CHAPTER 4

CELLULOSE STRUCTURAL TRANSFORMATION FOR HIGHER ENZYMATIC HYDROLYSIS BY IONIC LIQUIDS AND PREDICTING THEIR SOLVATING CAPABILITIES

Cellulose in LCB is bounded to hemicellulose and lignin via strong inter/intra molecular hydrogen bonding network, Van der Waals forces and dipole-dipole interaction and π- π interactions. This makes it highly recalcitrant for the production of fermentable sugars as compared with the sugars produced from starch-based (Corn, Kernels) feedstock. Two crystalline allomorphs of cellulose exist in biomass, i.e. cellulose I_α and I_β depending upon the orientation and type of hydrogen bonding. Cellulose I_a is the predominant and highly crystalline form present in nature. The solubilization of cellulose in Ionic Liquids (ILs) helps in transformation of cellulose I_a to I_β, which is more amenable for enzymatic attack. Recently, ILs have emerged as promising green solvents capable of dissolving cellulose. The attempts are made to understand the ILs properties, such as Kamlet-Taft parameters, viscosity, surface tension and their significance in cellulose solubilization with enzymatic saccharification.

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4.1 INTRODUCTION

Cellulose is the most abundant biopolymer in nature, contributing to 30–40 wt.% of lignocellulosic biomass. It is a linear polymer of glucose units linked via. strong β-(1-4)-glycosidic linkage (C-O-C) at the C_1 and C_4 positions with a degree of polymerization between 800-10,000. In nature, it exists in two highly crystalline allomorphs, i.e. cellulose I_α (in bacteria) and cellulose I_β (plants). In cellulose I_β, the β-1-4-glycosidic linkage is reinforced by strong intra and intermolecular hydrogen bonding and van der Waals interactions between the C_3-OH and adjacent ring oxygen and between C_2-OH and the hydroxymethyl group oxygen on C_6-OH as shown in Figure 4.1. This makes cellulose microfibrils extremely stiff and highly crystalline in nature.

Hence, due to this highly ordered and crystalline nature of cellulose, it is difficult to solubilize it in conventional solvents (organic solvents and water). Therefore, it poses challenges to effectively solubilize the crystalline cellulose by disrupting the inter/intramolecular hydrogen bonding for conversion into fermentable sugars and renewable chemicals. In this concern, ILs have proven to be potential, more sustainable, greener and environmentally responsible solvents.[10, 259-260]

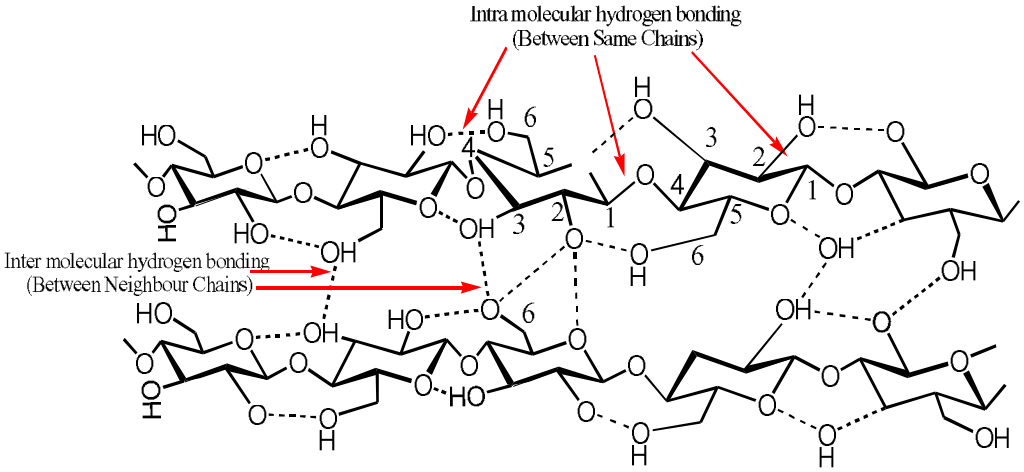


Figure 4.1 Inter/intramolecular hydrogen bonding in cellulose I

ILs are molten salts below 100 °C comprising organic cations and organic or inorganic anions.[261] These can be recycled to reduce their high cost and make the process more logical.[181, 262] Certain imidazolium-based ILs have demonstrated a promising ability for efficient dissolution of biomass cellulose,

which can be regenerated upon addition of an anti-solvent such as water/acetone/ethanol or mixture thereof.[263, 258, 262] IL pretreatment does not produce degradation products that inhibit enzymes or fermenting microorganisms unlike most of the commonly employed pretreatment methods as described in Chapter 1.[264] The initial hydrolysis rate for regenerated cellulose obtained after pretreatment with [C_4mim][Cl]was approximately 50-fold higher as compared to untreated cellulose as reported by Dadi et al. 2007.[42],which describes that [C_2mim][OAc] treatment of switchgrass gave more than 90% of hydrolysis yield after 12 h saccharification time as compared to dilute acid treatment, which gave 80% yield after 72 h of saccharification. Similarly, Singh S. et al. (2009)[118] examined the structural disturbance of switchgrass after pretreatment with [C_2mim][OAc], which enhanced the enzymatic saccharification from 16.5% to 72.5%. In a previous report, sugar cane treated with [C_2mim][OAc] resulted in 87.0% of enzymatic hydrolysis.[224] Table 4.1 shows the comparative sugar yield from biomass pretreated with different ILs.[164, 265]

ILs are able to interact with cellulose through a variety of different interactions, including dispersive, π–π, n–π, hydrogen bonding, dipolar, and ionic/ charge-charge interactions (Figure 4.2).[266] These interactions between the basic anion and the cellulose hydroxyl groups help to break the hydrogen bonds network between the cellulose microfibrils.

Table 4.1 Comparison of sugar yield % obtained after ILs pretreatment

Substrate	IL used	Sugar yield (%) post enzymatic saccharification	Reference
MCC	[C_4mim][Cl]	100[a], 97.95[b], 90.34[c]	265
MCC	[C_2mim][Cl]	90.72[a], 57.04[b], 57.04[c]	265
MCC	[C_4mim][Cl]	88.79[a], 81.92[b] 81.04[c]	265
Bagasse	[C_2mim][OAc]	99.8	164
Cellulose from bagasse	[C_2mim][OAc]	95.2	267
Switch grass	[C_2mim][OAc]	93.5	268
Switch grass	[C_2mim][OAc]	72.7	118

[a]Mass ratio of cellulose/IL (1:20); [b]Mass ratio of cellulose/IL (1:15); [c]Mass ratio of cellulose/IL (1:10); MCC is Micro crystalline cellulose

The imidazolium cation has been proposed to have hydrophobic interactions with the hydrophobic face of the cellulose. The C-2 proton in the imidazolium has been simulated to interact as a weak hydrogen bond donor with

the cellulose hydroxyl groups during dissolution and cation acidity has been suggested to be an important parameter for predicting cellulose solubility in certain cases.[269]

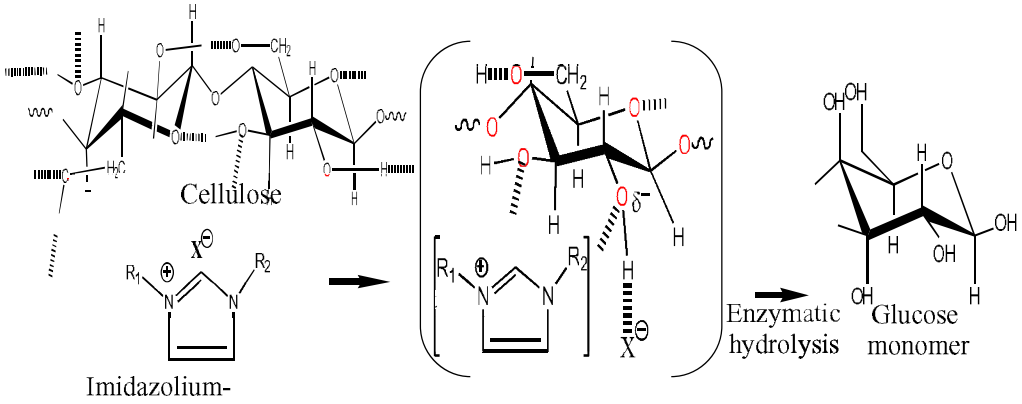


Figure 4.2 Mechanism of interaction of imidazolium cation and anion with cellulose chain

The ability of ILs to dissolve and transform cellulose efficiently depends on their physicochemical and solvent properties such as, the size of cation and anion, Kamlet-Taft parameters: hydrogen bond acidity (α), hydrogen bond basicity (β) polarizability (π^*) and viscosity (η) along with surface tension (σ). There are few reports on the properties of ILs and their impact on cellulose structural transformation and enzymatic saccharification and are limited to individual property such as K-T parameters.[270, 262, 268]

4.2 AIM OF STUDY

The current study is focused on five ILs namely; 1-ethyl-3-methylimidazoliumacetate [C_2mim][OAc], 1-butyl-3-methylimidazolium acetate [C_4mim][OAc], 1-ethyl-3-methyimidazolium chloride [C_2mim][Cl], 1-butyl-3-methyimidazolium chloride [C_4mim][Cl] and 1-butyl-3-methylimidazolium tetrafluoroborate [C_4mim][BF_4] as shown in Figure 4.3. The ILs were analyzed for their properties viz. K–T parameters, viscosity and surface tension.

