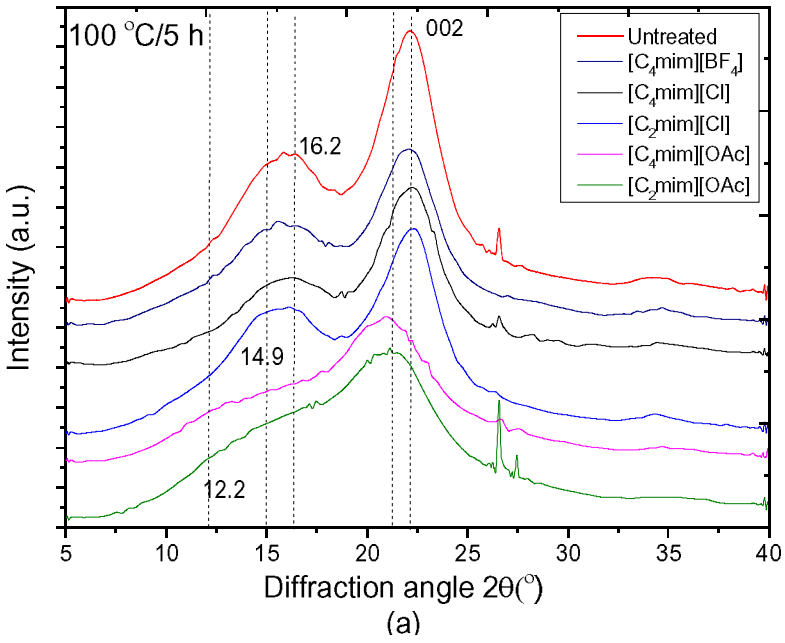
including crystallinity, the arrangement of Intra/interchain H-bonds, etc. In order to find out the structural changes favourable for better enzymatic hydrolysis, we have conducted the PXRD and FT-IR of the pretreated biomasses. PXRD show a sharp decrease in crystallinity (CrI) as given in Table 5.2 and shift in 2θ (Figure 5.9a, 5.9b). In general, it is observed from Figure 5.9 that there was a steeper decrease in CrI after pretreatment at 130 °C as compared with 100 °C resulting higher enzymatic hydrolysis at 130 °C. Moreover, after critical analysis of PXRD and Table 5.2 for all samples, it was observed that IL pretreatment significantly removes the amorphous portion (hemicellulose and lignin) from the biomass with cellulose I to cellulose II structural transformation.

Nevertheless, the decrease in CrI may be accompanied by an increase in crystal dislocations, increasing accessibility to cellulolytic enzymes by breaking inter/intra-chain hydrogen bonds in cellulose fibrils.[321-322] The CrI for untreated mustard stalk is found to be 62.5%, calculated based on the main peak intensity at 22.3°. After pretreatment, the intensity of the I_{002} peaks decreases and shifted to a lower degree, *i.e.* 14.9 and 12.2° respectively (Figure 5.9a 5.9b).
During [C_2mim][OAc] pretreatments, all of the cellulose I peak vanished and the I_{002} peak reduced to lower intensity and has the lowest CrI (32.3%) may be due to the swelling of the cellulose matrix and removal of >90% of the amorphous material. This shift in the peak of PXRD and decrease in CrI may be attributed to the formation of cellulose II, which is less crystalline in nature. Biomass pretreated at 130 °C/2 h with the other four ILs has decreased CrI compared to raw mustard stalk: [C_2mim][OAc] (42.0%) <[C_4mim][OAc] (46.9%) <[C_2mim][Cl] (47.4%) < [C_4mim][Cl] (55.6%) <[C_4mim][BF_4] (60.1%). Similar trends were also observed at 100 °C/5 h.

