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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6.1 INTRODUCTION

World energy demand is increasing continuously with an increase in industrialisation in the past two decades. The total world energy consumption in 2012 was 1.384 X 10^{19} million tonnes of oil equivalent (Mtoe) and is estimated to reach 1.586 X 10^{19} Mtoe and 2.055 X 10^{19} Mtoe in 2020 and 2040 respectively, almost a 48% increase.[3] In order to meet these demands, lignocellulosic biomass (LCB) is considered as a renewable, replenishable, abundant and potential source for production of biofuels alternative to fossil fuels, which helps in mitigation of GHG emissions.[326] LCB mainly consists of three biopolymers, *i.e.* cellulose (35-50%), hemicelluloses (20-35%), lignin (10-25%), apart from some amount of ash (1-20 %), extractives (1-15%) and proteins (1-2%).[327] These cell wall components are architect together into a complex, non-uniform, three-dimensional, heterogeneous matrix via strong inter/intramolecular hydrogen bonding, Van der Waal forces, dipole-dipole interaction, π-π bonding, covalent bonding and many different cross-links making it a rigid lignin-carbohydrate recalcitrance material making it to resist any biotic and abiotic degradation.[263] Hence, bioconversion of lignocellulosic biomass is significantly hindered by innate recalcitrance.

To extract fermentable sugars from LCB, pretreatment is a pre-requisite step, which involves disruption of the lignin-carbohydrate complex as one of the key steps in bioethanol production.[230, 328] An effective pretreatment helps to expose surface area, increases porosity, reduces crystallinity and decreases degree of polymerization of biomass, which in turn increases the accessibility of cellulases towards cellulose chain for hydrolysis to fermentable sugars.[329-331] Cellulose accessibility towards cellulases is associated with intimate contact between cellulose and cellulases for enzymatic hydrolysis, which is highly dependent on surface opening/internal slits, voids and spaces. It was reported earlier that small pores (i.e. those with diameters smaller than the diameter of cellulase, i.e. 2.4 to 7.7 nm in diameter, with a mean of 5.9 nm) restrict the accessibility and large pores enhance enzymatic saccharification.[332] Large pores formed during any pretreatment increases the probability of maximum enzyme access and subsequently significant to increase the sugar production from biomass.

As discussed in chapter 4 and 5, imidazolium-based ionic liquids (ILs) can be efficient in transforming of LCB, which helps to increase the surface area after pretreatment.[333-334] ILs are organic salts with melting points of <100 °C, and are mainly composed of organic cations[335] (pyridinium, imidazolium and quaternary ammonium species) and organic/inorganic anion (halogen, acetate and some other polyatomic inorganic species).[8-12] Certain imidazolium-based ILs with varying degree of hydrogen bond basicity has emerged as a "green solvent" to solubilize cellulose and helps to mitigate the LCB recalcitrance due to unique solvation properties.[336]

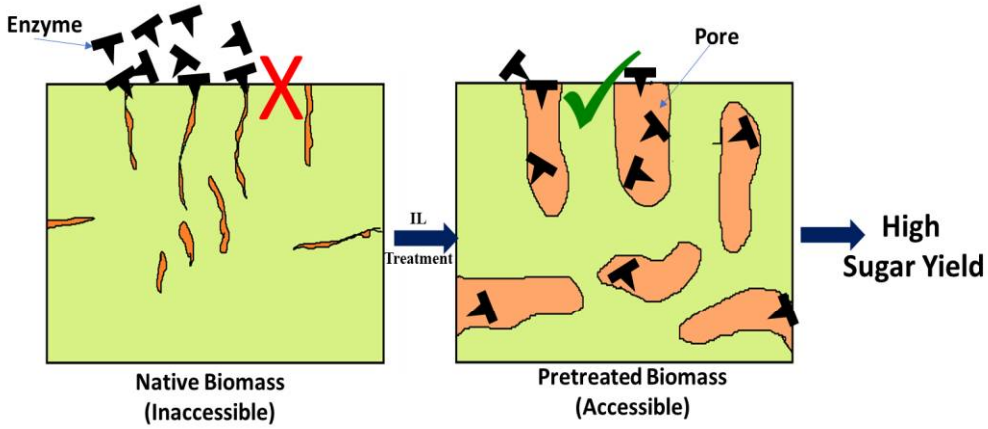

Figure 6.1 Pore formation in biomass during IL pretreatment

In natural biomass, the pores present are very small and are inaccessible for enzyme adsorption as shown in Figure 6.1. During, ionic liquid pretreatment, a portion of lignin and hemicellulose were liberated with cellulose structure transformation from cellulose I to cellulose II as described in Chapter 4 and 5, which creates voids and pores of larger size increased the overall surface area. Structural characteristics such as specific surface area, pore volume, crystallinity significantly affect the hydrolysis yield for ethanol production.[42,337]. Pore properties, i.e. pore size and pore volume distribution are important features of any pretreated biomass, however, these properties in pretreated biomass are significantly altered upon drying of biomass. Claudia et al. (2007)[24] have shown that porosity is the limiting factor for cellulose digestibility and have determined the porosity of dilute sulphuric acid using 1H NMR and thermometry to measure the water in pores ranging from 20 to 200 Å.

6.2 AIM OF STUDY

The aim of current study was to evaluate the effect on characteristics of biomass pretreated with an ionic liquid and its impact on enhancement for enzymatic digestibility. For this, five imidazolium-based ILs, viz. 1-ethyl-3-methylimidazolium acetate [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] were employed for mustard stalk pretreatment at 100 °C/5h and 130 °C/2h, the regenerated cellulose-rich biomass was subjected to enzymatic hydrolysis for sugar release. Accessibility of cellulases towards cellulose is affected by biomass crystallinity, surface area, and pore volume. Hence, the structural features of pretreated biomasses were measured by chemical analysis, Brunauer-Emmett-Teller (BET), Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy (FT-IR) respectively and were correlated with sugar released after enzyme hydrolysis. FT-IR proved to measure the cellulose-related properties, viz. lateral order index (LOI), total crystallinity index (TCI) and hydrogen bond index (HBI), acetyl content and cross-linked lignin (CLL) for improved enzymatic hydrolysis. DSC was used to measure the cellulose accessibility/porosity of ILs pretreated biomass. DSC helps to determine freezing and non-freezing water, which was used to quantify the pore volume, pore diameters. BET helps to determine the specific surface area of pretreated biomass to further measure the accessibility of pretreated biomass. Altogether these properties of pretreated LCB were found as a rate determining step in improved enzymatic hydrolysis with varying capacity.

6.3 EXPERIMENTAL SECTION

6.3.1 MATERIALS

All ILs and other chemical were purchased from Sigma-Aldrich as described in materials and methods section of Chapter 5.

Mustard (*Brassica juncea*) stalk 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.

6.3.2 IONIC LIQUID PRETREATMENT

The method of IL pretreatment was discussed in experimental section in Chapter 5 in Section 5.2.