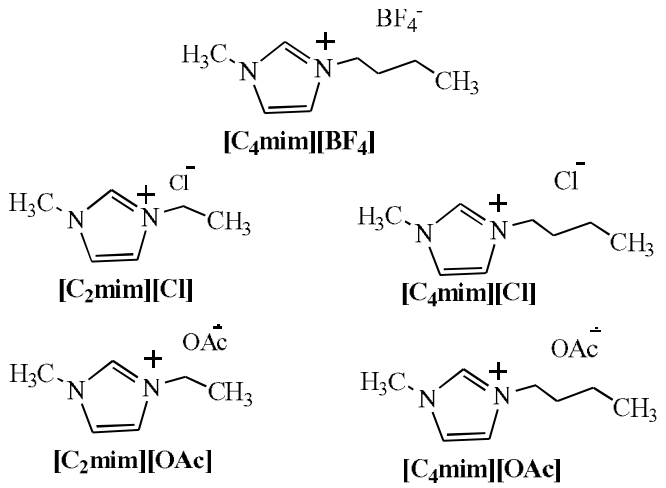


Figure 4.3 Ionic liquid structure

The study was started on Avicel PH 101 cellulose as a model substrate, which reflects the properties of biomass up to some extent. After treatment and regeneration of cellulose (Avicel PH 101) using all these ILs, the regenerated cellulose was subjected to enzymatic hydrolysis and the attempts were made to find the condition of ILs properties with enzymatic hydrolysis. Regenerated cellulose, thus obtained was characterised by PXRD and FT-IR to find out the structural transformation. This is one of the few such studies which will help to choose best IL for biomass pretreatment, wherein a comprehensive analysis of ILs properties, i.e. K-T parameters, viscosity and surface tension were analysed under various treatment conditions and their impact on the structure of cellulose and enzymatic hydrolysis was evaluated.

4.3 RESEARCH METHODOLOGY

Scheme of overall research methodology is shown in Figure 4.4. It showed that at first solvatochromic properties of ionic liquids were studied for K-T parameters, viscosity and surface tension. After that, ionic liquid treatment optimisation was conducted to determine the most appropriate condition for IL treatment of Avicel. The dissolved cellulose was regenerated using water as anti-solvent and undergone for enzymatic saccharification for sugar recovery.

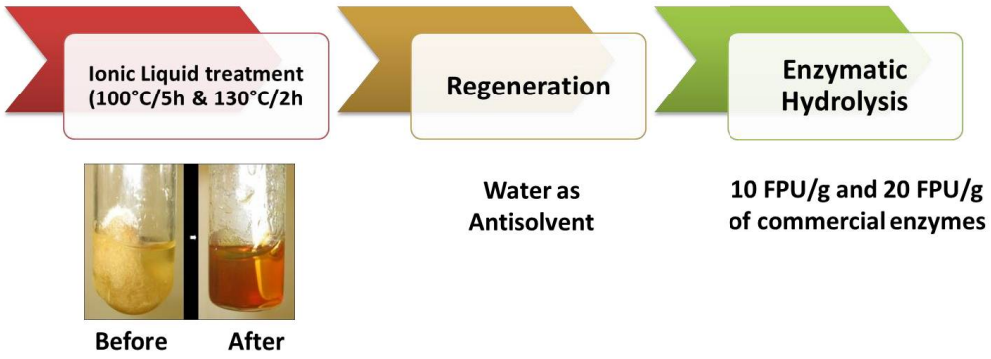


Figure 4.4 Scheme of over all research methodology used

4.3.1 MATERIAL AND METHODS

4.3.1.1 MATERIAL

1-Ethyl-3-methylimidazolium acetate [C_2mim][OAc], (≥ 98%), 1-ethyl-3-methyimidazolium chloride [C_2mim][Cl], (≥ 97%), 1-butyl-3-methyimidazolium acetate [C_4mim][OAc], (≥ 98%), 1-butyl-3-methyimidazolium tetrafloroborate[C_4mim][BF_4], (≥ 98%), 4-Nitroaniline (4NA) (≥ 99%), Reichardt's dye (RD) (dye content ≥ 90%), imidazole (≥ 97%), butyl chloride and Avicel® (PH 101) (> 98%) were purchased from Sigma-Aldrich (India) and used without any further purification. N, N-Diethyl-4-nitroaniline (DENA) (≥ 97%), was purchased from Oakwood Chemical Products (West Columbia, USA). 1-butyl-3-methyimidazolium chloride [C_4mim][Cl] was synthesised as per as the synthetic protocol [271]. All experiments were conducted using a single lot of Avicel® (PH 101). Cellulase enzyme was obtained from M/s Advanced Enzymes Technologies Ltd. (Mumbai, India).

4.3.1.2 IONIC LIQUID TREATMENT

A 10% (w/w) Avicel (PH 101) was prepared by combining 1 g of Avicel with 9 g of IL in a 50 mL round bottom flask under stirring condition. The mixture was then placed in a preheated silicone oil bath on a hot plate (IKA C-MAG H7 basic, Germany) equipped with a stirrer at 200-300 rpm for given

temperature (100 and 130 °C) and desired time (5 and 2 h) under sealed conditions, so as to avoid moisture uptake. After treatment, the regeneration of the dissolved cellulose was carried out by the precipitation of cellulose/IL slurry with 50 mL of hot deionized water (60 °C) under stirring as shown in Figure 4.5. The regenerated cellulose was collected by filtering through a Whatman no. 41 paper and thoroughly washed with deionized water (5 x 50 mL) to remove ILs. The residual ILs were checked after a fifth wash by measuring the absorbance at 240nm using Shimadzu UV-Vis 2401spectophotmeter of recovered liquid.[272] Regenerated cellulose was then stored in a freezer at 4 °C for further analysis and for enzymatic hydrolysis. A portion of regenerated cellulose was air dried at 30 °C for characterization.

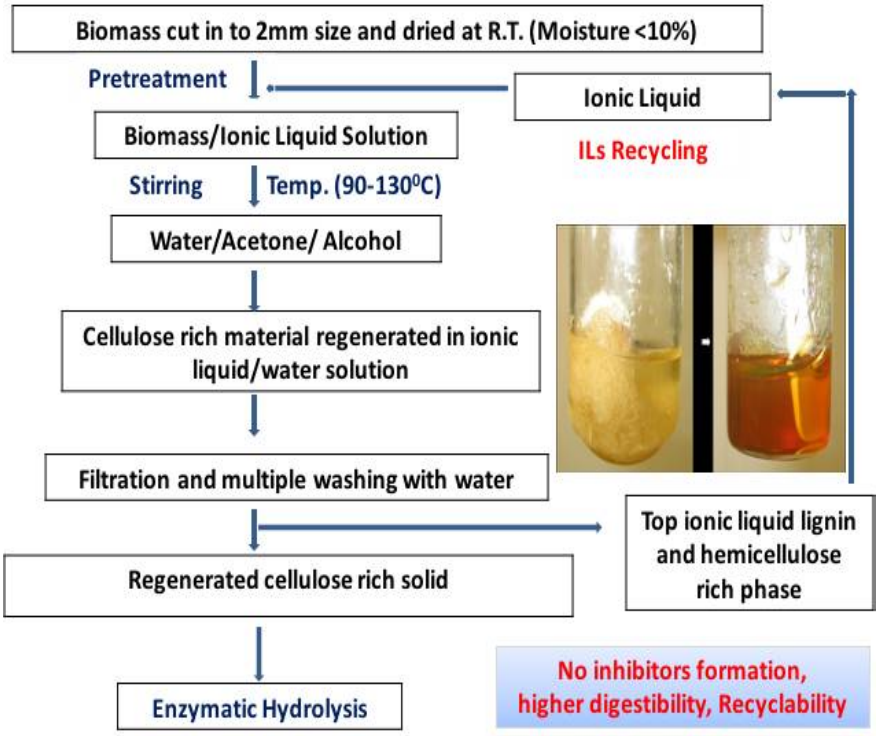


Figure 4.5 Scheme of IL treatment & downstream processing

4.3.1.3 ENZYMATIC SACCHARIFICATION

Enzymatic saccharification of untreated and regenerated cellulose was carried out using commercially available cellulases. All reactions were conducted at 10% loading by placing 100 mg of regenerated cellulose (on dry weight) in 15 mL glass vials. 0.05 M Sodium citrate buffer (0.05 M, pH 5.0)

containing 0.02% sodium azide was used to make the reaction volume up to 1mL to prevent the growth of any microorganism. The cellulase preparation was found to contain, β-glucosidase activity: 5930 IU/g, filter paper unit: 578 FPU/g, endo-cellulases activity: 4625 IU/g and endo-xylanase activity: 64867 IU/g. β-glucosidase activity was determined as described previously.[273] Filter paper units (FPU) and endoglucanase (CMCase) activity were determined according to the method described.[274] The enzyme dosage used for the reaction was 10 & 20 FPU/g biomass. The reaction was carried out at 50 °C for 72 h in a rotatory incubator with a shaking speed of 150 rpm. Samples were withdrawn at 0, 1, 2, 4, 6, 24, 48 and 72 h. Each aliquot was sealed and incubated for 5 min in a boiling water bath to denature the cellulase enzyme. The aliquot was then centrifuged at 10,000 g for 5 min. The diluted supernatant was then filtered through a 0.45 μm of the nylon syringe filter before being injected into HPLC (Waters) for sugar analysis. on 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.

4.3.1.4 KAMLET-TAFT (K-T) PARAMETERS

Kamlet-Taft parameters were determined according to the reported protocol [268]. The three dyes, 4-nitroaniline (4NA), N, N-diethyl-4-nitroaniline (DENA) and Reichardt's dye (RD) solution were prepared in ethanol to a concentration of 1 mg/mL. 2 μl of 4NA, 2 μl of DENA and 20 μl of RD were pipetted into four separate glass vials and the ethanol was evaporated under stream of dry nitrogen. Dye concentration of 12, 8 and 29 mM respectively, were obtained by adding 1.25 mL of the ILs to each vial and mixing on a shaker at 300 rpm at 25 °C for 30 min. The absorbance spectra at 30, 50 and 60 °C of each IL-dye solution was measured from 350 to 700 nm using a UV-Vis dual beam spectrophotometer, Spectra Max M5 molecular devices, S/N MV 06585 (North America, US) equipped with a temperature controller. These parameters are temperature dependent and were measured between 30 to 60 °C, which was then extrapolated by a linear fit to estimate the values at 100 and 130 °C.[275]