There are mainly two factors responsible for reduction in CrI of the recovered solids: (1) deformation of the crystalline structure of cellulose I to II, and (2) removal of amorphous lignin and hemicellulose. The increased in CrI with a higher temperature for [C_2mim][OAc] and [C_4mim][OAc] ILs (32.3 to 42.0 % and 37.7 to 46.9%) respectively indicates that the higher amorphous components removal with the formation of cellulose II is the dominant mechanism for higher enzymatic hydrolysis. This is consistent with the

compositional analysis (Table 5.1), which shows that 130 °C/2 h treatment with [C_2mim][OAc] results in 71.4% of xylan and 20.4% lignin removal and [C_4mim][OAc]: 75.4% and 23.1% respectively. $[OAc]^-$ containing ILs at 100 °C/5 h and 130 °C/2 h results in disappearance of 101 and $10\bar{1}$ the broad peak of cellulose I at 2θ=15–16°, again represents the transformation to cellulose II.[322]

The material is highly amorphous with a broad peak around 21° assigned to the 002 plane for cellulose II lattice.[323] This indicates that $[OAc]^-$ based ILs significantly disrupted the crystal structure of cellulose during the higher temperature pretreatment with the removal of recalcitrant xylan and lignin.



(a)

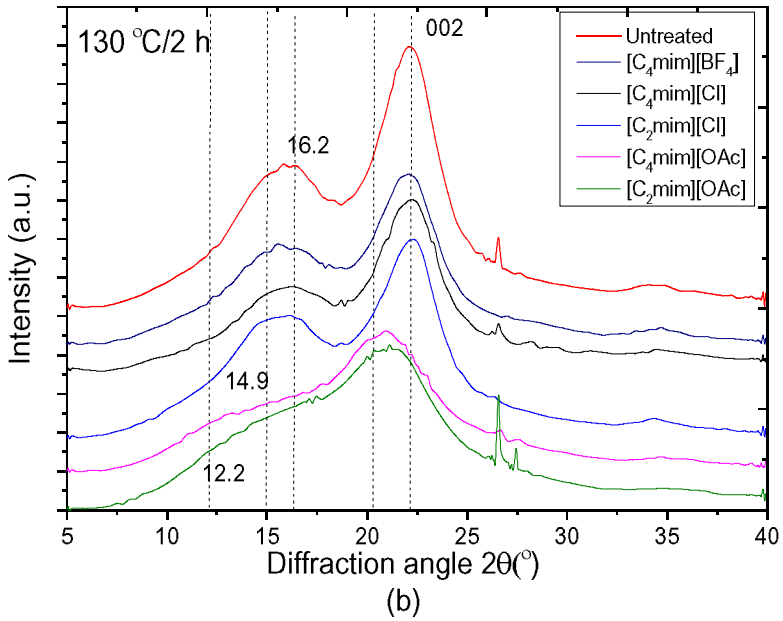


Figure 5.8 X-Ray diffraction patterns and CrI value (noted on the right side the spectrum) of the pretreated mustard stalk with different pretreatment conditions: (a) 100 °C/5 h and (b) 130 °C/2 h.

This finding explains the higher enzymatic hydrolysis (97.7%) by using [C_2mim][OAc] than [C_4mim][OAc] (72.8). However, [C_2mim][Cl], [C_4mim][Cl] and [C_4mim][BF_4] pretreated mustard stalk and wheat straw retains their highly crystalline cellulose I structure (Figure 5.9a and 5.9b), suggesting the removal of amorphous components still dominates the process even under higher pretreatment temperature. Moreover, [C_2mim][OAc] or [C_4mim][OAc] ILs pretreatment at 100/5 h and 130/2 h °C display cellulose II structural transformation with characteristic diffraction peaks at ~12.2°, 20.0° and 21.7° with a drastic reduction in CrI (Table 5.2). A similar observation is also reported by Sun et al. (2014).[268] Conversely, no significant effects on CrI were observed after pretreatment with [C_4mim][BF_4] and [C_4mim][Cl] and remained the same as untreated mustard stalk (*i.e.* cellulose I). Slight decrease in CrI with ILs compared with native (mustard stalk: 62.5%; [C_4mim][BF_4]: 59.7, 55.6%; [C_4mim][Cl]: 61.0, 60.1% respectively at 130°C/2 h and 100°C/5 h) may be due to removal of slightly less xylan and lignin as compared to $[OAc]^-$ ILs. Figure 5.10 showed the correlation of CrI vs. glucose yield, which

