STUDIES IN THE HYDROLYSIS OF CELLULOSE USING CELLULASE IN IMIDAZOLIUM BASED IONIC LIQUID

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For the Award of

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BY

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CERTIFICATE

I declare that the dissertation entitled **"STUDIES IN THE HYDROLYSIS OF CELLULOSE USING CELLULASE IN IMIDAZOLIUM BASED IONIC LIQUID"** has been prepared by me under the guidance of Administrative Guide, Prof. P. Rama Rao, Dean, Centre for Environmental Science and Technology and Dr. J. N. Babu, Assistant Professor, Centre for Environmental Science and Technology, School of Environment and Earth Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

STUDIES IN THE HYDROLYSIS OF CELLULOSE USING CELLULASE IN IMIDAZOLIUM BASED IONIC LIQUID

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Ionic liquids are the modern day revelations as a green solvent. These solvents have found many applications in biofuel strategy. The cellulose pretreatment using ionic liquid is currently being studied for future biofuels from lignocellulosic biomass. The strategy involves the dissolution of cellulose in these solvents, followed by precipitation by an anti-solvent like water or acetone, leading to the formation of amorphous cellulose which is easy to hydrolyze by biocatalytic methods. Further studies had been conducted in the inhibitory effect of ionic liquid traces present in pretreated cellulose, on the biocatalytic hydrolysis by cellulase. Apart from this, biocatalytic hydrolysis had been studied in binary aqueous-ionic liquid solution for a one-pot process for simultaneous pretreatment & hydrolysis. These process involved the study of biocatalytic hydrolysis in imidazolium based ionic liquids namely, [EMIM]OAc & [BMIM]Cl, as hydrophilic solvents. These solvents have anions with predominantly strong hydrogen bond acceptor capacity. The study reveals the deactivation of the enzyme in presence of these ionic liquids. Thus in an effort to increase the hydrolysis efficiency of the enzyme without loosing the hydrogen bond acceptor capacity of the IL, was essentially required. Thus in the present study, we have investigated one-pot biocatalytic hydrolysis of cellulose in IL with variable alkyl chain length of the cation leading to hydrophobic environment around the biocatalyst. The ILs used in the present study are [BMIM]Cl, [HMIM]Cl & [OMIM]Cl.

The biocatalysis was studied in both homogenous as well as heterogeneous conditions. In homogenous conditions dissolution of cellulose was maintained throughout the reaction period. The homogenous biocatalysis was studied with or without the presence of surfactants. The results indicate that [HMIM]Cl is a promising solvent for cellulase catalyzed hydrolysis of cellulose in both homogenous & heterogeneous condition, resulting in more than 70% hydrolysis in presence of non-ionic surfactant PEG-1500 and in the binary mixture of 40% *w/w* [HMIM]Cl in citrate buffer, respectively.

(**Rabindra Kumar**) (**Dr. J.N.Babu**) (**Prof. P. Ramarao**)

DEDICATION

This dissertation is dedicated to all the people who never stop believing me and who along with God, have been my 'footprints in the sand'

My mother

My father

My sisters & brother- who have supported me all the way since the beginning of my studies.

To my Teacher, who taught me to get up after a fall and start again

Finally, this dissertation is dedicated to all those who believe in the richness of learning.

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At this moment of accomplishment, first of all I pay honour to my guide, **Dr. J. N. Babu,** Assistant Professor, Centre for Environmental Science & Technology, School of Environment and Earth Sciences. This work would not have been possible without his guidance, support and encouragement. Under his guidance I successfully overcame many difficulties and learned a lot. I can't forget his hard times. Despite of his busy schedule, he used to review my dissertation progress, give his valuable suggestions and made corrections. His unflinching courage and conviction will always inspire me, and I hope to continue to work with his noble thoughts. I am very much thankful to him for picking me up as a student at the critical stage of my M.Phil. I can only say a proper thanks to him through my future work.

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Last but not least, I would like to pay high regards to my **parent**, **sisters** & **brother** for their sincere encouragement and inspirational throughout for my research work and lifting me uphill this phase of life. I owe everything to them. Besides this, several people have knowingly and unknowingly helped me in the successful completion of this dissertation and I would like to thank everybody who was important to the successful realization of dissertation, as well as expressing my apology that I could not mention personally one by one.

(Rabindra Kumar)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES / SCHEMES

LIST OF ABBREVIATIONS

Full form	Abbreviation
Ammonia fiber explosion	AFEX
bis (trifluoromethyl sulfonyl)-imide	$[NTf_2]^-$
Bovine Serum Albumin	BSA
β-glucosidases	BG
1-Butyl-3-methylimidazolium chloride	[BMIM]Cl
1-butyl-3-methylimidazolium bromide	[BMIM]Br
1-Butyl-3-methylimidazolium acetate	[BMIM]OAc
Cellobiohydrolases	CBH
Cellulose-binding domain	CBD
Centimetre	cm
Cetyl Trimethyl Ammonium Bromide	CTAB
Degree of Polymerization	DP
Differential Scanning Calorimetry	DSC
3,5-Dinitrosalicylic acid	DNS
Distilled water	D.W.
1-Dodecyl-3-methylimidazolium	[Dmim]
Endonuclease	EG
1-Ethyl-3-methylimidazolium bromide	[EMIM]Br
Filter Paper	FP

Fourier Transformed Infrared	FTIR
Gallon	gal
Gram	g or gm
1-Hexyl-3-methylimidazolium chloride	[HMIM]Cl
Hour	h
Ionic Liquids	ILs
Lignin-Carbohydrate Complex	LCC
Methyl *tert*-Butyl Ether	MTBE
Metric tons	MT
Milligram	mg
M,N-Dimethylethanolammonium alkylcarboxylate	DMEAA
Nuclear Magnetic Resonance	NMR
1-Octyl-3-methylimidazolium chloride	[OMIM]Cl
Polyethylene Glycol	PEG
revolutions per minute	rpm
Scanning electron microscope	SEM
Simultaneous saccarification & fermentation	SSF
Sodium Dodecyl Sulphate	SDS
Sodium bis(2-ethyl-1-hexyl) sulfosuccinate	AOT
Total Reducing Sugar	TRS
Tris-(2-hydroxyethyl)-methylammonium methylsulphate	$[HEMA]^{+}\ MeSO_4^{-}$

Tris (perfluoroalkyl) trifluorophosphate	FAP
Volume / Volume	*v/v*
Weight / Weight	*w/w*
With respect to	w.r.t.
X-ray diffraction	XRD

CHAPTER – I

INTRODUCTION

CHAPTER - I

INTRODUCTION

Biomass to biofuel conversion is one of the most relevant technologies for the energy sustainability (Gomez *et al*., 2008; Goldemberg, 2007). The production of biofuels from lignocellulosic feedstocks (Perlack *et al*., 2005; Lee, 1997), such as agricultural waste or wood, provides a means to meet the energy demand in a manner that reduces the emission of greenhouse gases. In contrast to the present biofuel paradigm, these feedstocks do not contribute to the world's food supply, and using them avoids the food *vs.* fuel debate (Balat and Balat*,* 2009). Biomass is primarily constituted of three sub-constituents namely cellulose, hemicellulose and lignin. Cellulose is a semi-crystalline biopolymer, which constitutes approximately one third of the total weight of the biomass. The conversion of this cellulosic component to bioethanol is a suitable alternate, as bioethanol finds application as fuel additive to the extent of 5% in petroleum (Wang *et al.,* 2011a). The production of bioethanol for this purpose is estimated to be about 92 Million Gallons in India which is far lower than that of Brazil (16 Billion Gallons) and USA (10.7 Billion Gallons) (Sorda *et al.,* 2010). The Brazilian policies support 25% ethanol additive gasoline as fuel supplemented with the flex-fuel technology for the automotive usage [UNICA (2010a); UNICA (2010b) and ALCOPAR (2010)]. Presently, in Indian scenario, 5% blending of bioethanol is being carried out. Further Indian government intends to use 10% ethanol blend [Planning Commission, Eleventh five year plan, 2008] and the standards are already made for the E10 fuel. BIS proposal for a 20% blending of bioethanol to be implemented by 2017 is under review (GAIN, 2011).The total projected bioethanol production would have to keep pace with this increasing demand. The bioethanol blending has various advantages like increased octane number of fuel, significantly reduction in Methyl *tert*-Butyl Ether (MTBE), SO_2 and CO emission for the same energy (Malca and Friere, 2006).

The bioethanol desired for the present consumption has mostly been recovered from the hydrolysis followed by fermentation of oligosaccharides and starch polysaccharide, based on staple-foods especially corn and sugarcane (Klein-Marcuschamer *et al*., 2012). The cost of the bioethanol has currently been placed

at $ 0.68 /gal, whereas the estimated cost of the lignocellulosic bioethanol is $ 1.47 /gal. A number of factors influence the economics of cellulosic ethanol and an efficient commercialization strategy will be required to account for the following factors: cost of feedstock, pre-treatment technologies, and cellulase, fermentation configuration, hexose and pentose sugar utilization, ethanol tolerance, water requirements, side product utilization and integration with various other clean fuel technologies.

The major cost factors in lignocellulosic bioethanol strategy are the pre-treatment technologies and the enzyme used in the hydrolysis (Alvira *et al.,* 2010). Pre-treatment is an essential step for the removal of lignin and hemicellulose and/or escalating the accessibility of cellulose by enzymes during hydrolysis process. The pre-treatment processes (Saha, 2003) investigated can be categorized (da Costa et al., 2009: Hendriks and Zeeman, 2009; Sun and Cheng, 2002) into physical method (milling and steam explosion by Fernandez-Bolanos *et al.,* 2001), chemical methods (acidic by Saha and Bothast, 1999 or alkaline hydrolysis by Koullas *et al*., 1993), physicochemical methods (AFEX, supercritical fluid by Gao *et al.,* 2010) or biological methods (Xylanase or cellulase, white rot fungi by Sanchez*,* 2009). Recent addition to pretreatment is ionic liquid solubilization method (Swatloski *et al.,* 2002). The key features of an effective pre-treatment strategies are, breaking of lignocellulosic complex, decreasing the cellulose crystallinity, preserving the hemicellulose sugars, limited degradation products having inhibitory effect on hydrolysis and fermentation step, energy efficient and atom economy process, simple set-up, value added lignin co-product isolation and minimum wastewater generation (Limayem and Ricke*,* 2012; Banerjee *et al*., 2009). Hydrolysis on the other hand is carried out in either enzyme catalyzed conditions (Bose *et al.,* 2010) or acid catalyzed conditions (Suganuma *et al*., 2008). In the enzymatic catalysis, consortiums of enzymes known as cellulases are used for the hydrolysis of cellulose and hemicellulases and/or xylanases for the hydrolysis of hemicelluloses (Binder and Raines, 2009). Cellulases are obtained from various species of fungi namely *Tricoderma reesei, Aspergillus niger, T. longibrachiatum* etc. (Ahamed and Vermette, 2008; Bara *et al*., 2003). However, the major cost to the production of bioethanol is contributed by the enzyme utilized for the hydrolysis (Alvira *et al*., 2010). Thus, to lower the cost of

the bioethanol production from lignocellulose, various strategies are under consideration including the use of alternate process with good recyclability (Zhao, 2010), preparation of thermophilic and halophilic enzymes (Gao *et al*., 2010), integration of processes like the pretreatment-hydrolysis (Kim *et al*., 2008; Bose *et al*., 2012) or Simultaneous Saccharification and Fermentation (SSF) (Lee, 1997) and whole cell biocatalysis (Fukuda *et al*., 2008) etc. However, these strategies are at the higher end research and it would require time to improve the commercial utilization of these processes. One of the promising technologies being studied involves integration of pre-treatment and hydrolysis of lignocellulose in ionic liquids. The technology is viable due to the fact that cellulose dissolves in this class of green solvent (Alvira *et al*., 2010) and biocatalytic pathways in ionic liquid are being explored and established (Park and Kazlauskas, 2003).

Ionic Liquids (ILs) are modern day green solvent, comprising of organic cations and/or anions, with the salient feature of being ionic as well as liquid at temperatures below 100^{o}C. Their unique properties such as non-volatility, non-flammability, and excellent chemical and thermal stability have made them an environmentally attractive alternative to conventional organic solvents (Dupont *et al*., 2002). The near limitless potential to combine anions and cations in order to tailor solvent properties as per the requirement of application, has led to ILs being termed “designer solvents” (Pezoa *et al*., 2010). Ionic liquids have found applications in chemical transformations (Miao and Chan, 2006), separations/chromatography (Tian *et al*., 2005), electrochemistry (Zhao *et al*., 2004), solar cell technologies (Singh and Bhattacharya, 2010) and biocatalysis (Rantwijk and Sheldon, 2007). These ILs are being studied for the pre-treatment of lignocellulosic materials due to their increased affinity for the dissolution of the cellulose and lignin components, followed by the precipitation of the cellulose and lignin upon addition of water and ethanol as anti solvents, respectively (Verma and Banarjee, 2010; Cabeza *et al*., 2011). The high cost of these solvents for pre-treatment is compensated by the easy recoverability of these ILs. Of the various cost reduction in lignocellulosic bioethanol, there have been integration of the pre-treatment and acid (Galbe *et al*., 2006) / heavy metal chloride catalyzed (Su *et al*., 2009) hydrolysis had been explored. The results indicate excellent hydrolysis in presence of mineral acid (H_2SO_4) (Li *et al*., 2008) and $CrCl_3$ (Li *et al*., 2009). It is

further envisaged that the integration of pre-treatment with hydrolysis of cellulosic material would significantly support the green solvent for the one-pot method for bioethanol production, in particular, from agricultural crop residues. During present investigation, we are interested in the integration of pre-treatment and biocatalytic hydrolysis of cellulose in frequently used imidazolium ionic liquids.

OBJECTIVES

The objectives of the present study are

1. To study the effect of alkyl group of imidazolium ionic liquid on biocatalytic hydrolysis of cellulose in presence of cellulase.
2. To study the effect of alkyl group of imidazolium ionic liquids on the performance of water-ionic liquid binary solutions for enzymatic hydrolysis of cellulose.
3. To study the effect of surfactant on the stability of cellulase in imidazolium based ionic liquid and its efficacy for hydrolysis.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER - II

REVIEW OF LITERATURE

2.1 Cellulose Structure and its Association to Lignin and Hemicellulose:

Cellulose is found almost exclusively in plant cell walls. It is a linear polymer of glucose, composed of thousands of molecules of anhydroglucose linked by *β*-(1,4)-glycosidic bonds (**Figure 2.1**). The basic repeating unit, the disaccharide is known as cellobiose. The properties of cellulose are essentially determined by the polymer chain length i.e. the degree of polymerization (DP). A typical cellulose polymer has DP in the range of 1,000-17,000 with an average of 10,000 (Young, 2007). This polymer when threaded together form long, rigid and insoluble microfibrils, which are approximately 3.5 nm in diameter. These microfibrils are the backbone of the cell walls. Naturally occurring cellulose has both amorphous and crystalline forms. The crystalline cellulose comprises approximately 50-70% of the total cellulose. Cellulose has seven crystal polymorphs designated as I_α, I_β, II, III_I, III_{II}, IV_I and IV_{II} (Marchessault and Sarko, 1967; Walton and Blackwell, 1973; Marchessault and Sundararajan, 1983). In nature the I_α and I_β forms are the most abundant crystal forms. The crystallinity of cellulose with its secondary and tertiary conformation, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose recalcitrant in nature.

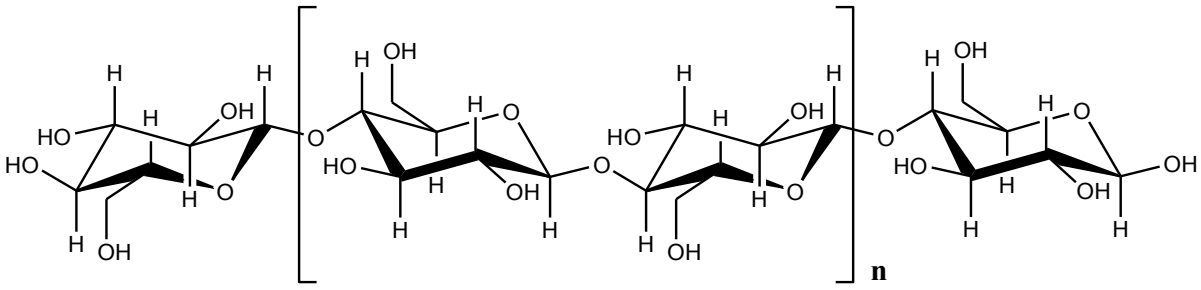

Figure 2.1: The structure of single cellulose molecule with *β*-(1,4-glycoside linkage and the basic disaccharide repeating unit cellobiose.

The nature of *β*-1,4-glycosidic bond between the glucose molecules allows the polymer to be arranged in long straight chains. The latter arrangement of the molecule, together with the fact that the hydroxides are evenly distributed on both sides of the monomers, allows for the formation of hydrogen bonds between the

molecules of cellulose (**Figure 2.2**). The hydrogen bonds in turn, result in the formation of a compound that is comprised of several parallel chains attached to each other (Faulon *et al.,* 1994).

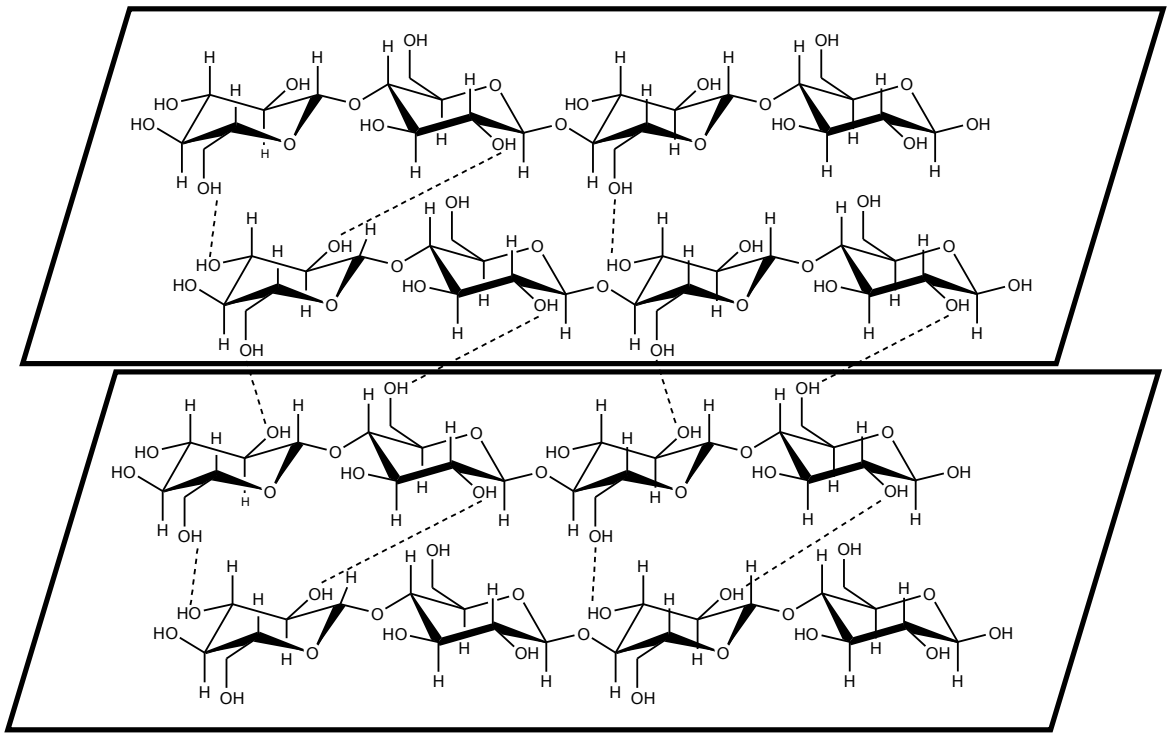

Figure 2.2: Showing three dimensional arrangements of cellulose and its hydrogen bonding interaction with neighboring cellulose unit.

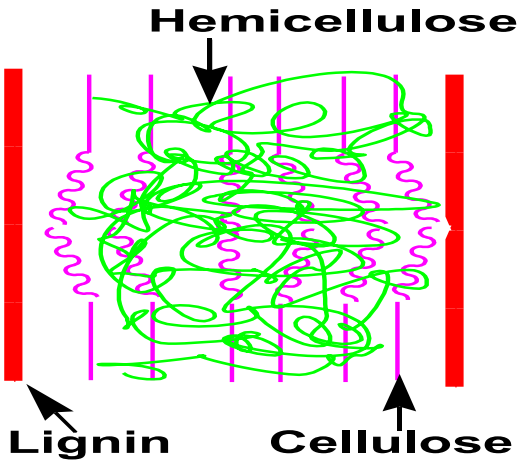

Figure 2.3: Generalized arrangement of Cellulose, Hemicellulose and Lignin in the cell wall of wood.

Cellulose is a relatively hygroscopic material absorbing 8-14% water under normal atmospheric conditions (20^{o}C, 60% relative humidity). Nevertheless, it is insoluble in water, however it swells in water. Cellulose is also insoluble in dilute acid solutions at low temperature. Cellulose does not melt with temperature, but its decomposition starts at 180^{o}C (Helsinki, 2003).

The wood cells are composed of different layers, which differ from one another in to their structure and chemical composition. Basically, cellulose forms a skeleton which is surrounded by other substances functioning as matrix (hemicelluloses) and encrusting (lignin) materials (**Figure 2.3**) (Lee, 1997). Cellulose, hemicelluloses and lignin are closely associated and covalent cross linkages exist between lignin and polysaccharide (Lignin-carbohydrate complex, LCC). The side group arabinose, galactose and *O*-methyl-glucouronic acid are frequently perceived as connecting links to lignin (Fengel and Wegener, 1984). It is also agreed upon that the hemicellulose molecules are oriented parallel to the cellulosic fibrils.