6.3.3 COMPOSITIONAL ANALYSIS AND ENZYMATIC SACCHARIFICATION

The method of compositional analysis and enzymatic saccharification of ionic liquid pretreated biomass was discussed in experimental section in Chapter 5 section 5.2.

6.3.4 DSC MEASUREMENT

Lignocellulosic biomass is rich in hydrophilic sites (OH groups of cellulose and hemicellulose). Water in biomass is adsorbed in pores, bulk and surrounding during the swelling process by interacting with these hydrophilic sites after ionic liquid pretreatment and are considered as non-freezing water. Washed pretreated biomass wet samples were stored in the refrigerator (4 °C) and moisture content was determined using Thermo Gravimetric Analyzer (TGA). For this, approximately, 10 mg sample was placed in TGA and temperature was increased from 30 to 105 °C at a rate of 10 °C/min maintained at 105 °C for 15 minutes to ensure the constant weight of the dry biomass. Moisture ratio of the sample was adjusted in the range 45-55% by gently pressing the sample between filter paper and repeating the TGA analysis till the desired level of moisture content was achieved, since drying of biomass collapse the pores structure.

Thermometry method using differential scanning calorimetry (DSC) can be helpful for determination of water bound in the pore present in pretreated biomass, which can be correlated with the accessibility of enzymes to substrates. DSC measurements were conducted using Perkin Elmer DSC 2000. The samples of approximately 10 mg were weighed into hermetically sealed aluminium pans. The weight of the sample was checked before and after the

DSC measurements to ensure that the pan was well sealed. Temperature calibration of DSC was done using ultrapure water. Heat calibration was done by carefully weighing about 3-4 mg of ultrapure water in a sealed pan. Freezing bound water was measured using isothermal melting process by slowly raising the temperature of a frozen pretreated sample from -40 °C to a preset value say -30 °C, where it is held constant until the melting transition is completed. The melting of the sample is then repeated at a slightly higher temperature in sequential way, i.e. (-30, -15, -10, -6, -4, -2, -1.5, -1.0, -0.5, -0.2, and -0.1 °C) and hold for certain time until heat flow comes to baseline. The heat absorbed at each temperature (T_m), *i.e.* melting enthalpy (H_m) is calculated by subtracting the sensible heat (C_p. ΔT) from the total heat of corresponding endotherm (H_t) as shown in Eq. 2:

$$H_m = H_t - C_p.\ \Delta T \qquad \text{Eq. 2}$$

The melting enthalpy (H_m) is assumed to correlate directly with the amount of melted water and the amount of non-freezing bound water was calculated by subtracting the total freezable water (both freezing bound water and unbound water). Quantitative determination of freezing bound water can be done by integration of endotherm (melting of water). Freezing bound water melts at a lower temperature than unbound free water, and the melting temperature depression (T_m) has a reciprocal relationship with the pore diameter (D). The pore size distribution (PSD) is calculated from the melting temperature depression of the water in pores of a given diameter.[332] Based on the Eq.3, each melting temperature depression (ΔT) represents a specific pore diameter assuming that pores biomass were cylindrical in shape.

$$\Delta T = T_0 - T_m = -4\ T_0\ \Upsilon_{ls}\ \text{Cos}\ \theta / D\ \rho\ H_f \qquad \text{Eq. 3}$$

Where T_0 is the melting temperature of water (273.15 K), Υ_{ls} is the surface energy at the ice-water interface (12.1 mJ/m^2), ρ and H_f are the density and the specific heat of fusion of freezing bound water, respectively assumed to be the same as that of unbound water (1,000 kg/m^3, 334 J/g).[338] Thus, Eq. 3

showed that water in a small pore has larger melting temperature depression as compared to the larger pore. In a real sense, the pores are irregular in shape and size.

6.3.5 FT-IR

Fourier transforms infrared spectroscopy (FT-IR) spectra of biomass samples were recorded using Prestige-21, Model No. A21004802514. All spectra were recorded in the absorbance mode from an accumulation of 128 scans at a $4cm^{-1}$ resolution over 4000–400 cm^{-1} range. The zero-baseline correction with 10 points smoothing using KubelkaMunk correction was used. The FT-IR spectra were normalised with respect to1504 cm^{-1}. Samples were analysed by grinding with KBr (1:100, w/w) in drift mode. Microcrystalline cellulose (Avicel PH 101) was used as the reference material. The quantitative relationship between infrared spectral bands of cellulose, hemicellulose, and lignin in pretreated biomass was conducted in term of Total crystallinity index (TCI), Lateral order index (LOI), Hydrogen bond index (HBI), Acetyl content and Cross-linked lignin (CLL) by measuring the peak intensities, *i.e.* 3400, 2920, 1708, 1600, 1510 1430, 1375, 1323 and 898 cm^{-1} using Nelson and O'Connor Method.[339]

6.3.6 BET SPECIFIC SURFACE AREA

Native and ILs pretreated mustard stalk were placed in degassing module of Quantachrome autosorb® IQ surface and pore area analyser and degassed at 90 °C for 12 h before analysis. All the samples were reweighed to account for the mass loss (if there was any). The degassed samples were then placed in the analyser for multipoint Brunauer–Emmett–Teller (BET) analysis. The adsorption isotherm was recorded for the pressures ranging from 0–1 (P/Po) using nitrogen as an adsorbate gas at the liquid nitrogen temperature using a method reported earlier.[76]

6.3.7 SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy (SEM) of native and pretreated biomass at different magnifications was conducted using HITACHI S-3400 (USA). The

specimens were mounted on a conductive tape and coated with gold using a fine coater (4 nm) and observed at an accelerating voltage of 2.0 kV.

6.3.8 STATISTICAL ANALYSIS

Statistical analysis was conducted using one-way ANOVA Tukey's HSD post hoc tests for multiple comparisons using JMP software (USA) and statistical significance was determined at 0.05 levels ($p \leq 0.05$).

6.4 RESULTS AND DISCUSSION

The change in physicochemical properties of MS after ILs pretreatment was measured in terms of compositional analysis, specific surface area, pore size/volume and FT-IR (LOI, TCI, HBI, CLL and acetyl content). Their correlation with subsequent enzymatic saccharification would give an insight into the mechanism of ILs action and potential indicators to measure pretreatment efficiency.

6.4.1 CHEMICAL COMPOSITION AND ENZYMATIC HYDROLYSIS

The chemical composition of native and IL-treated mustard stalk are summarised in Table 6.1. In general, glucan content increased with pretreatment severity from 100 °C/5h to 130 °C/2h concomitants with the reduction in xylan content. Focusing on the anion part, Table 6.1 showed that ILs with $[Cl]^-$ as anion were less effective in solubilizing xylan and lignin evident by the insignificant change in glucan content as 38.9, 39.5% and 40.5, 40.5% for 100 °C/5h and 130 °C/2h treated mustard stalk. On the other hand, IL with $[OAc]^-$ anion and small alkyl chain, *i.e.* $[C_2mim][OAc]$ effectively removed xylan and lignin with maximum enrichment of glucan content, *i.e.* 46.4% for 100 °C/5h and 50.1% for 130 °C/2h treated mustard stalk. Table 6.1 also showed that non-coordinating anion, *i.e.* $[BF_4]^-$ was not competent to change glucan/xylan content from native biomass. Removal of xylan and increase in glucan content is expected to correlate with the enzymatic saccharification of the respective biomass.