reveals that reduced CrI enhanced the glucose release, which in turns related to higher hydrogen bond basicity (β) of ILs. As shown in Figure 5.10, with a low CrI, the biomass pretreated at 130 °C/2 h with [C_2mim][OAc] and [C_4mim][OAc] exhibit higher glucose yield as compared to those pretreated at 100 °C/5 h. Hence, higher temperature has promoted the transformation of cellulose I to cellulose II structure.

Comprising of differently arranged H-bonds, this reorganisation of hydrogen bonds along with the removal of lignin and hemicellulose are responsible for the reduction of CrI resulting in enhanced enzymatic hydrolysis.

Figure 5.11a and 5.11b illustrates the FT-IR spectra of native and regenerated mustard stalk for different ILs treatments with their PXRD (CrI) on the extreme left. The intramolecular hydrogen bonds in cellulose I are formed between O(2)H---O(6) and O(3)H---O(5) of glucose units,[131] and can be identified by the presence of bands with the wavenumbers of 3400–3490 cm^{-1}. Similar observations are reported in the literature.[258] [C_2mim][OAc] and [C_4mim][OAc] ILs have the greatest shift on this vibration from 3348 to 3500 cm^{-1} with a reduction in intensity of this peak, indicating that these hydrogen bonds of cellulose were disrupted.[267]

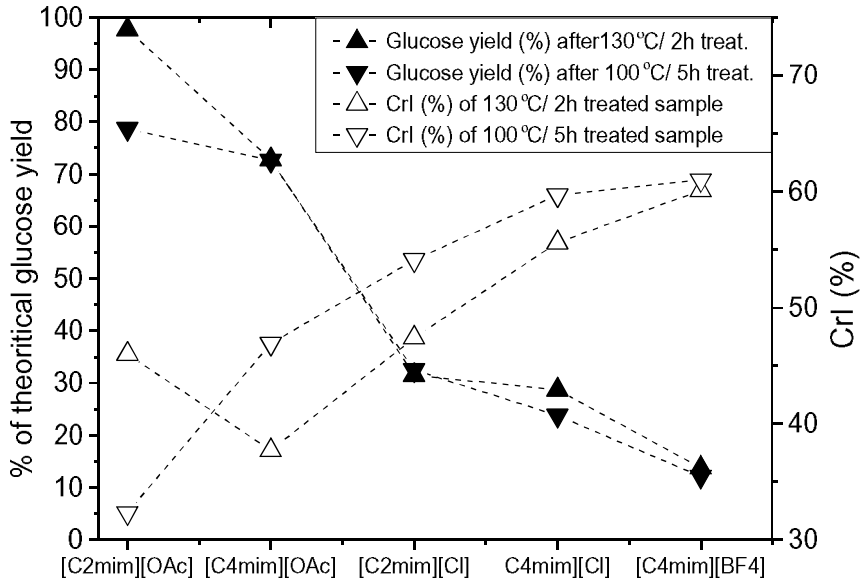


Figure 5.9 Correlation of CrI of pretreated biomass vs. glucose yield (%) after 72 h enzymatic hydrolysis with 10FPU/g of SacchariSEBC6enzymes.