E_T^N scale expresses the solvent polarity arising from the overall interaction between a solvent and the dye. Therefore, the E_T^N polarity is obtained by measuring the maximum absorption in a solvent by using following Eq. 4.1.[276-277]

$$E_T^N = \frac{E_T(30) - 30.7}{32.4} \quad \text{Eq. 4.1}$$

$$\pi^* = \frac{\upsilon_{max} - \upsilon_o}{s} \quad \text{Eq. 4.2}$$

Where, $E_T(30)$ in kcal.mol^{-1} is $28591/\lambda_{max}$ (nm); λ_{max} is the wavelength of RD dye in nm.[275] K-T parameter (π^*) is obtained (Eq. 4.2) by measuring the wavelength of maximum absorbance, υ_{max} in kilo Keyser (kK, 10^{-3}cm^{-1}), of N, N-diethyl-4-nitroaniline dye.[277] υ_0 = 27.52 kK and s = −3.182; here, υ_0 is the regression value for a reference solvent system and s is the susceptibility of the intensity of spectrum absorption due to the changing solvent polarity. π^* provides the measure of polarizability. Hydrogen-bond acidity, α was determined using Eq. 4.3

$$\alpha = \frac{E_T(30) - 14.6\,(\pi^* - 0.23) - 30.31}{16.5} \quad \text{Eq. 4.3}$$

$$\beta = \frac{1.035 * \upsilon(DENA)_{max} - \upsilon(4NA)_{max} + 2.64}{2.8} \quad \text{Eq. 4.4}$$

K-T parameter, (β) hydrogen bond basicity was obtained by measuring the relative difference of solvatochromism between 4NA and DENA. Where, $\upsilon(DENA)_{max}$ and $\upsilon(4NA)_{max}$ are the wavelength of maximum absorbance of dissolved DENA and 4NA respectively using Eq. 4.4.[276-277]

4.3.1.5 KINEMATIC VISCOSITY

The kinematic viscosity of ILs was measured using 20 mL of ILs at 100 and 130 °C directly with a Cannon-Fenske Viscometer (USA) with 300 and 400 bulb sizes and double bubble. The measurements were performed in triplicates. The temperature of the sample was maintained at ±0.1 °C via an external temperature controller. The viscometer was placed into a silicone oil bath with a temperature controlled system within ±0.2 °C using a bath circulator. The equilibrium time was about 15 min. The efflux time of the liquid solution through the capillary was measured manually with a stopwatch.[278]

4.3.1.6 SURFACE TENSION

Surface tension was conducted by using a model DCAT 11EC Tensiometer and Wilhelmy plate purchased from Data Physics. Before every use, the plate was first rinsed with acetone to remove any material sticking to the plate and washed with Millipore water. Finally, the plate was heated to red hot with a Bunsen burner and then cooled. The instrument works from 25 to 80 °C.[279] Surface tensions of ILs were measured at 25, 50 and 75 °C and the graph was plotted. The surface tension values for 100 and 130 °C was obtained by extrapolation of the linear fit of the data obtained.

4.3.1.7 POWDER X-RAY DIFFRACTOMETRY (PXRD)

The regenerated cellulose was air dried and characterised with horizontal Rigaku PXRD in Panalytical (Netherland), X-pert pro diffractometer with acceleration voltage at 40 kW and current at 30 mA. The radiation was Cu Kα at (λ=1.54Å) and grade range 2θ=30−50° with a step size of 0.003°. Crystallinity index (CrI) of cellulose was calculated according to the empirical method as given in Eq. 4.5 proposed by Segal et. al (1959).[280]

$$CrI = \frac{I_{002} - I_{amp.}}{I_{002}} \qquad \text{Eq. 4.5}$$

Where, CrI is the crystallinity index, (I_{002}) is the highest peak intensity at (002) plane at an angle of diffraction 2θ = 22.4° and (I_{amp}) is the intensity diffraction for amorphous cellulose at 2θ = 16.0°.

4.3.1.8 FT-IR

Fourier Transforms Infrared Spectroscopy (FT-IR) of cellulose samples were recorded using Prestige-21, Model No. A21004802514, FT-IR instrument. All spectra were recorded in the absorbance mode from an accumulation of 128 scans at 4 cm^{-1} resolution over 4000−400 cm^{-1} range. Samples were analysed by grinding with KBr (1:100, w/w) and pressing into pellets.

4.3.1.9 STATISTICAL ANALYSIS

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

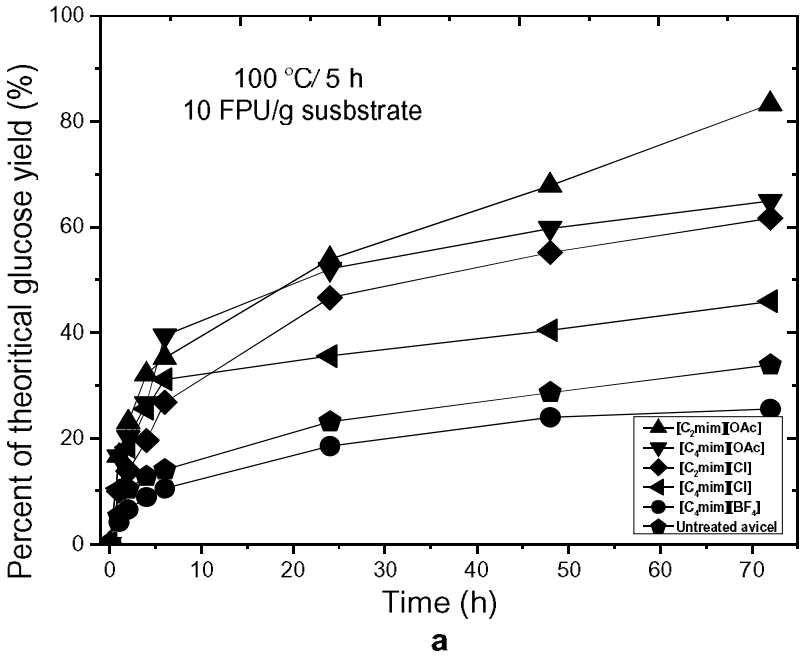
4.4 RESULTS AND DISCUSSION

4.4.1 TREATMENT AND ENZYMATIC SACCHARIFICATION

ILs treatment is usually carried out between 80−150 °C with a time interval of 1 h to few hrs, above 150 °C, cellulose solubilizes rapidly, however, this also results in loss of sugars. Therefore, two treatment conditions, i.e. 100 °C for 5 h and 130 °C for 2 h were chosen based on literature.[268] Crystalline cellulose was treated with five ILs at 10% loading (w/w) in a 50 mL round bottom flask and was regenerated by Milli-Q water as anti-solvent. Solid recovery was in the range of 86.3 to 97.8% and the data is summarised in Table 4.2. Solid recovery refers to the percent mass of cellulose (on dry basis) recovered from native after treatment and regeneration. Table 4.2, shows that treatment at 130 °C/2 h, resulted in slightly lower solid recovery (86.3−95.5%) as compared to 100 °C/5 h (92.2−97.8%). [C_4mim][BF_4] at 100 °C/5 h resulted in the highest (97.8%) solid recovery, while [C_4mim][OAc] at 130 °C/2 h resulted in lowest (86.3%). Similarly, [C_4mim][Cl] and [C_2mim][Cl] resulted in 96.0 and 97.4% of solid recovery after 100 °C/5 h treatment respectively.

Enzymatic saccharification of untreated and treated Avicel was carried out using 10 and 20 FPU/g substrate of cellulase enzymes at 10% (w/w) solid loading. Table 4.2, indicates that after ILs treatment, higher glucose yields were achieved as compared to untreated cellulose except in the case of [C_4mim][BF_4]. Cellulose treated at 100 °C/5 h using a 10FPU/g substrate of enzyme resulted in glucose yields in the order of: [C_2mim][OAc] (83.3%) > [C_4mim][OAc] (64.9%) > [C_2mim][Cl] (61.6%) > [C_4mim][Cl] (45.9%) > [C_4mim][BF_4] (25.5%). Higher treatment temperature, 130 °C/2 h resulted in higher glucose yields with the similar order, as was in the case of 100 °C/5 h. Glucose yields of [C_4mim][BF_4] at both temperatures were found to be lower than the untreated cellulose, even with 20 FPU/g substrate of enzyme loading. The glucose yields using 10 and 20 FPU/g substrate of the enzyme and its time course at different

intervals are represented in Figure 4.6a. Figure 4.6b clearly shows that an increase of the enzyme dose resulted in an increase of glucose yields at a faster rate. Figure 4.7a and 4.7b shows that $[OAc]^-$ containing ILs resulted in higher glucose yields and a higher rate of reaction at both 10 and 20 FPU/g substrate of the enzyme.



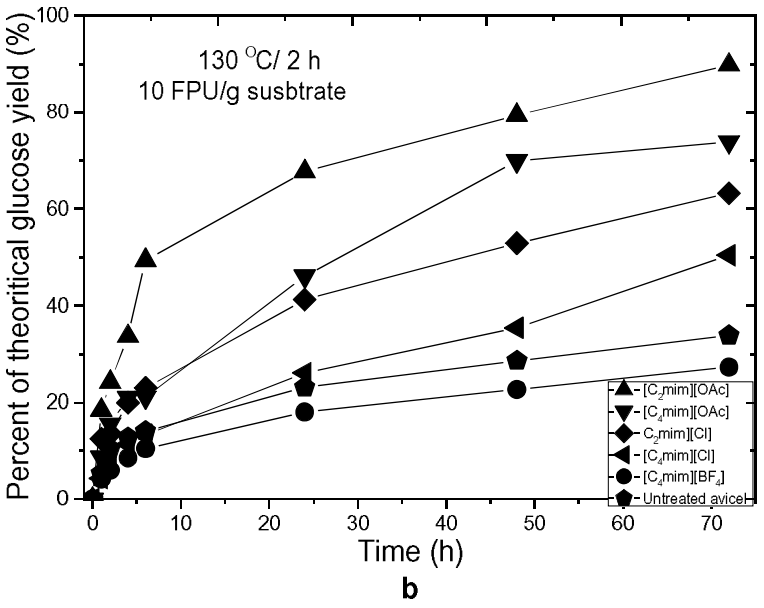


Figure 4.6 Time course for enzymatic hydrolysis and glucose yield (%) of untreated and ILs treated cellulose. Conditions: 100 mg untreated/regenerated cellulose, 0.8 mL of sodium citrate buffer 0.05M, 10 FPU/g substrate, 50 °C, 150 rpm, time, 72 h.