Table 6.1 summarizes the glucose yield (%) after 72h of enzymatic hydrolysis using 10 FPU/g at 10% biomass loading of IL pretreated mustard

stalk. As expected, corresponding to the increased glucan content, glucose yield (%) increased with pretreatment severity, *i.e.* 100 °C/5h and 130 °C/2h and was found in the order of [C_2mim][OAc] (97.7%)> [C_4mim][OAc] (72.8%)> [C_2mim][Cl] (31.5%)> [C_4mim][Cl] (28.7%)> [C_2mim][BF_4](13.8%)> Native (11.3%) for 130 °C/2h treated mustard stalk.

Table 6.1 Compositional analysis of untreated and ILs pretreated biomass

Pretreatment		Composition of pretreated biomass (%)			Enzymatic hydrolysis
ILs	*T/t*	Glucan[a]	Xylan[b]	Lignin[c]	Glucose yield (%)[d]
Mustard Stalk	**N/A**	**39.94**	**22.3**±0.5	**20.8**±0.5	**11.3±1.1**
[C_4mim][BF_4]	100/5	38.7 ± 0.5^{A}	20.9 ± 0.4^{A}	20.9 ± 0.4^{A}	12.1 ± 0.4^{A}
	130/2	$39.2\pm0.2^{A*}$	$21.9\pm0.4^{A*}$	$19.9\pm0.2^{A*}$	$13.8\pm0.8^{A*}$
[C_4mim][Cl]	100/5	38.9 ± 1.0^{A}	20.3 ± 0.9^{A}	21.7 ± 0.2^{A}	23.8 ± 0.4^{B}
	130/2	$39.5\pm0.8^{A*}$	$19.3\pm0.6^{B*}$	$20.0\pm0.3^{A*}$	$28.7\pm0.9^{B*}$
[C_2mim][Cl]	100/5	40.5 ± 0.7^{A}	21.8 ± 0.8^{A}	22.6 ± 0.5^{A}	32.5 ± 1.2^{C}
	130/2	$40.5\pm0.2^{A*}$	$20.7\pm0.2^{B*}$	$20.4\pm0.5^{A*}$	$31.5\pm1.2^{C*}$
[C_4mim]OAc]	100/5	45.4 ± 0.8^{B}	11.2 ± 0.3^{B}	23.8 ± 0.4^{B}	72.6 ± 0.9^{D}
	130/2	$48.8\pm0.2^{B*}$	$9.4\pm0.8^{C*}$	$20.4\pm0.5^{A*}$	$72.8\pm1.5^{D*}$
[C_2mim]OAc]	100/5	46.7 ± 0.5^{B}	8.5 ± 0.5^{B}	24.5 ± 0.6^{C}	78.7 ± 1.4^{E}
	130/2	$50.1\pm0.7^{B*}$	$8.7\pm0.5^{C*}$	$22.5\pm0.4^{B*}$	$97.7\pm2.3^{E*}$

[a,b,c]Glucan/Xylan/Lignin content was determined using NREL LAP protocols as described in material and methods. [d]Glucose yield (%) is the sugar yield after 72 h of enzymatic hydrolysis using 10 FPU/g of the enzyme (calculated based on total glucan present in pretreated mustard stalk). 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 $P \leq 0.05$. Letter with a star (*) superscript show statistical significance at 130 °C.

Similar trends were observed for 100 °C/5h treated mustard stalk, however, the glucose yields were comparatively lower, *i.e.* [C_2mim][OAc] (78.7%)> [C_4mim][OAc] (72.6%)> [C_2mim][Cl] (32.5%)> [C_4mim]Cl] (23.8%)>[C_2mim][BF_4] (12.1) respectively. In conclusion, we have demonstrated that $[OAc]^-$ based IL, *i.e.* [C_2mim][$OAc]^-$ at 130 °C/2h treatment gave maximum glucose yield (97.7%) as compared to other ILs treated mustard stalk. Besides the ILs, the reaction temperature has a very important role in improving the digestibility.

6.4.2 DSC ANALYSIS OF IL TREATED BIOMASS

In the current study, DSC thermometry was adopted for determination of pore size based on the depression in freezing points of water bound in pores. Water absorbed in lignocellulosic cell wall matrix is categorised as non-freezing

bound and freezing water and unbound/free water.[340] In moist biomass samples, bound water is the water retained in the capillaries/pores of biomass particles to prevent the irreversible collapse of pores. Figure 6.2 shows the representation of water molecules in and around the biomass cell wall. Non-freezing bound water is retained in the first 1-3 layers of water adjacent to the surface and does not freeze due to the restricted motion in association with the surface.[338] Freezing bound water, *i.e.* water held in submicroscopic pores, cracks, and crevices has different thermodynamic enthalpic interaction due to high tension from the surface, thus has the depressed freezing point. Free water is the unbound water with thermodynamic properties similar to those of pure bulk water. ILs pretreatment helps to generate the number of pores and water retained in the capillaries of pretreated biomass has a depressed melting temperature due to lower vapour pressure at the curved surface in pores, assuming all pores are cylindrical in nature.

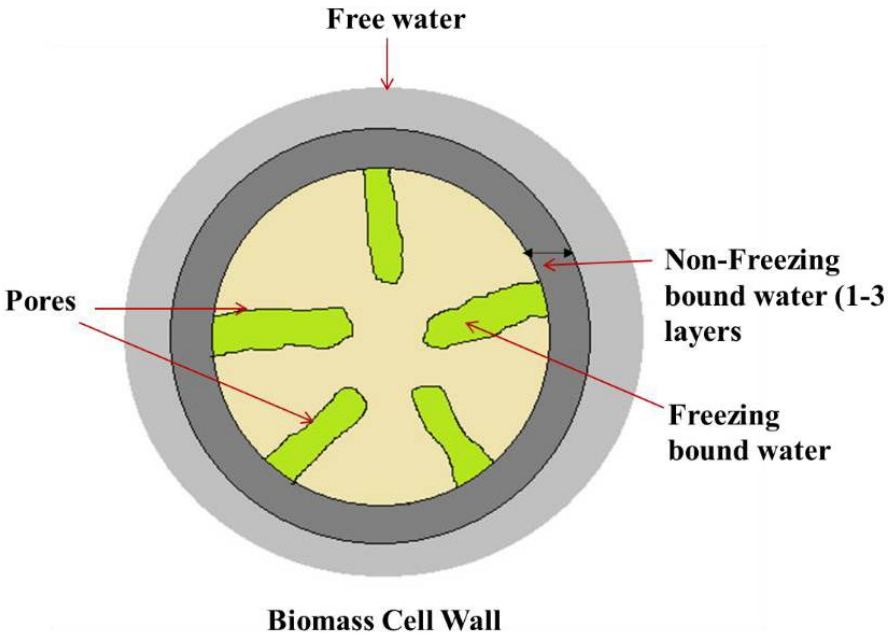

Figure 6.2 Freezing and non-freezing bound water in biomass cell wall

Table 6.2 showed the pore size and pore volume of mustard stalk pretreated by five imidazolium-based ILs and ranged from 1.7 to 396 nm, and 112.5 mg/g to 487.7 mg/g of biomass respectively. In nature, most of the cellulose fibrils have a diameter of 3-4 nm, which resist the accessibility of