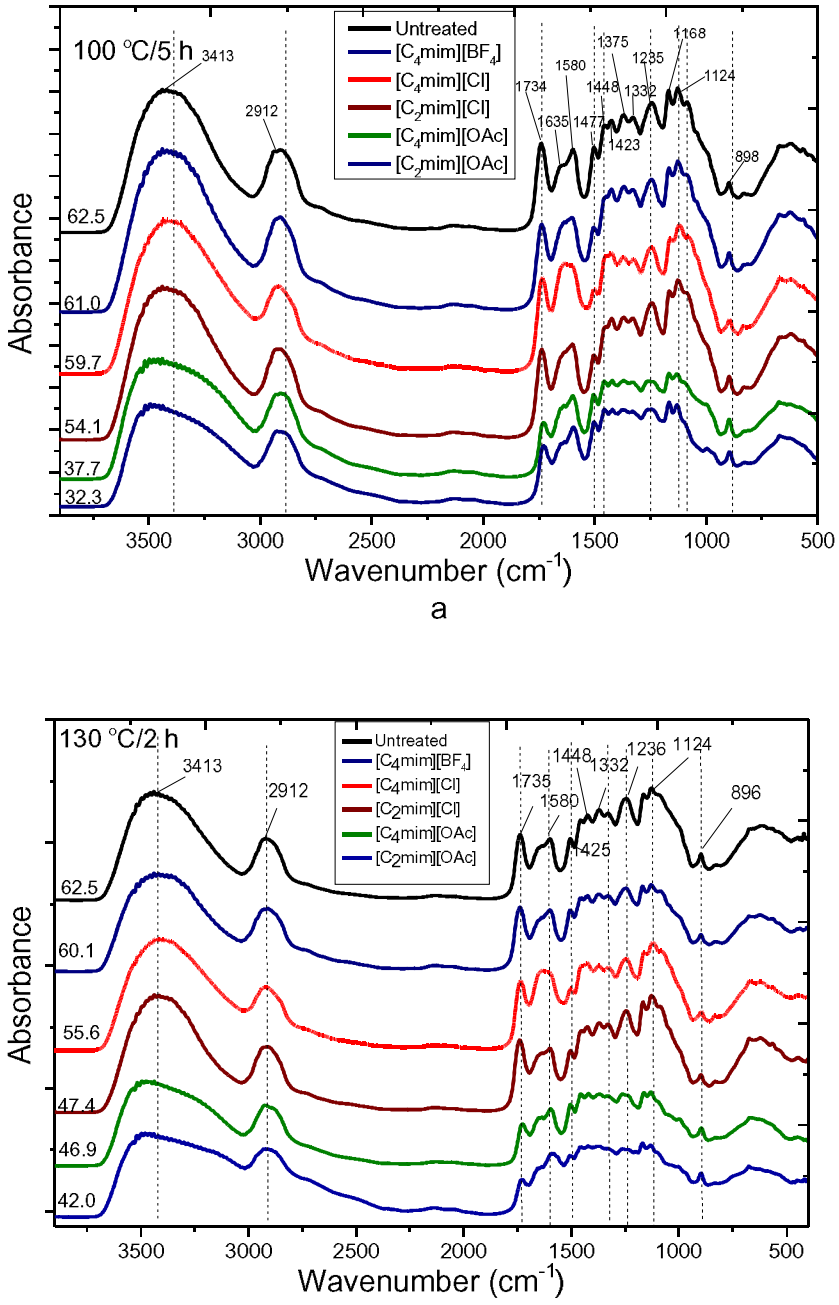


Figure 5.10 FT-IR spectrum with CrI (noted on the right side of the spectrum) of the pretreated mustard stalk with different pretreatment conditions: **(a)** 100 °C/5 h and **(b)** 130 °C/2 h.

The band intensity positioned at 2912 cm^{-1} is attributed to C–H stretching, which decreased with [C_2mim][OAc] pretreated biomass, indicating that the methyl and methylene portions of cellulose were ruptured. The band positioned at 1734-1738 cm^{-1} is attributed to hemicellulose acetyl content and