The recalcitrant property of lignocellulose stems from the crystalline cellulose skeleton with *β*-1,4-glycosidic linkages. However, in nature certain enzymes particularly secreted by fungi, lead to degradation of cellulose in natural environment. This class of enzyme is known as cellulase has been isolated and studied for their tendency to hydrolyze cellulose (Mansfield *et al*., 1999). Cellulase is a class of enzyme comprising of three subclasses of enzymes namely:

1) Endoglucanase (EC 3.2.1.4)
2) Exoglucanase (EC 3.2.1.91)
3) β-glucosidase (EC 3.2.1.21)

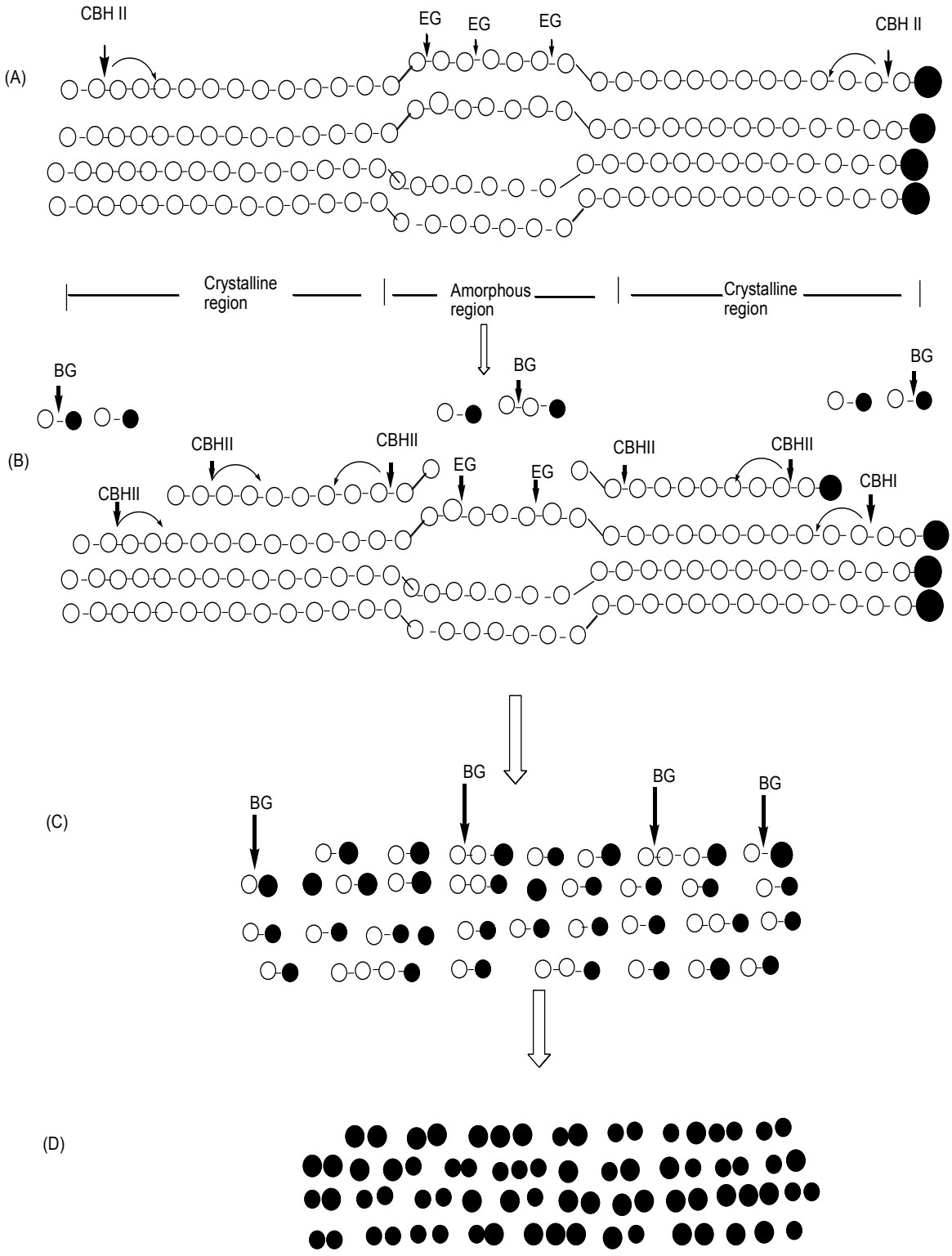

Figure 2.4: Scheme of the enzymatic hydrolysis of cellulose catalyzed by a fungal cellulase system. **(A)** Initial cellulose consisting of crystalline and amorphous regions. **(B)** Partially hydrolyzed cellulose. **(C)** Outer solution containing cellobiose (disaccharide) as a major intermediate product, together with minor amounts of higher oligosaccharides and glucose. **(D)** Final monomers. The open circles represent anhydroglucose residues in cellulose and oligosaccharides; the solid circles represent reducing ends of cellulose and oligosaccharides or glucose.

Cellulose is homopolymer requiring number of enzymes to degrade it. Endoglucanases (EG), which hydrolyse internal *β*-1,4-D-glycosidic linkages in the cellulose chain, cellobiohydrolases (CBH or exoglucanases) which cleaves cellobiose units from the cellulose ends, *β*-glucosidases (BG) hydrolyse cellobiose to glucose and also cleaves glucose unit from cello-oligosaccharides. Therefore, these three types of enzymes play significant role to degrade cellulose synergistically by creating new sites for each other and preventing product inhibition. Cellulose hydrolysis by cellulase enzyme is schematically represented in **Figure 2.4**.

For complete hydrolysis of cellulose to glucose, the three enzymes must be present in right proportions. *T. reesei* produces at least five endoglucanases (EGI, EGII, EGIII, EGIV and EGV), two exoglucanases (CBHI and CBHII) and two *β*-glucosidases (BGI and BGII). An *exo-exo* synergism between the two cellobiohydrolases (CBH) was observed (Igarashi *et al.,* 2011). Cellobiohydrolase is the key enzyme for the degradation of native cellulose (Divne *et al.,* 1994). The catalytic site of the enzyme is covered by long loop. The cellulose binding domain (CBD) is responsible for bringing the catalytic domain in the correct position for the breakdown of cellulose. *β*-glucosidase produces glucose from cellobiose and it reduces cellobiose inhibition of the cellulolytic enzyme due to cellotriose (Saha and Bothast,1996). Guimaraes *et al.* (2002) studied crystal structure and catalytic mechanism of cellobiohydrolase (CBH), in which CBH plays an important role in cellulosome (an active cellulose system produced by the thermophilic anaerobe *Clostridium thermocellum.*

2.2. Pre-treatment of Cellulose in Ionic Liquid:

Ionic liquids have been shown to be highly effective at solvating cellulose to technically useful concentrations (Lee, 1997). For attempting to dissolve carbohydrates, many different ionic liquids were discovered and it was concluded that good dissolution of cellulose may be obtained using Imidazolium based ionic liquids. More than twenty Ionic Liquids are known to dissolve cellulose and lignin (Pinkert *et al.,* 2009). In general, the inter- and intra-molecular hydrogen bonds of cellulose are disrupted, replaced by hydrogen bonding between the anion of ionic liquid and the carbohydrate hydroxyls (Remsing *et al.,* 2006). As a result, the

basicity of the ionic liquid anions can alter cellulose solubility. Solubility of cellulose decreases with the basicity of anion as follows OAc^- > Cl^- > Br^- > I^- (Holm and Lassi, 2011).

Few reports between 2009-2011 have been tabulated for study of various ILs and their cellulose / lignocellulose solubility (**Table 2.1**). The studies have extensively found the solubilization followed by precipitation upon addition of water. Most of the cases we have seen that studies for enzymatic hydrolysis in IL have shown to have loss of hydrolysis activity due to the presence of ionic liquid as impurities.

Doherty *et al*. (2010) described the correlation between the Kamlet-Taft solvent polarity parameters namely, solvent polarizability (π^*), hydrogen bond donor capacity (α) and hydrogen bond acceptor capacity (β) as the descriptors for predicting the pre-treatment of maple wood flour in ionic liquids. However, a strong correlation was observed between the hydrogen bond acceptor property of ionic liquid and the biomass crystallinity index of cellulose, upon ionic liquid pre-treatment. Further, SEM analysis of pretreated wood samples showed a tremendous decrease in the size of the fibres in case of [BMIM]OAc (IL with a strong hydrogen bond acceptor anion) whereas, in case of [BMIM][$MeSO_4$] (Mora-Pale *et al*., 2011), no change in the fiber size was observed, indicating better enzymatic hydrolysis of IL-pretreated wood in acetate based ionic liquids. A correlation was also observed between the addition of water to ionic liquid and the increased crystallinity of wood sample, which indicates that the added water reduces the ability of anions to affect both intra- and inter-crystalline swelling, as well as fiber size reduction.

Table 2.1: Room Temperature Ionic Liquids (RTILs) used for dissolution of lignocellulosic biomass subcomponents:

S.N.	RTILs	SOLUBILITY	USES	REFERENCES
1	[EMIM]OAc		Dissolution of cellulose	Kosan *et al.,* 2008; Vitz *et al.,* 2009; Zavrel *et al.,* 2009.
			Extraction of lignin from maple wood flour	Lee *et al.,* 2009.
			Dissolution of variety of carbohydrates such as sugar, starch and cellulose	Zhao *et al.,* 2008.
2	[EMIM]Cl		Dissolution of Cellulose	Kosan *et al.,* 2008; Vitz *et al.,* 2009; Zavrel *et al.,* 2009.
3	[AMIM]Cl	14.5%[a]	Dissolution of cellulose	Fukaya *et al.,* 2008; Zavrel *et al.,* 2009.
		8%[a]	Dissolution of hard wood and softwoods	Kilpelainen *et al.,* 2007.
			Extraction of lignin from maple wood flour	Lee *et al.,* 2009.
4	[BMIM]Cl	10%[a]	Dissolution of cellulose	Erdmenger *et al.,* 2007; Kosan *et al.,* 2008; Vitz *et al.,* 2009; Zavrel *et al.,* 2009.
			Pre-treatment of cellulose for enhancing enzymatic hydrolysis	Dadi *et al.,* 2006.
			Dissolution of hard wood and soft woods	Fort *et al.,* 2007; Kilpelainen *et al.,* 2007.
			Extraction of lignin from maple wood flour	Lee *et al.,* 2009.
		10%[a]	Dissolution of lignin	Pu *et al.,* 2007.
5	[BMIM]OAc		Extraction of lignin from maple wood flour	Doherty *et al.,* 2010.
6	[BMIM]BF_4		Dissolution of cellulose	Swatloski *et al.,* 2002; Zavrel *et al.,* 2009.
			Extraction of lignin from maple wood flour	Lee *at al*., 2009.
7	[BMIM]PF_4		Dissolution of cellulose	Zavrel *et al.,* 2009.
			Extraction of lignin from maple wood flour	Lee *at al*., 2009.

8	[BMIM]$MeSO_4$		Extraction of lignin from maple wood flour	Doherty *et al.,* 2010.
		312 [b]	Dissolution of lignin at 50^0C	Pu *et al.,* 2007.
9	[OMIM]Cl		Dissolution of cellulose	Vitz *et al.,* 2009; Zavrel *et al.,* 2009.
10	[AMIM][HCO_3]	10% [a]	Dissolution of cellulose heating at 60^0C	Fukaya *et al.,* 2006.
11	[EMIM][(MeO)HPO_4]	10% [a]	Dissolution of cellulose on room temperature within 3~5 hours	Fukaya *et al.,* 2008.
12	[EMIM][Et_2PO_4]	14% [a]	Dissolution of cellulose heating at 100^0C within 1 h	Vitz *et al.,* 2009.
13	[BMIM]OAc	15.5% [a]	Dissolution of cellulose heating at 70^0C	Xu *et al.,* 2010.
14	[BMIM][$HSCH_2CO_2$]	12% [a]	Dissolution of cellulose heating at 70^0C	Xu *et al.,* 2010.
15	[HMIM][CF_3SO_3]	275 [b]	Solubility of lignin heating at 70^0C	Pu *et al.,* 2007.
16	[B_zMIM]Cl	5% [a]	Dissolution of hard wood and soft woods heating at 130^0C, 8h	Kilpelainen *et al.,* 2007.
17	[BMPy]Cl		Dissolution of cellulose heating at 105^0C	Olivier-Bourbigou *et al.,* 2010.
18	[$HO(CH_2)$MI]Cl		Dissolution of cellulose heating at 70^0C	Olivier-Bourbigou *et al.,* 2010.
19	[MMIM][$MeSO_4$]	344 [b]	Solubility of lignin from southern pine kraft pulp	Pu *et al.,* 2007.
20	[Bm_2im][BF_4]	15% [a]	Solubility of lignin from southern pine kraft pulp at 70-120^0C	Pu *et al.,* 2007.
21	[EMIM][XS]	71 [c]	Lignin separation from bagasse and wood pulp	Upfal*,* 2005.
22	[TBA][XS]	71% [c]	Lignin separation from bagasse and wood pulp	Upfal*,* 2005.
23	[TBA][Bz]	68% [c]	Lignin separation from bagasse and wood pulp	Upfal*,* 2005.
24	[CMIM]Br		Separation of cellulose from cellulose / lignin mixture	Lateef *et al.,* 2009.
25	[PMIM]Br		Separation of cellulose from cellulose / lignin mixture	Lateef *et al.,* 2009.

[a] Solubility (*w/w* %)

[b] Solubility (g / L)

[c]Lignin removed (%)

Singh *et al.* (2009) have studied the biomass solubility and cellulose regeneration by pre-treatment of switch grass with ionic liquid [EMIM]OAc, using confocal microscopy, SEM, ATR-FTIR and X-ray diffraction methods. The study included the monitoring of the lignin fluorescence as an indicator of biomass solubilization. The study indicated that the cell wall of the biomass swells upto eight times within 10 minutes of IL pre-treatment. The swelling was attributed to the breaking of inter and intra-molecular hydrogen bond responsible for the rigid and highly compact crystalline cellulose polymer structure within biomass. Further, upon addition of water to the IL solution, non-fluorescent fibres were formed with a background fluorescence of IL-water-Lignin solution, which clearly indicated the separation of cellulose from the lignocellulosic material. The FTIR, SEM and XRD studies indicate an increase in the amorphous nature of the cellulose thus generated from [EMIM]OAc.

Liu *et al.* (2010) and Jiang *et al.* (2007) have studied the interaction of ionic liquid with cellulose by MD stimulations in both crystalline and dissociated state. The study concluded that perturbation of solvent structure by the dissolved cellulose chains can be crucial factor in determining solubility. Further, the interaction mode was established with the strong interaction of chloride and $[BMIM]^+$ ions with cellulose. The chloride ion interacts by hydrogen bonding with the axial and equatorial hydroxyl group of the glycan chains. However, the cation interacts only with the axial hydroxyl group of the gylcan chain. Thus, the study strongly suggested that the chloride ion is responsible for the dissolution of cellulose, whereas cation can interact with the glycan only in the soluble state.

Further Liu *et al*. (2010), carried out a MD simulation of a binary mixture of [EMIM]OAc-Water and mixture of [EMIM]OAc-Water-Cellulose. The study revealed that in the binary solution, as the concentration of water increases, the hydrogen bonding between the water and anion becomes saturated, whereby water forms an aggregate. Thus, the subsequent addition of water in the ternary system induces changes in the structural organization of [EMIM]OAc and disrupts the interactions between cellulose and [EMIM]OAc by saturating the anion and the hydroxyl group of the cellulose, forming hydrogen bonding networks and displaces the cation beyond the solvation shell. Liu *et al.* (2010) have reported a strong

interaction between imidazolium NC^5HN with cellulose through hydrophobic interactions as studied by molecular dynamic (MD) stimulations. Further NMR studies have revealed a strong interaction between the imidazolium C_5-H with the oxygen moiety of cellulose hydroxyl group.

Thus, cellulose solubility and its amorphous nature in ionic liquid pre-treatment has been studied in detail. Bearing these in mind, a lot of efforts have been devoted to producing valuable chemicals from biomass *via* hydrolysis (Kumar *et al*., 2009), dehydration (Chheda *et al*., 2007), hydrogenolysis (Zhou *et al*., 2008), etc., taking the advantages of the homogenous platform of IL. However, when enzymatic hydrolysis of the IL pretreated biomass is carried out, the results show a loss in the hydrolytic activity of the enzyme, due to the presence of residual imidazolium IL traces.

2.3. Biocatalysis in Ionic Liquid:

Ionic liquid biocatalysis is fairly new, Erbeldinger *et al*. (2000) were the first to report on the use of ionic liquid in biocatalysis. Prior to this, the biocatalysis was significantly studied in non-aqueous solvents (Yang *et al*., 2005). Due to the repulsive interaction of the enzyme and the non-polar solvents, enzyme stabilization was achieved in many cases leading to enhanced activity of the enzymes (Dadi *et al*., 2006). Since the potential application of ionic liquid for enzymes biocatalysis was revealed in 2000, many of the enzymes have been studied for their catalytic activity in these green solvents including, the lipases (Diego *et al*., 2009), alcohol dehydrogenase (Sheldon, 2005), proteases and oxidoreductase (Park and Kazlauskas, 2003) to name some. There are many advantages of preferring ionic liquid in biocatalysis over the organic solvents (Daneshjoo *et al*., 2011), which include:

- ILs are tailor-made solvents, and can be designed for particular bioprocesses: there have been three generation of ILs:
    1. **$AlCl_3$ based ionic liquids**: These are first generation ILs. These are hygroscopic liquids, liberating HCl and variety of oxo-chloraaluminate upon exposure to moisture. $AlCl_3$ based ILs require controlled inert

gas atmosphere with low water concentration with limited contamination. So, it has little importance in practice.

2. **Imidazolium and ammonium based ILs**: They are second generation of ILs. Ammonium based ILs synthesized from ammonium salts and dialkyl carbonates. They are environmentally benign solvents.
3. **Ether functionalized ILs**: Physicochemical properties of the ether- and alcohol functionalized ILs, highlight the impact of ionic structure on features such as viscosity, phase behaviour / transitions, density, thermostability, electrochemical properties, and polarity. The attractive applications of these functionalized ILs, include their use as electrolytes or functional fluids for electrochemistry, extractions, biphasic systems, gas separations, carbon capture, carbohydrate dissolution (particularly, the lignocelluloses), polymer chemistry, antimicrobial and anti-electrostatic agents, organic synthesis, biomolecular stabilization and activation, and nanoscience.

- In number of cases, enzymes show excellent solution and temperature stability, thus many processes can be conducted at higher temperature.
- IL as a reaction media for biotransformation has the advantage of biocatalyst recycling and product recovery schemes that are not feasible with traditional organic solvent.

The solvent properties like the hydrophobicity, viscosity and polarity have a significant role to play in biocatalysis (Illanes *et al*., 2011). Hydrophobicity is essential parameter which has been the driving force for the biocatalysis in organic reaction media, and is parameterized in terms of log P value of that particular solvent. Similarly, ionic liquids could be classified as hydrophilic and hydrophobic ionic liquids. The parameters for hydrophobicity (Log P) has been studied by Kaar *et al*. (2003) and have showed that the ionic liquid Log P values were extremely low at -2.90 to -2.39, showing hydrophilic tendency. However, in general the hydrophilicity of ionic liquid depends on the anion present in the ionic liquid. The anions like chloride, nitrate and acetate strongly interacts with water by forming hydrogen bonds and contribute significantly to the hydrophilicity of the ionic liquid, whereas the anions like PF_6^- and NTf_2^- have weak interaction with water and

result in hydrophobic nature of IL. Generally, it is believed that the hydrophobicity of the ionic liquid contributes to the increased stability of enzyme and hydrophilicity contributes to increased interaction with protein structure leading to the inactivation of the enzyme. Thus, the enzymes that dissolve in ionic liquid do not show catalysis and the enzymes that do not dissolve in ionic liquid show catalytic activities. Thus, most of the significant studies have been carried out in hydrophobic ionic liquids like [BMIM]PF_6, [BMIM]NTF_2^- etc. Carrying PF_6^- and NTf_2^- as the counter anion.

Similarly, viscosity has a vital role to play in the enzyme biocatalysis (Naushad *et al.*, 2012). Viscosity generally controls the enzyme activity by affecting mass-transfer limitations. On the contrary, the ionic liquid viscosity may lead to slowing down of the change in enzyme conformation leading to better stability of the enzyme (Rantwijk and Sheldon, 2007). Polarity of the ionic liquids has been studied to influence the activity of enzyme, in few cases, the increase in activity is observed in ionic liquid with higher polarity. However, no trends can be suggested, but the polarity relates to viscosity which is a broader parameter and could explain for the results obtained in different ionic liquids. Apart from the solvent properties, water, surfactants, pH and impurities have a considerable effect on enzyme catalysis in ionic liquid. To mention particular, PEG has been reported by Maruyama *et al.* (2004) to enhance the activity of lipase by 14 fold in a PEG-lipase complex.

Thus, after a general overview of biocatalysis is in ionic liquid, a more intense study of the effect of alkyl chain length of imidazolium ionic liquid is relevant to the present study. Thus, few such literatures have been put forward to bring this salient feature of these designer solvents.

Yang, (2009) have reported that the increase in the chain length of alkyl group of imidazolium cation leads to increase in chaotropicity of ionic liquid whereas the change towards hydrophobic anion leads to kosmotropic effect. These are anomalies in the behaviour of ionic liquids in these biocatalysis.

Dabirmanesh *et al*. (2011) have studied the activity of BAA and BLA in aqueous buffer upon addition of ionic liquid [BMIM]Cl and [HMIM]Cl. The fluorescence study

reveals an interesting phenomenon of the formation of aggregates in [BMIM]Cl whereas in [HMIM]Cl the aggregates were dispersed. The results were confirmed from the Differential Scanning Calorimetry (DSC). This allowed the use of [HMIM]Cl in catalysis.

Biocatalytic esterification of glycerol using feruloyl esterase has been studied in ionic liquids [BMIM]PF_6, [OMIM]PF_6, [C_2OHMIM]PF_6 and [C_5OMIM]PF_6. The result indicated mild improvement in the conversion yield in the second generation imidazolium ionic liquid comprising of methyl diethylenediglycol derivative than the hydroxyethyl derivative, whereas the catalysis in [BMIM]PF_6 and [HMIM]PF_6 was not significant.

Pinto *et al*. (2012) studied the β-galactosidase activity in mixed micelles of imidazolium ionic liquids and sodium dodecyl sulphate (SDS) by a sequential injection kinetic study. The variation in particle size of the micelle formed in various concentrations of imidazolium ionic liquids namely, [HMIM]Cl and [BMIM]BF_4, with sodium dodecylsulphate (SDS) was studied. In [HMIM]Cl, particularly when used with various concentration of SDS showed larger particle size of the mixed micelles in aqueous solution. Further, the enzyme activity was increased in presence of lower concentration of ionic liquids. Catalytic enhancements to a similar extent were observed in aqueous SDS solution in both the ionic liquids.

Hydrophobic ionic liquids with different alkyl substituents and / or anion were studied for biphasic whole cell biocatalysis using *E. coli* with an over expression of the genes of *lactobacillus brevis* alcohol dehydrogenase (LB-ADH) and a *Candida boidinii* formate dehydrogenase (CB-FDH) for cofactor regeneration, for asymmetric reduction of ketones. The ionic liquids based on pyrrolidinium and piperidinium cation were found to show better activity and control over the stereoselective conversion of 2-octanone to (R)-2-octanol. It was observed that in pyrrolidinium based ionic liquid [HMPL]NTf_2 had better activity that [BMPL]NTf_2. However, in case of imidazolium based ionic liquid [BMIM]NTf_2 had better activity than [HMIM]NTf_2^-. The enzyme was found to be inactivated by the presence of MIM. Generalization could be made on the anion and its effect on the biocatalysis,

whereby, NTf_2^- and PF_6^- based ionic liquids had better activity than the tris (perfluoroalkyl) trifluorophosphate (FAP) based ionic liquids (Keskin *et al.*, 2007).

Lipase catalyzed synthesis of glucose fatty acid ester were studied by Lee *et al*. (2009) in ionic liquid mixtures. The activity of the lipase was found to be higher in water miscible [BMIM]OTf, whereas, lipase stability was highest in the water immiscible ionic liquid $[BMIM]NTf_2$. In a mixture of 1:1 *v/v* of [BMIM]OTf in $[BMIM]NTf_2^-$, the stability and activity were optimum with recovery and reuse of enzyme upto 86% after 5 time usage of the sample repeatedly.

Thomas *et al.* (2011) studied the activity of various cellulases, hemicellulases and *β*-glucosidases in 0-20% [EMIM]OAc, [EMIM]DEP or [EMIM]DMP in aqueous solution. The enzyme activity was found to be very high in [EMIM]DEP followed by [EMIM]OAc. Generally it was observed that the activity of the enzyme decreased with the increase in the concentration of ionic liquid above 10% *v/v*.

