SELECTIVE EXTRACTION OF LIGNIN FROM LIGNOCELLULOSIC BIOMASS USING IONIC LIQUIDS

Submitted in fulfilment of the academic requirements of the degree of Master of Applied Sciences (Chemistry) in the Faculty of Applied Sciences at the Durban University of Technology, Chemistry Department, Durban, South Africa

THANDEKA MKHIZE

JANUARY 2016

PREFACE

The work described in this thesis was performed by the author under the supervision of Professor. N. Deenadayalu at Durban University of Technology, Durban, South Africa, from 2013 – 2015. The study presents original work by the author and has not been submitted in any form to another university. Where use is made of the work of others, it has been clearly stated in the text.

Signed: Date:

Thandeka Mkhize

Signed: Date:

Prof. N. Deenadayalu (Supervisor)

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the:

- God almighty who always gives me strength.
- Durban University of Technology for an MAppSc Scholarship and for giving me the opportunity to undertake my research at the institution.

I am forever grateful to my supervisor **Prof. N. Deenadayalu** and co-supervisor Dr. P. Reddy for guidance, words of encouragement, valuable suggestions and support throughout the period of my studies.

I am expressing my most deep and sincere gratitude to my family especially my Fiancé Phumlami Gumede and daughter Siphokazi Gumede for standing by my side always, seeing potential in me, helping me in trying times and for understanding that “mommy is in school”; you are a blessing in my life. To my dearest mother, father and siblings, thank you for inspiring me, words of encouragement, support and instilling good values in me.

My sincere thanks to the Department of Chemistry and staff of Durban University of Technology, Durban, South Africa; Department of Chemical Engineering of Imperial College, London, United Kingdom and Department of Microbiology, University of Delhi, New Delhi, India for collaboration, mentorship, providing the facilities and resources to carry out my studies.

ABSTRACT

Globally there is a drive for the use of renewable materials for the production of biofuels or high-end value chemicals. The current production of chemicals from crude oil refining is unsustainable and leads to global warming effects. Biomass is the most attractive renewable energy source for biofuel or fine chemical production. Sugarcane bagasse is a by-product of the sugar milling industry and is abundantly available.

In this study lignin was sequentially extracted using ionic liquids. The ionic liquids (ILs) 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) and triethylammonium hydrogen sulfate ($[HNEt_3][HSO_4]$) were used to fractionate the sugarcane bagasse. The pre-treatment of sugarcane bagasse was carried out at different temperatures ranging from 90 - 150 ^{0}C and reaction times ranging from 1 - 24 h in a convection oven at a 10 % biomass loading.

Both ILs were able to dissolve the raw bagasse samples at 120 ^{0}C with [Emim][OAc] giving a lignin maxima of 28.8 % and a low pulp yield of 57 % after 12 h; $[HNEt_3][HSO_4]$ gave a lignin recovery of 17.2 % and low pulp yield of 58.5 % after 6 h. Regenerated lignin was obtained by adding ethanol/ water to the mixture followed by vacuum filtration. The regenerated pulp materials were characterized by Scanning Electron Microscope (SEM) to study the morphology; Fourier Transform Infrared Spectroscopy (FTIR) to study the characteristic bands and thermal analysis to study the thermal stability.

CONTENTS **Page**

Chapter 3 EXPERIMENTAL METHODS

Chapter 4 EXPERIMENTAL

Chapter 6 DISCUSSION

APPENDICES

1. Papers in preparation/ submitted

    (i) Enzymatic saccharification of acid/ alkali pre-treated mill run and depithed sugarcane bagasse

    (ii) Pre-treatment of South African sugarcane bagasse using a low-cost ionic liquid: Comparison of whole bagasse and depithed, fibrous and pith fractions

Abbreviations

AFEX	Ammonia Fibre Explosion
AGU	Anhydroglucose unit
AIL	Acid Soluble Lignin
ASL	Acid Insoluble Lignin
Br^-	Bromide
BSA	Bovine Serum Albumin
$Ca(OH)_2$	Calcium Hydroxide
CH_4	Methane
CO_2	Carbon Dioxide
COSLIF	Cellulose Organic Solvent-based Lignocellulose Fractionation
cP	Centipoise
CS	Calibration Standard
DA	Dilute Acid
DB	Depithed Bagasse
DBP^-	Dibutylphosphate
DMBQ	2, 6-dimethoxy-1, 4-benzoquinone
DMSO	Dimethyl sulfoxide
DSC	Differential Scanning Calorimetry
ECB	Energy Cane Bagasse
Fe^{3+}	Iron Oxide
FTIR	Fourier Transform Infrared Spectroscopy
GFP	Green Fluorescent Protein