enzymes.[341] However, the discrimination between the extent of enzyme accessibility was expressed by the quanta of pores as depicted by pore volume. Interestingly, Figure 6.3a and 6.3b showed that all the pretreated biomasses predominantly contained two pore sizes, viz. 19.8 nm and 198 nm. Pore size 19.8 nm matches closely with the inter-chain distance (~20 nm) in cellulose II fibrils indicating a possible transition of cellulose I (crystalline) to cellulose II (amorphous) during IL pretreatment.

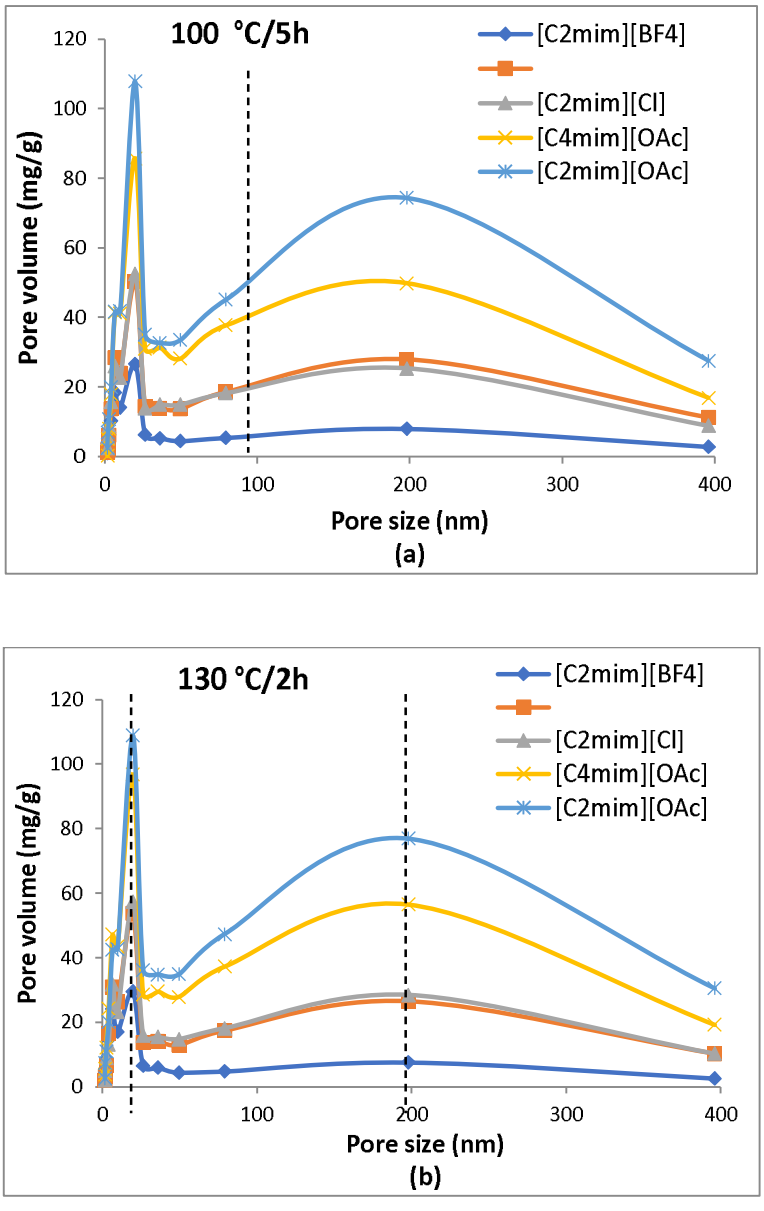

Figure 6.3: Pore volume distribution of IL pretreated mustard stalk at; (a) 100 °C/5h; (b) 130 °C/2h

Table 6.2: Pore size and pore volume of ionic liquid treated mustard stalk

	$[C_2mim][BF_4]$		$[C_4mim][Cl]$		$[C_2mim][Cl]$		$[C_4mim][OAc]$		$[C_2mim][OAc]$	
	100/5h	130/2h	100/5h	130/2h	100/5h	130/2h	100/5h	130/2h	100/5h	130/2h
Pore Size, nm	**Pore volume (mg/g)**									
1.7	0.9	3.4	1.2	2.0	2.5	2.0	0.2	3.8	2.5	2.5
2	3.9	5.5	2.1	3.2	5.4	4.9	5.1	5.6	6.9	7.2
2.6	6.1	8.7	6.1	6.5	8.8	8.9	7.9	12.2	10.7	10.9
4	10.2	14.7	13.7	16.5	15.7	13.1	18.0	24.2	19.9	20.1
6.6	18.4	24.1	28.5	30.9	25.9	31.7	41.4	47.2	41.7	42.5
9.9	14.1	17.0	23.9	26.2	22.7	23.2	41.2	43.5	41.8	42.7
19.8	26.6	29.5	50.3	53.7	52.5	57.2	85.7	96.7	107.8	108.9
26.4	6.3	6.5	14.4	13.8	13.8	15.8	31.0	28.6	35.1	36.1
36	5.3	5.9	13.8	14.1	15.0	15.5	31.4	29.5	32.7	34.8
49.5	4.4	4.4	13.7	12.9	14.9	14.8	28.1	27.8	33.5	34.9
79.2	5.3	4.8	18.7	17.4	18.2	18.1	37.8	37.2	45.2	47.3
198	8.0	7.6	27.9	26.5	25.4	28.4	49.8	56.5	74.4	76.9
396	2.8	2.6	11.2	10.2	8.9	10.2	16.9	19.2	27.5	30.5
Total Freezing bound water (pores), mg/g	112	134	225	233	229	243	405	420	479	487

The pore size 198 nm corresponds to the observed size of lignin particles (~200 nm in length), which might get ripped off resulting in limiting lignin-carbohydrate bonding. Both this phenomenon could result in facilitating cellulose accessibility to enzymes yielding 97.7 % glucan conversion for [C_2mim][OAc] treated mustard stalk. Effect of pretreatment temperature was also found to have a positive effect on pore volume, for example, [C_2mim][OAc] at 130 °C/2h showed higher pore volume (108.9 mg/g) compared to 100 °C/5h (107.8 mg/g) resulting in 97.7 % and 78.7% glucose yield respectively. Figure 6.4 showed a high positive correlation between glucose yield and total freezing bound water with R^2=0.84.