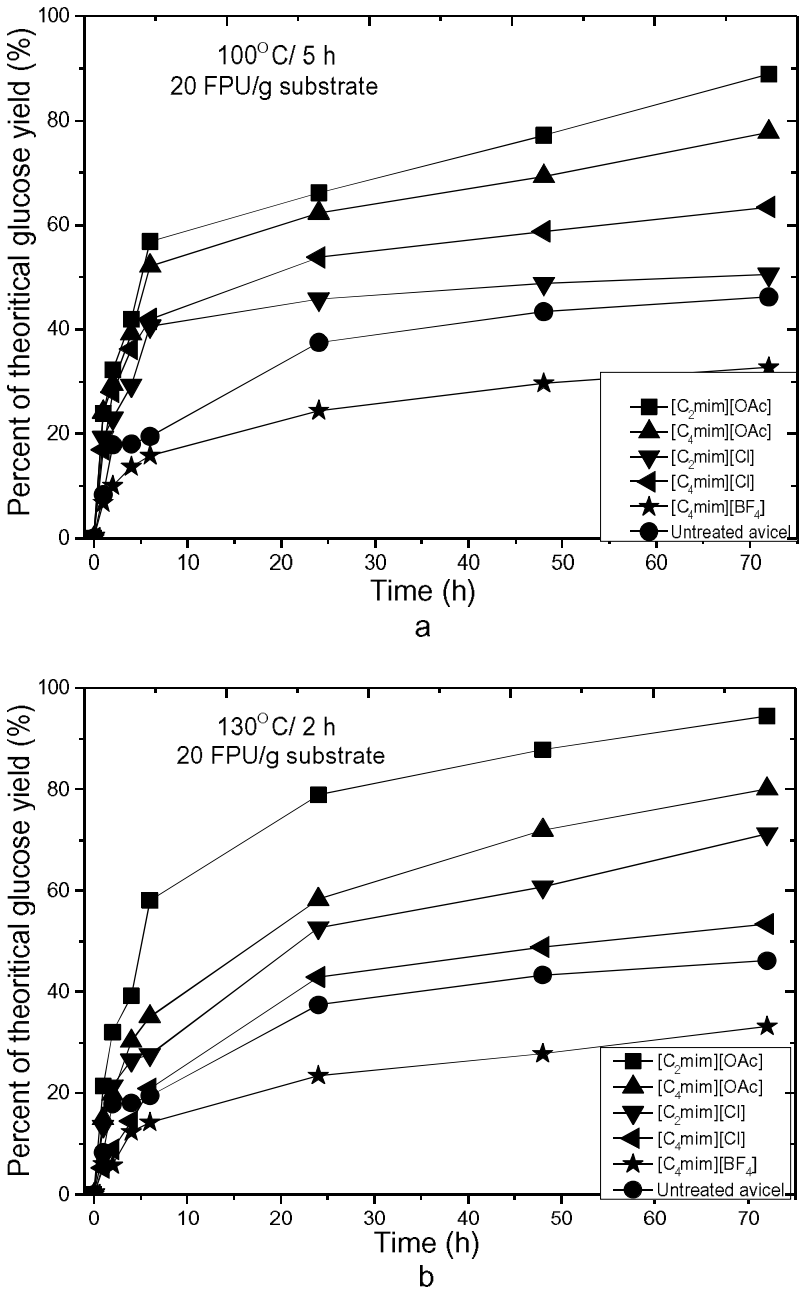


Figure 4.7 Time course for enzymatic hydrolysis and glucose yield (%) of untreated and treated cellulose. Conditions: 100 mg untreated/regenerated cellulose, 0.8 mL of citrate buffer 0.05M, 20 FPU/g substrate of Advance enzyme, 50 °C, 150 rpm.

Table 4.2 also indicates a direct relationship between lowering of crystallinity by ILs treatment and the extent of enzymatic hydrolysis. Thus, [C_2mim][OAc] was more effective in lowering the crystallinity of Avicel, from 89.0 to 57.4% and also showed best enzyme digestibility of 94.4% at 20 FPU/g substrate (discussed in the later part).

Table 4.2 Solid recovery and % glucose yield of regenerated cellulose obtained

	T/t	Solid recovery (%)[a]	Glucose yield (%)[b]		Overall glucose recovery (g/g avicel)	Crystallinity Index (CrI (%)
			10 FPU/g substrate	20FPU/g substrate		
Untreated Avicel	N/A	N/A	33.86±0.5	46.19±0.9	0.46±0.01	89.0
[C_2mim][OAc]	100/5	93.30 ± 0.5^{A}	83.28 ± 0.9^{A}	88.80 ± 1.0^{A}	0.83 ± 0.02^{A}	67.3
	130/2	$89.69\pm0.5^{A*}$	$89.83\pm0.8^{A*}$	$94.48\pm1.0^{A*}$	$0.85\pm0.02^{A*}$	57.4
[C_4mim][OAc]	100/5	92.23 ± 0.2^{B}	64.92 ± 0.2^{B}	77.77 ± 0.9^{B}	0.72 ± 0.01^{B}	75.3
	130/2	$86.32\pm0.9^{B*}$	$73.87\pm1.2^{B*}$	$80.10\pm0.7^{B*}$	$0.69\pm0.03^{B*}$	70.6
[C_2mim][Cl]	100/5	96.00 ± 0.4^{C}	61.67 ± 0.7^{B}	63.44 ± 1.2^{C}	0.61 ± 0.02^{C}	73.4
	130/2	$94.74\pm0.4^{C*}$	$63.27\pm1.2^{C*}$	$71.24\pm1.3^{C*}$	$0.67\pm0.02^{C*}$	72.5
[C_4mim][Cl]	100/5	97.41 ± 0.1^{D}	45.96 ± 1.5^{C}	50.54 ± 1.0^{D}	0.49 ± 0.01^{D}	75.0
	130/2	$94.50\pm0.5^{C*}$	$50.82\pm1.0^{D*}$	$63.44\pm1.6^{D*}$	$0.60\pm0.02^{D*}$	73.0
[C_4mim][BF_4]	100/5	97.83 ± 0.3^{E}	25.53 ± 2.0^{D}	32.72 ± 0.8^{E}	0.32 ± 0.02^{E}	88.5
	130/2	$95.45\pm0.3^{C*}$	$27.33\pm1.9^{E*}$	$33.20\pm1.9^{E*}$	$0.32\pm0.03^{E*}$	87.8

N/A means not applicable, Reaction condition: Cellulose sample (1 g) was incubated in ILs (9 g) under a sealed condition at 100 °C/ 5h and 130 °C/ 2h under stirring. [a]Calculated based on the mass of regenerated cellulose divided by the mass of cellulose multiply by100 (dry weight based). Enzymatic hydrolysis condition: 100 mg untreated/regenerated cellulose, 0.8 mL of citrate buffer (0.05M, 10 and 20 FPU/g substrate enzyme, 50 °C and 150 rpm). [b]Calculated based upon the mg of glucose released after 72 h of enzymatic hydrolysis divided by initial cellulose (0.1g) multiply by 100 (dry weight basis) using anhydro correction of 0.9 for glucose. All experiments were done in triplicate and the mean is reported with ± S.D. Values in the same column for same treatment 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. [c]Overall glucose recovery g/g avicel is calculated using solid recovery after treatment based upon total glucose released after 72 h of enzymatic hydrolysis of 1g cellulose.

Results presented in Table 4.2 show that ILs having the same cation with a different anion, i.e. [C_2mim][OAc] and [C_2mim][Cl] resulted in 83.3% and 61.7% glucose yields at 100 °C/5 h treatment using 10 FPU/g substrate of enzyme respectively. Whereas, [C_2mim][OAc] and [C_4mim][OAc] ILs comprising different cation and same anion resulted in 83.3% and 64.9% glucose yields respectively under similar process parameters. It is evident from Table 4.2, that ILs having a different combination of the cation with the same anion resulted in different glucose yield. Under similar treatment and saccharification conditions, $[Cl]^-$ based ILs performed moderately, while $[BF_4]^-$ containing IL was ineffective. Therefore, both anion and cation of ILs are the key players to influence the treatment efficiency. Hence, in order to find out the role of cation and anion, we have measured the characteristic parameters viz. K-T parameters, viscosity and surface tension to find the correlation of these properties with glucose yields. In order to differentiate better and for economic aspects, lower enzyme loading, i.e. 10 FPU/g substrate was considered for further studies and in detailed discussion.

4.4.2 IMPACT OF K-T PARAMETERS ON ENZYMATIC SACCHARIFICATION

K-T solvent parameters have been in past used to measure the ability of a solvent to donate a hydrogen bond (α), accept a hydrogen bond (β).[281] It has earlier been shown that basicity (β) correlates well with an IL's ability to dissolve lignocellulose.[269] A study on the range of cations in combination with the same anion demonstrated that cation acidity is also important for cellulose solubility.[270] The K-T parameters describe the hydrogen bond acidity (α), hydrogen bond basicity (β) and polarizability (π^*) of ILs.[277] K-T parameters of all ILs were measured at 100 and 130 °C as described in equation 4.3 and 4.4 and are presented in Table 4.3. Since [C_2mim][Cl] has a high melting point (*ca.* 77 °C), its K-T parameters could not be determined.