uronic ester groups or linkages in lignin and/or ester hemicellulose ferulic and p-coumaric acid carboxylic groups.[321] The intensity of this band gets reduced in case of [C_4mim][OAc] and [C_2mim][OAc] treated biomass due to removal of >65% of xylan as compared to $[Cl]^-$ and $[BF_4]^-$ containing ILs, which causes 9.1, 24.5 and 32.2% of xylan removal in case of [C_4mim][BF_4], [C_2mim][Cl] and [C_4mim][Cl] treated biomass respectively. The band at 1428cm^{-1} and 1332 cm^{-1} were assigned as symmetric CH_2 bends, CH_2 wagging and are shifted to lower wave numbers such as 1417 and 1327 cm^{-1} respectively. The shift from 1428 to 1417 cm^{-1} indicates the development of new arrangement of intra-molecular hydrogen bonding,[131] hence, reduction in CrI and formation of cellulose II (Table 5.2). The band at 898 cm^{-1} assigned to C-O-C stretching (anti-symmetric out of plane ring stretch of amorphous cellulose due to β-(1-4) glycosidic linkage) shifted from 898 to 893 cm^{-1} in case of [C_4mim][OAc] and [C_2mim][OAc] pretreated biomass due to transformation of cellulose I to II allomorph. When compared to the untreated mustard stalk, the bands at 1635 cm^{-1} (aromatic skeletal from lignin) and 1332 cm^{-1} (syringyl and guaiacyl condensed lignin) are significantly weaker for [C_2mim][OAc] and [C_4mim][OAc] treated biomass due to removal of >20% lignin as compared to $[BF_4]^-$ and $[Cl]^-$ containing ILs. For instance, [C_4mim][BF_4], [C_4mim][Cl] and [C_2mim][Cl] ILs treatments at 130 °C/2 h causes 6.8, 8.2 and 19.8% of lignin removal respectively. Furthermore, a significant decrease in intensity is observed at 1235 cm^{-1} (C-O stretching in lignin and hemicellulose), 1375 cm^{-1} (C-H deformation in cellulose, lignin and hemicellulose) and 1745 cm^{-1} (carbonyl C-O stretching) after $[OAc]^-$ pretreatment, which is likely due to the cleavage of ester linkages in lignin and hemicelluloses well as cleavage of lignin side chains and the fact that no significant lignin removal was observed in case of [C_4mim][BF_4] pretreatment. The reduction in intensity of bands at 1124 and 1168 cm^{-1}, which represents the C-O and C-O-C stretching vibration with a shift of 1124 cm^{-1} to higher wavenumber, *i.e.* 1138 cm^{-1} shows the cellulose II structure after [C_2mim][OAc] pretreatment. In addition to that, a strong peak at 1033 cm^{-1} was more pronounced in [C_2mim][OAc] and [C_4mim][OAc] treated biomass, which represents cellulose II structure. $[OAc]^-$ containing ILs pretreatment showed a clear reduction in intensity at 1246 cm^{-1} with the

appearance of a newer peak at 1234 and 1265 cm^{-1} due to cellulose II formation (Figure 5.11a, 56.11b). From the above discussion, it is evident that the removal of lignin/or hemicellulose along with the transformation of cellulose I to II increases the enzymatic hydrolysis, however, the transformation of cellulose I to II only happens once the amorphous lignin/xylan is removed up to some extent.

5.6 PLAUSIBLE MECHANISM FOR BETTER ENZYMATIC HYDROLYSIS

It is evident that cellulose allomorphs play a very important role in enzymatic hydrolysis. After ionic liquid pretreatment, cellulose I is converted to cellulose II as established by PXRD and FT-IR. It is also evident that cellulose II structure is kinetically favoured as seen by the enhanced rate of enzymatic hydrolysis and higher glucose yields than the cellulose I. Cellulose I was found to be present in the native biomass whereas, cellulose II was the result of ILs pretreatment as discussed above. However, no mechanism has been established, why cellulose II is chemically more reactive towards cellulases for enzymatic hydrolysis than the Cellulose I. In the current work, high glucose yield and improved rate of hydrolysis were achieved post ILs pretreatment and we here postulate the plausible mechanism for better reactivity of cellulases towards the cellulose II than the cellulose I.