Lipase catalyzed ester synthesis was studied in various alkyl (butyl, hexyl and octyl) imidazolium ionic liquids comprising of counter anions BF_4^-, PF_6^- or ethylsulphate, with ammonium ionic liquid [CPMA]MS. The lipases used in the study were obtained from *Candida antarctica* (CALA and CALB), *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML). Activity of the biocatalyst in general were found to be higher in water soluble [CPMA]MS. In case of $[BMIM]BF_4$, $[HMIM]]BF_4$ and $[OMIM]BF_4$, the intial rate and the product concentration, was found to decrease with the increase in the alkyl chain length.

Daneshjoo *et al*. (2011) studied the lipase activity in water miscible imidazolium based ionic liquid [BMIM]Cl and [HMIM]Cl. The study was limited to 0-50% aqueous environment in these ionic liquids. The lipase activity was compared with that in the organic solvents. The study indicated a strong enhancement in the catalytic activity of lipase in 30% aqueous-[HMIM]Cl solvent as compared to that in aqueous-[BMIM]Cl. Fluorescence and Light Scattering experiments confirmed the destabilization of the enzyme in 20% aqueous ionic liquid solutions.

Rodriguez *et al*. (2010), studied the Baeyer-Villiger Monoxygenase (BVMO) for oxidation of racemic benzylketones to esters in 10-30% of ionic liquid in water. The

ionic liquids used in the study were [EMIM], [BMIM], [HMIM], [BMP], [THMA], [TBMA] and AMMOENG® as cations and in combination with any of the anions like BF_4^-, PF_6^-, OTf^-, RSO_4^- (R=Me, Et), Cl^-. The results indicated that catalysis was evidently best in AMMONENG® based ionic liquids, however, on comparing the [BMIM] and [HMIM] with similar counter anion, it was observed that [HMIM]Cl showed excellent activity at pH 9, whereas [BMIM]Cl showed good but lower activity at pH 8.

Based on these studies on biocatalysis and the effect of alkyl chain length, it is observed that the effect of alkyl chains have not been documented sufficiently.

2.4. Cellulase based Biocatalysis in Ionic Liquid:

Cellulose solubility and precipitation from ionic liquid have been studied extensively. Further, there have been studies carried out on the effect of residual ionic liquid upon the IL-pretreatment of cellulose on the enzymatic hydrolysis in aqueous media. However, recently with the realization of biocatalysis in ionic liquid, few reports have been carried out in the biocatalytic hydrolysis of cellulose using cellulase in ionic liquid and aqueous ionic liquid binary solution. The results of these studies are discussed below.

Turner *et al*. (2003) studied ionic liquid salt-induced inactivation and unfolding of cellulase from *T. reesei* in ionic liquids [BMIM]Cl and [BMIM][BF_4], and dimethylacetamide-LiCl. The study indicated that [BMIM]Cl and dimethyl acetamide-LiCl denatured the enzyme in salt-induced conditions. Further, it was observed that the anion played a significant role in the inactivation of cellulase. The studies had indicate refolding of denatured cellulase in the most diluted solvents (0-5%) in [BMIM]Cl as detected by fluorescence spectroscopy. Therefore, observations indicates that the refolding tendency of unfolded cellulase is inversely proportional to [BMIM]Cl concentration.

Cellulase activity and denaturation of cellulase from *T. reesei* were investigated in N, N-dimethylethanolammonium alkylcarboxylate (formate, acetate and octanoate) ionic liquids. The activities in 20% and 40% v/w solution of [DMEA]OAc were equal

to citrate buffer controls. Lower enzymatic activity and denaturation was observed in formate and acetate ionic liquids. (Rayne and Mazza, 2007).

The study carried out on enzyme-catalyzed hydrolysis of cellulose in ILs for the production of biofuels (**Figure 2.5**). The calorimetric techniques have been used for the reactivity and stability of a commercial mixture of cellulase (from *T. reesei*) in ILs. Study indicated that HEMA is a novel, green medium for biocatalysis of cellulose to convert lignocellulosic biomass to biofuels. Therefore, HEMA has been an ideal design of ILs for hydrolysis of biomass and used with a wide spectrum of enzymatic reactions (Bose *et al*, 2010).

Bose *et al*. (2011) studied the hydrolysis of cellulose using a pure cellulase, endo-1, 4-glycosidases (EG) from the fungus, *Aspergillus niger,* in buffered, IL, tris-(2-hydroxyethyl)-methylammonium methylsulphate (HEMA), and various mixtures of the two at different temperatures. They observed percentage conversion to glucose from pretreated cellulose by [BMIM]Cl was increased when the temperature increased from 45-60^{o}C. Higher yield was reported in buffer HEMA for the hydrolysis of pretreated cellulose at 60^{o}C. The report also indicated that commercial *A. niger* enzyme showed higher tolerance to ILs and also enhanced thermostability in the presence of the IL (Bose *et al.,* 2010).

Zhi *et al.* (2012) studied one pot process of enzymatic hydrolysis of filter paper was studied in [MMIM]Me_2PO_4, [EMIM]Et_2PO_4 and [BMIM]Me_2PO_4. The hydrolysis of filter paper upto the extent 56.4% (24h) in 10% [MMIM]Me_2PO_4 aqueous solution was observed, which did not substantially increase and was found to 64% upon 96 h of stay. The results indicated that [MMIM]Me_2PO_4 had no side effect on cellulase as compared with aqueous buffered solution. The solution carrying 10% load of ionic liquid was found to show 2.1 times better rate of hydrolysis for filter paper than untreated cellulose. Sharp decline in activity of cellulase was found in the ionic liquids upon increase of temperature. Wang *et al*. (2011a) studied the hydrolysis of cellulose without pre-treatment and with pre-treatment in ionic liquid. [EMIM]OAc with simultaneous hydrolysis in 5-30% ionic liquid solution in citrate buffer. The catalysts used were enzyme mixture of cellulase isolated from *Trichoderma reesei* supplemented with *β*-glucosidase or Novozyme 188 isolated from *Aspergillus niger* was used in the studies. Further the hydrolysis of yellow

poplar was carried out and found to be highest at 0^0C showed a 55% cellulose conversion when subjected to hydrolysis. The yellow poplar was activated at 60^0C for 72 h.

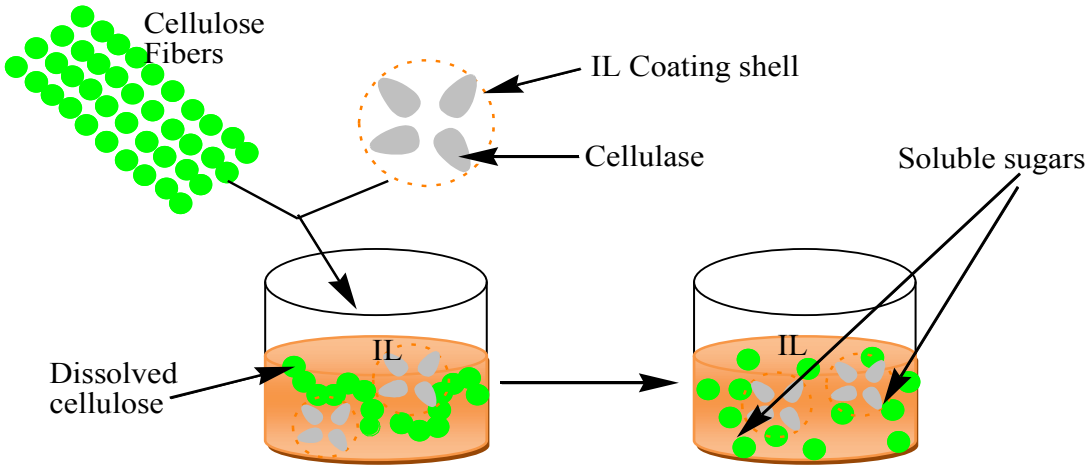

Figure 2.5: Saccharification of cellulose dissolved in ILs catalysed by cellulase enzyme coated with a hydrophobic IL.

Muhammad *et al*. (2012) studied the delignification of wood by Laccase in 5-20% ionic liquid in buffer. The studies were compared with the lignin extraction by ionic liquid pretreated lignocellulose in 20% ionic liquid in buffer solution. The enzymatic delignification upto 50% efficiency was reported in 5% *w/w* ionic liquid in buffer. In biocatalytic hydrolysis of cellulose, kosmotropicity of anions of the ILs stabilize the protein and hydrophobic aggregates and increased the stability of hydrophobes in cellulose and cellulase interactions, whereas, chaotropicity of cations unfolded protiens, destabilise hydrophobic aggregates and increase the solubility of hydrophobic moiety of cellulose structure.

2.5. Surfactants and Their use in Ionic liquid biocatalysis:

Surfactants are surface active agents, amphiphilic compounds containing a polar head and a non-polar tail. The self-assembly of the surfactant has been the key feature that segregates this class of molecules. Surfactants have found application for enhancement of biocatalytic activity in various solvents (Eriksson *et al*, 2002). Cellulase catalyzed hydrolysis of cellulose and wood, have extensively been investigated in past two decades in aqueous buffered conditions (Zhang *et al*., 2005). The mechanism for the enhancement in the hydrolysis of cellulose or wood

is due to the effects categorized as follows (Eriksson *et al*, 2002; Kaar and Holtzapple, 1998):

1. Surfactant may act as enzyme stabilizer and prevent denaturation.
2. Surfactant may have an effect on the substrate structure i.e. a surface structure modification or disruption that increases enzyme accessibility.
3. Surfactant may affect enzyme-substrate interactions, in particular by preventing non-productive binding adsorption of enzyme.

Recently, Kapu *et al*. (2012) studied Tween 20 and PEG have been studied simultaneous saccharification and fermentation of rice straw to lactic acid in presence of Tween 20 and PEG. The loading of 20% *w/w* loading of PEG w.r.t. substrate showed a 22% and 12% increase in the conversion of cellulose to yield sugar was observed for 24 h, in presence of Tween-20 and PEG, respectively.

Ouyang *et al*. (2010) studied the effect of order of addition of PEG-4000 in the hydrolysis of lignocellulose under aqueous conditions. Addition of PEG before addition of cellulase leads to an increase upto 52%. This is attributed to the adsorption of 80% of PEG-4000 on lignocelluloses. However, without the addition of lignocelluloses, it was observed that PEG-4000 interacts strongly with CBH I by hydrogen bonding. Further, it was observed that the activity of CBH I decreased by increasing interaction time and amount of PEG-4000 added in saccharification process.

Qing *et al*. (2010) studied the pre-treatment of corn stover by hot water or dilute acid pre-treatment at 140-220^{0}C in presence of surfactant Tween-80, dodecylbenzene sulphonic acid and PEG-4000. All these surfactants showed an increase in lignin removal during pre-treatment and increased hydrolysis yields in the next stage. However, Tween-80 increased enzymatic hydrolysis yields and enhanced total sugar recovery more than other two surfactants. In a similar study, Zhang and Tang (2011) studied the effect of PEG-4000 on cellulase catalyzed hydrolysis in various lignin and cellulose substrate by XRD and ATR-FTIR. The studies accounted the enhanced activity of cellulase in PEG-4000 either due to the unproductive adsorption of cellulase on both cellulose and lignin, thus prevent

cellulase deactivation induced by cellulose or promoting removal of amorphous cellulose.

Brethauer *et al*. (2011) studied the effect of BSA on stabilizing cellulase in the enzyme catalyzed hydrolysis of microcrystalline cellulose and corn stover. Addition of BSA improved the yield and time for hydrolysis. It reduces the deactivation of the exoglucanases and facilitates reduction in particle size and crystallinity during the preincubation conditions. Lavenson *et al*. (2011) had investigated the adsorption of BSA on cellulosic substrates using MRI.

Wang *et al*. (2011b) compared the effect of biosurfactant rhamnolipid and chemical surfactant Tween X-100 by studying the production of cellulases and xylanases from *Pencillium expansum* in untreated, acid and alkali-pretreated wheat straw submerged fermentation. The study found that the biosurfactant increased cellulase and xylanase quiet substantially in comparison to the Triton X-100. Apart from this, the acid pretreated straw broth was the most active with about 22.5% increase in the cellulase activity. Thus, these studies affirm that the non-ionic surfactants are more suitable for the stabilization of enzyme and for enhanced catalytic activity. However, there exists no report on the interaction of cellulase and cellulose with surfactants in ionic liquids.

Thus, from the above review of Cellulose and its Biocatalysis in Ionic liquids the following parameters can be delineated

1. Cellulose is made up of Inter- and Intra-molecular hydrogen bonding.
2. Ionic liquids with high polarity are desirable for cellulose dissolution.
3. Biocatalysis in ionic liquid is favoured in hydrophobic solvents like BF_4^-, PF_6^- and NTf_2^- anion based ionic liquids.
4. The role of alkyl group on the biocatalytic activity is not clear and varies with the properties of enzyme.
5. One pot saccharification of cellulose in ionic liquids have been studied in binary solution of ionic liquid in buffer condition. The studies have shown some promise in ammonium and imidazolium based ionic liquid.
6. The aqueous IL solution of [BMIM]Cl, [HEMA]OAc, [EMIM]OAc has been studied for cellulase biocatalysis.

7. The use of surfactants in enzyme stabilization and decreasing the cellulase adsorption on to the surface of lignin and cellulose component in heterogeneous conditions of aqueous phase have been studied.

From the above factors, it is clear that for a biocatalytic hydrolysis of cellulose, it is essential to desirable to meet two ends of the issue i.e.

a) The requirements of hydrophobic environment for better cellulase activity.
b) The requirement of hydrophilic environment for better solubility of cellulose.

High loading of cellulose would lead to better cost effectiveness and commercial viability of the process. Whereas the increased hydrophobicity can control the cost of cellulase enzyme and energy efficiency. However, to meet both ends, we have studied the biocatalytic activity of cellulase in ionic liquid solutions. The results of the investigation would be discussed as given below.

A. Studies of the biocatalytic hydrolysis of cellulose in ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl.
B. Studies of the biocatalytic cellulose hydrolysis in ionic liquid-aqueous buffer solution.
C. Studies of the effect of surfactant on IL biocatalysis.

CHAPTER III

MATERIALS AND METHODS

CHAPTER - III

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3.1. Chemicals Required:

1-Methylimidazole for synthesis was obtained from Spectrochem Pvt. Ltd. (Mumbai, India), *n*-Butyl chloride, Toluene, Ethyl acetate, Polyethylene Glycol (PEG)-1500, Polyethylene glycol (PEG)-4000, Sodium dodecyl sulphate (SDS) and 3,5-Dinitrosalicylic acid were obtained from Loba Chemie Pvt. Ltd. (Mumbai, India). D-Glucose anhydrous Extrapure, 1-Chlorohexane, 1-Chlorooctane and Dichloromethane were obtained from SD fine-chem Ltd. (Mumbai, India). CTAB was obtained from Merck specialties Pvt. Ltd. (Mumbai, India). Cellulase (5 g, *Aspergillus niger*, ›60,000 unit/g solid) was obtained from MP Biomedicals (LLC, Ohio). Whatmann filter paper (A grade) was obtained from Fisher scientific UK Ltd (Bishop Meadow Road, Loughborough), Avicel® PH-101 (CAS No.9004-34-6, Lot No.#BCBD6133) was obtained from Sigma-Aldrich (St. Louis, USA). Double distilled water was used for chemical and reagent preparation.

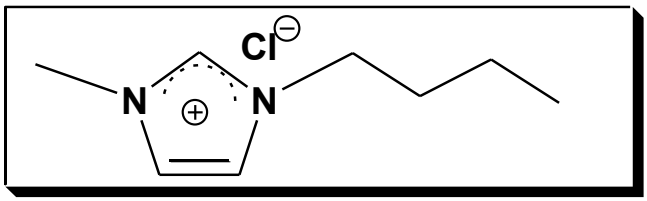
3.2. Instruments Used:

UV-Vis absorption were measured on a PC based Double Beam UV-VIS Spectrophotometer 2202 (Systronics) in a 1 cm quartz cuvette. All chemicals and reagents were weighed on weighing balance (Precisa Gravimetrics AG, Swiss made, 1 mg-120 g ± 0.1 mg). All pH were maintained on Mettler Toledo AG pH meter (FE20-I, Schwerzenboch, Switzerland). Characterization of all Ionic Liquids were done on 400 MHz Bruker Fourier Transform-Nuclear Magnetic Resonance (FT-NMR) and Fourier Transform-Infrared Spectrometer (FTIR) using chloroform for ionic liquids. The NMR spectra were recorded on Avance-II (Bruker) instrument, which operated at 400 MHz for ^{1}H NMR, in $CDCl_3$ as solvent with Tetramethylsilane (TMS) as internal reference. IR spectra were recorded on a Bruker Tensor 27 FTIR spectrometer for the compounds in the liquid state using chloroform as solvent. All centrifugation were done in CPR-24 REMI centrifuge (REMI Electronic Limited). Fluorescence studies measured on Shimadzu 2401 PC based Fluorescence Spectrophotometer.

3.3. Synthesis of methylimidazolium based ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl:

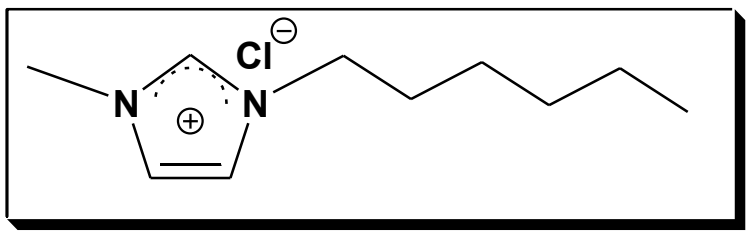
Synthesis of all methylimidazolium based ionic liquid was done as reported in literature with some modifications (Crowhurst *et al*., 2003).

3.3.1. Synthesis of 1-Butyl-3-methylimidazolium chloride; [BMIM]Cl (3a):



To a solution of 1-Chlorobutane (**2a**) (13.02 g, 0.140 mol) in 19 ml toluene was added N-Methylimidazole (**1**) (10.25 g, 0.125 mol) in ice cold water bath and mixed well at room temperature. The resulting solution was refluxed at 100^0C for 48 h. The reaction is characterized by formation of two layers in the solvent and was monitored using $CuSO_4$ solution (turns dark blue in presence of N-Methylimidazole). Upon completion of the reaction, the mixture was transferred to a separating funnel and two layers were separated. The denser layer was repeatedly washed with ethyl acetate (2x30 ml), dried in *vacuo* resulting into pale yellow product (Yield: 22.59 g; %Yield: 97), sealed and kept in deep freezer till further utilization. ^{1}H NMR (400 MHz, $CDCl_3$): δ (in ppm) 0.87 (t, J=2.4 Hz, 3H, CH_3), 1.10-1.47 (m, 2H, CH_2), 1.70-1.97 (m, 2H, CH_2), 4.07 (s, 3H, N-CH_3), 4.30 (t, J= 7.2 Hz, 2H, CH_2), 7.57 (s, 1H, Imidazole ArH), 7.73 (s, 1H, Imidazole ArH), 10.37 (s, 1H, Imidazole ArH). FTIR ($CHCl_3$) cm^{-1}: 3052, 2961, 2869, 1673, 1568, 1461, 1379, 1271, 1169, 755.

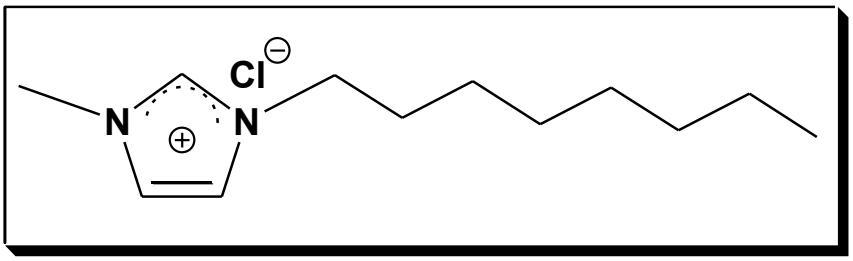
3.3.2. Synthesis of 1-Hexyl-3-methylimidazolium chloride; [HMIM]Cl (3b):



To a solution of 1-Chlorohexane (**2b**) (16.60 g, 0.140 mol) in 19 ml toluene, N-methylimidazole (**1**) (10.25 g, 0.125 mol) in ice cold water bath and mixed well then set it in room temperature. The resulting solution was refluxed at 100^0C for 48 h. The reaction was characterized by formation of two layers in the solvent and was monitored using $CuSO_4$ solution.

Upon completion of the reaction, the reaction mixture was transferred to a separating funnel (100 ml). Two layers formed during the reaction were separated and the denser layer was repeatedly washed with ethyl acetate (2x30 ml), dried in *vacuo* to yield pale yellow coloured product (Yield: 25.51 g; %Yield: 95), sealed and kept in deep freezer till further utilization. ^{1}H NMR (400 MHz, $CDCl_3$): δ (in ppm) 0.81 (t, J=2.4 Hz, 3H, CH_3), 1.09-1.52 (m, 6H, CH_2), 1.87-2.00 (m, 2H, CH_2), 4.10 (s, 3H, N-CH_3), 4.27 (t, J= 8.0 Hz, 2H, CH_2), 7.50 (s, 1H, Imidazole ArH), 7.76 (s, 1H, Imidazole ArH), 10.40 (s, 1H, Imidazole ArH). FTIR ($CHCl_3$) cm^{-1}: 2998, 1463, 1268, 756.

3.3.3. Synthesis of 1-Octyl-3-methylimidazolium chloride; [OMIM]Cl (3c):



To a solution of 1-Chlorooctane (**2c**) (20.40 g, 0.140 mol) in 19ml toluene was added N-Methylimidazole (**1**) (10.25g, 0.125 mol) in ice cold water bath and mixed well then set it in room temperature. The resulting solution was heated under reflux at 100^{0}C for 48 h. The reaction is characterized by formation of two layers in the solvent and was monitored using $CuSO_4$ solution. Upon completion of the reaction, the mixture was transferred to a separating funnel (100 ml). Two layers formed during the reaction were separated and the denser layer was repeatedly washed with ethyl acetate (2x30ml), dried in *vacuo* to yield pale yellow coloured product (Yield: 28.15 g; %Yield: 92), sealed and kept in deep freezer till further utilization. ^{1}H NMR (400 MHz, $CDCl_3$): δ (in ppm) 0.87 (t, J=6.0 Hz, 3H, CH_3), 1.24-1.31 (m, 10H, CH_2), 1.80-1.90 (m, 2H, CH_2), 4.13 (s, 3H, N-CH_3), 4.32 (t, J= 7.2 Hz, 2H, CH_2), 7.45 (s, 1H, Imidazole ArH), 7.66 (s, 1H, Imidazole ArH), 10.45 (s, 1H, Imidazole ArH). FTIR ($CHCl_3$) cm^{-1}: 2999, 2857, 1463, 1268, 1171, 756.

3.3.4. Preparation of DNS Solution:

DNS reagent was prepared by adding 3, 5-dinitrosalicylic acid (AR) (1.87 g), Sodium hydroxide (3.50 g), Rochelle salt (63.56 g), Phenol (1.34 ml) and sodium metabisulfite (1.46 g) in 250 ml distilled water.

3.3.5. Preparation of Citric Acid Buffer:

Citric acid buffer (pH 4.8) was prepared using citric acid monohydrate (AR) (21.00 g), sodium hydroxide (5.00 g) in 100 ml distilled water. 5 ml stock solution diluted to 100 ml of D.W. and used as C.B. (pH 4.8).