This showed that ionic liquid pretreatment helps to increase the porosity of pretreated biomass and total pore volume distribution was found to decrease in the order of [C_2mim][OAc] (487)> [C_4mim][OAc] (420)> [C_2mim][Cl] (243) [C_4mim][Cl] (233) and [C_4mim][BF_4] (134 mg/g) respectively for 130 °C/2h treated mustard stalk, with improved cellulose digestibility. A similar trend was also observed for 100 °C/5h treated mustard stalk. Thus, the results show the efficiency of sugar hydrolysis of pretreated biomass was proportional to the population of large pore size, which is in comparable to the size of the enzyme. We have found that the distribution of larger pore is comparatively more in $[OAc]^-$ ionic liquids, which might be attributed to cellulose II transformation and higher lignin and xylan removal.

6.4.3 BET SURFACE AREA ANALYSIS

For hydrolyzing the cellulose, cellulase needs to be adsorbed on the biomass surface to interact with cellulose fibrils. Surface area and pore volume were significant factors affecting enzymatic hydrolysis, because, to some extent, they reflected the polysaccharide accessibility. The more available surface is reflected as increased specific surface area determined by BET. Native mustard stalk showed the lowest surface area as 1.58 m^2/g due to rigid and packed arrangement of cell wall components. Table 6.3 showed that pretreatment with ILs having small alkyl chain and $[OAc]^-$ anion was most effective in enhancing the specific surface area. Effective removal of xylan and

lignin during pretreatment correlates well with increased surface area.

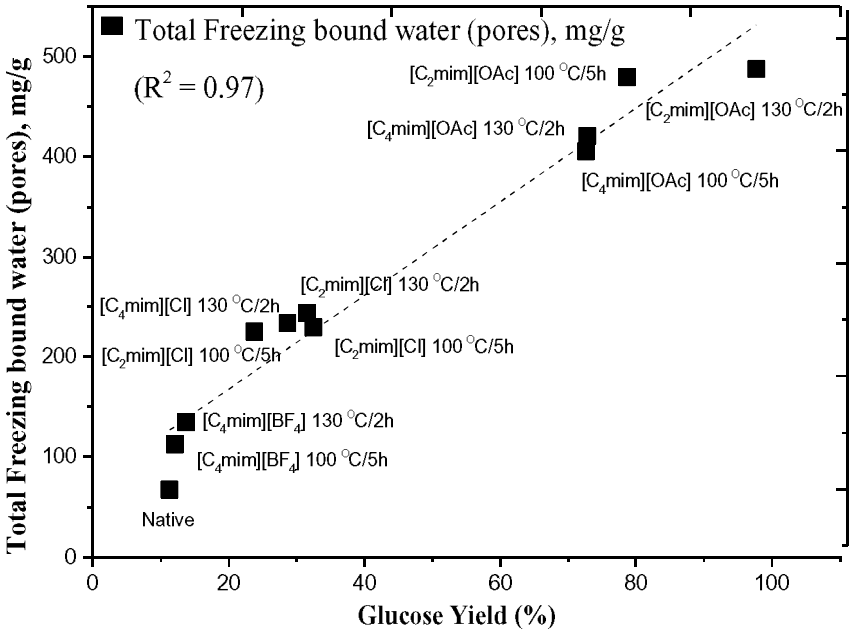

Figure 6.4: Co-relation of between total freezing bound water (mg/g) vs. glucose yield (%)

The effect was more pronounced at a higher temperature, *i.e.* 130 °C/2h (7.05 m^2/g) as compared to 100 °C/5h (5.85 m^2/g). The order of surface area was: [C_2mim][OAc] (7.05 m^2/g)> [C_4mim][OAc] (6.05 m^2/g)> [C_2mim][Cl] (2.93 m^2/g)> [C_4mim][Cl] (2.63 m^2/g)> [C_2mim][BF_4] (1.93 m^2/g)> Native (1.58 m^2/g) for 130 °C/2h treated mustard stalk. Similar trends were also found for 100 °C/5h treated biomass. The higher specific surface area must result in adsorption of a higher amount of cellulases resulting in higher glucose yield. A high R^2 value of 0.97 for a linear correlation between specific surface area and glucose yield (%) was found as shown in Figure 6.5a. We conclude that larger surface area made polysaccharides more easily accessible to enzymes, and led to the substantial improvements in the digestibility of polysaccharides.

After establishing the correlation between the surface morphology, i.e. pore size/volume and surface area. It was interesting to find a relation between these two. Hence, Figure 6.5b was drawn to find the correlation between pore volume and specific surface area, which showed a linear positive correlation with R^2=0.95). The increased specific area was attributed to increased pore size,

Table 6.3: LOI, TCI, HBI, CLL, Acetyl content and specific surface area

ILs treated Biomass	*T/t*	LOI[a] 1430/898	TCI[b] 1375/2920	HBI[c] 3400/1323	CLL[d] (1510/1600)	Acetyl Content[e] 1738	Specific surface area (m^2/g)
Untreated Mustard stalk	N/A	2.14±0.11	1.42±0.01	1.34±0.01	1.13±0.01	1.13±0.01	1.58±0.11
$[C_2mim][BF_4]$	100/5h	2.10±0.01	1.45±0.01	1.33±0.01	0.92±0.02	1.12±0.01	1.88±0.11
	130/2h	2.0±0.010	1.32±0.02	1.30±0.03	1.02±0.02	1.06±0.02	1.93±0.12
$[C_4mim][Cl]$	100/5h	2.04±0.12	1.39±0.01	1.30±0.01	0.98±0.01	1.02±0.01	2.13±0.13
	130/2h	1.98±0.01	1.34±0.01	1.26±0.02	1.00±0.01	1.02±0.03	2.63±0.15
$[C_2mim][Cl]$	100/5h	2.07±0.01	1.47±0.01	1.34±0.02	1.16±0.01	1.10±0.01	2.83±0.14
	130/2h	1.89±0.01	1.47±0.02	1.22±0.01	1.08±0.03	1.07±0.04	2.98±0.12
$[C_4mim][OAc]$	100/5h	1.87±0.02	1.34±0.02	1.18±0.01	0.99±0.01	0.66±0.01	5.75±0.15
	130/2h	1.77±0.01	1.28±0.01	1.12±0.02	1.07±0.01	0.58±0.02	6.05±0.10
$[C_2mim][OAc]$	100/5h	1.76±0.02	1.27±0.01	1.15±0.01	0.98±0.01	0.21±0.01	5.85±0.12
	130/2h	1.65±0.01	1.21±0.01	1.09±0.01	1.02±0.01	0.16±0.01	7.05±0.14

[a]Lateral order index (LOI) was calculated using the ratio of intensity of 1430/898 cm^{-1} stretching vibrations, [b]Total crystallinity index (TCI) was calculated using the ratio of intensity of 1375/2920 cm^{-1}, [c]Hydrogen bond index (HBI) was calculated using the ratio of intensity of 3400/1323 cm^{-1}, [d] Cross linked lignin (CLL) was calculated from the ratio of 1510/1600 cm^{-1}, [e]Acetyl content was calculated from the intensity of 1738 cm^{-1}. All the values are shown with standard deviation having $p<0.05$ within the same column.