H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
HEAF	2-hydroxyl ethyl ammonium formate
HPLC	High Performance Liquid Chromatography
ILs	Ionic Liquids
LFB	Long Fibre Bagasse
$Mn(NO_3)_2$	Manganese Nitrate
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance
NREL	National Renewable Energy Laboratory
ODW	Oven Dry Weight
OPB	Oil Palm Biomass
SCB	Sugarcane Bagasse
SEM	Scanning Electron Microscope
SFB	Short Fibre Bagasse
SO_2	Sulfur Dioxide
TGA	Thermo-gravimetric Analysis
WB	Whole Bagasse
WIS	Water Insoluble Solids

Ionic Liquids (ILs)

$[AlK(SO_4)_2.12H_2O]$	Aluminium potassium sulphate dodecahydrate
[Amim][Cl]	1-Allyl-3-methylimidazolium chloride
$[Bm_2im][BF_4]$	1-butyl-2,3-dimethylimidazolium tetrafluoroborate
$[Bmim][BF_4]$	1-butyl-3-methylimidazolium tetrafluoroborate
$[Bmim][BF_6]$	1-butyl-3-methylimidazolium hexafluorophosphate
[Bmim][Br]	1-butyl-3-methylimidazolium bromide
[Bmim][Cl]	1-butyl-3-methylimidazolium chloride
$[Bmim][HSO_4]$	1-butyl-3-methylimidazolium hydrogen sulfate
$[Bmim][MeSO_4]$	1-butyl-3-methylimidazolium methylsulfate
[Bmim][OAc]	1-butyl-3-methylimidazolium acetate
$[Bmpy][PF_6]$	1-butyl-4-methylpyridinium hexafluorophosphate
$[C{=}C_2C_1im][Cl]$	1-allyl-3-methylimidazolium chloride
$[C{=}C_2C_1im][MeCO_2]$	1-allyl-3-methylimidazolium acetate
$[C_1C_1im][MeSO_4]$	1-methyl-3-methylimidazolium methylsulfate
$[C_1mim][MeSO_4]$	1-methyl-3-methylimidazolium methylsulfate
$[C_2C_1im][MeCO_2]$	1-ethyl-3-methylimidazolium acetate
$[C_2mim][CF_3SO_3]$	1-ethyl-3-methylimidazolium trifluorosulfonate
$[C_2mim][EtSO_3]$	1-ethyl-3-methylimidazolium ethylsulfonate
$[C_2mim][MeSO_3]$	1-ethyl-3-methylimidazolium methylsulfonate
$[C_2min][DEP]$	1-ethyl-3-methylimidazolium diethylphosphate
$[C_4C_1C_1im][BF_4]$	1-butyl-3-methylimidazolium ethyl tetrafluoroborate
$[C_4C_1im][BF_4]$	1-butyl-3-methylimidazolium tetrafluoroborate
$[C_4C_1im][BF_4]$	1-butyl-3-methylimidazolium tetrafluoroborate