It is reported that conversion of cellulose I to cellulose II through ILs pretreatment, there occurs a flip-flop in the structure and reorganisation of hydrogen bonds.[289] Some hydrogen bonds break while some new bonds are formed (Figure 5.12). Initiation of the cellulose hydrolysis is driven by the nucleophilic attack of endoglucanases at the C-1 carbon of the glucose unit in a glucan chain followed by the action of exo-glucanases and β-glucosidase.[324] Therefore, a better accessibility is a prerequisite for glucan hydrolysis. Critical analysis of structural aspects of cellulose I (Figure 5.11) show an existence of 6 and 8 membered rings on both sides of β-(1-4) glycosidic linkage due to two intra-chain H-bonding, viz. 3-OH---O5 and 2-OH---O6 respectively.

Additionally, cellulose I comprise another ten-membered ring between two consecutive glucan chains consisting of two intra H-bonds (between 2-OH-

---O-6 and 3-OH---O-pyranose ring) and two interchain H-bonds (between 3-OH---O-6).[313] 6, 8 and 10 membered rings are known to have strain energies of 0.1 and 12.4 kcal/mol respectively, assuming these rings are formed by hydrocarbon in real time situation. The structures with lower strain energy are more resistant to reorganise. After conversion of cellulose I to II, the 6 membered rings are retained due to lowest strain energy while the 8 membered and 10 membered rings, having relatively higher strain energy are lost. This resulted in the formation of new 16 membered rings having strain energy of 2.0 kcal/mol (lower than 8 and 10 membered) comprising three types of H-bonds viz. one intra H-bonds between 3-OH---O-pyranose ring and two new interchain H-bonds between 6-OH---O-2. This explains that the 6 membered rings having lower strain energy (0.1 kcal/mol) sustained the structural deformation of cellulose during the conversion process. Considering the ring strain energies, the loss of 8 membered rings and deformation of 10 membered rings to 16 membered rings is an energetically favourable transition possibly making the cellulose more open and rough.

After rearrangement, more OH groups are open and the distance between the two chains, A and B of glucan is higher in Cellulose II than the Cellulose I due to 16 members ring present against the 10 members ring in Cellulose I. Moreover, larger rings are more flexible, therefore, may help fundamentally, endo-glucanases to bind on the rough surface and facilitate the cleavage of the glycosidic bond. Due to the presence of 6 and 8 membered rings above and below the β-1-4-glycosidic bond will have more hindrance for endo-gluconases to attach at C_1 carbon in cellulose I, whereas in cellulose II, 6 membered rings are retained and 8 membered rings are destroyed, this making the better access of endo-glucanases to attach the C_1 of glycosidic bond. This further explains the better rate of hydrolysis after ILs pretreatment.

Kosan et al. (2008)[325] reported that the charged species of ILs and oxygen and hydrogen atoms of cellulose forms electron donor-acceptor complexes. This interaction occurs between C-6 and C-3 hydroxyl groups of the neighbours of cellulose chains resulting in the dissolution of cellulose in ionic liquid. Therefore, IL which has a better capability of formation of donor-acceptor complexes show the improved dissolution of cellulose and better

enzymatic hydrolysis.