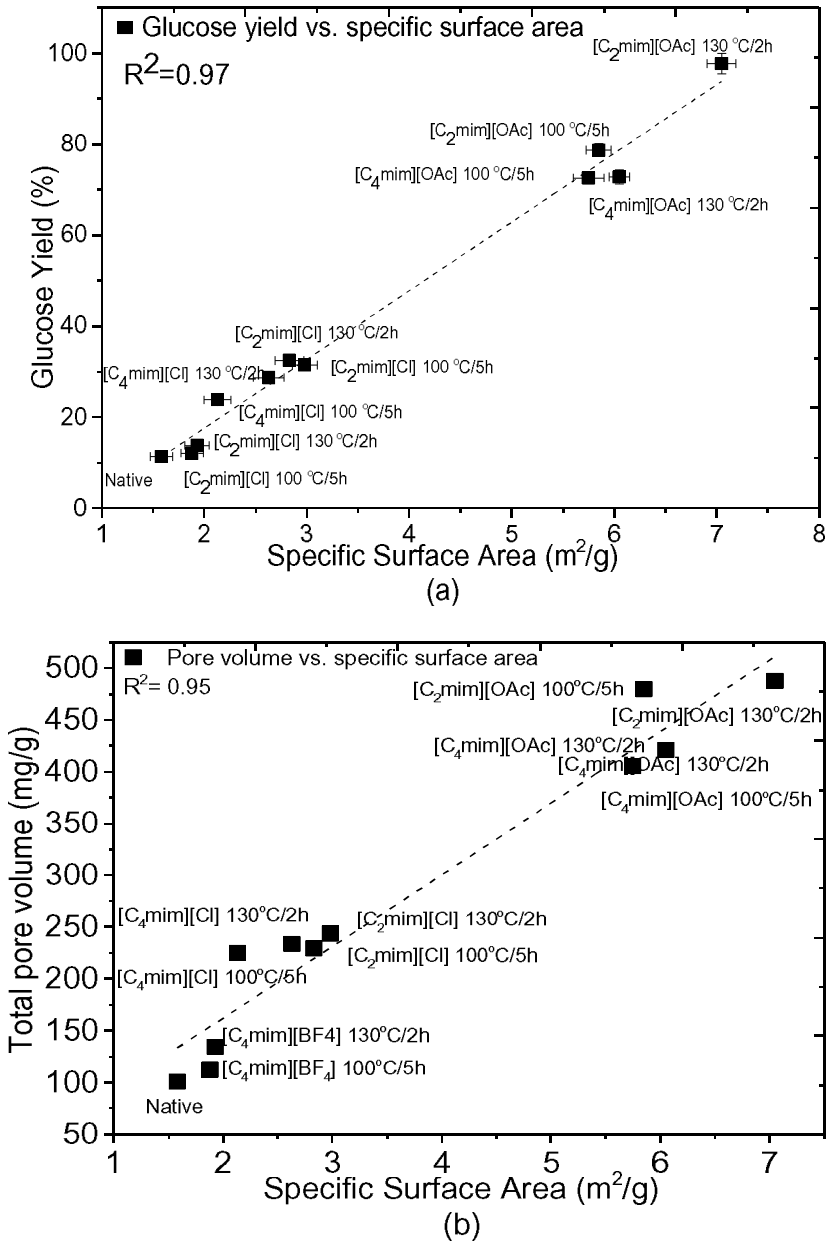

Figure 6.5: Co-relation between: **a**) Glucose yield (%) vs. specific surface area (m^2/g); **b**) Total pore volume (mg/g) vs. specific surface area (m^2/g)

lignin removal with cellulose II transformation as discussed in above section. Exposed surface in $[OAc]^-$ IL-treated mustard stalk leads to increased number of active sites for enzyme adsorption for enhanced enzymatic saccharification, which resulted in 97.7% sugar yield.

6.4.4 FT-IR ANALYSIS

FT-IR analysis was conducted to examine the biomass related properties (Figure 6.6). The absorption band at 1430 cm^{-1} is attributed to CH_2 scissoring motion and that at 898 cm^{-1} to vibrational mode involving C_1 of C-O-C stretching. Later is the characteristic of β-(1-4)-

glycosidic linkage in cellulose and indicate amorphous region. $[OAc]^-$ 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. The shift from 1428 to 1417 cm^{-1} indicates the development of new arrangement of intra-molecular hydrogen bonding.[131] Formation of cellulose II was also evident by the reduction in intensity of bands at 1124 and 1168 cm^{-1}, which represents the C-O and C-O-C stretching vibration. A shift of band at 1124 cm^{-1} to higher wave number, *i.e.* 1138 cm^{-1} showed the cellulose II structure after $[C_2mim][OAc]$ pretreatment. In addition, a strong peak at 1033 cm^{-1} was more pronounced in $[C_2mim][OAc]$ and $[C_4mim][OAc]$ treated biomass also represent the formation of cellulose II. The 2912 cm^{-1} band represents C–H stretching, which decreased with $[C_2mim][OAc]$ pretreated biomass, indicating that the methyl and methylene portions of cellulose were ruptured. As 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) were significantly weaker for $[C_2mim][OAc]$ and $[C_4mim][OAc]$ treated biomass indicating removal of lignin. Furthermore, a significant decrease in intensity was 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, likely due to the cleavage of ester linkages in lignin and hemicelluloses.

The above Table 6.3 showed that crossed linked lignin (CLL) in pretreated mustard stalk ranged between 0.92 and 1.16, and was changed from native (1.13) to $[C_2mim][OAc]$ (0.98) and (1.16) for 100 °C/5h and 130 °C/2h pretreated biomass respectively. However, we have not found any direct correlation with glucose yield, which might be due to the removal of lignin without altering the structure of lignin.

The total crystallinity index (TCI), lateral order index (LOI) and hydrogen bond intensity (HBI) were calculated from FT-IR spectroscopy and are represented in Table 6.3. LOI represents the overall degree of order of the cellulose chains and was calculated from the ratio of peak area of 1430/898 cm^{-1}.[50] TCI is proportional to the crystallinity index of cellulose and was calculated from the ratio of absorbance of 1375/2920 cm^{-1}, by Nelson and O'Connor method.[339] HBI of cellulose is related to the crystal system, chain mobility, bond distance and degree of intermolecular regularity, which was calculated from the ratio of the peak at 3400/1323 cm^{-1}. We have found that LOI, TCI, HBI and an acetyl content of mustard stalk decreased with respect to pretreatment severity and IL hydrogen bond basicity. Hydrogen bond basicity of $[C_2mim][OAc]$ $[C_4mim][OAc]$ $[C_4mim][Cl]$ and $[C_4mim][BF_4]$ ILs was quantified

as 1.27, 1.19, 1.16, 0.67 and 1.32, 1.23, 1.22, 0.72 respectively at 100 and 130 °C as mentioned in previous report.[315]