$[C_4C_1im][Br]$	1-butyl-3-methylimidazolium bromide
$[C_4C_1im][Cl]$	1-butyl-3-methylimidazolium chloride
$[C_4C_1im][HSO_4]$	1-butyl-3-methylimidazolium sulfate
$[C_4C_1im][MeCO_2]$	1-butyl-3-methylimidazolium acetate
$[C_4C_1im][MeSO_4]$	1-butyl-3-methylimidazolium methyl sulfate
$[C_4C_1im][MeSO_4]$	1-butyl-3-methylimidazolium methylsulfate
$[C_4C_1im][PF_6]$	1-butyl-3-methylimidazolium hexafluorophosphate
$[C_4C_1pyr][PF_6]$	1-butyl-3-methylimidazoliumpyrrolidium hexafluorophosphate
$[C_6C_1im][OTF]$	1-hexyl-3-methylimidazolium trifluoromethyl sulfonate
[Ch][AA]	cholinium amino acid
[Emim][ABS]	1-ethyl-3-methylimidazolium alkylbenzenesulfonate
[Emim][Cl]	1-ethyl-3-methylimidazolium chloride
$[Emim][EtSO_4]$	1-ethyl-3-methylimidazoilum ethylsulfate
[Emim][Gly]	1-ethyl-3-methylimidazoilum glycinate
$[Emim][MDEGSO_4]$	1-ethyl-methylimidazolium-2-(2-methoxyethoxy)ethyl-phosphate
[Emim][OAc]	1-ethyl-3-methylimidazolium acetate
[Emim][OTf]	1-ethyl-3-methylimidazolium triflate
[Emim][TFA]	1-ethyl-3-methylimidazolium trifluoro-acetate
[Emim][XS]	1-ethyl-3-methylimidazolium xylene
[Hmim][Cl]	1-hexyl-3-methylimidazolium chloride
$[HNEt_3][HSO_4]$	Triethylammonium hydrogen sulfate
$[HSO_3\text{-}pmim][Cl]$	1-(1-propylsulfonic)-3-imidazolium chloride
[Hxmim][Cl]	1-hexyl-3-methylimidazolium chloride
[Na][XS]	Sodium xylate

[P4441][$MeSO_4$]	Tributyl (methyl) phosphonium methyl sulfate
[P66614][Cl]	Tetradecyl (trihexyl) phosphonium chloride

IL cations

$[Bbim]^+$	1, 3-dibutylimidazolium
$[Bmim]^+$	1-butyl-3-methylimidazolium
$[BmPyr]^+$	1-butyl-3-methylpyrrolidinium
$[Btma]^+$	butyl trimethyl ammonium
$[Bu_3NH]^+$	tri butyl ammonium
$[Bu_4N]^+$	tetra butyl ammonium
$[Bzmim]^+$	1-benzyl-3-methylimidazolium
$[Chol]^+$	choline
$[Emim]^+$	1-ethyl-3-methylimidazolium
$[Et_3NH]^+$	tri ethyl ammonium
$[Et_4N]^+$	tetra ethyl ammonium
$[Gua]^+$	guanidinium
$[Hmim]^+$	1-hexyl-3-methylimidazolium
$[Me_3NH]^+$	tri methyl ammonium
$[Me_4N]^+$	tetra methyl ammonium
$[Mmim]^+$	1, 3-dimethylimidazolium
$[MTOA]^+$	methyl trioctyl ammonium
$[OH\text{-}emim]^+$	1-(2-hydroxyethyl)-3-methylimidazolium
$[Omim]^+$	1-octyl-3-methylimidazolium
$[Pmim]^+$	1-propyl-3-methylimidazolium

$[Pr_4N]^+$	tetra propyl ammonium
$[C_2C_1im]^+$	1-ethyl-3-methylimidazolium
$[C_4C_1im]^+$	1-butyl-3-methylimidazolium
$[C_4C_2im]^+$	1-butyl-3-ethylimidazolium
$[C_4C_3im]^+$	1-butyl-3-propylimidazolium
$[C_6C_1im]^+$	1-hexyl-3-methylimidazolium
$[C_8C_1im]^+$	1-octyl-3-methylimidazolium

IL anions

$[BF_4]^-$	tetrafluoroborate
$[CF_3OO]^-$	trifluoro acetate
$[CF_3SO_3]^-$	trifluoro sulfonate
$[dca]^-$	dicyanamide
$[dhp]_2^-$	dihydrogen phosphate
$[dmp]^-$	dimethyl phosphate
$[EtSO_4]^-$	ethyl sulfate
$[MDEGSO_4]^-$	2-(2-methoxyethoxy) ethyl phosphate
$[MeBP]^-$	dimethyl dibutyl phosphate
$[MeBr]^-$	methyl bromide
$[MeCO_2]^-$	methyl acetate
$[MeSO_3]^-$	methyl sulfonate
$[MeSO_4]^-$	methyl sulfate
$[OTf]^-$	trifluoromethane sulfonate
$[PF_6]^-$	hexafluorophosphate

$[Tf_2N]^-$	bis(trifluromethane)sulfonimide
$[TMA]^-$	trimethyl acetate

List of Tables

List of Figures

CHAPTER **1**

INTRODUCTION

1.1. Background

An increasing demand for energy, the rise of unnatural weather changes and increase in global warming by utilization of fossil fuels have revived the search for alternate options for the production of chemicals and renewable energy sources. The fossil fuel-based economy has challenges and difficulties which include the increased emissions of carbon dioxide, diminishing oil reserves, increasing oil costs and non-renewability. A potential answer for these issues could be the use of lignocellulosic biomass as an alternate and manageable energy source for the future. It can be utilized to synthesise chemicals, biofuels, which don't compete with food supply (Lee *et al*., 2008).