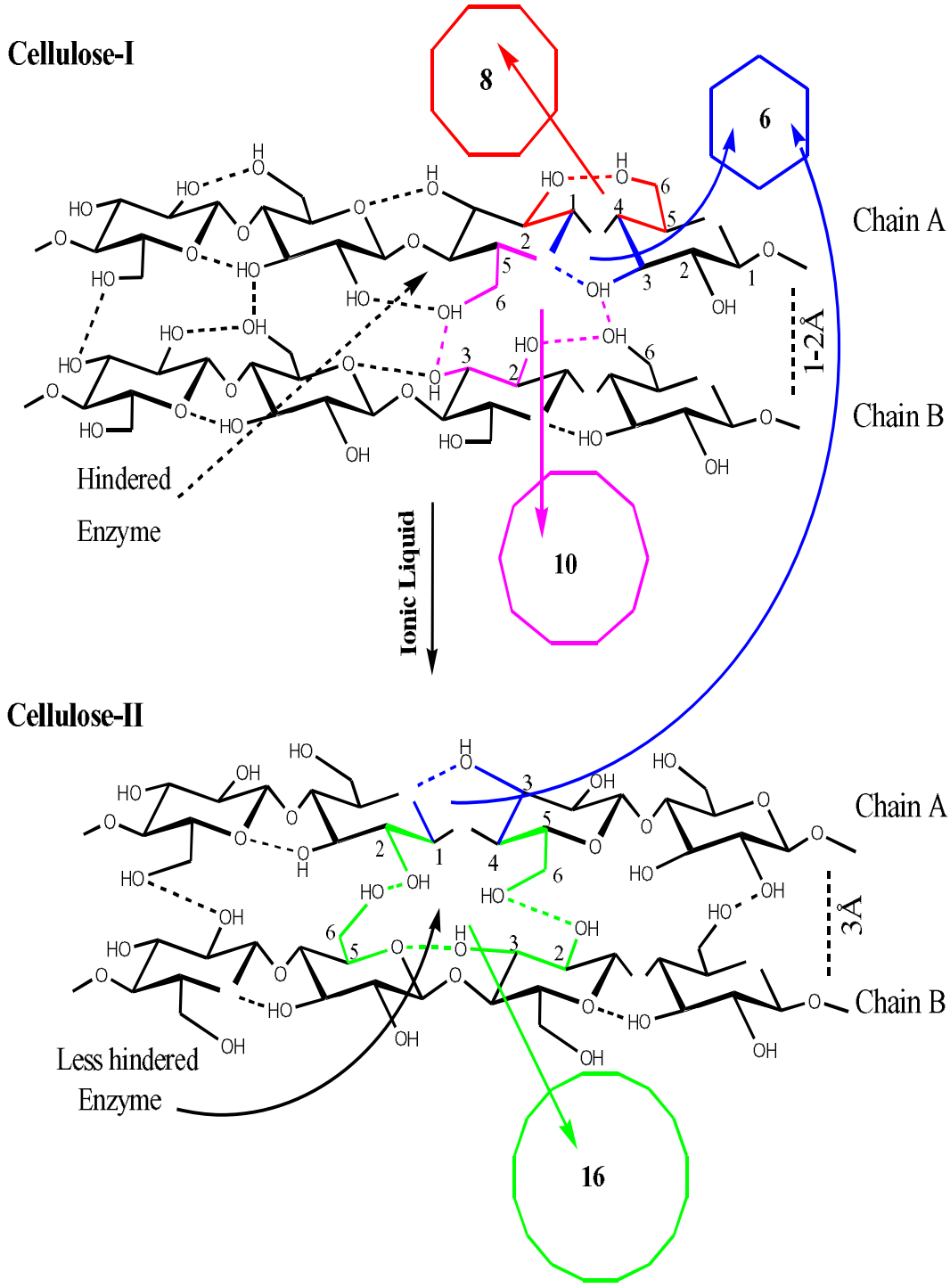


Figure 5.11 Inter/intrachain hydrogen bonding and postulated mechanism of cellulose I to cellulose II transformation

This donor–acceptor capacity of a given ILs is indicated by β-value in K-T parameters. Hence, out of five ILs studied [C_2mim][OAc] shows best results in enzymatic hydrolysis as it has better donor-acceptor complex formation capability as evidenced by its higher β-value of 1.32. Xylan and lignin removal experiments were conducted on wheat straw, which shows almost the same observation as obtained on the mustard stalk.