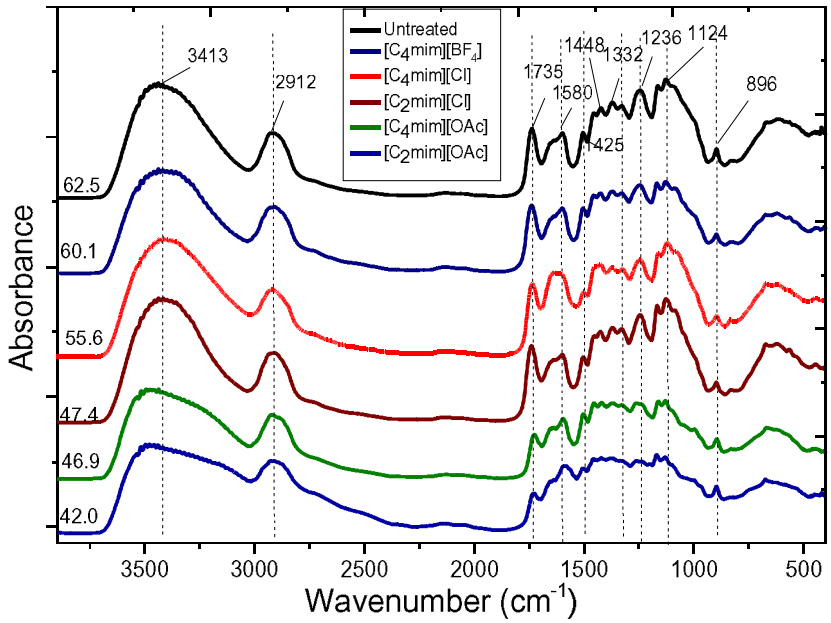

Figure 6.6 Fourier Transform spectrum (FT-IR) of ionic liquid treated biomass

Table 6.3 showed that both LOI and TCI decreased significantly post IL pretreatments from native to [C_2mim][OAc] treated biomass. LOI decreased from 2.14 to 1.65 and TCI decreased from 1.42 to 1.21. [C_4mim][OAc] and [C_2mim][OAc] treated mustard stalk resulted in lowest LOI, *i.e.* 1.87, 1.76 and 1.77, 1.65 for 100 °C/5h and 130 °C/2h post treated biomass respectively, which was attributed to highest hydrogen bond basicity and hence resulted in maximum transformation of cellulose I to cellulose II crystal structure. Figure 6.7a showed a positive correlation between LOI and glucose yield (%), with R^2=0.86 for 100 °C/5h and 130 °C/2h treated mustard stalk. Similarly, Figure 6.7a showed the negative correlation of TCI with glucose yield (%) with an R^2= 0.58. Table 6.3 showed that [C_2mim][OAc] treated mustard stalk have a maximum reduction in total crystallinity index, *i.e.* 1.27, 1.21 for 100 °C/5h and 130 °C/2h treated biomass respectively. Similarly, it showed the negative correlation between HBI vs. glucose yield (%) and an acetyl content of IL pretreated biomass vs. glucose yield (%) with a R^2 =0.87 and 0.89 respectively. A step forward, Figure 6.7b showed a negative correlation between the IL K-T property, i.e. "β" values and post-pretreatment biomass properties viz. HBI, LOI and TCI supporting our previous observations that K-T parameter undoubtedly serves as a very good indicator of ILs pretreatment efficiency

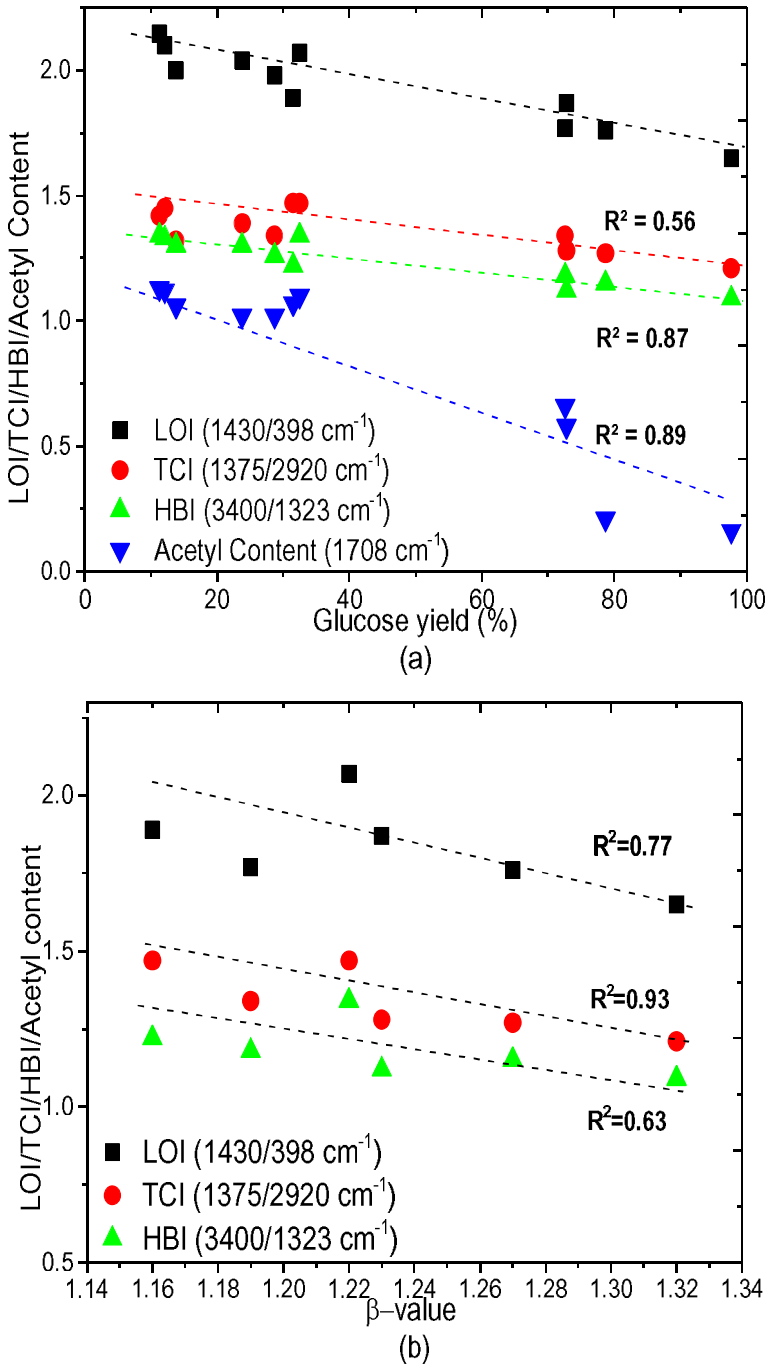

Figure 6.7 Correlation graphs: **a)** LOI/TCI/HBI/Acetyl content vs. glucose yield (%); **b)** β-values vs. LOI, TCI and HBI