3.3.6. Preparation of Phosphate Universal Buffer (pH 3, 5, 7, 8 and 10):

Universal phosphate buffer was prepared by dissolving KOH (0.1 M, 5.6 g), Tris-hydrochloride (0.1 M, 15.76 g), Citric acid (0.1 M, 19.21 g), di-Sodium tetraborate (0.1 M, 38.14 g), K_2HPO_4 (0.1 M, 17.42 g) and Potassium hydrogen phthalate (0.4M, 81.68 g) in 1000 ml double distilled water. pH was adjusted by NaOH (0.4 M) and HCl (0.4 M) for the preparation of 3, 5, 7, 8 and 10 pH solutions (Perrin and Dempsey, 1974).

3.4. Total Reducing Sugar (TRS) assay by DNS method:

In four test tubes, an aliquot of 4 ml, 2 ml, 1 ml and 0.5 ml of 0.05 M sodium citrate having pH 4.8 were added. To these solutions 1ml of 1000 ppm glucose standard solution is added to each of the above test tube and mixed well. 0.5 ml of the final glucose solutions from each test tube was added to 3ml of DNS. The sample thus prepared was kept on water bath at 100°C for 5 min. and transferred to ice cold water to cease reaction. The samples were analyzed for absorbance at 540 nm using UV-Vis spectrophotometer. The amount of reducing sugar was expressed in terms of glucose, against the calibration graph prepared (**Figure 4.2**).

3.5. FPU (Total Cellulase Assay) Determination:

To perform FPU, rolled filter paper (50 mg) were put in five test tubes. Diluted C.B. (1 ml) was added to each test tube so as the paper strip be submerged in C.B., the enzyme dilution series (E1-E5) were prepared by dissolving 50, 25, 10, 5 and 1 mg of cellulase enzyme per ml of C.B. Glucose standards (GSs) were prepared by dissolving 1 ml glucose standard solution diluted to 4, 2, 1 and 0.5 ml of C.B. (GS1-GS4). An aliquot of 0.5 ml from GS1-GS4 were mixed with 1 ml C.B. in another test tubes, respectively. Reagent blank of 1.5 ml of C.B. was used for the analysis. An aliquot of 0.3 ml solution from E1-E5 test tubes were mixed with 0.6ml

C.B. for enzyme controls (EC1-EC5) preparation. Filter paper strip was submersed in 1.5 ml C.B. in a test tube and used as a substrate control (SC).

To the pre-warmed enzyme solutions, blank and controls, were added 0.5 ml of enzyme dilution series to the test tubes with filter paper substrate (E1-E5). Incubated the test tubes of E1-E5, GS1-GS4, RB, SC, EC1-EC5 and SC in a water bath at 50^0C for 1 h. After completion of incubation period, added 3 ml of DNS reagent in all test tubes and mixed well. Further, all test tubes were boiled on water bath at 100^0C for 5 min. The test tubes were transferred to an ice-cold water bath. 0.5 ml of aliquot was transferred to a 1.5 ml micro centrifuge tubes and subsequently centrifuged at 10,000 g for 3 min. 200 µl of supernatant was withdrawn, diluted with 2.5 ml distilled water (D.W.) and mixed well in cuvette. All measurements were done at 540 nm, where the absorbance of RB was used as a blank.

Calculation of FPU: Plotted a standard sugar curve (sugar along the x-axis vs. absorbance at 540 nm along the y-axis). Therefore, calculated the δ in absorbance of diluted enzyme solutions (ΔE1-4) for E1-5 by subtraction of the sum of the absorbance of EC1-5 and SC. Calculated the real glucose concentrations released by E1-5 according to a standard sugar curve. Plotted the relationship between the real glucose concentrations and their respective enzyme dilution rates (EDRs). Linked the points <2 mg and >2 mg by a line, and identified the EDR by using the point for 2 mg glucose based on the line.

$$\text{FPA} \Longrightarrow \frac{0.37}{\text{EDR}}$$

Where, 2 mg glucose = 2 mg / [(0.18 mg/µmol) X 0.5 ml X 60 min] = 0.37 µmol/min/ml.

3.6. Hydrolysis experiments:

3.6.1. TRS in ionic liquid:

Enzymatic activity was measured on soluble Filter Paper or Avicel®, using the 3, 5-dinitrosalicylic acid (DNS) reducing sugar assay. 40 mg FP or Avicel mixed with ([BMIM]Cl, [HMIM]Cl or [OMIM]Cl) on magnetic stirrer at 50^{o}C and 320 rpm. The cellulase enzyme (5 mg) added in the solution and mixed well at 50^{o}C. The reaction mixture was analyzed with different time intervals for reducing sugar. The solution was prepared by mixing of 100 mg sample and 200 µl distilled water. The mixture centrifuged at 10000 rpm for 10 min, 100 µl supernatant of centrifuged solution was withdrawn and diluted with with 300 µl of DNS solution. The reactants were incubated on boiling water bath for 5 min and transferred to in cooled water and the absorbance was read at 540 nm on UV-spectrophotometer by preparation of 100 µl sample added with 2.5 ml D.W. Distilled water used as a blank. The reducing sugar concentration in the sample was calculated from its absorbance using the standard calibration curve of D-glucose.

3.6.2. Hydrolysis of Avicel (Cellulose) in Ionic Liquids using different amount of Cellulase enzyme:

All hydrolysis experiments were performed with laboratory based synthesized 1 g Ionic Liquids ([BMIM]Cl, [HMIM]Cl and [OMIM]Cl, 40 mg Avicel, 10% C.B. (pH 4.8) and Cellulase enzyme (1 mg, 2 mg, 3 mg, 4 mg and 5 mg). The experiments were performed on a magnetic hot plate at 50°C and 320 revolutions per minute (rpm). The 100 mg aliquot was taken in different time intervals to added 200 µl distilled water and centrifuged it on 10000 rpm for 10 min. 100 µl centrifuged supernatant was added with 300 µl DNS reagent and mixed well. DNA mixed solution was heated on boiling water bath for 5 min then transferred the solution in ice-cold water bath. All measurements were done at 540 nm on spectrophotometer using 100 µl sample added with 2.5 ml D.W. in a cuvette. Total Reducing Sugar (TRS) were measured in mg/ml using the dinitrosalicylic acid (DNS) method at time intervals.

3.6.3. Hydrolysis of cellulose in Ionic Liquids:

Hydrolysis of Avicel and Whatmann Filter paper using as cellulose were performed in concentration of [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The reactions were initiated by addition of 100 µl of cellulase enzyme solution in citrate buffer at pH 4.8. The experiments were performed on a magnetic hot plate at 50°C and 320 revolutions per minute (rpm). The hydrolysis was terminated after several hours. The 100 mg of aliquot was withdrawn at in different time intervals, diluted with 200 µl distilled water and centrifuged it on 10000 rpm for 10 min. 100 µl centrifuged supernatant was added with 300 µl DNS reagent and mixed well. The solution thus obtained and was heated on boiling water bath for 5 min then transferred the solution in ice-cold water bath. All measurements were done at 540 nm on spectrophotometer using 100 µl sample added with 2.5 ml distilled water (D.W.) in a cuvette. Total Reducing Sugar (TRS) were measured in mg/ml using the dinitrosalicylic acid (DNS) method at time intervals.

3.6.4. Hydrolysis of cellulose in binary aqueous solution:

Hydrolysis of Avicel and Whatmann Filter paper using as cellulose were performed in 0%, 20%, 40%, 60%, 80% and 100% [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The binary aqueous solutions were prepared by weighing known amount of the ILs i.e. 200, 400, 600, 800 and 1000 mg and diluting the samples with 800, 600, 400, 200 and 0 mg of citrate buffer maintained at pH 4.8. The reactions were initiated by mixing of cellulase enzyme solution with citrate buffer at pH 4.8. The experiments were performed on a magnetic hot plate at 50°C and 320 revolutions per minute (rpm). The hydrolysis was terminated after several hours. The 100 mg aliquot was taken in different time intervals to added 200 µl distilled water and centrifuged it on 10,000 rpm for 10 min. 100 µl centrifuged supernatant was added with 300 µl DNS reagent and mixed well. DNA mixed solution was heated on boiling water bath for 5 min, transferred the solution in ice-cold water bath. All measurements were done at 540 nm on spectrophotometer using 100 µl sample added with 2.5 ml D.W. in a cuvette. Total Reducing Sugar (TRS) were measured in mg/ml using the dinitrosalicylic acid (DNS) method at different time intervals.

3.6.5. Hydrolysis of cellulose in Ionic Liquids with Universal Buffer (U.B.):

Hydrolysis of Avicel using as cellulose were performed in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The reactions were initiated by mixing of cellulase enzyme stock solution in different pH (pH 3, 5, 7, 8, 10) using universal buffer. The experiments were performed on a magnetic hot plate at 50°C and 320 revolutions per minute (rpm). The hydrolysis was terminated after several hours. The 100 mg aliquot was taken in different time intervals diluted with 200 µl distilled water and centrifuged at 10,000 rpm for 10 min. 10 µl centrifuged supernatant was diluted with 300 µl DNS reagent and mixed well. DNA mixed solution was heated on boiling water bath for 5 min, transferred the solution in ice-cold water bath. All measurements were done at 540nm on spectrophotometer using 100 µl sample added with 2.5 ml D.W. in a cuvette. Total Reducing Sugar (TRS) were measured in mg/ml using the dinitrosalicylic acid (DNS) method at different time intervals.

3.6.6. Hydrolysis of cellulose in Ionic Liquids with surfactants:

Hydrolysis of Avicel® using as cellulose were performed in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. Four different surfactants (PEG-1500, PEG-4000, SDS and CTAB) were evaluated for their ability to enhance enzymatic hydrolysis. The reactions were initiated by mixing of cellulase enzyme solution with citrate buffer at pH 4.8. The experiments were performed on a magnetic hot plate at 50°C and 320 revolutions per minute (rpm). The 100 mg aliquot was taken in different time intervals diluted with 200µl distilled water and centrifuged at 10,000 rpm for 10 min. 100 µl centrifuged supernatant was diluted with 300 µl DNS reagent and mixed well. DNA mixed solution was heated on boiling water bath for 5 min, transferred the solution in ice-cold water bath. All measurements were done at 540 nm on spectrophotometer using 100 µl sample added with 2.5 ml D.W. in a cuvette..Total Reducing Sugar (TRS) were measured in mg/ml using the dinitrosalicylic acid (DNS) method at different time intervals.

3.6.7. Fluorescence based study of Hydrolysis of cellulose in Ionic Liquid with cellulase enzyme and Surfactants (PEG-1500 and 4000):

For these experiments, vials containing a total volume of 2.7 g IL ([BMIM]Cl, [HMIM]Cl and [OMIM]Cl]) and 0.3 g citrate buffer having pH 4.8 were prepared at room temperature. To each solution, 50 µl freshly prepared cellulase stock solution (150 mg cellulase and 5 ml C.B.) was added and mixed well on magnetic stirrer for proper mixing at room temperature. Two stock solutions of PEG-1500 and PEG-400 were prepared. To the cellulase in IL solution PEG solutions were added in small aliquots and mixed before measuring fluorescence. After each loading of PEG emission scans were then taken from 280-700 nm (λ_{ex} =280nm) with a slit width of 10.10 nm. For extended experiment 40 mg/g Avicel was used as a cellulose for same condition in each IL mixed solution and after each PEG loading emission scans were then taken from 300-700 nm (λ_{ex} =nm) at excitation slit width 5 and 10 nm and emission 300 nm slit width 5 and 10 nm.

CHAPTER IV

RESULTS

CHAPTER - IV

RESULTS

Cellulose dissolution and pre-treatment in ionic liquid, has been a promising method for bioethanol production from lignocellulosic biomass (Doherty *et al*., 2010). The pre-treatment involves dissolution of lignocellulose in ionic liquids followed by precipitation of cellulose using water as anti-solvent. Hydrolysis has been studied after repeated washing with water to remove ionic liquid (Mora-Pale *et al*., 2011). However, there have been studies in the tolerance of cellulase for the biocatalytic hydrolysis of cellulose in presence of traces of IL. The results indicated hydrolysis of cellulose is inhibited even at lower concentration of ILs. However, recently there have been reports confirming the stability of cellulase in upto 20% *v/v* IL in water (Zhi *et al*., 2012). Further no study exists on the cellulase activity in presence of surfactant in imidazolium ionic liquid biocatalytic hydrolysis of cellulose.

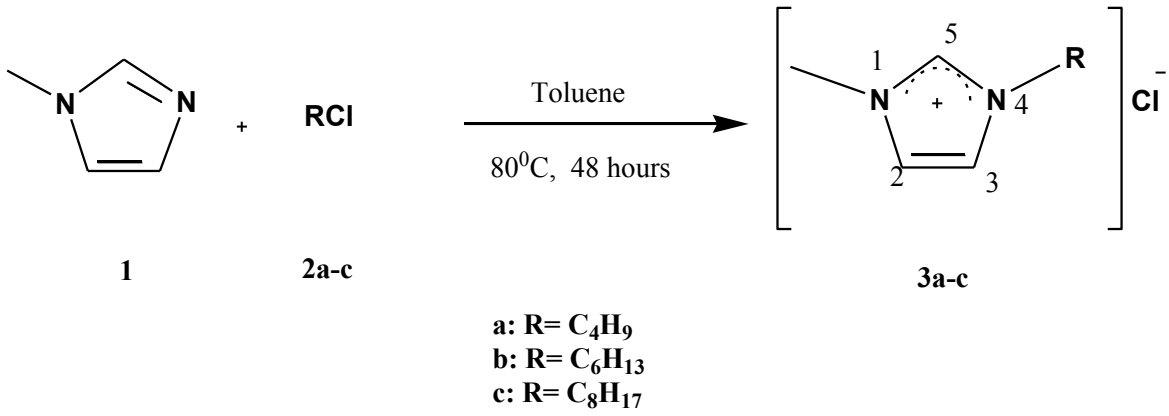
In the present investigation, study of the biocatalytic hydrolysis of cellulose in presence of cellulase in imidazolium based ionic liquids has been carried out. The ILs used in the study have alkyl groups with varying alkyl chain length i.e., [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. Thus the present study has been discussed as follows

a) Optimization of conditions for IL based biocatalysis
b) Study in the biocatalysis in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl
c) Study of biocatalysis in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl-aqueous buffer solution
d) Study of biocatalysis in IL in presence of surfactant
e) Fluorescence study of enzyme stability in IL

4.1. Synthesis of Ionic Liquid:

Ionic liquids were synthesized using the method reported by Crowhurst *et al*. (2003), the synthesis involved the addition of alkyl halides namely *n*-chlorobutane, *n*-chlorohexane and *1*-chlorooctane (**2a-c**) to N-methylimidazole **(1)** in toluene as solvent, the reaction flasks were stirred and kept at 80^0C (**Scheme-4.1**). Both the

reactants were soluble in toluene and with progress of the reaction, ionic liquid get settled down as a separate layer. The dense layer in the reaction comprised of ionic liquid, whereas, the lighter portion comprised of toluene. Upon completion of reaction, the ionic liquid (**3a-c**) were separated, washed with ethyl acetate and dried *in vacuo* to give desired ionic liquid in quantitative yield with 97%, 95% and 92% of ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively. The ionic liquids were characterized by ^{1}H NMR and FT-IR studies. The ^{1}H NMR of the ionic liquids showed a triplets (3 protons) at δ 0.87, 0.81 and 0.87 ppm for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively, corresponding to the terminal methyl protons of the n-alkyl groups.



Scheme 4.1: Synthesis of *N*-Methylimidazolium based Ionic Liquids.

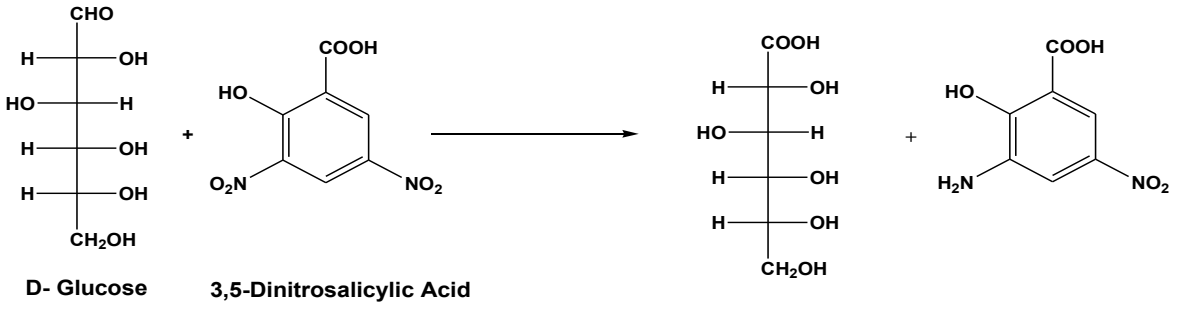
A singlet (3 protons) at δ 4.07, 4.10 and 4.13 ppm, corresponding to the protons of N-methyl group of [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively. The alkyl methylenes were observed as multiplets in range of δ 1.24-1.31(4 protons), 1.09-1.52 (8 protons) and 1.10-1.47 (12 protons) ppm for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The methylene attached to the imidazolium show a triplet (2 protons) at δ 4.30, 4.27 and 4.32 ppm for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively. Two doublets (2 proton each) at δ 7.57 and 7.73 ppm corresponds to the aromatic imidazolium protons of [BMIM]Cl. Two doublets (1 proton each) at δ 7.50 and 7.76 ppm corresponding to the aromatic imidazolium protons of [HMIM]Cl. Similarly, two doublet at δ 7.45 and 7.66 ppm corresponding to the aromatic imidazolium

protons of [OMIM]Cl. A singlet (1 proton) at δ 10.37, 10.40 and 10.45 ppm for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively, corresponding to the C-5 methylene proton, was observed to be shifted downfield.

The FTIR spectrum of compound (**3a-c)** were recorded in $CHCl_3$ solution and showed strong absorption bands at 1568, 1461-63, and 1169-1171 cm^{-1} characteristic of 1,3-dialkyl imidazolium cation (Talaty *et al.,* 2004). A strong absorption peak around at 3147 and 3097 cm^{-1} were attributed to C-H stretching vibration of imidazolium ring, whereas, C-H stretching bands of the alkyl chain on the imidazolium ring were observed at 2926 and 2855 cm^{-1}. Thus, the spectroscopic data of the ILs **(3a-c)** corroborate with the literature, confirming formation of the addition compound.

4.2. Total Reducing Sugar (TRS) Analysis using DNS method:

Total reducing sugar (TRS) analysis in the present work, was carried out using UV-Vis spectrophotometer based 3,5-Dinitrosalicylic acid (DNS) method (Miller, 1959). The standardization of DNS method was carried out in the laboratory based on experiments performed in triplicate. The reaction involved in the method is given in **Scheme 4.2**. The colorimetric reaction involved, the reduction of 3,5-dinitrosalicylic acid by glucose or any other reducing sugar leading to the formation of 3-amino-5-nitrosalicylic acid. The formation of the reduction product is characterized by a change in colour from light yellow to dark brown, which strongly absorbs in visible region at λ_{max}=540 nm. The result of the calibration experiments is given in **Figure 4.1.**



Scheme 4.2: Reaction of Reducing Sugar (D-Glucose) with 3, 5-dinitrosalicylic acid.

For TRS, different concentrations of standard D-glucose solutions were mixed with citrate buffer having pH 4.8. 0.5 ml aliquot from each test tube was mixed with 3 ml DNS reagent and incubated at 100^0C for 5 min. The reaction mixture was transferred and kept in ice-cold water bath before the absorbance was measured at 540 nm using UV-Vis Spectrophotometer. The absorbance of the samples at 540 nm was directly proportional to the TRS concentration expressed as concentration of glucose in mg/ml as shown in **Figure 4.1**.

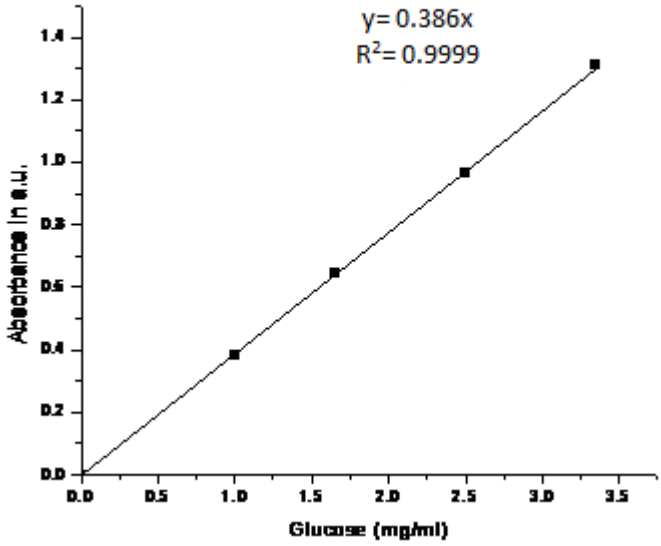


Figure 4.1: Calibration curve for Total Reducing Sugar (TRS) expressed as concentration of glucose (mg/ml) versus absorbance at 540 nm.

The sample absorbance increased linearly with the increase in the concentration of glucose. The absorbance of the solution to the concentration of reducing sugar is related by the equation.

$$y = 0.386x$$

where, 0.386 is the slope of the graph and regression coefficient R^2=0.9999 indicates excellent linear curve fitting for the method of analysis of total reducing sugar using DNS method (**Figure 4.1**).

In the present investigation, ionic liquid is the base matrix used and could interfere with analysis of reducing sugar. Thus, the addition of ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl to a standard glucose solution followed by subsequent

analysis by DNS method were carried out. No significant deviation in reducing sugar analysis was observed in presence of these ionic liquids.

4.3. Cellulase Activity in terms of Filter Paper Unit (FPU):

FPU (filter paper unit) is the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC) for reporting of cellulase activity (Ghose, 1987). One unit of filter paper (FPU) activity is defined as the amount of enzyme required for releasing 1 μmole of reducing sugar from filter paper per ml per min. The cellulase that was used in our experiments has been isolated from *Aspergilus niger*, commercial activity of these enzymes was reported to be >60,000 FPU/g. However, experiment was carried out to find the FPU activity of this commercial cellulase. The FPU activity of cellulase enzyme was studied by the approved NREL method (1996). The study involved the preparation of four dilution of cellulase in citrate buffer, cellulose filter papers were added and the dilution incubated at 50^0C. The incubated solutions were removed after one hour and analyzed by DNS method for the liberated total reducing sugar (TRS). The calculation was further reported as given below:

$$FPA = \frac{0.37}{EDR} = \frac{0.37}{0.0058} = 63.79 \text{ FPU / ml}$$

$$\text{FPU in g} = 63.79 \times 1000 = 63790 \text{ FPU / g}$$

where, 2 mg glucose = 2 mg / [(0.18 mg/ μmol) X 0.5 ml X 60 min] = 0.37 μmol/min/ml.

The activity of the commercial cellulase isolated from *Aspergillus niger* provided was found to be 63790 FPU / g (**Figure 4.2**).