Much research has been done on lignocellulosic biomass worldwide. Lignocellulosic biomass, for example, agricultural waste, waste paper, and vitality crops, has long been perceived as a potential practical source of sugars for biotransformation into biofuels and quality included bio-based items (Himmel *et al*., 2007; Li *et al*., 2008). Before biomass can be used it has to be fractionated into its components, which requires pre-treatment of the biomass. In the last decade different sorts of pre-treatments were researched, including dilute acids (Gomez *et al*., 2010), steam explosion (Ramos, 2003), heated water, organic solvents, hydrogen peroxide, yet all had their own limitations for large scale applications. Some of these strategies were not selective and created undesirable products that interfered with the fermentation and transformation of monosaccharides into ethanol and other products (Wang *et al*., 2011). Ionic liquids (ILs) have been considered as “green solvents” which are an environmentally friendly alternative to current pre-treatment methods, since ILs can also be recycled (Bose *et*

al., 2010). Figure 1.1 shows the possible products that can be obtained from lignocellulosic biomass after pre-treatment.

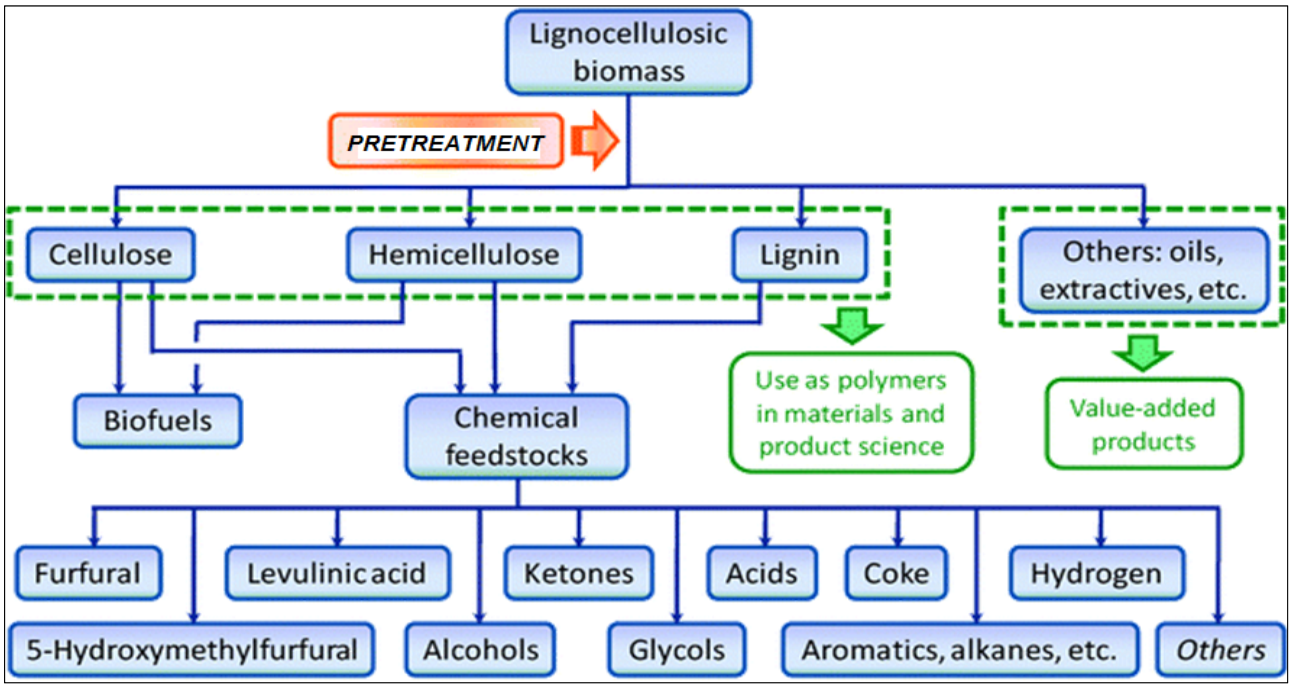


Figure 1.1 Possible products from lignocellulosic biomass (Sun *et al.*, 2011)