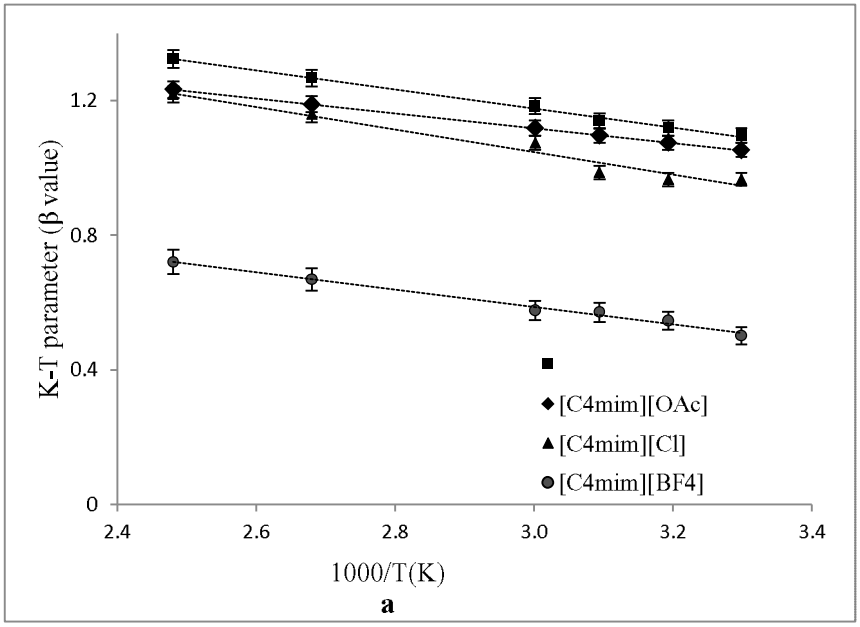
The polarizability (π^*) of ILs slightly decreased with an increase in temperature except for [C_4mim][BF_4] and was found to be in the range of 0.88−0.99. With an increasing alkyl chain length attached to the cation, a decrease in π^* values was observed. $[BF_4]^-$ containing IL are considered as poor

solvents due to low acidity and basicity.[282] Hydrogen bond acidity (α) and hydrogen bond basicity (β) were found to increase with increasing temperature (Table 4.3). The β values of ILs at both temperatures were in the order of: [C_2mim][OAc]> [C_4mim][OAc]> [C_2mim][Cl]> [C_4mim][Cl]> [C_4mim][BF_4]. In a plot of K-T parameters *vs.* 1000/temperature (Figure 4.8a), the slope of $[OAc]^-$ containing ILs is steeper, showing that β values of ILs increase faster with an increase in temperature as compared to π^* and α values (Figure 4.8b, 4.9).

Table 4.3 Solvatochromic K-T parameter of the ILs

	α		β		π^*	
ILs	100 °C	130 °C	100 °C	130 °C	100 °C	130 °C
[C_2mim][OAc]	0.72 ± 0.03^{A}	$0.79\pm0.02^{A*}$	1.27 ± 0.0^{A}	$1.32\pm0.02^{A*}$	0.99 ± 0.01^{A}	$0.98\pm0.01^{A*}$
[C_4mim][OAc]	0.60 ± 0.04^{B}	$0.63\pm0.01^{B*}$	1.19 ± 0.0^{B}	$1.23\pm0.01^{B*}$	0.95 ± 0.01^{B}	$0.88\pm0.02^{B*}$
[C_4mim][Cl]	0.72 ± 0.01^{A}	$0.77\pm0.01^{A*}$	1.16 ± 0.0^{B}	$1.22\pm0.01^{B*}$	0.91 ± 0.01^{C}	$0.90\pm0.02^{B*}$
[C_2mim][Cl]	N/A[a]	N/A[a]	N/A[a]	N/A[a]	N/A[a]	N/A[a]
[C_4mim][BF_4]	0.59 ± 0.01^{B}	$0.63\pm0.2^{B*}$	0.67 ± 0.0^{C}	$0.72\pm0.01^{C*}$	0.99 ± 0.02^{A}	$0.99\pm0.01^{A*}$

K-T parameters using the following set of dyes: Reichardt's dye, N, N-diethyl-4-nitroaniline and 4-nitroaniline: (α), hydrogen bond acidity, (β), hydrogen bond basicity, (π^*), polarizability. All experiments were done in triplicate and the mean is reported with ± S.D. Values in the same column for the same 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. [a]N/A means not applicable, a K-T parameter for [C_2mim][Cl] could not be determined since the melting point of 1-ethyl-3-methyl imidazolium chloride is 77-75 °C.



a

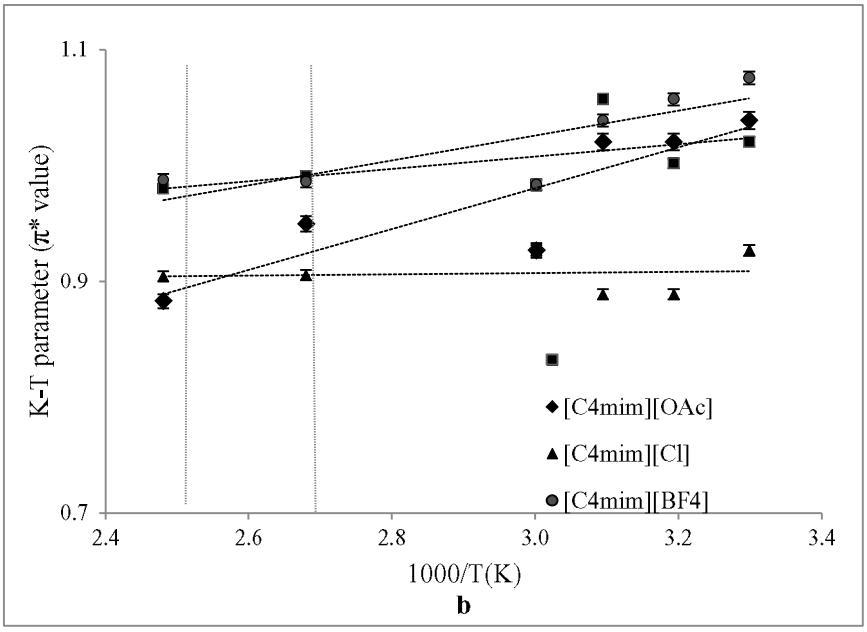


Figure 4.8 K-T parameters of various ILs using the following set of dyes: Reichardt's dye, N, N-diethyl-4-nitroaniline and 4-nitroaniline. (a) Hydrogen bond basicity (β), (b) polarizability (π*).

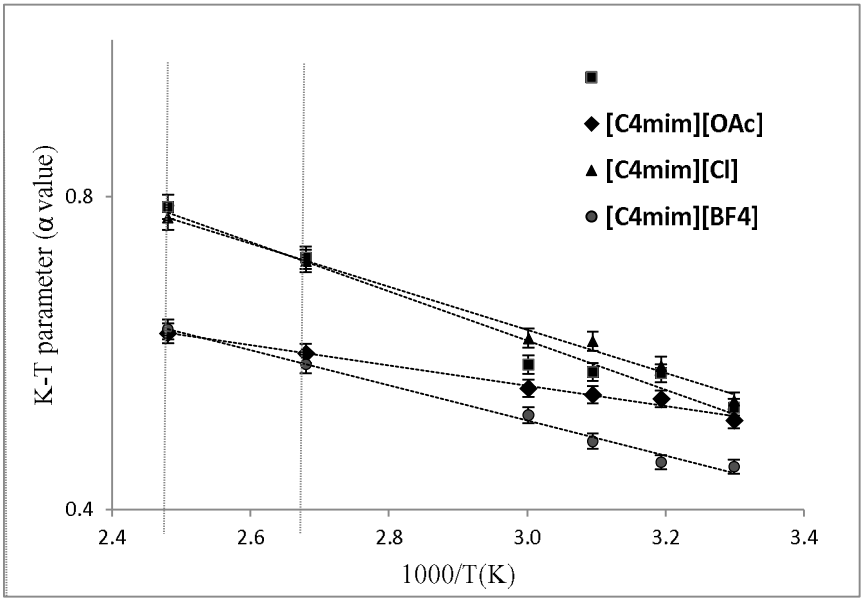


Figure 4.9 K-T parameters: Hydrogen bond acidity (α) as a function of temperature in the range of 25 to 130 °C.

Cellulose treatment in ILs with $\beta > 0.72$ resulted in more than 55% glucose yield after 24 h of hydrolysis and ILs with $\beta \leq 0.72$, neither decrease cellulose

crystallinity (discussed in the later part) nor improve glucose yields. For instance, higher hydrogen bond basicity of [C_2mim][OAc] (β = 1.27) gave highest glucose yield (83.3%) as compared to [C_4mim][OAc] (β = 1.19), which resulted in 64.9% glucose yields. Similarly, [C_4mim][Cl] with β = 1.16 gave 45.9% of glucose yield whereas, [C_4mim][BF_4] having lower β value (0.67) gave only 25.5% glucose yield at 100 °C/5 h. Similar trends were also observed for 130 °C/2 h (Table 4.2). Figure 4.10 shows the correlation between glucose yield and β-values concluding that cellulose treated with ILs with higher β value leads to higher glucose yields. Higher hydrogen bond basicity (β) of [C_2mim][OAc] could stabilise the solvation ion causing further higher solvation of cellulose thus facilitating subsequent enzymatic. In literature, [283-284]; it is mentioned that hydrogen bond basicity (β) of anion attracts hydroxyl protons of cellulose, disrupting the crystal lattice and hydrogen bonding interaction of crystalline cellulose.

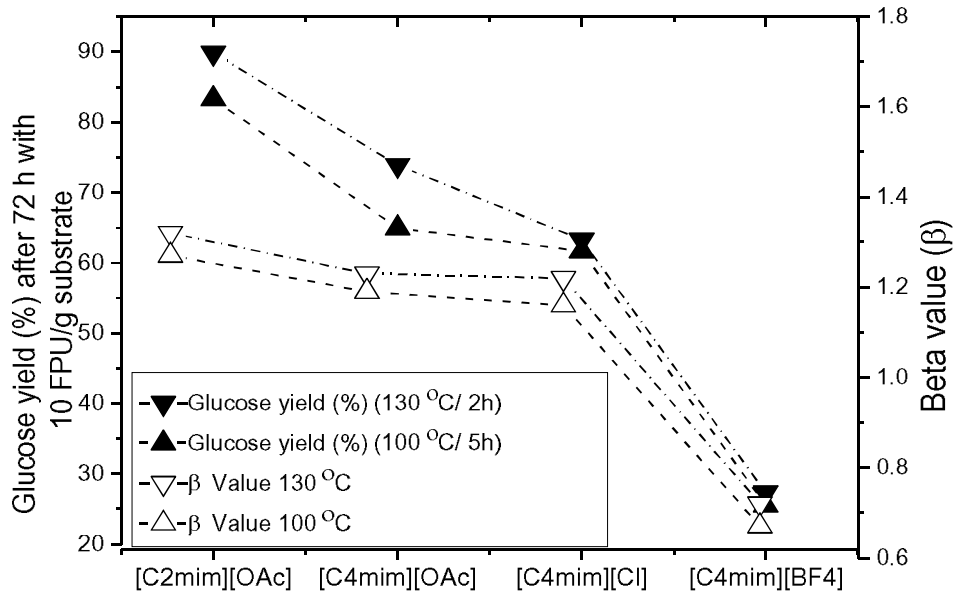


Figure 4.10 Effect of hydrogen bond basicity (β) of ILs and correlation between β vs. glucose yield (%) of the treated cellulose at 100 °C/5 h and 130 °C/2 h.