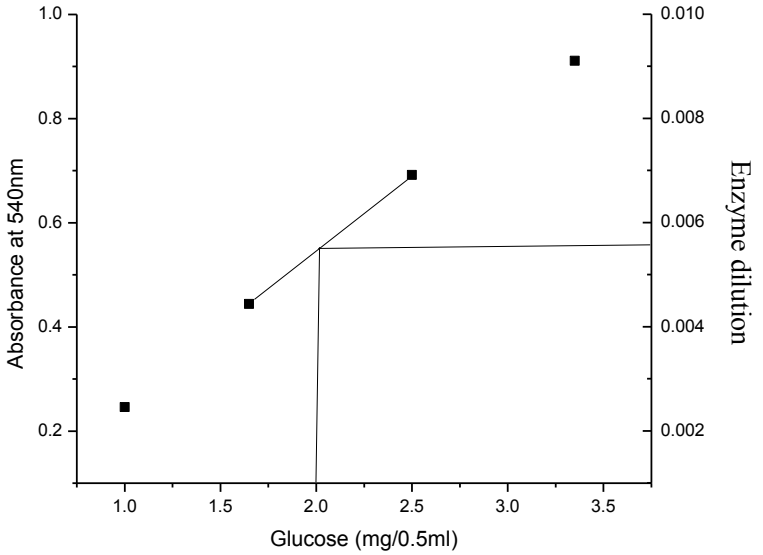


Figure 4.2: Graph plotted for enzyme dilution series against glucose concentration (mg / 0.5 ml)

4.4. Optimization of Condition for Biocatalysis in Ionic Liquid using Cellulase enzyme:

4.4.1. Dissolution of Cellulose for Optimized Condition:

Cellulose dissolution in all the three ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl have been reported (Pinkert *et al*., 2009). However, in our laboratory for the hydrolysis experiments, solubility of cellulose substrates in ionic liquids at 50^{o}C was carried out. The study of dissolution of cellulose filter paper and Avicel were undertaken in all the three ionic liquids namely [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The result of the solubility of cellulose in these ionic liquid is summarized in **Table 4.1**.

Table 4.1: Dissolution of cellulose filter paper and Avicel in ionic liquid at 50^{o}C.

Ionic liquid	Dissolution of Cellulose Filter Paper (in mg/g of IL)	Dissolution of Avicel (in mg/g of IL)
[BMIM]Cl	40 $\pm$ 5	55 $\pm$ 5
[HMIM]Cl	50 $\pm$ 5	60 $\pm$ 5

[OMIM]Cl	50 ± 5	60 ± 5

The results show that the dissolution of filter paper were upto 40 ± 5, 50 ± 5 and 50 ± 5 mg/ml and Avicel were upto 55 ± 5, 60 ± 5 and 60 ± 5 mg/ml for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively.

4.4.2. pH Optimization studies:

Cellulase activity is optimum in aqueous media at pH 4.8. Thus, citrate buffer is ideally suited for the application. However, as the aqueous environment is changed to IL-aqueous binary mixture, the solution pH would have a significant effect on the cellulase activity. Thus, we studied the effect of pH on the cellulase activity of 10% water in IL solution with cellulase concentration of 5 mg/g. Universal phosphate buffer was used for maintaining pH of 10% water in ionic liquid for [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The pH of the aqueous buffer solutions added to ionic liquid were maintained at pH 3.0, 5.0, 7.0, 8.0 and 10.0 at 50^{o}C and 320 rpm. A citrate buffer solution for pH 4.8 was also analyzed simultaneously. The hydrolysis were carried out at small scale with 1 g of buffered ionic liquid solutions. Further, the high viscosity of these ionic liquids aggravated the stirring problems of the reaction. Thus, a magnetic stirrer maintained at 320 rpm was used in all these reaction. The reactions were monitored at various time intervals and the results are as given in **Figure 4.3 (A-C)**.

The pH of the solution showed certain general trends

- The reducing sugar formed upon hydrolysis of cellulose was higher in case of solution having acidic pH, with the exception of [OMIM]Cl, where the optimum pH for enzyme catalysis was at pH 7.0. The high cellulose hydrolysis rate at pH 3.0 could be attributed to the hydrolysis of cellulose by H^{+} ions in the acidic medium.
- The study indicates the fact that the optimum biocatalytic cellulose hydrolysis was obtained at pH 4.8.
- At pH >5.0, the solution show decreased hydrolytic activity.

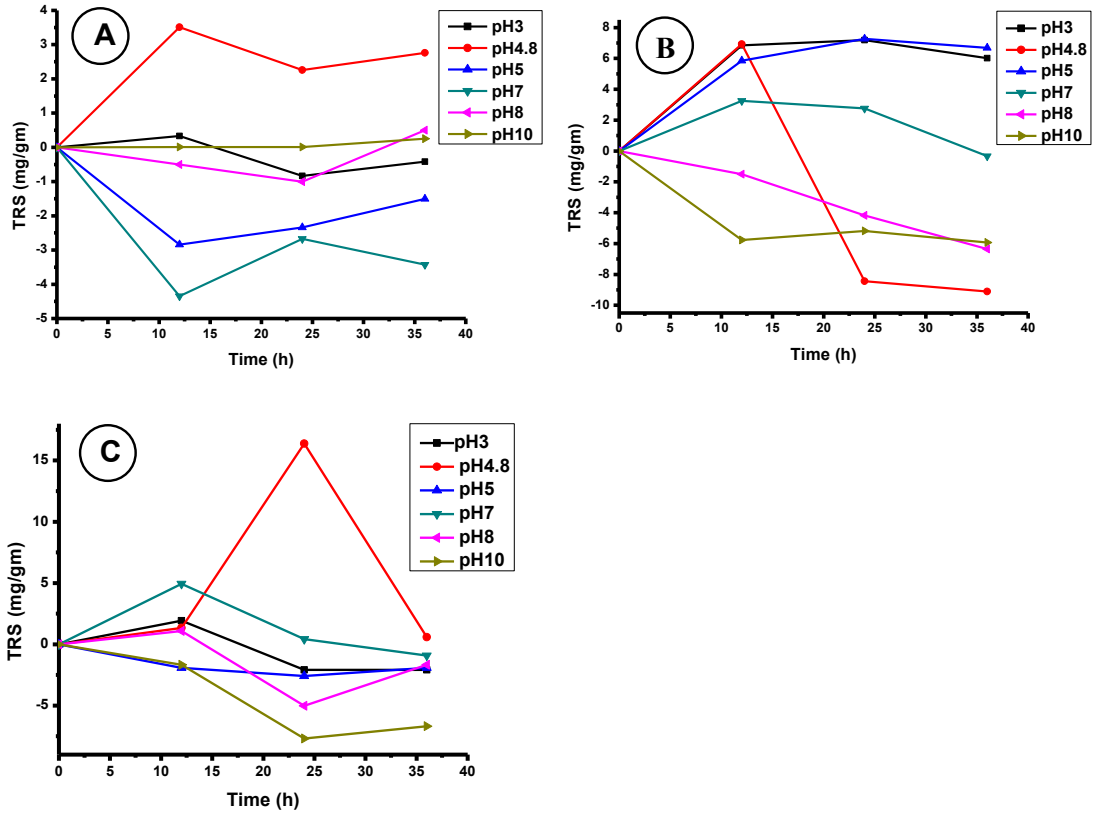


Figure 4.3: Effect of pH variation on TRS generated by biocatalytic hydrolysis of Avicel (40 mg) and Cellulase (5 mg) in 1 g binary aqueous buffered-IL solution (10% buffer) **(A)** [BMIM]Cl **(B)** [HMIM]Cl **(C)** [OMIM]Cl]

The results indicate that optimum pH for the maximum cellulase activity was found to be pH 4.8, which is same as that of the hydrolysis under aqueous condition. Thus, in further experiment, we have used citrate buffer maintained at pH 4.8 as a standard condition for carrying out the biocatalytic hydrolysis of cellulose.

4.4.3. Optimization of cellulase enzyme concentration:

The linearity of increase in biocatalysis with catalyst concentration might not be valid in case of system where degradability of the desired product plays a key role. It was observed that a significant drop in the reducing sugar concentration occurs during the progress of biocatalytic hydrolysis of cellulose, which could be accounted to the significant rate of degradation of the reducing sugar formed during the progress of the reaction.

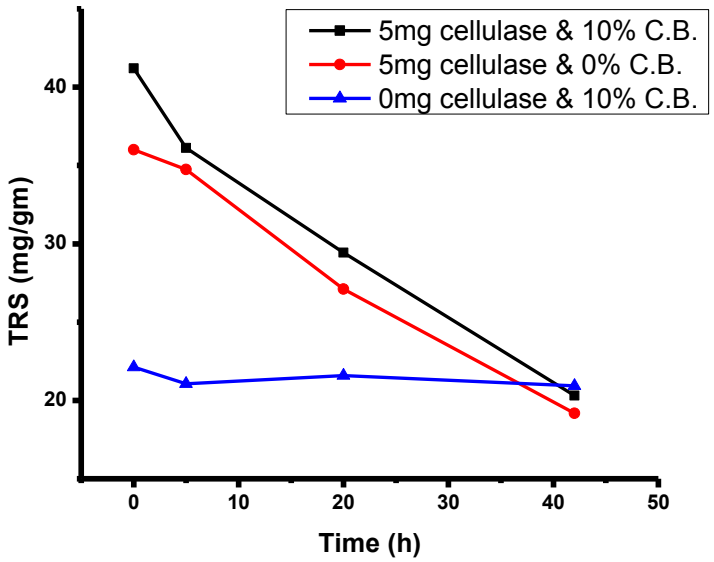


Figure 4.4: TRS assay performed at different time interval of glucose (40 mg) dissolved with and without cellulase enzyme in 1 g *w/w* pure and aqueous buffered-IL [BMIM]Cl binary solution.

To investigate the effect of ionic liquid and cellulase on the solubility of glucose, studies were carried out time dependent degradation of glucose, by dissolving known amount of glucose in IL-cellulase mixture with or without citrate buffer, maintained at 50^0C with progress in time it was observed that the glucose concentration got depleted upon dissolving known amount of glucose in IL containing cellulase. Upon dissolving known amount of glucose in IL containing only citrate buffer, there was no change in concentration of glucose observed (**Figure 4.4**). Thus degradation of product is evident during the hydrolysis of cellulose in presence of the biocatalyst.

Studies were carried out to investigate the optimum concentration of enzyme for biocatalytic hydrolysis in IL. Thus, the formation of the TRS with time upon variation of cellulase concentration was carried out at 1-5 mg/g IL for various 10% aqueous citrate buffer in IL solution **(Figure 4.5 A-C)**. All the solutions were kept at 50^0C with a constant stirring at 320 rpm. The solutions were continuously monitored for TRS by DNS method for 42 h.

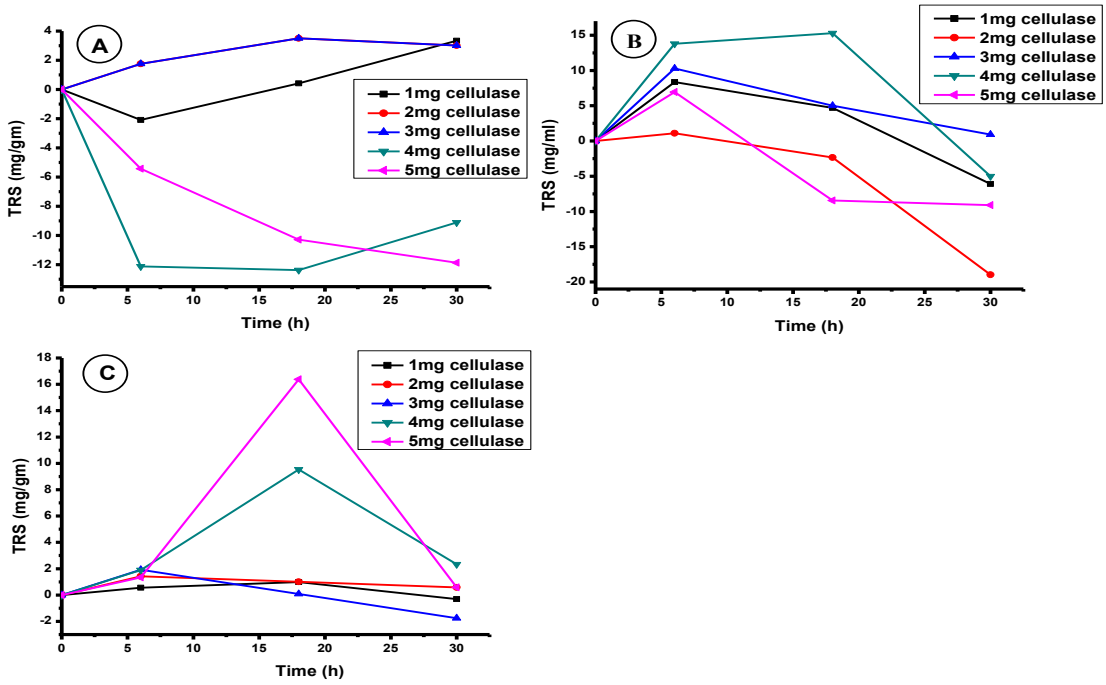


Figure 4.5: TRS generated by biocatalytic hydrolysis of Avcel cellulose substrate upon changing cellulase (1-5 mg) concentration in 1 g *w/w* 10% C.B. in ILs **(A)** [BMIM]Cl **(B)** [HMIM]Cl **(C)** [OMIM]Cl.

In [BMIM]Cl, the aliquots were analyzed for TRS at various time intervals. The results indicate that the reaction in optimum condition, show better stability to decomposition with a good TRS and rate of hydrolysis at 2-3 mg cellulase/g of IL solution. Interestingly, in [HMIM]Cl the results show better rate of hydrolysis at a cellulase concentration of 4 mg/g. In [OMIM]Cl, the studies show better TRS concentrations at a cellulase concentration between 4-5 mg/g. However, in cellulase concentration apart from those mentioned, it is evident that the rate of degradation is much higher than the rate of formation of reducing sugar. However based on these results, cellulase concentration were studied at 2, 3 and 5 mg/g in all the experiments further carried out.

Finally, the outcome of these optimization studies can be summed up for an ideal study reaction condition as follows:

- 40 mg cellulose / g of IL
- 2, 3 and 5 mg cellulase / g of IL
- pH 4.8 maintained using citrate buffer

The cellulose concentration was kept on the lower side at 40 mg/g of IL (i.e., a loading of 4% cellulose w.r.t. ionic liquid), to have better homogenous conditions for hydrolysis in 10% and 20% citrate buffer in IL solutions. The cellulase concentration for comparison could not be arrived, but the catalysis was enhanced in presence of 4 and 5 mg of cellulase in [OMIM]Cl and [HMIM]Cl, however, in [BMIM]Cl, 2-3 mg/g of cellulase suited better than other concentrations of enzyme in the hydrolysis experiments.

4.5. Study of hydrolysis in Ionic Liquids: Effect of cation on the hydrolysis rate:

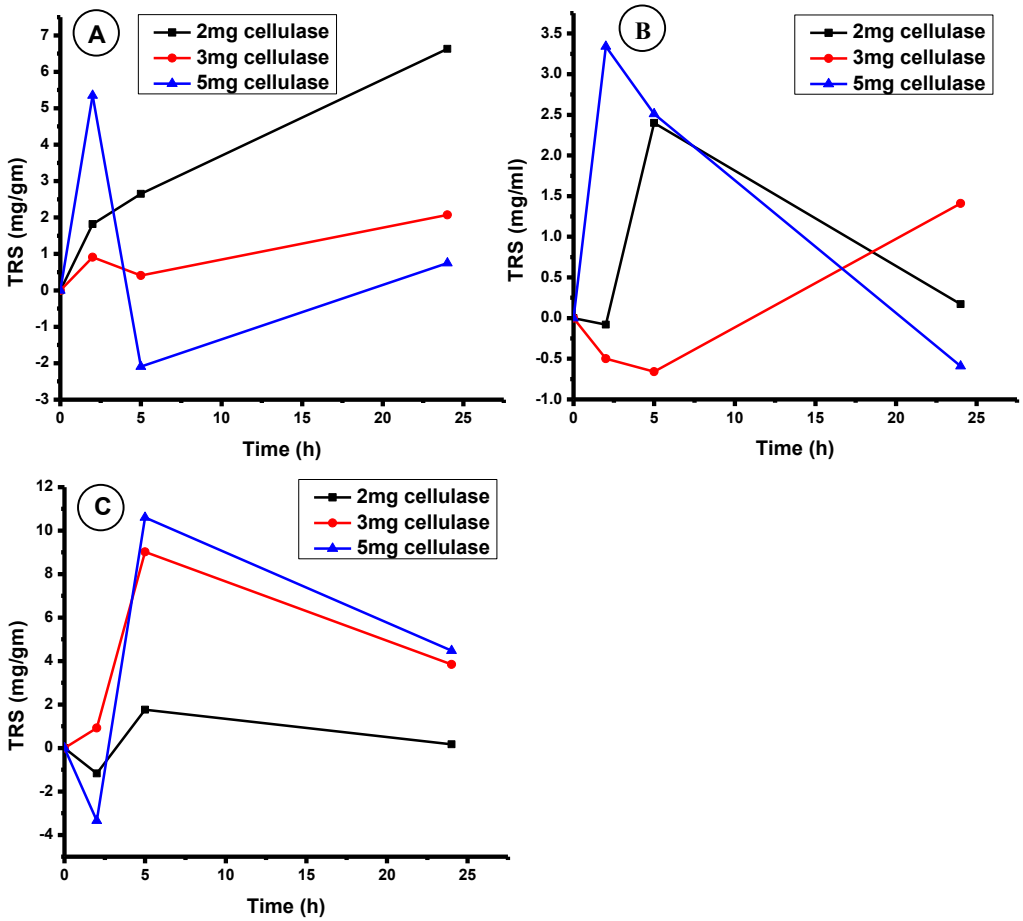


Figure 4.6: TRS generated by biocatalytic hydrolysis of Avicel cellulose substrate (40 mg) in different concentration of cellulase (2, 3 and 5 mg/g IL) in the ILs **(A)** [BMIM]Cl **(B)** [HMIM]Cl **(C)** [OMIM]Cl.

The study of cellulose hydrolysis in IL has been reported by Bose *et al*. (2010) in [BMIM]Cl, [BMIM]$MePO_4$, [HEMA]OAc, [EMIM]OAc and in imidazolium carbamate ionic liquids (Rayne and Mazza, 2007).

Bose *et al.* (2010) had studied the hydrolytic activity with cellulose azure as the substrate and monitoring the release of the fluorophore from the substrate upon hydrolysis. However, the study indicated better hydrolysis in tris-(2-hydroxyethyl)-methylammonium methylsulphate (HEMA)OAc, The present work, involves the study of behaviour of imidazolium IL carrying chloride counter ion as a low toxic solvent. Thus, we have studied the effect of hydrophobicity of IL based on the alkyl group introduced in the imidazolium cation. The studies were carried out by monitoring TRS at various time intervals for a 40 mg cellulose substrate in 2, 3 and 5 mg cellulase catalyzed reaction per g of ionic liquids ([BMIM]Cl, [HMIM]Cl or [OMIM]Cl) as the solvent (**Figure 4.6**).

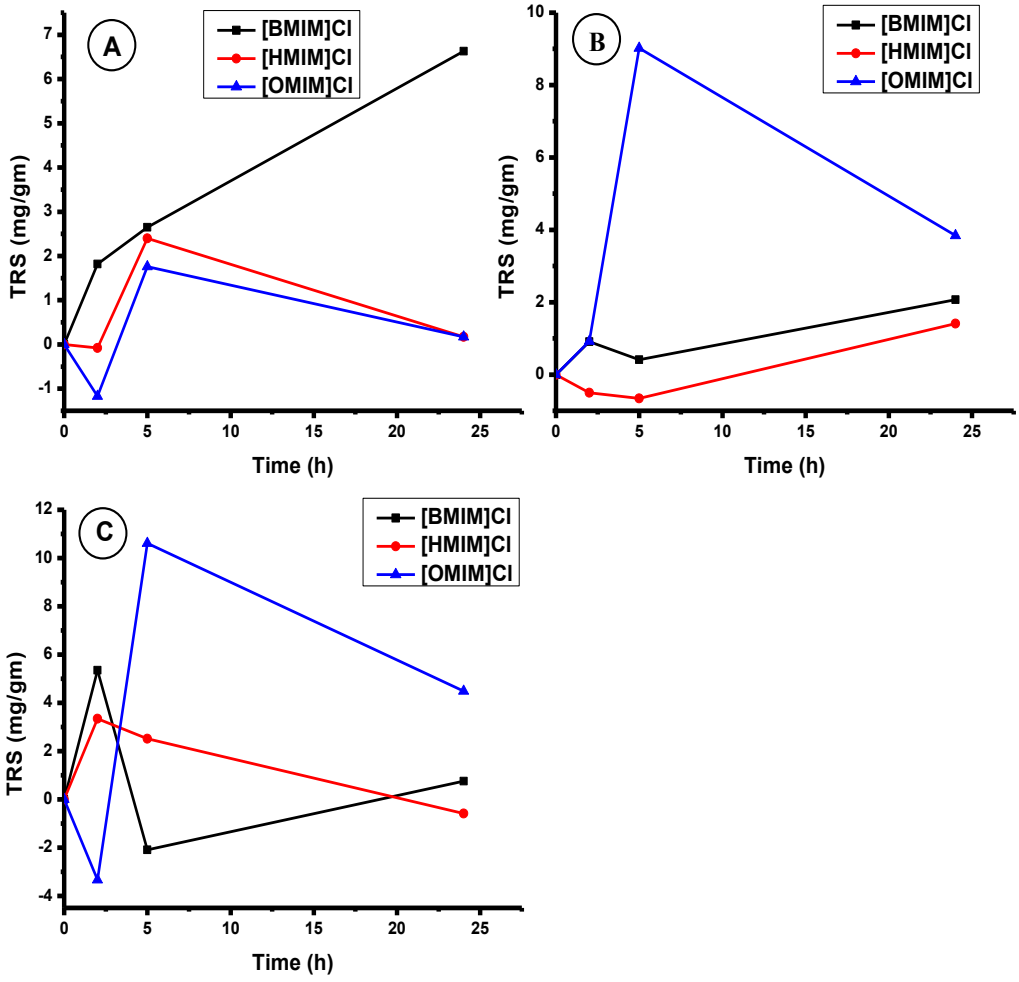


Figure 4.7: TRS generated by biocatalytic hydrolysis of Avicel cellulose substrate (40 mg) in presence of different concentration of cellulase (1-5 mg) **(A)** 2 mg cellulase **(B)** 3 mg cellulase **(C)** 5 mg cellulase obtained.

The cellulase catalyzed hydrolysis of cellulose in IL [BMIM]Cl, at concentration of 2, 3 and 5 mg of cellulase/g of IL showed a maximum reducing sugar concentration of 6.63 (24 h), 2.1 (24 h) and 5.35 (2 h) mg/g of IL, respectively. Similar study carried out in [HMIM]Cl at concentration of 2, 3 and 5 mg/g of cellulase / g showed minimum reduced sugar concentration of 2.4 (5 h), 1.41 (24 h) and 3.34 (2 h) mg/g of IL, respectively. However, under similar condition in [OMIM]Cl as solvent a maximum reducing sugar concentration 1.76 (5 h), 9.02 (5 h) and 10.61(5 h) mg/g of IL, respectively were obtained upon addition of 2, 3 and 5 mg of cellulase / g of IL.