1.2. Biomass

Biomass is an organic material that is derived from living or recently living organisms. It is carbon based and is made out of a blend of natural atoms containing hydrogen generally including molecules like oxygen, nitrogen and different particles like salt and antacid earth metals in small amounts. The carbon used to build biomass is assimilated from the climate as carbon dioxide (CO_2) by vegetation utilizing energy from the sun. Plant material that is not eaten is either separated by smaller scale creatures or blazed. In the event that it is separated, it discharges the carbon once again into the environment, mostly as either CO_2 or methane (CH_4). When carbon is burnt, it returns to the atmosphere as carbon dioxide. These procedures form part of the carbon cycle. Biomass sources include agricultural crops, forestry crops, industrial residues, animal residues, municipal solid waste and sewage as illustrated in figure 1.2.

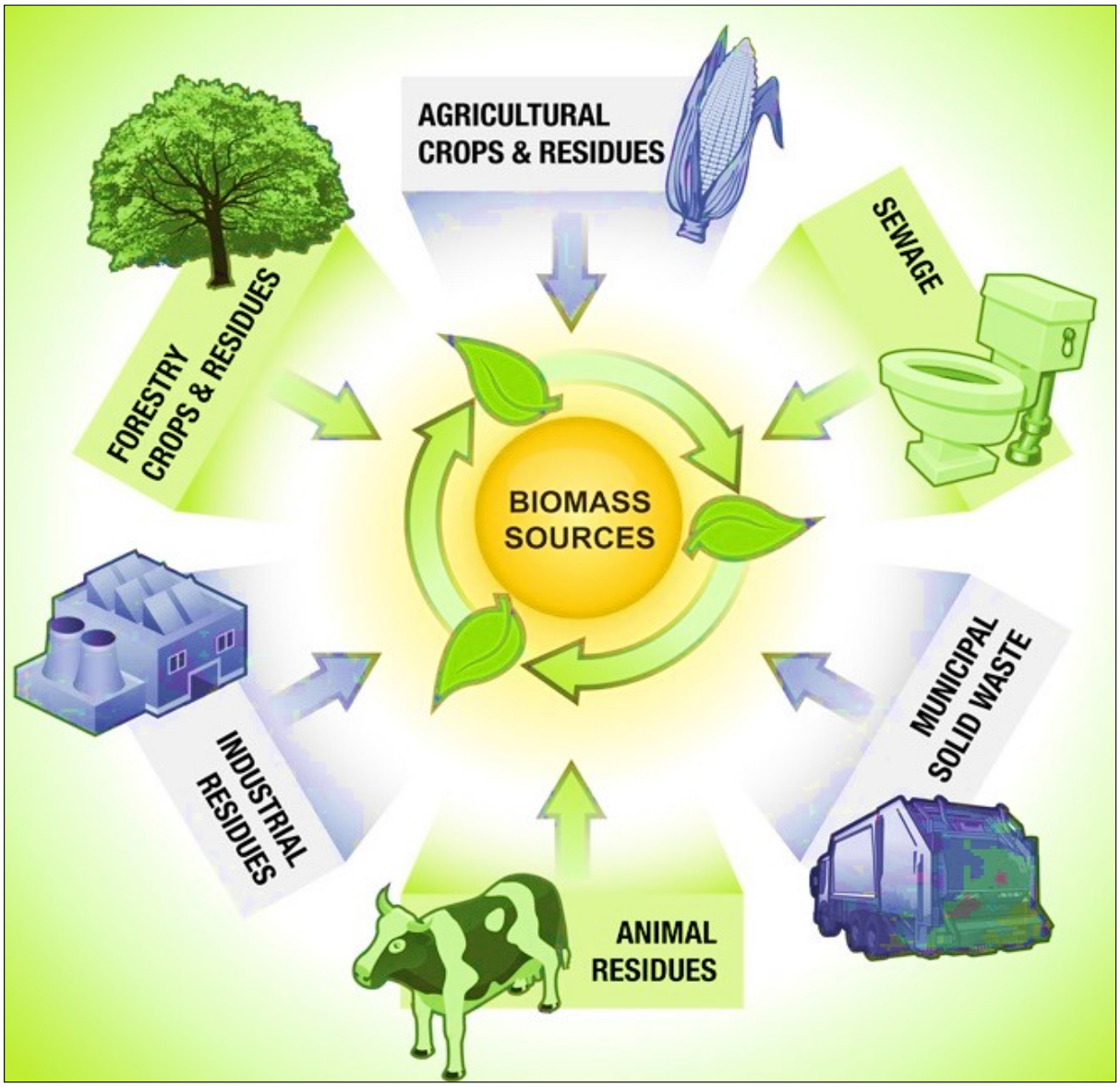


Figure1.2 Biomass sources (pinterest.com/biomass sources)

1.2.1. Lignocellulosic biomass

The auxiliary materials that plants produce to shape the cell walls, leaves, stems, stalks, and woody segments of biomass are fundamentally composed of three bio-based chemicals called cellulose, hemicellulose, and lignin. Together, they are called lignocellulose - a composite material of unbending cellulose fibres implanted in a cross-

connected framework of lignin and hemicellulose that tie the strands. Lignocellulose plant structures also contain other chemicals in the framework, called extractives (gums, phenolics and different chemicals) and minerals (calcium, magnesium, potassium). Lignocellulose materials are impervious to physical, chemical and organic attack, yet it is of interest to bio-processing the cellulose and hemicellulose through hydrolysis to produce fermentable basic sugars. Lignocellulosic biomass is a waste material of the food processing (e.g. sugar mills) and forestry (e.g. saw and paper mills). Figure 1.3 shows examples of lignocellulosic biomass.

Figure1.3 Lignocellulosic biomass examples (epilson-econ.gr/en)

Lignocellulosic biomass is the most abundantly available raw material for the production of bio-fuels, mainly bio-ethanol. It is composed of carbohydrate polymers (cellulose and hemicellulose) and an aromatic polymer (lignin). Amongst the lignocellulosic biomasses sugarcane bagasse, an agriculture residue, possesses a high potential for the conversion to biofuels and is one of the most important agro-industrial crops produced abundantly in South Africa. Typical lignocellulosic biomass contains 40 - 50 % cellulose, 20 - 40 % hemicellulose and 18 - 35 % lignin as shown in figure 1.4.