4.4.3 IMPACT OF VISCOSITY ON ENZYMATIC SACCHARIFICATION

Kinematic viscosity (η) of ILs could also be an important factor which can influence the glucose yields. The viscosity of all five ILs were analysed at 100 and 130 °C and the data is presented in Table 4.4. The data shows that, as expected an increase in temperature, a significant decrease in viscosity of ILs was obtained, i.e. viscosity of [C_2mim][OAc] at 100 °C is 8.4 cSt/sec, whereas, 4.4 cSt/sec at 130 °C. Viscosity of ILs at 100 °C and also at 130 °C was following the same order as: [C_2mim][OAc] ≤ [C_4mim][BF_4] < [C_4mim][OAc] < [C_2mim][Cl] < [C_4mim][Cl]. An increase in viscosity of ILs with various anion/cation combinations may be attributed to the increased strength of van der Waals forces over hydrogen bonding. The viscosity of ILs was also increased with increasing alkyl chain length of the cation of ILs with the same anion. Similar results are reported by Huddleston et al. (2001).[285-286]

Table 4.4 Viscosity and surface tension of different ionic liquids

	Kinematic viscosity (η, cSt/sec)[a]		Surface tension (σ, mN/m)[b]	
ILs	T/K (373.15)	T/K (403.15)	T/K (298.15)	T/K (403.15)
[C_2mim][OAc]	8.35±0.5^{A}	4.41±0.3^{A*}	32.45±0.1^{A}	30.25±0.5^{A*}
[C_4mim][OAc]	14.08±0.4^{B}	6.73±0.4^{B*}	37.34±0.2^{B}	36.42±0.4^{B*}
[C_2mim][Cl]	21.47±0.3^{C}	9.31±0.1^{C*}	42.42±0.3^{C}	40.48±0.5^{C*}
[C_4mim][Cl]	25.71±0.2^{D}	11.44±0.1^{D*}	45.07±0.1^{D}	43.81±0.4^{D*}
[C_4mim][BF_4]	8.15±0.4^{A}	4.53±0.2^{A*}	41.68±0.2^{E}	40.71±0.2^{C*}

[a]Kinematic viscosity (η) was calculated by Cannon-Fenske Viscometer with 300 and 400 bulb sizes directly at 100 and 130 °C. [b]Surface tension (σ) was calculated at 25, 50 and 75 °C and then extrapolated by the linear fit to get value for 100 and 130 °C. All experiments were done in triplicate and the mean is reported with ± S.D. Values in the same column for the same temperature with different superscripts letter indicate the significance difference at $P \leq 0.05$. Letter with star (*) superscript show statistical significance at 403.15K.

In order to find the correlation between the viscosity of ILs and glucose yields, Figure 4.11 was drawn, which shows that the glucose yield was in the reverse order of the viscosity. Highest glucose yield (83.2 and 89.8%) was achieved with [C_2mim][OAc], having a lower viscosity (8.3 and 4.4 cSt/sec) at 100 and 130

°C respectively. Similarly, [C_4mim][Cl] and [C_2mim][Cl] showed highest viscosity, i.e. 25.71 and 21.71 cSt/sec at 100 °C leading to 45.9 and 61.7% glucose yields respectively. This is in consistence with the previous results that ILs with lower viscosity can dissolve more amount of cellulose.[282] However, [C_4mim][BF_4] having a very low viscosity 8.2 and 4.5 cSt/sec at 100 and 130 °C does not improve the enzymatic hydrolysis. This may be attributed to the low hydrogen bond basicity ($\beta < 0.72$), $[BF_4]^-$ being a non-coordinating anion, which is unable to interrupt the hydrogen bonding in cellulose hence, almost no solubilization of cellulose. It can be argued, based on the effect of viscosity and β value on glucose yields, β value is preferred than the viscosity when comparing the ILs treatment efficacy. From this study, it is also observed that ILs having $\beta > 0.72$, with low viscosity results in higher glucose yields.

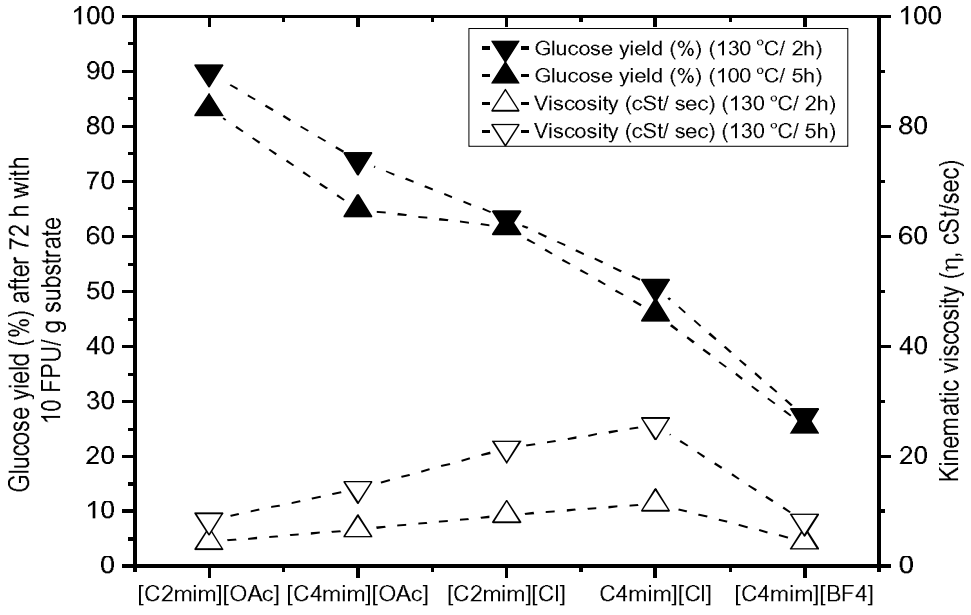


Figure 4.11 Effect of the viscosity of ILs and correlation with glucose yield (%) of the treated cellulose at 100 °C/5 h and 130 °C/2 h.

4.4.4 IMPACT OF SURFACE TENSION ON ENZYMATIC SACCHARIFICATION

The surface tension of all the ILs was measured at 25, 50 and 75 °C and extrapolated by the linear fit to get values for 100 and 130 °C. As shown in Table 4.4 and Figure 4.12 the surface tension decreases with an increase in temperature. ILs with same anion and cation of different alkyl chain length, show 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 substituted on cation the order is $[OAc]^- > [Cl]^- > [BF_4]^-$.

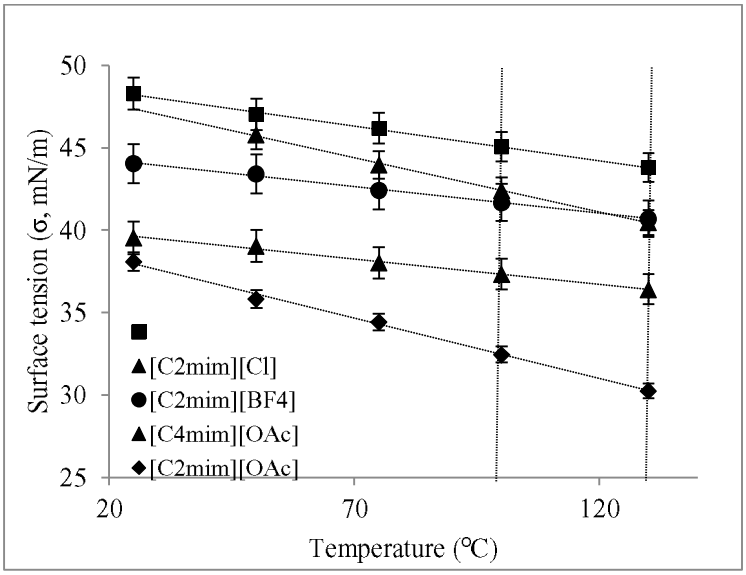


Figure 4.12 Surface tension (σ) of different ILs as a function of temperature for the range of 25 to 75 °C and extrapolated to obtain the values at 100 and 130 °C.

Glucose yields after enzymatic saccharification of ILs treated and regenerated cellulose was drawn against the surface tension in Figure 4.13 and it showed a negative correlation, i.e. ILs with lower surface tension gave higher glucose yields except that of $[C_4mim][BF_4]$. $[C_2mim][OAc]$ with low surface tension, i.e. 32.5 mN/m gave 83.3%, whereas, $[C_4mim][OAc]$ (37.3, mN/m) gave 64.9% of glucose yield at 100 °C/5 h. Similarly, $[C_2mim][Cl]$ with a surface tension of 42.4, mN/m gave 61.7% and $[C_4mim][Cl]$ (45.1, mN/m) gave 45.9% glucose yield at 100 °C/5 h. Similar trends of surface tension vis-à-vis glucose yields were

observed at 130 °C/2 h.

However, surface tension of [C_4mim][BF_4] is 41.7 mN/m, lower than [C_4mim][Cl] (45.17 mN/m), which gave poor glucose yield (25.5%). It may again be attributed to the lower hydrogen bond basicity (β = 0.72) of the former. Therefore, it is clear from the above discussion that, surface tension and viscosity, both are showing a negative correlation with glucose yields and a positive correlation is obtained between natural logarithms of surface tension and viscosity as given in Figure 4.14.[287] Also, from the above data, it can be noticed that the viscosity and surface tension has 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 surface tension may be that liquids with lower surface tension have low surface forces and could easily penetrate into the material resulting in more interaction with cellulose hydroxyls.