The result can be put in significant percentage hydrolysis of cellulose. In [BMIM]Cl the percentage hydrolysis of cellulose was found to be as 15% (24h), 5% (24h) and 12% (2h) in presence of 2, 3 and 5 mg of cellulase in 1 g of IL solution, respectively. The percentage hydrolysis of cellulose in [HMIM]Cl was found to be 5% (5 h), 3% (24 h) and 8% (2 h) in presence of 2, 3 and 5 mg of cellulase in 1 g IL solution, respectively. However, the percentage hydrolysis of cellulose in [OMIM]Cl was 4% (5 h), 20% (5 h) and 23% (5 h) in the presence of 2, 3 and 5 mg of cellulase in 1 g of IL solution, respectively (**Figure 4.6 and 4.7**). Thus, a maximum 23% hydrolysis was observed in [OMIM]Cl based system upon addition of 5 mg of cellulase.

4.5.1. Cellulose Hydrolysis Behavior of Cellulase in [BMIM]Cl –Water Binary Mixture:

The investigation of the cellulose hydrolysis by cellulase enzymes was carried out in binary solutions of w/w [BMIM]Cl-water. To the binary solvent prepared 0-100% IL in buffer at room temperature, cellulose were added and stirred for one hour at 50^0C before the addition of cellulase in citrate buffer. The amounts of cellulase added to these solutions were varied as 2, 3 and 5 mg cellulase in 1 g of the binary solvent keeping all other conditions constant. Aliquots of the sample were taken and analyzed for the presence of total reducing sugars (TRS) by DNS method (Miller, 1959). The study in presence of 5 mg cellulase indicates the highest cellulase activity in aqueous citrate buffer solution producing 38.94 mg of reducing sugar upon the continuation of the reaction till 90h, indicative of 80% hydrolysis of cellulose. The activity of the cellulase was completely lost in 100%

[BMIM]Cl sample, showing a negative curve for the cellulose hydrolysis. Upon subsequent increase in the citrate buffer concentration in the binary solution, it was observed that the activities increased, but to insignificant levels. The results indicate that minimum hydrolysis was found in the sample with 40% [BMIM]Cl in aqueous citrate buffer binary mixture. The binary mixture in 40% [BMIM]Cl showed an increase in the hydrolysis of cellulose upto15.04 mg/g of IL, which is about a 34% hydrolysis of cellulose. The optimum cellulose hydrolysis was obtained at 20 h. (**Figure 4.8**).

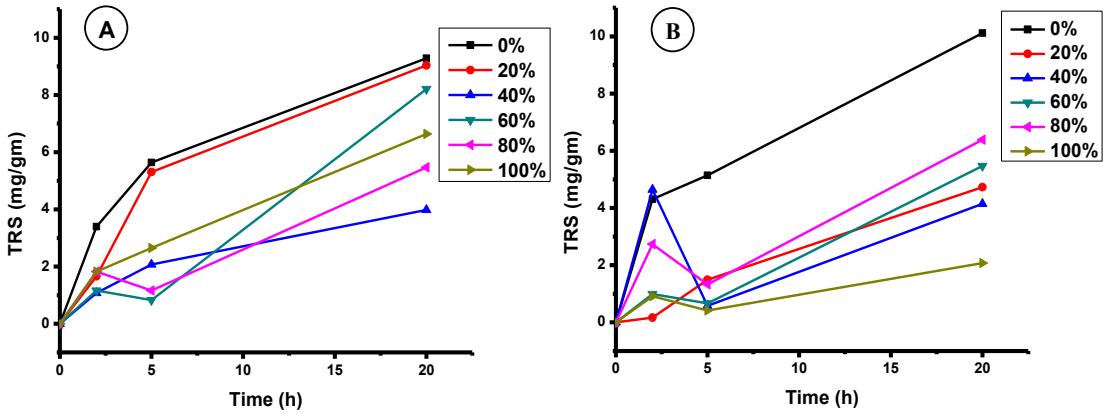


Figure 4.8: TRS generated by biocatalytic hydrolysis of Avicel cellulose substrate (40 mg) in presence of **(A)** 2 mg cellulase and **(B)** 3 mg cellulase, in (0-100%) 1 g *w/w* [BMIM]Cl-water binary solution.

The studies were carried for filter paper as a substrate instead of Avicel cellulose subsrate, the results indicate that the filter paper undergo slow hydrolysis than Avicel in 100% aqueous citrate buffer solution leading to complete hydrolysis upto 99.9% in 90 h. This result is indicative of higher amorphous nature of cellulose filter paper. Further, the hydrolysis behavior of cellulose filter paper was completely different from that of the microcrystalline cellulose in binary solution of aqueous citrate buffer-ionic liquid. The cellulose filter paper showed a maximum TRS upon hydrolysis in 20% binary mixture of [BMIM]Cl in aqueous citrate buffer. The hydrolysis was found to increase till 48 h furnishing reducing sugar at maximum of 16.72 mg/g of IL-binary solutions. However, upon subsequent increase in the concentration of [BMIM]Cl in the binary mixture, a decrease in the TRS is observed with the formation of reducing sugar to the maximum extent (time period) of 8.86 (90 h), 8.77 (72 h), 9.11 (90 h) and 6.18 mg/g (48 h) in 1 g of the

binary mixture with concentration of [BMIM]Cl 40%, 60%, 80% and 100%, respectively. These results indicate that there is a decrease in the extent of hydrolysis upon the increase in the ionic liquid concentration in the binary mixture (**Figure 4.9 A-B**).

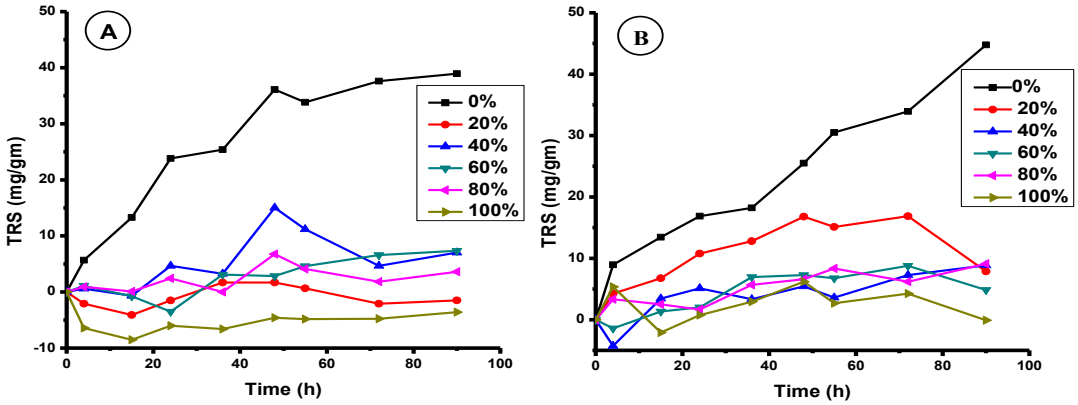


Figure 4.9: TRS generated by biocatalytic hydrolysis of **(A)** Avicel cellulose substrate (40 mg) and **(B)** Filter paper (40 mg) with cellulase (5 mg) in different concentration mixture (0-100%) of 1 g *w/w* [BMIM]Cl and C.B. obtained..

Further studies were carried out by varying the concentration of the enzyme used in the study. The studies were carried out in presence of 2 mg/g and 3 mg/g cellulase in various binary mixtures. The TRS released from the hydrolysis of cellulose in ionic liquid [BMIM]Cl-water binary solution in presence of 2 mg/g of cellulase in 1 g of aqueous buffered-IL, was found to be upto the maximum extent (time period) of 9.03 (20 h), 3.98 (20 h), 8.21 (20 h), 5.47 (20 h) and 6.63 mg/g (20 h) in the binary mixture concentration of [BMIM]Cl 20%, 40%, 60%, 80% and 100%, respectively. Similarly, the extent of reducing sugar (time period) formed in the binary mixture of [BMIM]Cl-water in presence of 3 mg/g of cellulase in 1 g of aqueous buffered-IL solution was found to be 4.73 (20 h), 4.64 (2 h), 5.47 (20 h), 6.38 (2 h) and 2.07 mg/g (20 h) at the binary solution concentration of [BMIM]Cl at 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.8 A-B**).

4.5.2 Cellulose Hydrolysis Behavior of Cellulase in [HMIM]Cl –Water Binary Mixture:

Biocatalytic hydrolysis of cellulose was carried out in the presence of [HMIM]Cl-water binary mixture. In [HMIM]Cl-water binary mixture (0-100%) the solution showed a decrease in the concentration of total reducing sugar after 48 h. Thus,

no further study of TRS was carried out above this time period. The hydrolysis of cellulose in ionic liquid [HMIM]Cl-water binary solution in presence of 5 mg cellulase in 1 g of binary solution of aqueous buffered-IL and 40 mg of Avicel microcrystalline cellulose was found to attained a maximum (time period) of 17 (5 h), 33.42 (48 h), 16.13 (36 h), 19.89 (36 h) and 3.34 mg/g (2 h) in 1 g of IL binary mixture, with the concentration of [HMIM]Cl 20%, 40%, 60%, 80% and 100%, respectively. The maximum activity in case of [HMIM]Cl based aqueous binary mixture, is found to be greater in case of 40% [HMIM]Cl binary solution with subsequently high TRS concentration observed at 48 h. On comparison of these results with the hydrolysis in various binary mixtures of [BMIM]Cl, results indicate that the hydrolysis rate is enhanced in case of [HMIM]Cl better than [BMIM]Cl based binary mixture. The results further indicate that in the [HMIM]Cl binary solution, maximum reducing sugar was found to be of 33.42 mg/g at 40% [HMIM]Cl in water binary solutions. There was a 75% hydrolysis of cellulose in 40% [HMIM]Cl-aqueous binary solution. It is further observed that the cellulase becomes inactive at higher concentration of [HMIM]Cl, particularly in 100% [HMIM]Cl. However, it has been further observed that at 60% [HMIM]Cl binary solution, there is an increase in the total reducing sugar upto 18.9 mg/g, which is equivalent to about 45% hydrolysis of the cellulose present in the solution (**Figure 4.10 A**).

Similarly, the hydrolysis of filter paper cellulose in ionic liquid [HMIM]Cl-water in presence of 5 mg of cellulase in 1 g of binary [HMIM]Cl solution was carried out and found to be upto the maximum extent (time period) of 15.88 (48 h) and 9.12 mg/g (36 h), in 20% and 40%, respectively. In the binary mixture with [HMIM]Cl concentration 60%, 80% and 100%, hydrolysis of cellulose was not significant (**Figure 4.10 B**).

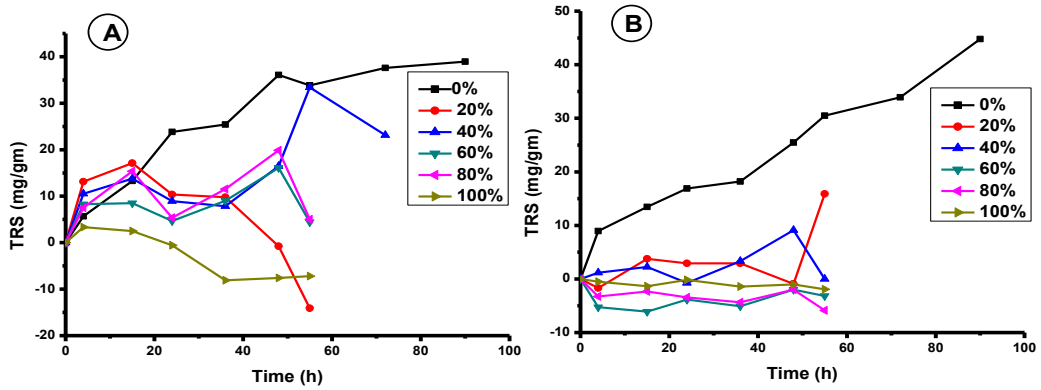


Figure 4.10: TRS generated by biocatalytic hydrolysis of **(A)** Avicel cellulose substrate (40 mg) and **(B)** Filter paper (40 mg) with cellulase (5 mg) in different concentration mixture (0-100%) of 1 g *w/w* [HMIM]Cl and C.B. obtained.

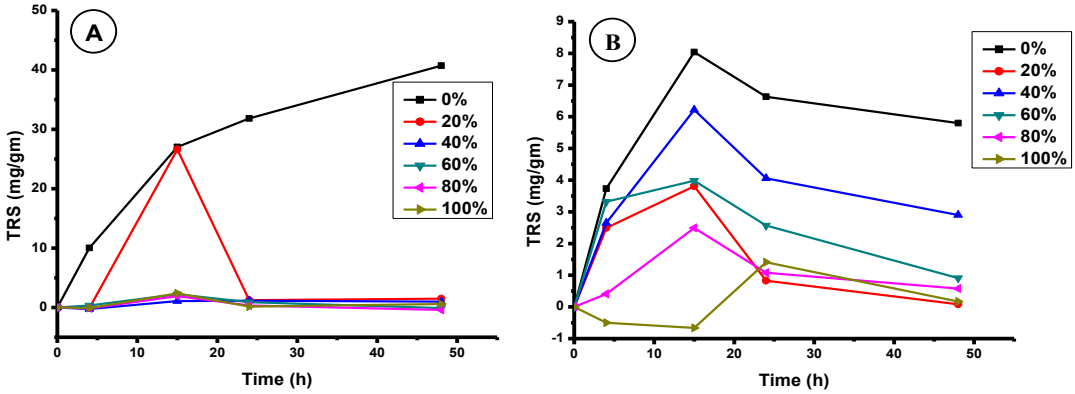


Figure 4.11: TRS generated by biocatalytic hydrolysis of cellulose (Avicel, 40 mg) in 1 g *w/w* [HMIM]Cl-water binary solutiom (0-100%) in presence of **(A)** 2 mg cellulase **(B)** 3 mg cellulase.

The hydrolysis of cellulose in 20-100% ionic liquid [HMIM]Cl-water binary solution in presence of 2 mg/g of cellulase was found to be upto the maximum extent (time period) of 26.61 (15 h), 1.16 (24 h), 6.22 (15 h), 3.98 (15 h) and 2.49 (15 h) mg/g in the binary mixture with concentration of [HMIM]Cl at 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.11 A**).

Similarly, the extent of reducing sugar (time period) formed in the binary mixture of [HMIM]Cl-water in presence of 3 mg/g of cellulase was found to be 3.81 (15 h), 1.16 (24 h), 3.32 (4 h), 1.91 (15 h) and 1.41 mg/g (24 h) in 1 g of binary mixture with the concentration of [HMIM]Cl at 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.11 B**).

4.5.3. Cellulose Hydrolysis Behavior of Cellulase in [OMIM]Cl –Water Binary Mixture:

Similarly, biocatalytic hydrolysis of cellulose was studied in [OMIM]Cl-water binary mixture(0-100%). The hydrolysis of cellulose in ionic liquid [OMIM]Cl-water binary solution in presence of 5 mg cellulase per g of IL-binary solution was carried out to a maximum extent (time period) of 21.22 (55 h), 18.47 (55 h), 10.53 (36 h), 7.10 (24 h) and 10.61 mg (15 h) per g in binary mixture concentration of [OMIM]Cl 20%, 40%, 60%, 80% and 100%, respectively. The results indicate that the maximum activity in case of [OMIM]Cl based aqueous binary solution, is found to be greater in case of 20% [OMIM]Cl binary solution. On comparing these results with the maximum hydrolysis in various binary mixtures of [BMIM]Cl, the results indicate that the hydrolysis rate is enhanced in case of [OMIM]Cl better than the [BMIM]Cl based binary mixtures. It is further observed that the cellulase becomes inactive at higher concentration of [OMIM]Cl particularly the 80% [OMIM]Cl in binary solution. However, it has been further observed that at 40% [OMIM]Cl solution there is an increase in the total reducing sugar upto 18.47 mg/g hydrolysis of the cellulose substrate present in the solution (**Figure 4.12 A**).

Similarly, the hydrolysis of filter paper cellulose in ionic liquid [OMIM]Cl-water binary solution in presence of 5 mg of cellulase in 1 g of IL-binary solution was carried out and found to be hydrolyzed to a maximum extent (time period) of 14.04 (55 h), 9.86 (55 h), 12.45 (48 h), 14.29 (36 h) and 12.45 mg/g (48 h) in binary mixture with concentration of [OMIM]Cl 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.12 B**).

The hydrolysis of cellulose in ionic liquid [OMIM]Cl-water binary solution in presence of 2 mg of cellulase / g of IL was found to be upto the maximum extent (time period) of 4.17 (18 h), 7.69 (18 h), 1.67 (6 h), 1.00 (6 h) and 1.76 mg (6 h) per g in the binary mixture with concentration of [OMIM]Cl 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.13 A**).

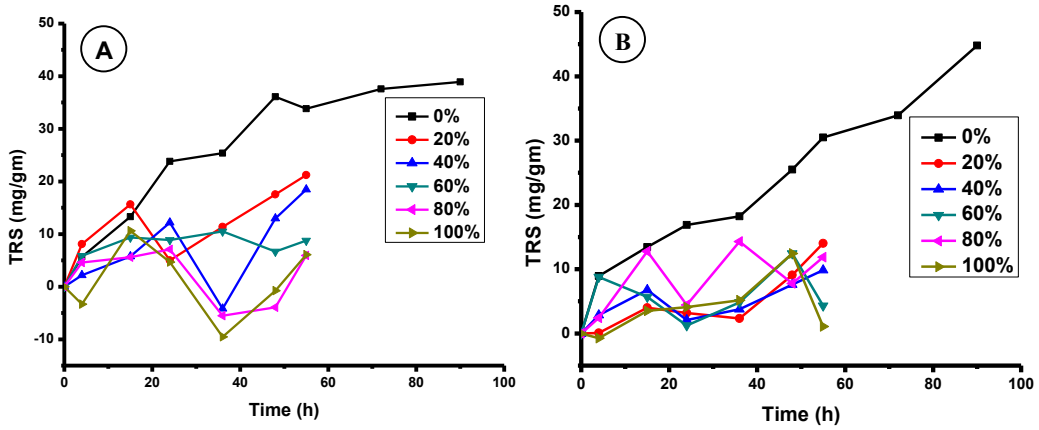


Figure 4.12: TRS generated by biocatalytic hydrolysis of **(A)** Avicel cellulose substrate (40 mg) and **(B)** Filter Paper (40 mg) in presence of 5 mg cellulase in 1 g *w/w* [OMIM]Cl-water binary solution (0-100%).

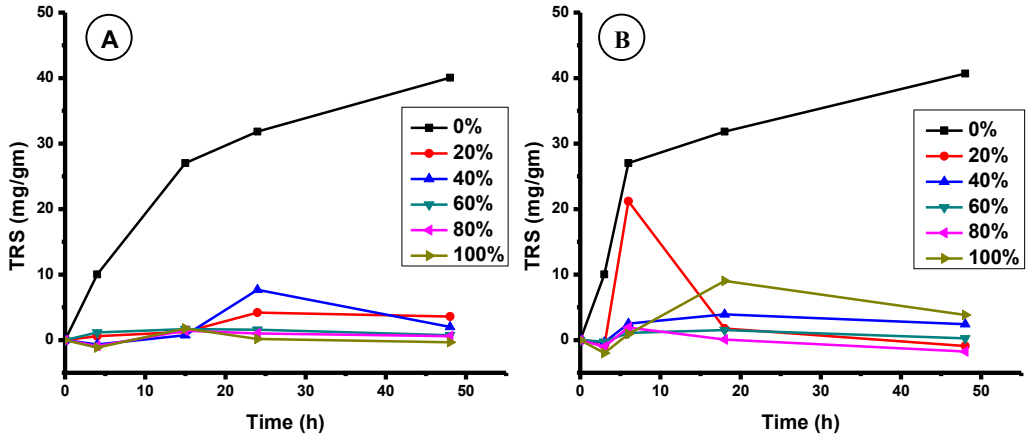


Figure 4.13: TRS generated by biocatalytic hydrolysis of cellulose (Avicel 40 mg) in presence of **(A)** 2 mg cellulase **(B)** 3 mg cellulase in 1 g *w/w* [OMIM]Cl-water binary solution (0-100%).

Similarly, the amount of reducing sugar (time period) formed in presence of of 3 mg of cellulase per g of the [OMIM]Cl in water binary mixture, was found to be 21.19 (6 h), 3.93 (18 h), 1.50 (18 h), 1.90 (6 h) and 9.00 mg (18 h) per g in the binary solution with concentration of [OMIM]Cl at 20%, 40%, 60%, 80% and 100%, respectively (**Figure 4.13 B**).

4.6. Surfactant screening for enhanced enzymatic hydrolysis:

Biocatalysis in ionic liquid have been studied extensively and surfactants have been found to stabilize the enzyme in ionic liquid. Anionic surfactant namely

sodium bi(-2ethyl-1-hexyl) sulfosuccinate (AOT) has been frequently used for the studies in ionic liquid and found to stabilize and enhance the activity of the enzyme in ionic liquid based biocatalysis (Brown *et al.*, 2011). Biocatalytic hydrolysis of cellulose in lignocellulose components have been enhanced by addition of surfactant in water. The mechanism of surfactant based enhancement in biocatalysis in IL is accounted to the increased stability of enzyme or by affecting unwanted cellulase adsorption on lignin component (Alkasrawi *et al.,* 2003; Zhang and Tang*,* 2011). There exist no study on stabilization of cellulase and henceforth the impact of surfactant on the activity of cellulase in ionic liquids. Thus, in the present studies, investigations are carried out on the effect of surfactant on the hydrolysis of cellulose in ionic liquid. In the present study, four surfactants namely Sodium Dodecyl sulphate (SDS), Cetyltrimethylammonium Bromide (CTAB), Polyethylene Glycol-1500 (PEG-1500) and Polyethyelene Glycol-4000 (PEG-4000), were evaluated for their ability to enhance enzymatic hydrolysis of Avicel® Cellulose substrate. The surfactants were compared for enzymatic hydrolysis (5 mg cellulase / g of IL) by adding 1, 5 and 10 mg of surfactant having a loading of 20%, 100% and 200% w.r.t. the enzyme, respectively, in 1 g of IL ([BMIM]Cl, [HMIM]Cl and [OMIM]Cl) having 10% citrate buffer. The aliquot samples were analyzed in different time intervals for yield of reducing sugar. Hydrolysis was measured by TRS expressed in glucose unit (mg/ml) by DNS method on UV-Vis spectrophotometer.

The maximum yield of the hydrolysis products from cellulose in presence of various surfactants in ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl have been studied are discussed as follows. In [BMIM]Cl, upon addition of 1, 5 and 10 mg of SDS, the hydrolysis sugar yields of 1.84 (33 h), 3.76 (8 h) and 2.76 mg (2 h), respectively, were obtained per g of IL. Similarly, upon addition of 1, 5 and 10 mg of cationic surfactant CTAB, the yield of hydrolyzed sugar yield was found to be 0.75 (24 h), 2.59 (24 h) and 0.84 mg (33 h), respectively. In case of non-ionic surfactant PEG-1500, upon addition of 1, 5, and 10 mg of the surfactant the yield of sugar was 0.59 (22 h), 4.51(30 h) and 4.6 mg (3 h), respectively, whereas addition of same amount of higher polymer PEG-4000, gave the hydrolysis product yield of 3.34 (6 h), 3.34 (3 h) and 1.25 mg (3 h), respectively, per g of IL.