5.7 Impact of variability of biomass

Biomass variability plays an important role in pretreatment due to the different architectural arrangement of its components. For this, wheat straw was also pretreated with some of the ILs. Table 5.1 shows that [C_2mim][OAc] pretreatment of wheat straw causes significant xylan and lignin removal, *i.e.* 11.3 and 42.9% at 100 °C/5 h and was enhanced with increase in pretreatment temperature, *i.e.* 58.9 and 50.6% at 130 °C/2 h, respectively, whereas, in case of the mustard stalk, the xylan and lignin removal was 69.5 and 5.1% at 100 °C/5 h and 71.7 and 20.4% at 130 °C/2 h respectively. Similarly, [C_4mim][Cl] pretreatment of wheat straw account for 28.3 vs. 27.4% of xylan removal in case of the mustard stalk, whereas the lignin removal was more pronounced for wheat straw, *i.e.* 14.9% as compared to 8.2% in the case of the mustard stalk. This is clear from the data the lignin removal was predominant in wheat straw and xylan removal was preferred in case of mustard stalk across the pretreatment conditions.

This variation leads to variation in enzymatic hydrolysis of IL pretreated biomass. Mustard stalk pretreated [C_2mim][OAc] releases 78.7 and 97.7% of glucose yield, when compared to wheat straw, *i.e.* 61.9 and 98.7% at 100 °C/5 h and 130 °C/2 h respectively. Considering the lower temperature, the higher xylan removal (69.5%) transforms the biomass for higher glucose yield (78.7%); however, this impact was insignificant at a higher temperature. This data concludes that the removal of recalcitrant varies from one biomass to another, hence, the variability of biomass within same IL pretreatment condition dictates the overall process or glucose yield. Therefore, an optimisation of process parameters of each biomass would be necessary.

5.8 Conclusion

In this work, we have systematically investigated the driving factors for effective ILs mediated pretreatment. A clear contribution of the ILs properties and operating conditions for efficient hydrolysis of pretreated biomass has been explained. The acceleration in enzymatic hydrolysis of pretreated biomass was derived by the higher hydrogen bond basicity of ILs, which facilitated the delignification, hemicellulose removal and significantly reduced the cellulose crystallinity by the transition from cellulose I to cellulose II resulting in more amorphous nature. A combination of temperature and the specific ILs was the factor responsible for allomorph transformation. Therefore, higher enzymatic hydrolysis was the consequence of primarily xylan removal supported by the lignin removal with cellulose structural transformation to cellulose II allomorph. Therefore, efforts should be made to customise ILs having more hydrogen bond basicity (β) either by external induction or by structural manipulation. Biomass pretreatment methods should be customised to target removal of hemicellulose and lignin both, as most of the methods developed, either target lignin or xylan preferentially. Removal of both of these recalcitrant could help to reduce the processing load and to improve glucose yields during commercialization. Moreover, researchers should lay emphasis to transform the cellulose structure conformation within the biomass towards the cellulose II allomorph, which is easily hydrolyzable. These steps, in turn, can help to reduce the enzyme doses per unit of LCB, which contribute a significant part of the operational cost.

CHAPTER 6

PHYSICOCHEMICAL ALTERATION OF IONIC LIQUID PRETREATED MUSTARD STALK FOR IMPROVED ENZYMATIC ACCESSIBILITY

In the previous chapters, we have demonstrated the dissolution capability to different imidazolium ionic liquid for cellulose and lignocellulosic biomass. In this chapter, we have investigated the alteration in physicochemical properties of the mustard stalk after ILs pretreatment, where, we have emphasised on changes in biomass structure, porosity, surface area, crystallinity and their impact on enzymatic saccharification. Differential Scanning Calorimetry (DSC) showed increased pore size coupled with increased population of pores evoked by certain ILs in better facilitating enzymatic accessibility. Interestingly, all the five ILs predominantly increased the propensity of two pore sizes formation; 19 and 198 nm, but the remarkable difference in the pore volumes of pretreated MS suggested the supremacy of $[OAc]^-$ based ILs, resulting in higher glucose yields. Cellulose I to II transition in pretreated MS was supported by the reduced total crystallinity index (TCI), lateral order index (LOI) and hydrogen bond index (HBI) values. An inverse relationship between Kamlet-Taft parameter with HBI, LOI and TCI suggested it to be a good indicator of IL pretreatment efficiency.

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