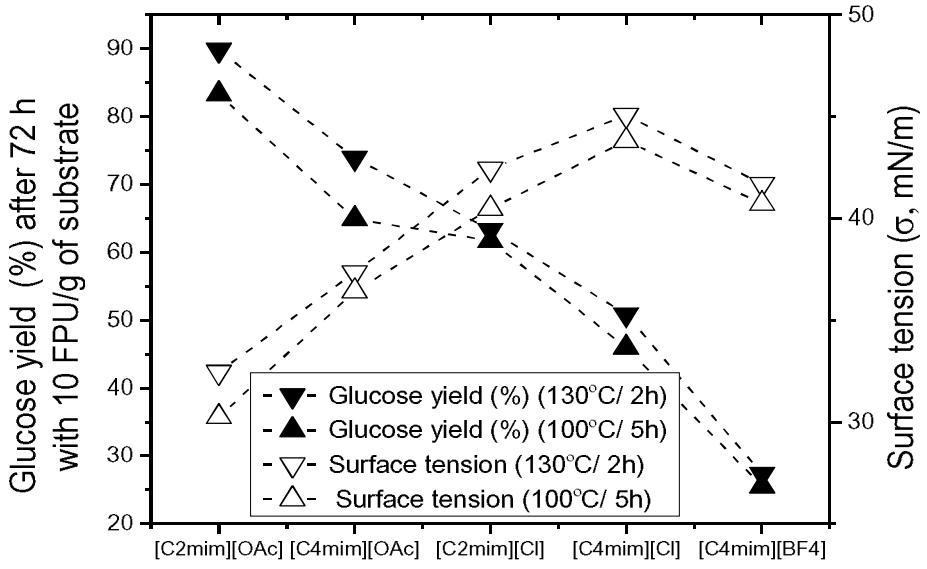


Figure 4.13 Effect of surface tension (σ) of ILs and the correlation between σ vs. glucose yield (%) at 100 °C/5 h and 130 °C/2 h.

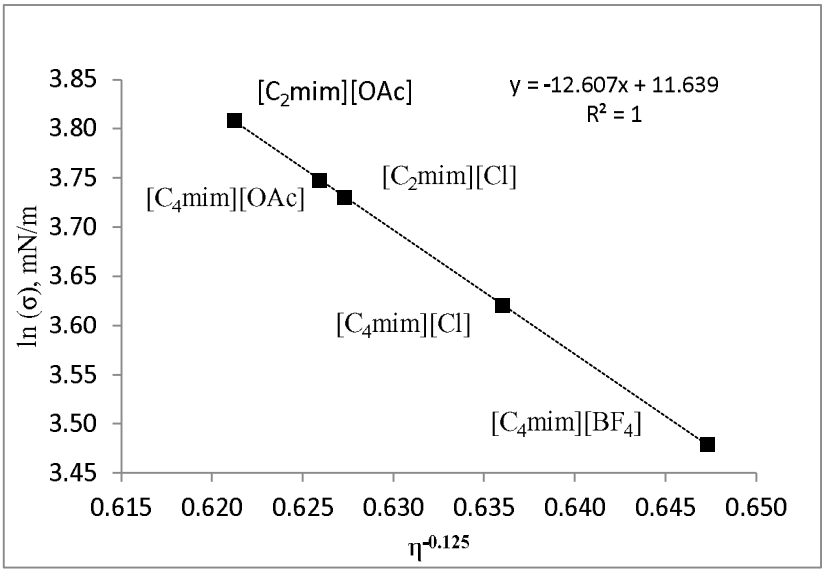


Figure 4.14 Relationship between ln (surface tension, σ) and kinematic (viscosity, η)$^{-0.125}$ for imidazolium-based ILs.

4.4.5 IMPACT OF SIZE OF CATION AND ANION

The impact of different anions $[OAc]^-$, $[Cl]^-$, $[BF_4]^-$ and cations having two different alkyl chain lengths, $[C_2mim]^+$ and $[C_4mim]^+$ were analysed to find out the impact on glucose yields. Results of enzymatic saccharification of regenerated cellulose (Table 4.1) indicate that, with increasing alkyl chain length of cation the glucose yields decreased. The size of the anion in five ILs follows the order of $[OAc]^- < [Cl]^- < [BF_4]^-$,[288] which is reverse to the order of glucose yields, thus anion with small size gives higher yields in enzymatic hydrolysis. $[C_2mim][OAc]$ and $[C_4mim][OAc]$ with same anion, i.e. $[OAc]^-$ with different cation, i.e $[C_2mim]^+$ and $[C_4mim]^+$ results in 89.8 and 73.9 % glucose yields after 72 h of enzymatic hydrolysis respectively. Similarly, $[C_4mim][OAc]$, $[C_4mim][Cl]$ and $[C_4mim][BF_4]$ with same cation $[C_4mim]^+$ and different anion, i.e. $[OAc]^-$, $[Cl]^-$ and $[BF_4]^-$ results in 73.9, 50.8 and 27.3% of glucose yields respectively at 130 °C/2 h. Thus, $[C_2mim][OAc]$ with the best combination of smaller size anion and smaller chain length on cation is quite powerful in cellulose structural deformation leading to highest glucose yields in enzymatic hydrolysis. The size of the cation or anion derives a degree of interaction with cellulose chain thereby disrupting inter and intramolecular hydrogen bonding in cellulose causing higher accessibility to

cellulases thus, giving higher glucose yield.

4.4.6 STRUCTURAL CHANGES IN TREATED CELLULOSE

PXRD and FT-IR analysis of native and treated cellulose were carried out to determine the structural transformations with respect to the treatment temperature, time and ILs. Native cellulose showed a characteristic peak of cellulose I polymorph at $2\theta = 15.2°$ (101) plane and 22.6° (002) plane. Peaks at $2\theta = 15.2°$ (101), 16.4° ($10\bar{1}$) and 22.6° (002) are characteristic of cellulose I. Peaks at 12.1° (110), 20.0° (021) plane represents amorphous cellulose, herein called as cellulose II. After ILs treatment at 100 °C/5 h, [C_2mim][OAc] and [C_4mim][OAc] displayed the reduction in CrI, which may be due to the conversion of cellulose I to cellulose II as shown in Figure 4.15.

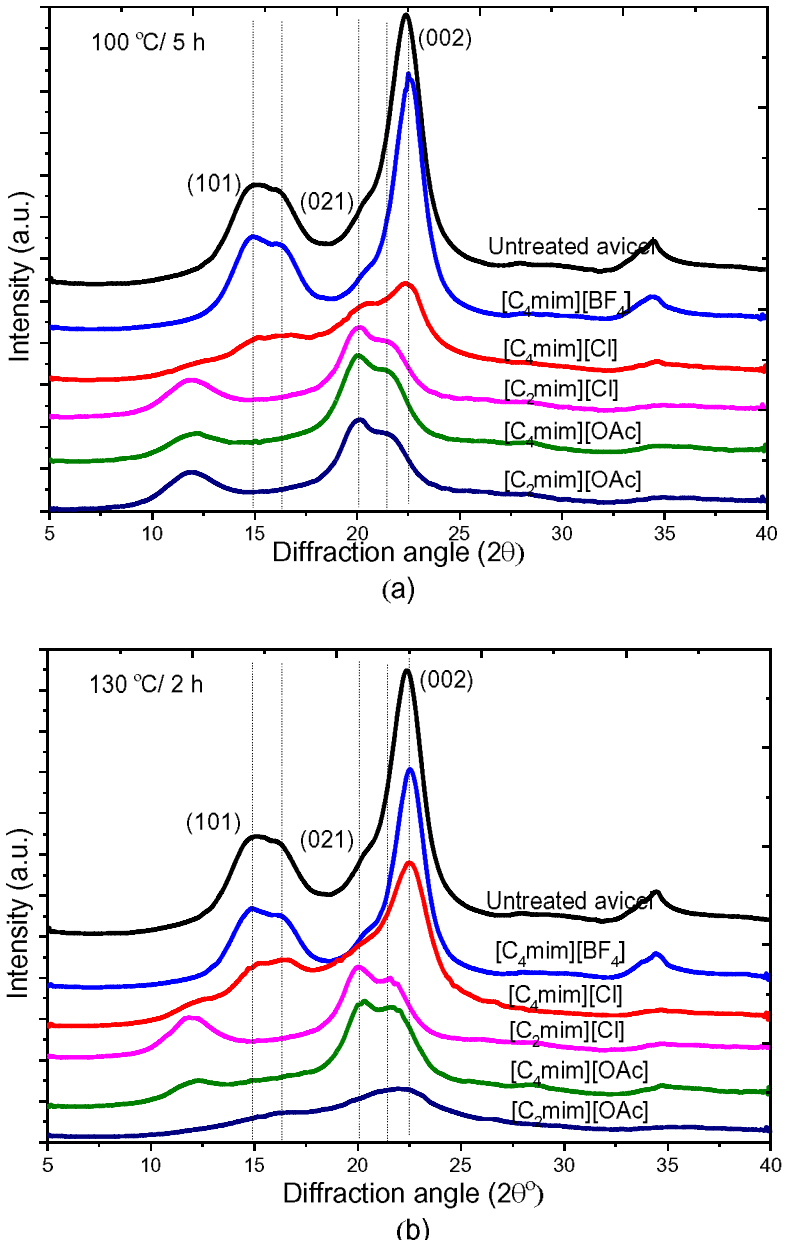


Figure 4.15 PXRD diffraction patterns for untreated and ILs treated cellulose treated by five ILs at 100 °C/5 h and 130 °C/2 h.

Cellulose treated with [C2mim][OAc] at 130 °C/2 h has the lowest CrI (57.4%), compared with [C4mim][OAc] (70.6%), [C2mim][Cl] (72.5%), [C4mim][Cl] (73.0%), [C4mim][BF4] (87.8%) and native cellulose (89.0%). This decrease in CrI clearly shows the transformation of cellulose I to cellulose II. Similar trends were observed at 100 °C/5 h, although the crystallinity index is

higher at 100 °C/5 h. Cellulose treated with [C_4mim][Cl] and [C_4mim][BF_4] retained the crystalline cellulose I structure exhibiting high CrI, i.e. 73.0 and 87.8% respectively even at 130 °C/2 h (Table 4.1), showing no structural changes and hence, resulted in lower enzymatic hydrolysis (Figure 4.16).