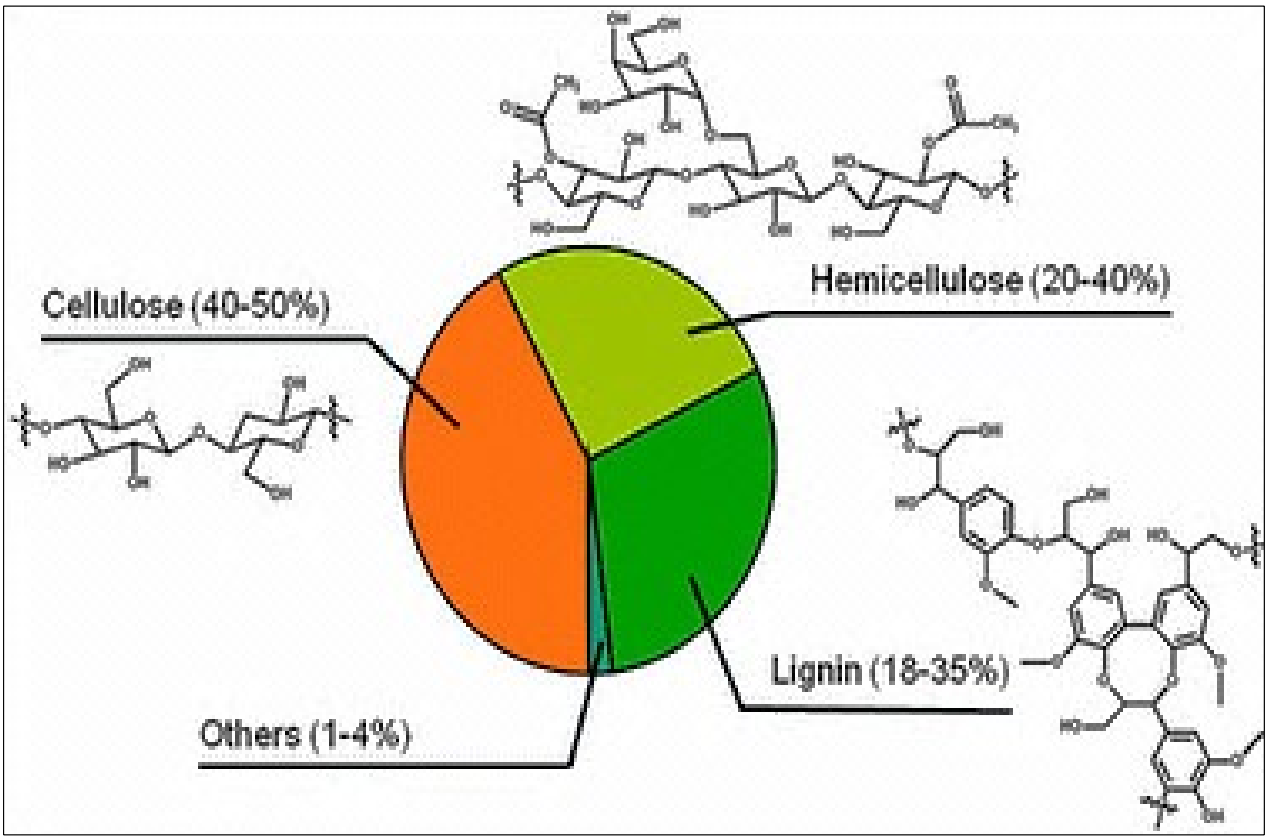


Figure 1.4 Biomass compositions (google/lignocellulosic biomass composition)

Bagasse is the fibrous remains of sugarcane stalks that remain after the crushing and extraction of juice and is one of the most abundant renewable and biodegradable feed stocks. It is estimated that approximately 270 kg of bagasse (50 % humidity) is produced per ton of sugarcane processed (Garcia-Perez *et al*., 2002) or 1.25 kg of

bagasse is produced per kilogram of sugar produced (Botha and Von Blottnitz, 2006; Singh *et al*., 2007). Bagasse consists of three components, namely pith, fiber and rind mixed in different proportions. Though sugarcane bagasse (SB) is an abundant source of fermentable carbohydrates, raw moist bagasse contains only 55 – 60 % of useful fiber. The non-fibrous 'pith' or parenchymatous tissue found predominantly in the center of the sugarcane stalk represents about 30 % and the dense non-fibrous 'rind' or epidermis surrounding the stalk accounts for the remaining 5 %. The high moisture content of whole bagasse after juice extraction, typically 40 - 50 % by weight, is detrimental to its use both as a fuel and as a substrate for bioprocessing (Carrier *et al*., 2012; Pandey *et al*., 2000). An efficient depithing process to remove as much of the rind and pith as possible, as well as other fine extraneous material, has until recently been considered key to cost-effective utilization of bagasse (Atchison, 1971). The pith is made of up non-fibrous, spherical particles and has a low density and high hygroscopic ability, contributing to various problems in processing (Rasul *et al*., 1999). Pith has a higher ash content than woody biomass, which is problematic for paper manufacture and many biofuels production processes (Anzaldo *et al*., 2001). Removal of the smaller pith fibers from bagasse prior to stockpiling is also used to reduce hazards through dust formation and spontaneous combustion (Rainey *et al*., 2013).

Depithed bagasse has a much lower water holding capacity than whole bagasse. As a result, commercial depithing has been in use for many years to increase the fiber content before bioconversion. Depithing is an industrial process performed in two stages (moist and wet depithing) to remove the pith and undesirable short fibers that are unsuitable for pulping, inevitably also reducing the ash content of the material (Lois-Correa, 2012). Depithed bagasse consists of approximately 40 - 45 % cellulose, 30 - 35 % hemicelluloses and 20 - 30 % lignin (Pandey *et al*., 2000). In contrast with other agricultural residues such as rice husk, rice straw and wheat straw which have very high ash contents of 20.0 %, 17.5 % and 11.0 %, respectively, depithed bagasse contains relatively little ash (1.0 - 5.0 %) (Cardona *et al*., 2010). Table 1.1 below lists the definition of different sugarcane bagasse substrates.