6.4.5 SEM

Figure 6.8 showed the SEM images of native and ionic liquid treated mustard stalk at 100 °C/5 h and 130 °C/2 h. It is observed that untreated mustard stalk (A) has compact, ordered, plane and smooth surface. After treatment, large pores and substantial surface disruption were observed in the case of [C_2mim][OAc] treated mustard stalk at 100 °C/5h and 130 °C/2h

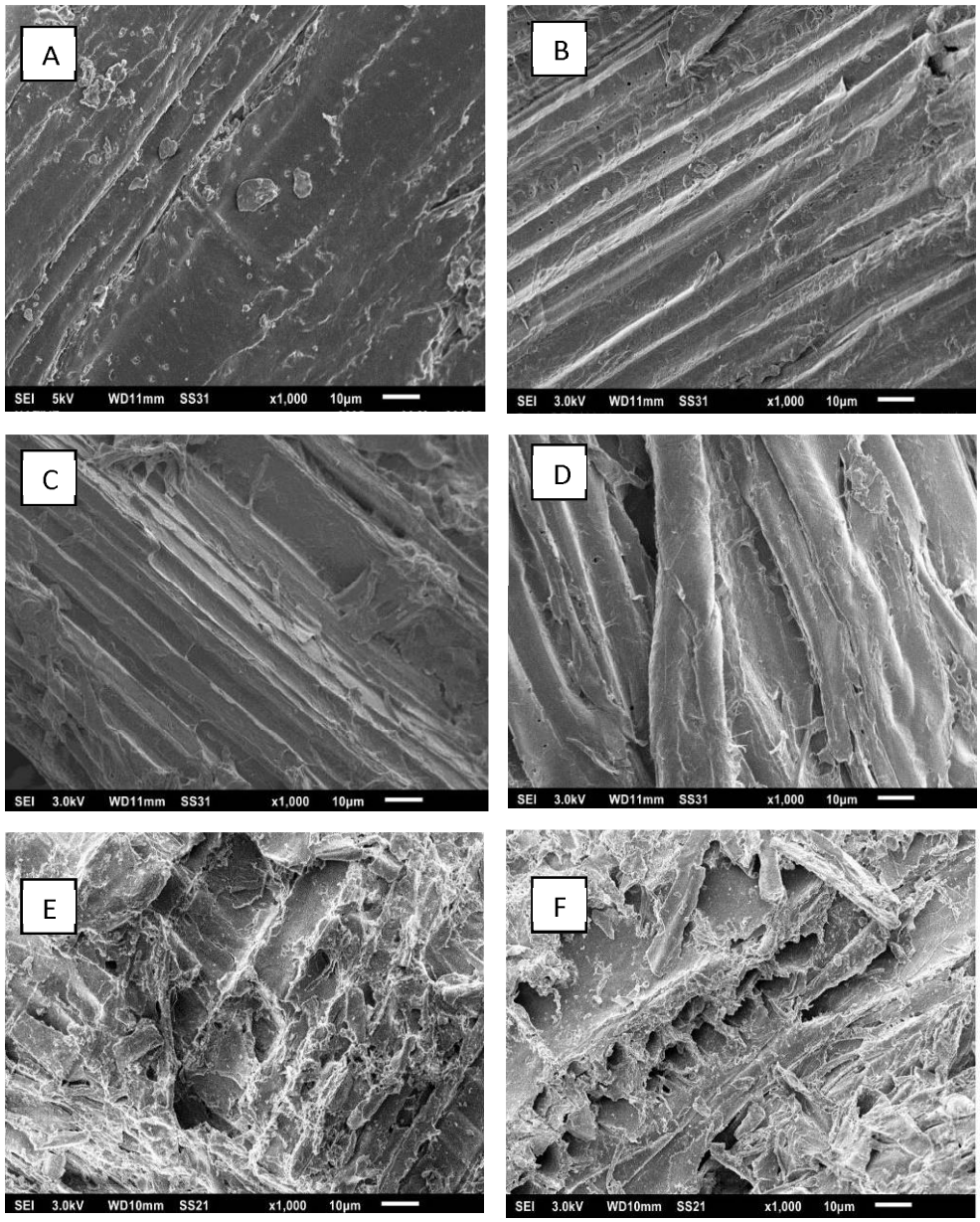

Figure 6.8 SEM images of native and IL treated biomass at 130 ºC/2h; A) Native mustard stalk; B) [C_4mim][BF_4]; C) [C_2mim][Cl; D) [C_4mim][OAc]; E) [C_4mim][OAc]; F) [C_2mim][OAc]

respectively (Figure 6.8C & 6.7D). ILs pretreatment helps to solubilize xylan and lignin, which resulted in the formation of a loose, rough, disordered, porous and open structure with increased numbers of pores of various sizes ranging from 1.7 to 396 nm in diameter. Figure 6.8 also showed that [C_2mim][OAc] (F) and [C_4mim][OAc] (E) treated mustard stalk have more ruptured and open structure compared to [C_4mim][BF_4] (D),

[C_2mim][Cl] (C) and [C_4mim][Cl] (D) treated biomass. This enhancement may be correlated with higher hydrogen bond basicity of $[OAc]^-$ ILs which results in removal of xylan and lignin as reported earlier[342], thus as of result, the polysaccharide accessibility to enzymes was significantly increased, which might be a significant contributor to the improved polysaccharide enzymatic digestibility due to adsorption of high amount of enzyme for hydrolysis and consequently resulted 72.8 and 97.2 % of glucose yield compare glucose released with and [C_2mim][Cl], [C_4mim][Cl], [C_4mim][BF_4] treated biomass.

6.5 CONCLUSION

Various physicochemical properties including the morphological structure, crystallinity, biomass composition, surface area and pore size/volume were studied in detail, and these physicochemical properties were correlated with the polysaccharide enzymatic digestibility. We found that IL pretreatment helps to reduce the cellulose crystallinity measured by LOI, TCI and HBI. LOI and TCI were decreased from 2.15 to 1.65 and 1.42 to 1.21 for native and [C_2mim][OAc] treated biomass respectively. A negative correlation of these parameters with glucose yield (R^2>0.80) was observed across five ILs at both the reaction temperatures 100 and 130 °C. Hemicellulose related feature, i.e. residual acetyl content also had a negative correlation with glucose yield (R^2=0.89). A positive relationship between biomass pore size distribution and enzymatic hydrolysis suggests that large pores enhance enzymatic hydrolysis. The highest surface area was also achieved (7.05 m^2/g) after [C_2mim][OAc] pretreatment resulting in highest glucose yield (97.7%) among the five ILs employed. The pore size distribution versus pore volume profile determined by DSC showed all the pretreated biomasses to be predominately containing two pore sizes viz. 19 nm and 198 nm. However, the discrimination between the extent of enzyme accessibility by an IL was expressed by the quanta of pores as depicted by the pore volume. The proficiency of $[OAc]^-$ containing ILs in pretreating mustard stalk was witnessed by highest pore volume, surface area with higher sugar recovery.