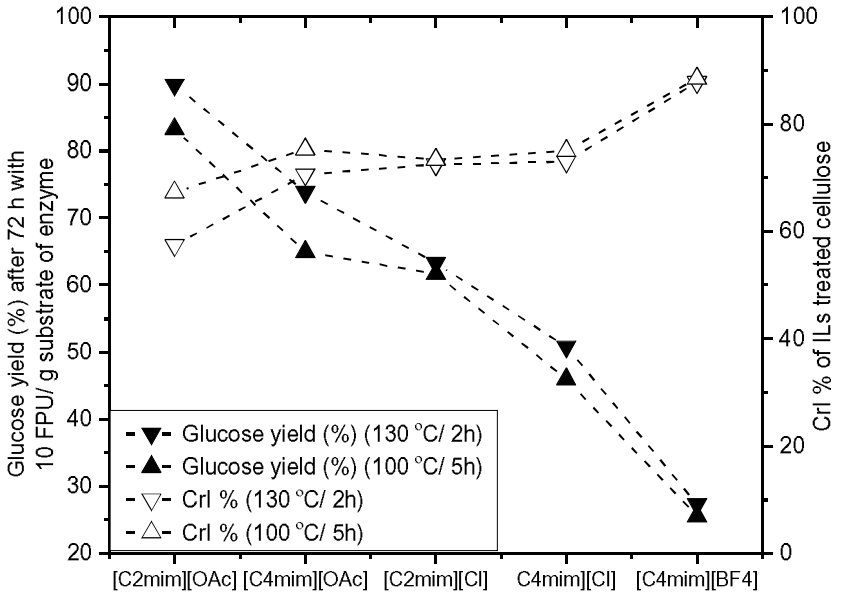


Figure 4.16 Correlation of CrI of treated cellulose vs. glucose yield (%) after 72 h of enzymatic hydrolysis.

The decrease in the X-ray peak intensity with the shift of the peak position to the left is observed with an increase in the treatment temperature. For cellulose treated at 130 °C/2 h, the intensity at 22.6° decreased significantly and disappeared at 15.2 and 16.4° with a peak appearing at 20.0° (110) plane for cellulose II. Cellulose treated with [C_2mim][OAc] at 130 °C/2 h showed highly amorphous nature with a broad peak around 21.4° assigned to the (I_{002}) plane of cellulose II lattice with peaks at ~15.2° and 21.5°. It may be argued that ILs treatment improves the enzymatic hydrolysis by transformation of cellulose I to cellulose II which is amorphous in nature.[289] [C_2mim][OAc] with high β value (1.32), low surface tension and low viscosity rapidly disrupted the inter and intramolecular hydrogen bonding and transformed the cellulose I crystalline structure to cellulose II resulting

in high glucose yields (89.7%). The formation of more amorphous cellulose (cellulose II) facilitated high glucose yields after enzymatic hydrolysis. Similarly, [C_4mim][OAc], [C_2mim][Cl] and [C_4mim][Cl] with moderate viscosity, surface tension and hydrogen bond basicity ($\beta > 0.72$), were also able to reduce cellulose crystallinity to an extent, resulting in moderate glucose yields (Table 4.2). However, [C_4mim][BF_4] with low viscosity (8.2 cSt/sec) and high surface tension (41.7 mN/m)) was unable to penetrate the cellulose structure. Thus, these ILs were not efficient in interrupting the intra and intermolecular hydrogen bonds of cellulose, which resulted in higher CrI with lower glucose yields after enzymatic hydrolysis.

FT-IR spectrum is given in Figure 4.17 for untreated and IL-treated cellulose. A strong absorption band at 3400 cm^{-1} is assigned to different O–H stretching modes due to inter and intramolecular hydrogen bonding. This band is very intense in untreated cellulose and the intensity of these bands decreased with ILs treatment and regeneration. Two bands at 2920 and 2850 cm^{-1} are related to asymmetric and symmetric methyl and methylene stretching. The overlapping features from 1480–1290 cm^{-1} region consist of a number of bands such as 1419, 1328 and 1312 cm^{-1} were mainly due to various deformation modes of C–H bonds in the methylene groups.

The absorption band at 1419 cm^{-1} assigned to the CH_2 scissoring motion was strong in untreated cellulose and is the characteristic peak of crystalline cellulose I. This band intensity was reduced in [C_2mim][OAc] and [C_4mim][OAc] treatment due to the structural transformation from cellulose I to cellulose II. This confirms the reduction in the crystallinity after ILs treatment of cellulose. As discussed above, [C_2mim][OAc] and [C_4mim][OAc] ILs, having higher β value, i.e. 1.27 and 1.19 caused higher transformation of cellulose than [C_4mim][Cl] and [C_4mim][BF_4]. The intensities at 3400, 2887, 1328, 1122, 1082, 1033 cm^{-1} peaks got reduced after treatment resulting in producing 89.8 and 73.9% glucose yield respectively (Table 4.2). The intensity of these peaks after [C_4mim][BF_4] treatment remained same as in the case of native, which confirms that [C_4mim][BF_4] did not

cause cellulose structure alteration, hence, only 27.3% of glucose yield was achieved. However, the absorption band at 898 cm^{-1} assigned to C−O−C stretching at the β−(1→4) glycosidic linkage is weak and broad in cellulose I but strong and sharp in cellulose II. Therefore, infrared spectra in the range of 850–1500 cm^{-1} were characteristic of cellulose samples regenerated from different treatments.

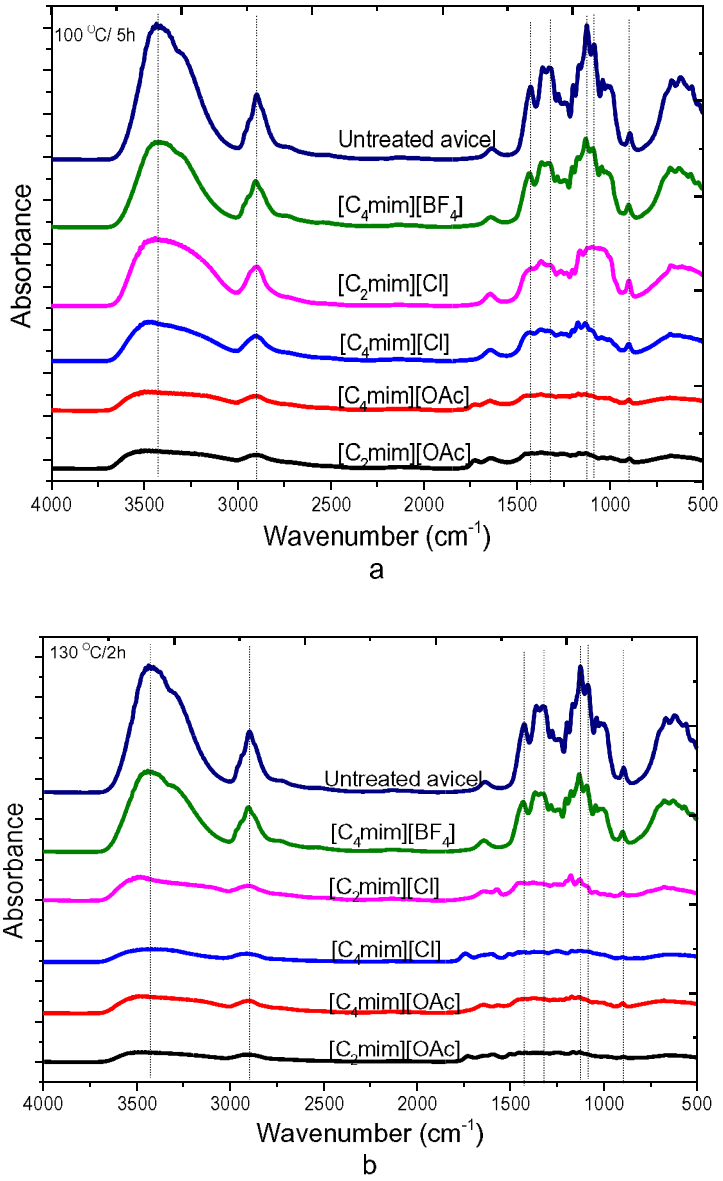


Figure 4.17 FT-IR spectrum of untreated and regenerated cellulose treated by five ILs; (a) 100 °C/5 h and (b) 130 °C/2 h.

It was observed that the untreated cellulose possessed cellulose I crystal type and transformed into cellulose II crystal structure after the treatment and regeneration of cellulose. The absorption bands at 1367 and 2900 cm^{-1} are for C–H and CH_2 stretching, respectively and the band at 1631 cm^{-1} relates to the bending mode of the water. The peak at 1382 cm^{-1} is attributed to the O–H bending and absorption band at 1122 and 1161 cm^{-1} corresponding to C–O antisymmetric bridge stretching. A strong peak at 1082 and 1033 cm^{-1} arises from C–O–C pyranose ring skeletal vibration.[5]

4.5 CONCLUSION

Cellulose transformation efficiencies for improving the enzyme hydrolysis by five ILs, i.e. [C_2mim][OAc], [C_4mim][OAc], [C_2mim][Cl], [C_4mim][Cl] and [C_4mim][BF_4] were investigated. A correlation between IL related K-T parameters including hydrogen bond acidity, hydrogen bond basicity and polarity, viscosity and surface tension and the subsequent impact on glucose yields was established. Among the K-T parameters, β value stands as the best predictor and the first parameter to be considered while checking ILs treatment efficiency. $[OAc]^-$ anion based ILs with $\beta > 0.72$ caused the maximum reduction of CrI and resulted in 89.7% glucose yield after 72 h of cellulase hydrolysis ILs with $\beta \leq 0.72$ like [C_4mim][BF_4] neither induced any structural transformation in cellulose nor improved glucose yields. Viscosity and surface tension of ILs have a negative correlation with glucose yields, lower the viscosity/surface tension higher the sugar yields in saccharification. This correlation is applicable only if IL has $\beta > 0.72$, hence, could also be an important parameter to predict the treatment efficiency. ILs with smaller anion and alkyl chain on cation is efficient for the treatment and enzymatic hydrolysis. If the variation is induced either in anion or cation, keeping the other constant, the surface tension and viscosity could be an indicator of treatment efficiency. [C_2mim][OAc] having higher β value, lower viscosity, lower surface tension and smaller size of anion and shorter alkyl chain gave highest glucose yields. The experimental correlation and detailed knowledge of multiple

properties presented in this work can be used as a tool for the rational design of ILs for lignocellulosic biomass treatment. However, these initial laboratory scale evaluations of IL-based treatment technology can be effectively scaled to larger operations. The pretreatment of lignocellulosic biomass using these ILs is being presented in Chapter 5..