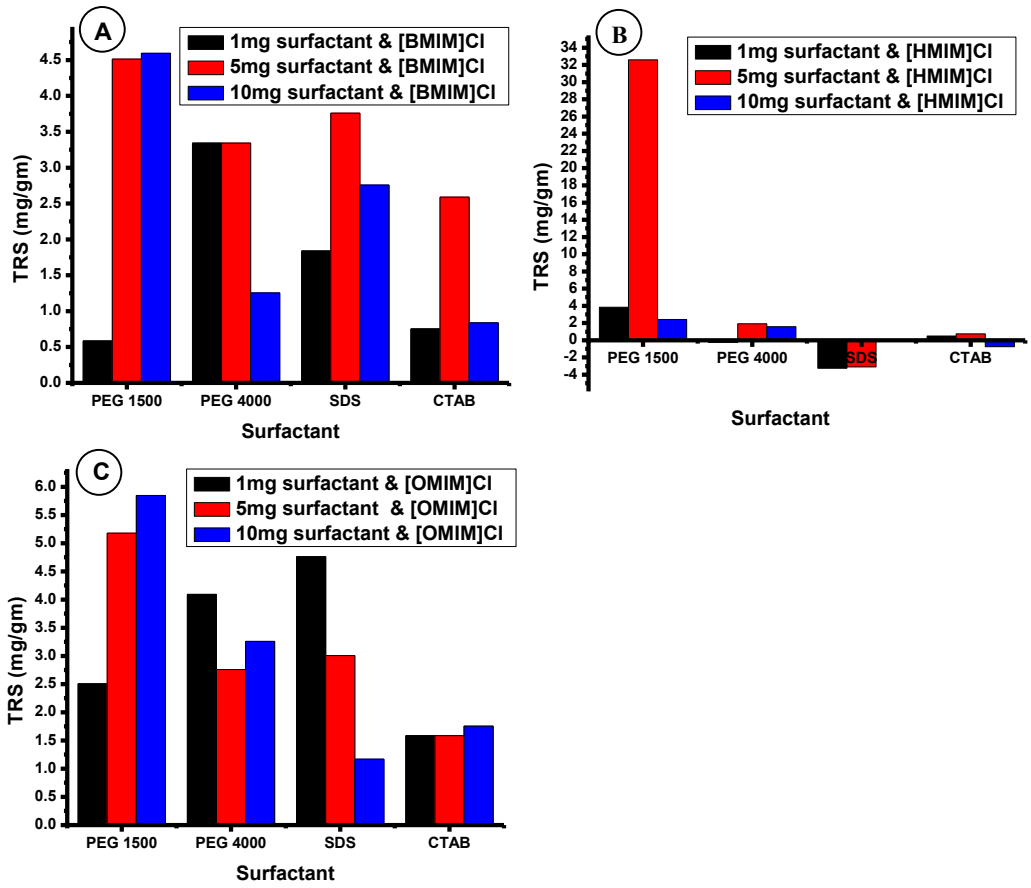


Figure 4.14: Screening of surfactant for TRS generated by biocatalytic hydrolysis of Avicel MCC at 50°C using cellulase (5 mg) in 1 g IL-water binary solution using 10% C.B. Surfactant concentrations (PEG-1500, 4000, SDS and CTAB) were 1, 5 and 10 mg. **(A)** Enzymatic hydrolysis in [BMIM]Cl solution **(B)** Enzymatic hydrolysis in [HMIM]Cl solution **(C)** Enzymatic hydrolysis in [OMIM]Cl solution.

Similarly, in [HMIM]Cl, upon addition of 1, 5 and 10 mg of SDS, no significant hydrolysis was observed. Similarly, upon addition of 1, 5 and 10 mg of cationic surfactant CTAB, the yield of hydrolyzed sugar was 0.5 (24 h), 0.75 (24 h) and 0.75mg (24 h), respectively. In case of non-ionic surfactant PEG-1500, upon addition of 1, 5 and 10 mg of the surfactant the yield of sugar was 3.84 (4 h), 32.59 (4 h) and 2.42 mg (10 h), respectively, whereas addition of same amount of higher polymer PEG-4000, gave the hydrolysis product yield of 0.25 (10 h), 1.92 (4 h) and 1.59 mg/ml (10 h), respectively.

Further, in [OMIM]Cl, upon addition of 1, 5 and 10 mg of SDS, the hydrolysis sugar yields of 4.76 (2 h), 3.12 (2 h) and 1.17 mg (2 h), respectively. Similarly, upon addition of 1, 5 and 10 mg of cationic surfactant CTAB, the yield of hydrolyzed sugar was 1.59 (2 h), 1.59 (2 h) and 1.76 mg (2 h), respectively. In case of non-

ionic surfactant PEG-1500, upon addition of 1, 5, and 10 mg of the surfactant, the yield of sugar was 2.5 (6 h), 5.18 (3 h) and 5.85 mg (6 h), respectively, whereas addition of same amount of higher polymer PEG-4000, gave the hydrolysis product yield of 4.09 (3 h), 2.75 (3 h) and 3.26 mg (22 h) per g of IL, respectively (**Figure 4.14 A-C**).

4.7. Study of cellulase enzyme activity in Ionic Liquid with surfactant through fluorescence:

Steady state fluorescence of enzyme is an essential tool for analysis of the microenvironment of enzyme in solution. It is one of the important tools for the study of biocatalysis by enzyme. Steady state fluorescence of cellulase has been studied either in aqueous solution, ionic liquid or in binary mixture of ionic liquid in water. Bose *et al.* (2010) had showed that the fluorescence of enzyme was perturbed and quenched by the cation of the ionic liquid and not by the anion in the microenvironment of the enzyme. Steady-state fluorescence of cellulase in ionic liquids, [BMIM]Cl, [HMIM]Cl and [OMIM]Cl containing 10% aqueous citrate buffer solution were carried out in the present study. The study involved the measurement of steady-state fluorescence of cellulase (0.5 mg/g of IL) in 10% citrate buffer solution in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The solutions were excited at λ_{ex}=280 nm and the emission was monitored in the spectral range of 300-700 nm. Interestingly, the solution showed a weak emission with maximum intensity at λ_{em}=532 nm, 534 nm and 543 nm in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively (**Figure 4.15**). The emission band of cellulase shows a small bathochromic shift of fluorescence when [BMIM]Cl is replaced with [HMIM]Cl ($\Delta\lambda$=2 nm) and [OMIM]Cl ($\Delta\lambda$=11 nm), with the quenching of fluorescence intensity.

However, in general it is observed that the bathochromic shift in the spectra is accounted to the interaction of the enzyme with the solvent microenvironment in various media (Zhang *et al*., 2005). Further, there is a constant decrease in the fluorescence intensity upon the increase in the alkyl chain from C_4 to C_6 and C_6 to C_8.

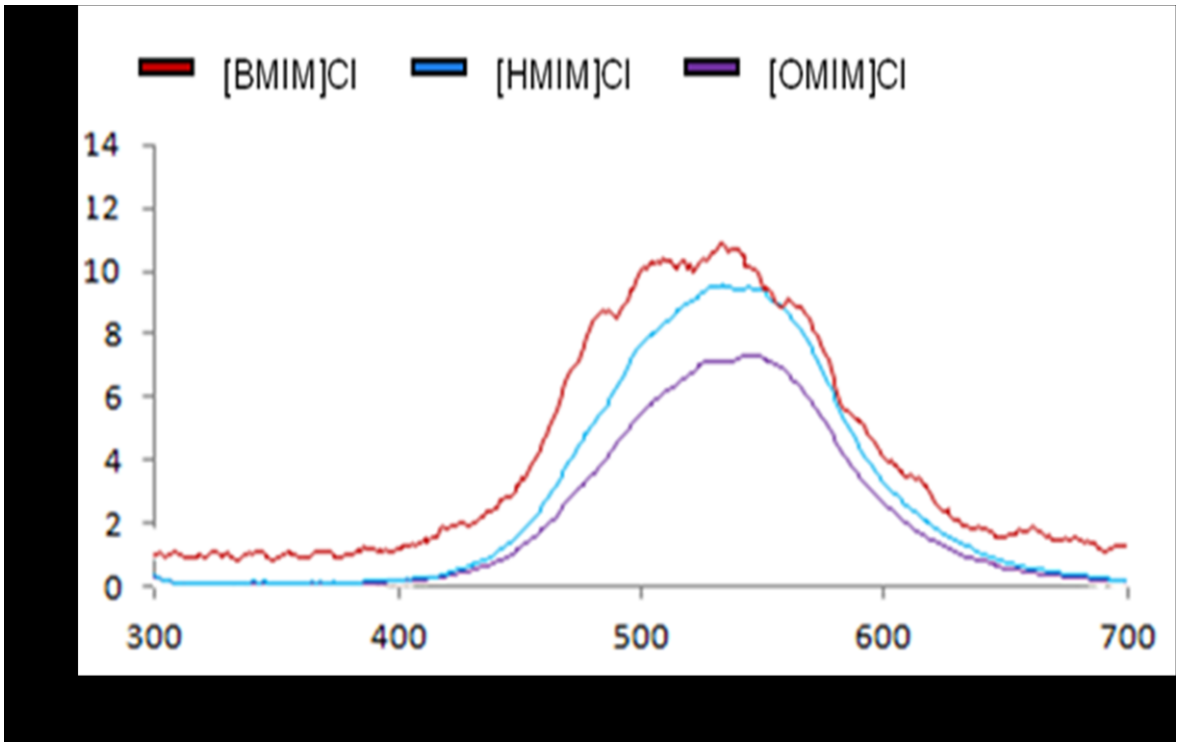


Figure 4.15: Fluorescence spectra of cellulase in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl with Cellulase (0.5 mg/g) on λ_{ex} =280 nm and wavelength from 300-700 nm excitation slit width 5 nm and emission slit width 5 nm.

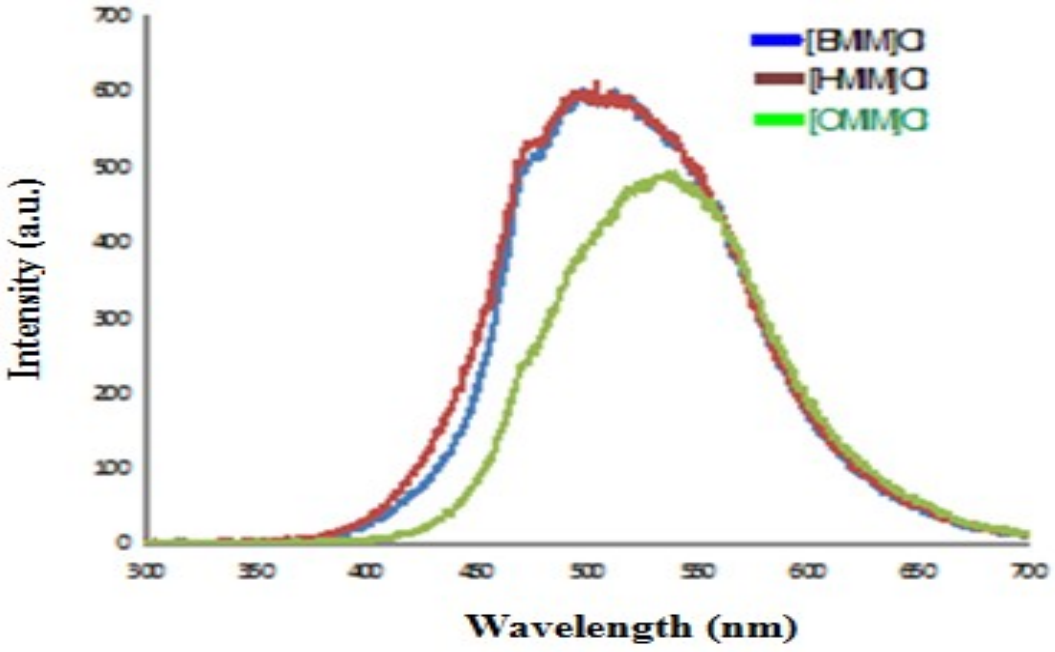


Figure 4.16: Fluorescence spectra of cellulase in [BMIM]Cl, [HMIM]Cl and [OMIM]Cl with Cellulase (0.5 mg/g) and Avicel (40 mg/g) on λ_{ex} =280 nm and wavelength from 300-700 nm, excitation slit width 10 nm and emission slit width 10 nm.

Recent reports on cellulase activity in aqueous buffered condition have being studied extensively in presence of lignocelluloses and cellulose (Zhao *et al*., 2009). However there exists no report on the study of the effect of surfactant on the cellulase activity in ionic liquid. Thus, in the present study, interaction of cellulase with microcrystalline cellulose and the kind of environment created by cellulose was investigated. Thus, to a 0.1 mg/g solution of cellulase in 10% citrate buffer in ionic liquids [BMIM]Cl, [HMIM]Cl or [OMIM]Cl, Avicel PH-101 was directly added to the solution, stirred till clear and fluorescence spectra were recorded at λ_{ex}=280 nm (**Figure 4.16**).

The fluorescence spectra of the solution showed an increase in the fluorescence intensity of all the samples. The fluorescence intensity was enhanced by a factor of 50, 60 and 55 approximately in the ionic liquid system of [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively. This enhancement in the intensity is further accompanied with a hypsochromic shift of the maximum emission peak to shorter wavelength of 512 nm ($\Delta\lambda$=20 nm), 504 nm ($\Delta\lambda$=30 nm) and 537 nm ($\Delta\lambda$=6 nm) in the solvents [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, respectively, compared to the system, where no cellulose was present in the solution. This hypsochromic shift is attributed to the increased polarity of the system, thereby increasing interaction between the enzyme and the solute-solvent media. Fendt *et al*. (2011) have studied the increased viscosity and, formation of hydrogen bonding of cellulose with the ionic liquid anion and cation, might be the factor responsible for the enhancement in the fluorescence intensity of these systems.

Shift of emission wavelength, $\Delta\lambda$=6 nm usually, upon increasing addition of PEG-1500 and PEG-4000 to a solution of cellulase in the [OMIM]Cl, a 1.29 fold and 1.58 fold enhancement of fluorescence was observed.

In [BMIM]Cl, upon addition of PEG-1500 there is an increase in the fluorescence intensity from 6.5 a.u. (arbitrary units) to 9.2 a.u. i.e., a 1.4 times fluorescence signal enhancement was observed (**Figure 4.17**). Similarly, fluorescence enhancement was observed in case the presence of surfactant PEG-4000 (**Figure 4.18**).

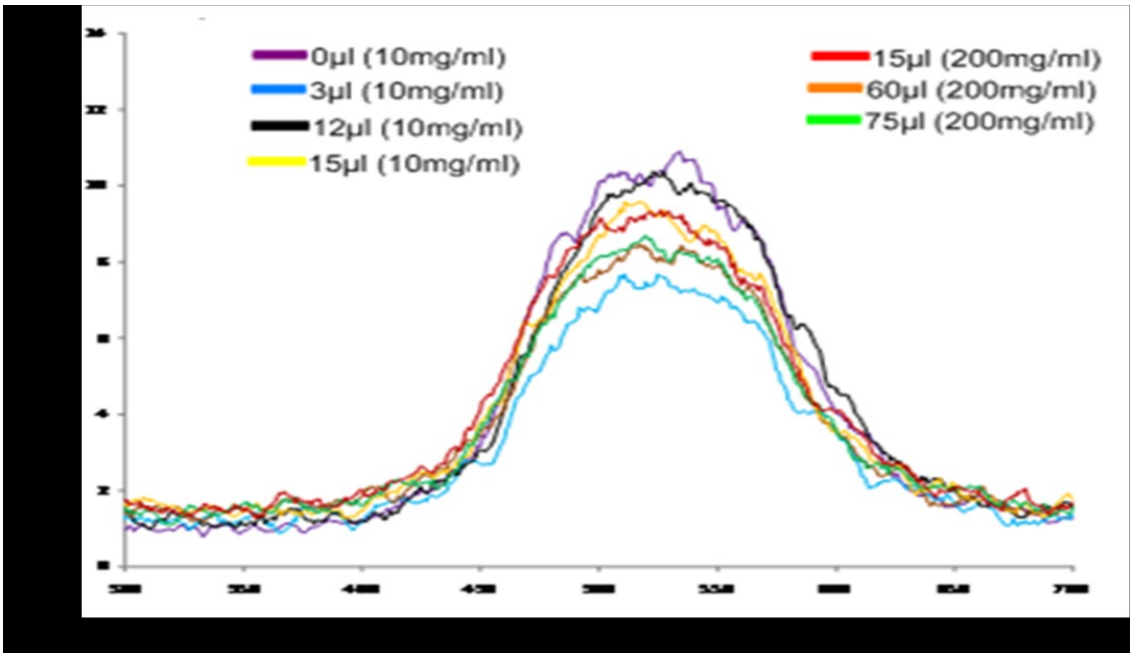


Figure 4.17: Fluorescence spectra of cellulase (0.1 mg/g IL) in [BMIM]Cl, 10% C.B., Cellulase (0.5 mg/g) and PEG-1500 on λ_{ex}=280 nm and wavelength from 300-700 nm at excitation slit width 5nm and emission slit width 5 nm.

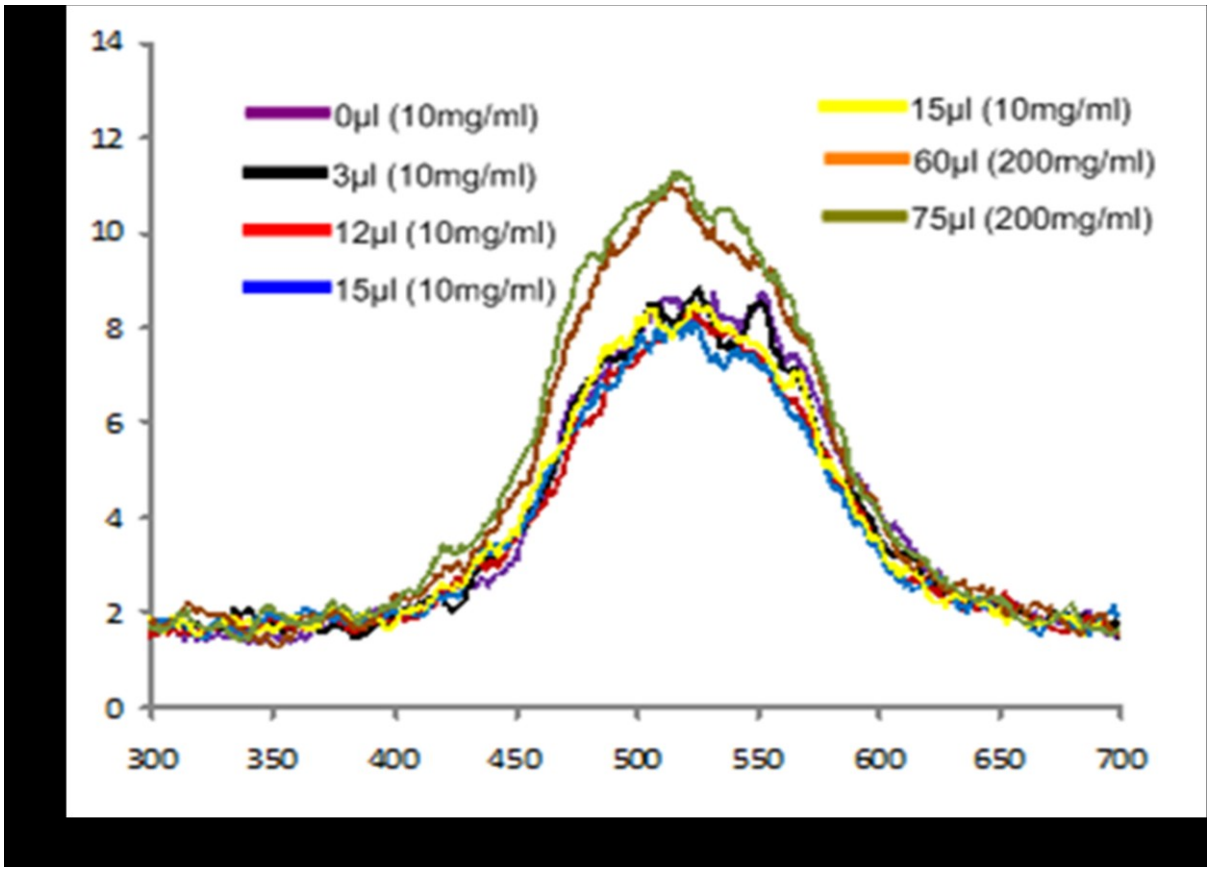


Figure 4.18 Fluorescence spectra of Cellulase in 10% C.B.-[BMIM]Cl , Cellulase (0.5 mg/g) and PEG-4000 on λ_{ex} =280 nm and wavelength from 300-700 nm at excitation slit width of 5 nm and emission slit width of 5nm.

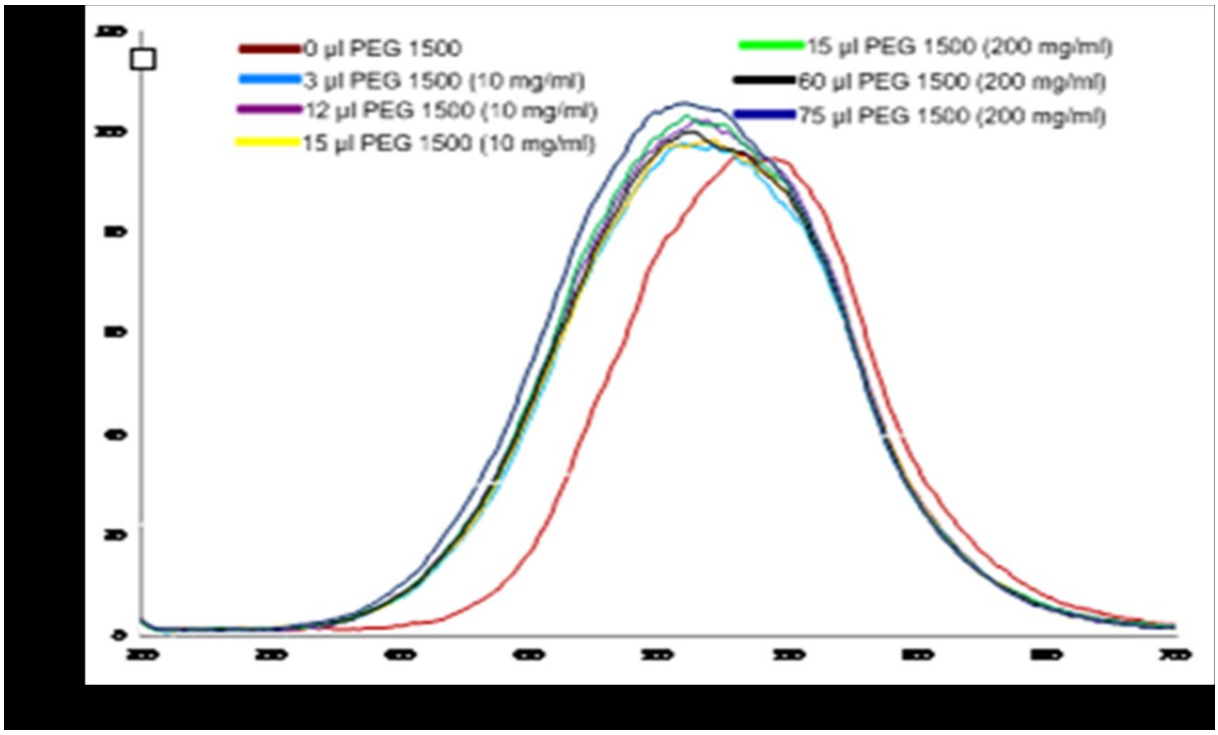


Figure 4.19: Fluorescence spectra of cellulase (0.5 mg/g in IL), 10% C.B.-[HMIM]Cl with PEG-1500 on λ_{ex} =280 nm and wavelength from 300-700 nm at excitation slit width 10 nm and emission slit width 10 nm.

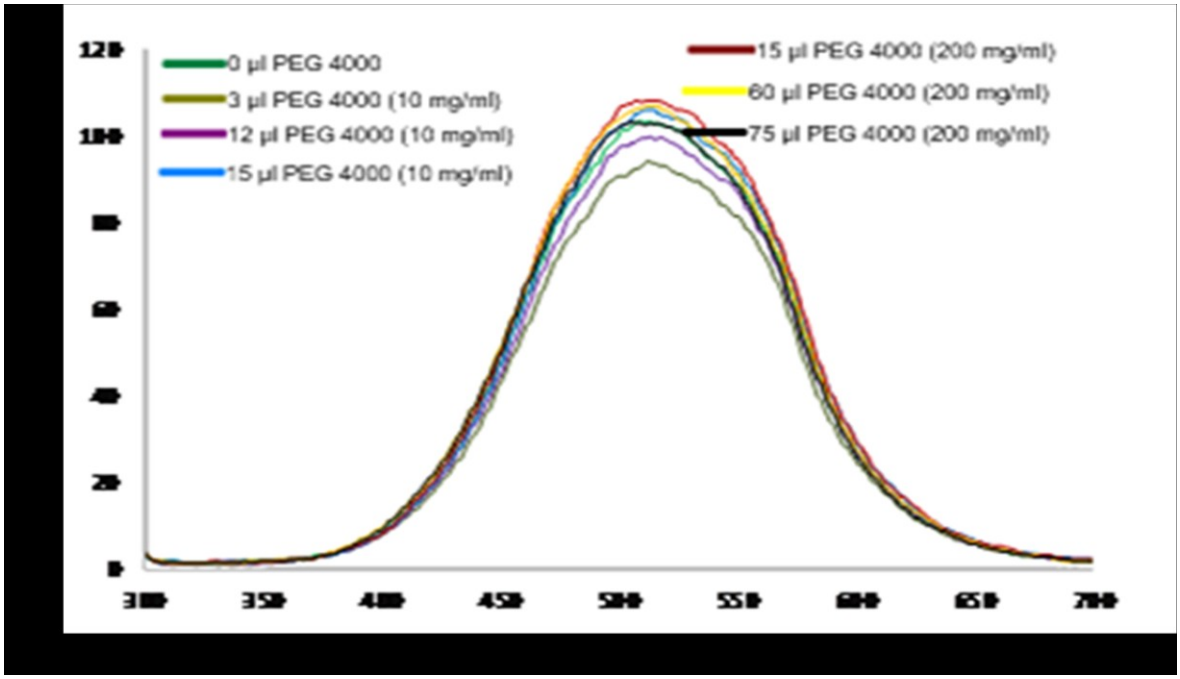


Figure 4.20 Fluorescence spectra of cellulase (0.5 mg/g in IL), 10% C.B.-[HMIM]Cl with PEG-4000 on λ_{ex} =280 nm and wavelength from 300-700 nm at excitation slit width 10 nm and emission slit width 10 nm.

Similarly fluorescence studies were carried with cellulase in 10% citrate buffer aqueous ionic liquids [HMIM]Cl or [OMIM]Cl) in presence of Polyethylene Glycol (PEG)-1500 and PEG-4000. The aliquot of stock solution of PEG-1500 and PEG-4000 were added to the cellulase in binary solution of 10%citrate buffer in ionic liquid. The fluorescence spectra of their solution were recorded at an excitation wavelength of λ_{ex}=280 nm and an emission wavelength 300-700 nm. In general, there was change in fluorescence spectra upon addition of PEG upto *w/w* of enzyme. However upon excess addition of PEG to the solution, there was a change in the fluorescence intensity due to the enzyme (**Figure 4.19**).

Further, fluorescence of cellulase in [HMIM]Cl binary solutions were studied in presence of PEG-1500 and PEG-4000 (**Figure 4.19 and 4.20**). Upon increasing addition of PEG-4000, the fluorescence enhancement was observed to be from 90 a.u. to 105 a.u. i.e. a 1.16 fold increase in the fluorescence intensity. However, upon addition of PEG-1500 the fluorescence maxima showed a hypsochromic or blue shift of $\Delta\lambda$=105 nm.

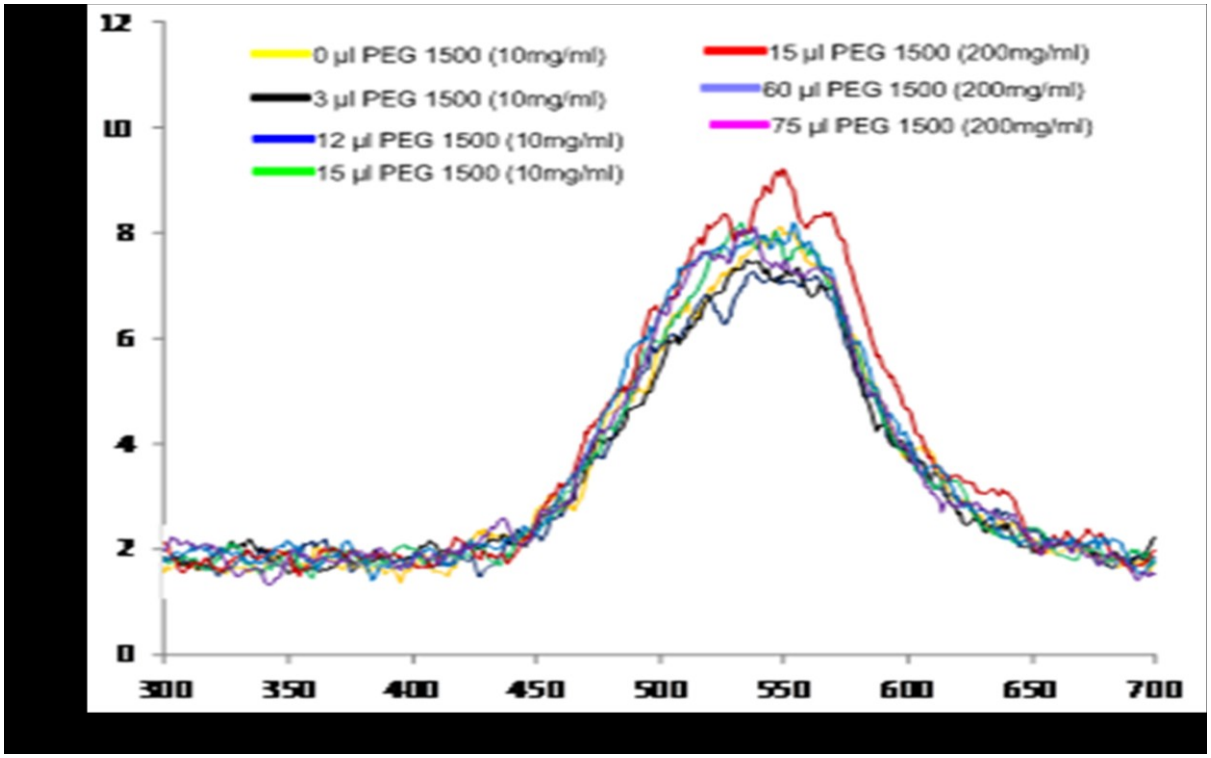


Figure 4.21: Fluorescence spectra of cellulase (0.5 mg/g in IL), 10% C.B.-[OMIM]Cl with PEG-1500 on λ_{ex} =280 nm and wavelength from 300-700 nm at excitation slit width 5 nm and emission slit width 5 nm.

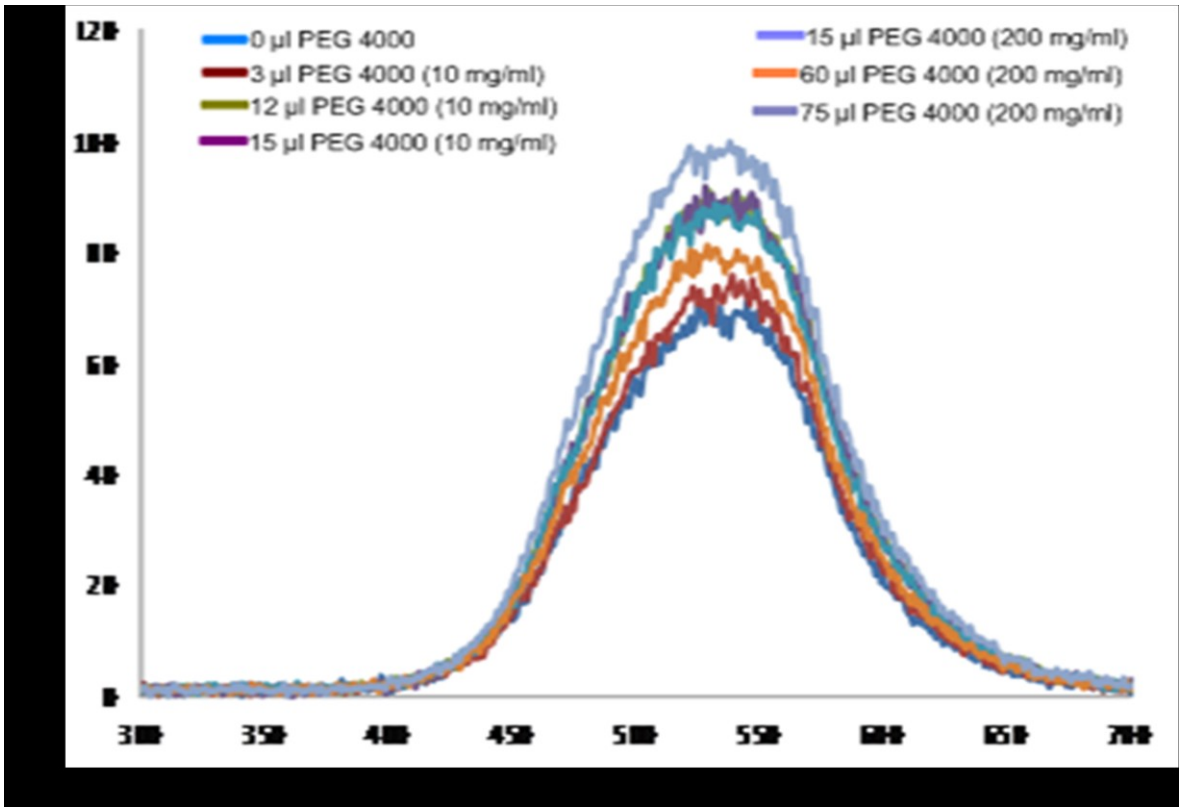


Figure 4.22 Fluorescence spectra of cellulase (0.5 mg/g in IL), 10% C.B.-[OMIM]Cl with PEG-4000 on λ_{ex} =280 nm and wavelength from 300-700 nm at excitation slit width 10 nm and emission slit width 10 nm.

Usually, upon increasing addition of PEG-1500 and PEG-4000 to a solution of cellulase in [OMIM]Cl, a fluorescence enhancement from 7 to 9 a.u. (1.29 fold) and 60 to 95 a.u. (1.58 fold) was observed (**Figure 4.21 and 4.22**).

Thus, the fluorescence studies revealed a correlation between the interactions of cellulase in IL and change in the microenvironment of the enzyme upon addition of cellulose and surfactant as additives.

CHAPTER V

DISCUSSIONS

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DISCUSSIONS

The study of the biocatalytic hydrolysis of cellulose has been studied in ionic liquids namely, [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The hydrolysis is studied using the DNS method for total reducing sugar concentration (Miller, 1959). Results of the study are discussed in the following sections:

(A) Study of biocatalytic hydrolysis of cellulose in ionic liquid
(B) Study of biocatalytic hydrolysis of cellulose in ionic liquid–buffered aqueous binary solution
(C) Study of biocatalytic hydrolysis of cellulose in ionic liquid in presence of surfactant

(A) Study of Biocatalytic Hydrolysis of Cellulose in Ionic Liquid: The study was carried out in a solution of 10% citrate buffer in ionic liquid as a representative of pure ionic liquid in biocatalytic hydrolysis of cellulose. Thus, in the present study, it is observed that as the alkyl group chain length increases from butyl to hexyl and subsequently to octyl, the rate of hydrolysis and the maximum amount of reducing sugar formed in the reaction increases. The study indicates that the maximum sugar yield is obtained in the first 5 h, and above this time period, the product formed might undergo degradation. The maximum amount of reducing sugar formed in the biocatalytic hydrolysis might be accounted to the viscosity of the ionic liquid (Fendt *et al*., 2011). The viscosity of ionic liquid increases with the increase in the alkyl chain length as arranged in the order:

[BMIM]Cl < [HMIM]Cl < [OMIM]Cl

As the viscosity of the ionic liquid increases, to associated parameters mass transfer and the protein unfolding both show a decrease in case of IL. However, upon decrease in the mass transfer, the biocatalytic rate should decrease with increase in alkyl chain length. However, the observation for hydrolysis is opposite. Thus, the stabilization of protein by the increase in the resistance to unfolding might be the dominant factor for the biocatalytic hydrolysis of cellulose in ionic liquid (Rantwijk and Sheldon, 2007).

Further, Bose *et al*. (2010) have indicated that the fluorescence of enzyme is quenched by the cation of the ionic liquid and not due to the couteranion. Thus fluorescence study of the solution of cellulase in ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl had been carried out. The studies are in agreement with the results of Bose *et al*. (2010). The fluorescence due to cellulase in 10% citrate buffer in ionic liquid was quenched upon increase in the alkyl chain length from C_4 to C_6 and subsequently to C_8-alkyl. Thus, with increased alkyl chain length, there is a subsequent interaction between the tyrosine and tryptophan moiety of enzyme, with the imidazolium cation of the ionic liquid. Particularly, the red shift in the spectra for [OMIM]Cl, could be explained based on the interaction of the ionic liquid positive charge with the benzene ring of the tryptophan moiety (Vivian and Callis, 2001). Bose *et al*. (2010) observed that the cellulase enzyme fluorescence is modulated by the presence of the cation of ionic liquid in the vicinity of the enzyme.

Further, fluorescence studies in presence of the ionic liquids and microcrystalline cellulose showed an enhancement in the fluorescence signaling of the enzyme. The fluorescence in case of ionic liquids [BMIM]Cl and [HMIM]Cl, were found to show a minor blue shift in the spectra, which could be attributed to the competitive binding of the cation $[BMIM]^+$ / $[HMIM]^+$ with the cellulose moiety. Whereas, the fluorescence of [OMIM]Cl is also revived, but there is no shift in the fluorescent spectrum. The fluorescence enhancement is similar to that in case of [BMIM]Cl and [HMIM]Cl. This observation suggests that the interaction between the cellulose and the enzyme could not be neglected, and might be an essential factor in reviving the enzyme fluorescence. Thus, fluorescence study reveals that the solvent cation interacts with the enzyme and this interaction is perturbed by a competitive cellulose-cation and cellulose-enzyme interaction.

(B) Study of Biocatalytic Hydrolysis of Cellulose in Ionic Liquid–Buffered Aqueous Binary Solution: Cellulase catalyzed hydrolysis has been performed in the presence of binary solution of buffered aqueous-ionic liquids [BMIM]Cl, [HMIM]Cl and [OMIM]Cl. The structure of IL systems exhibits unique spatial heterogeneity that results from their inherent polar / non-polar phase separation. Both experiments and computational work (Jiang *et al*., 2007) have shown that these ILs, with a moderate to long alkyl tail, show a prominent microphase

segregation structure both in the bulk and at the interface. Recently (Singh *et al*., 2009) have studied the aggregation of ionic liquids [BMIM]Cl and [OMIM]Cl in aqueous solution. The study assumes the ionic liquids to be made of hydrated anionic and cationic surfactant in aqueous solution. There aggregation was studied using NMR, pyrene fluorescence studies and refractive index. The study indicates the aggregation of [OMIM]Cl occurred at almost twice the concentration of [BMIM]Cl in aqueous solution.

There have been various studies on the physical properties of binary solution of ionic liquids in water like viscosity, aggregation behaviour, partial molar volume and density. Physical properties have been studied and reported in the binary solutions of ionic liquid containing [BMIM]Cl, [HMIM]Cl and [OMIM]Cl (Seddon *et al*., 2000). Generally, the viscosity of the binary solution of aqueous ionic liquids tends to increase as the concentration of ionic liquid in the solution increases (Fendt *et al*., 2011). Further, the viscosity of the binary solution at a particular concentration for a higher alkyl chain is more than that of the lower alkyl chain ionic liquid. In case of surface tension, generally, the surface tension of the binary solution decreases with the increase in the ionic liquid concentration. Surface tension of binary solution of higher member of [alkylMIM]Cl is lower than the binary solution of the lower member [alkylMIM]Cl. In case of conductivity, there are two regions observed, one is the water rich region and the other is the ionic liquid rich region. In the water rich region, the conductivity increases upto a maximum, which falls in the ionic liquid rich region. Conductivity of ionic liquid binary solution increases with the increase in the alkyl chain length of the ionic liquid cation, at a particular concentration.

Based on these few physical parameters, we could explain our observations in the experiments of hydrolysis carried out in ionic liquids. The most striking results obtained in binary solutions of [BMIM]Cl, [HMIM]Cl and [OMIM]Cl in aqueous solution, which requires mention is as follows:

1. The optimum hydrolysis was observed at a particular concentration of the binary mixture.
2. The concentration of binary mixture for optimum hydrolysis are not the same for all the ionic liquids, i.e. incase of [BMIM]Cl the reducing sugar

yield was obtained in 20% *w/w* [BMIM]Cl in aqueous buffer, however, the maximum hydrolysis in [HMIM]Cl is observed at 40% and for [OMIM]Cl there was no trend but the solution was observed to show a increased reducing sugar at 20% and 40%. Thus, the results have no part in common but could be explained based on the physical behaviour of these binary solutions as discussed earlier.

The results obtained in [BMIM]Cl binary solution is similar to results obtained in the ionic liquids [EMIM]OAc and [BMIM]Cl, reported earlier (Bose *et al*., 2010). However, it has been observed that in the presence of 40% [HMIM]Cl in aqueous solution, this result could be correlated to the Critical Aggregation Concentration (CAC) of ionic liquid. CAC of ionic liquid increases with the increase in the alkyl chain length of the imidazolium cation (Singh and Kumar, 2007). Thus, in the present study the results obtained are justified based on the aggregation of ionic liquid creating a hydrophobic core where the enzyme might be stabilized and result in better activity of the enzyme. This same could not be justified based on the viscosity as discussed in the previous section, because the viscosity of the solution increases only rapidly at higher concentration of ionic liquid. Further, the surface tension decrease and increased conductivity would have marginal effect on the enzymatic hydrolysis. However, it is further interesting that the ionic liquid [OMIM]Cl shows maximum hydrolyzed reducing sugar concentration at 20% and 40% ionic liquid in water. At the lower concentration 60%, the [OMIM]Cl aggregation might be solely be responsible for the hydrolysis, whereas in case of the higher concentration, 100% [OMIM]Cl, viscosity might be responsible for the hydrolysis of cellulose. Thus, the most suitable optimized biocatalytic hydrolysis of cellulose is observed at 40% *w/w* -[HMIM]Cl in aqueous buffer with a maximum hydrolysis percentage upto 73%.

(C) Study of Biocatalytic Hydrolysis of Cellulose in Ionic Liquid in presence of Surfactant: Hydrolysis studies in ionic liquid [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, showed a steady increase in the yield of total reducing sugar in the presence of PEG-1500 and PEG-4000, however the cationic (CTAB) and the anionic (SDS) surfactants had moderate to negative effect on the hydrolysis. There is a substantial increase in the total reducing sugar concentration upto 33 mg per 40 mg of cellulose dissolved in1 g IL used in the hydrolysis, which amount

to 75% hydrolysis of cellulose in presence of PEG-1500, using [HMIM]Cl as solvent.

Similarly, results had been reported by many authors in aqueous biocatalytic hydrolysis of cellulose and lignocelluloses in presence of non-ionic surfactants. However, under homogeneous conditions, cellulose is completely solubilized in ionic liquids leading to only one reason for the increased yield of reducing sugar i.e., the stabilization of cellulase by interaction with the non-ionic surfactants. Thus, to reiterate the same, we carried out the fluorescence studies and found that there is a 10- 20% enhancement in the fluorescence of the enzyme in all the ionic liquids.

A strong blue shift in the fluorescence emission of cellulase in [HMIM]Cl as solvents, whereas there was no shift in the fluorescence emission of the enzyme in [BMIM]Cl and [OMIM]Cl. This suggests that, in [HMIM]Cl, the cation $[HMIM]^+$ is in close vicinity of cellulase, is being substituted by PEG-1500 with electron pair donor oxygen moieties interacting strongly with the enzyme. This resulted in an increase in charge density around the benzene moiety of tryptophan, which lead to the blue shift in the spectra. The increase in the total reducing sugar upon hydrolysis is more correlated to the bathochromic shift in the enzyme fluorescence as compared to the less effective enhancement in the fluorescence of the enzyme which was observed in all the ionic liquids including [BMIM]Cl and [OMIM]Cl. Thus, an ideal situation for maximum hydrolysis for total reducing sugar yield was obtained in presence of PEG-1500 at a loading of 200% w.r.t. cellulase in [HMIM]Cl-buffered binary solution.

SUMMARY

SUMMARY

The hydrolysis of cellulose were studied in the homogeneous biocatalytic condition of ionic liquid with and without the surfactant additives and in heterogeneous conditions maintained by ionic liquid-water binary solutions. The ionic liquids used in the study are [BMIM]Cl, [HMIM]Cl and [OMIM]Cl, which differ in their alkyl chain length. The effect of the change in the alkyl chain length of the cation in ionic liquid was studied for the biocatalytic hydrolysis of cellulose. The biocatalytic hydrolysis in the homogeneous conditions of 10% buffered ionic liquid solutions, showed an increase in the hydrolysis upon increase in the alkyl chain length. The maximum hydrolysis percentage reached a high of 24% in [OMIM]Cl. However, this hydrolysis increase upon increase in the alkyl chain length is attributed to the increased viscosity leading to slow unfolding of the enzyme in the solution.

Further, hydrolysis were carried out in buffered aqueous-ionic liquid binary mixtures, the maximum hydrolysis percentage as high as 75% was observed at 40% *w/w* [HMIM]Cl in aqueous buffer solution. However, the reaction required prolonged time. The results obtained were significant and in general showed that the catalysis was dependent on the aggregation of ionic liquid in buffered aqueous solution.

The biocatalytic activity in ionic liquid in the presence of surfactant as additive is studied at a loading of 20-200% *w/w* concentration of the surfactant w.r.t. cellulase in ionic liquid. The study indicates that in general, the increased total reducing sugars were found in ionic liquid systems carrying non-ionic surfactants. Thus, particularly in presence of PEG-1500, the enzyme showed better activity with increase in the reducing sugar concentration. The maximum reducing sugar obtained by hydrolysis of cellulose was found to be 73% in [HMIM]Cl as solvent and at a 200% loading of PEG-1500 w.r.t. *w/w*, cellulase in the system.

Thus, from the above results we have found optimum conditions for the hydrolysis of cellulose, one in the homogeneous conditions and the other in the heterogeneous conditions. Both the conditions involve [HMIM]Cl as a solvent or part of the binary system.

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