Table 1.1 Definitions of different sugarcane bagasse substrates

Substrates	
Whole bagasse (WB)	Mixture of short fibers from the vascular bundle of sugarcane stalk (‘pith’) as well as longer fibers from the cortex of sugarcane stalks ‘rind’.
Depithed bagasse (DB)	High density, fibrous material left over after removal of pith particles by industrial depithing.
Pith bagasse (PB)	Small, spongy spherical particles from the vascular bundle of sugarcane stalk. Low density, porous material with a higher ash content; obtained from laboratory depithing process.
Short fiber bagasse (SFB)	Short fibers from the cortex of sugarcane stalk ‘rind’ with moderate length/width ratios; obtained from laboratory depithing process.
Long fiber bagasse (LFB)	Longer fibers from the cortex of sugarcane stalk ‘rind’ with high length/width ratios; obtained from laboratory depithing process.

Bagasse as a feedstock has variable compositions and heating values; Its characteristic properties mainly depends on the climate, type of soil on which cane was grown, cane variety, harvesting method, amount of cane washing and the efficiency of the milling plant (Janghathaikul and Gheewala, 2004).

South Africa produces sugarcane on a large scale since the 1860s when the first Indian labour brokers docked in Durban. The sugarcane was processed mostly to be used in

food and beverage production. However due to the increased sugarcane production costs, increasing global competition, weather conditions and cheaper sugar imports the industry is facing challenges. By diversifying into markets such as bioethanol and or value added chemicals from waste components (bagasse), the industry can be sustainable (Sunday Times: Business Times).

Holistic utilization of sugarcane bagasse for the production of value-added chemicals and materials is an important driver for diversification within the sugarcane industry. The abundance of sugarcane bagasse makes it an ideal low-cost feedstock for the second generation bio-refinery, with the potential to double bioethanol production per hectare without expanding cane fields or jeopardizing food and feed production (UNICA, 2013). Non-fuel value addition to lignin is already a priority in South Africa, where LignoTech South Africa have commercialized the production of low-value lignin-based products, including dust suppressants (Dustex Africa, 2015). The integral utilization of bagasse would produce an abundant supply of high quality lignin suitable for upgrading to fine chemicals, further boosting the economic and environmental viability of the sugarcane-based bio-refinery.

1.2.2. Lignocellulosic biomass Components

1.2.2.1. Cellulose

Cellulose is the largest component of lignocellulose which is an abundantly available biopolymer on this planet and is found in the cell walls of all the green plants to facilitate mechanical stability, and thus is a valuable renewable resource (Brandt *et al.,* 2013; Muhammad *et al.,* 2013; Casas *et al.,* 2012; Mahadeva *et al.,* 2012; Swatloski *et al.,* 2002; Mäki-Arvela *et al.,* 2010; Kubisa, 2009). It is a highly crystalline homopolysaccharide composed of the anhydroglucopyranose monomers formed *via* 1,4-β-D-glucose linkage of carbohydroglucose units and contains several intra and inter molecular hydrogen bonds (Abdulkhani *et al.,* 2013; Zhao *et al.,* 2012).

It is a polysaccharide in which D-glucose is linked uniformly by β-glucosidic bonds and has a molecular formula of $(C_6H_{12}O_6)_n$, (n represents the degree of polymerization which is broad due to its high molecular weight) while the regenerated cellulose fibres have a degree of polymerization of 250 - 600 (Kihlman 2012). Cellulose is mostly used in the pharmaceuticals and/ textile industries. The cellulose polymer chain is shown in figure 1.5.

Total hydrolysis of cellulose yields D-glucose (a monosaccharide), but partial hydrolysis yields a disaccharide (cellobiose) and polysaccharides in which n is in the order of 3 to 10. Cellulose has a crystalline structure and great resistance to acids and alkalis. Cellulose molecules are linear, unbranched, polymer with repeating units (Kraäsig 1993) and the supramolecular structure consists of cellulose organized into crystalline region (have well organized intermolecular hydrogen bonds) that can be arranged in different polymorphs (the ability of a solid material to exist in more than one form or crystal structure) named cellulose I (Iα and Iβ), II, III and IV (Kihlman 2012; Espinoza-Acosta *et al.,* 2014; Lindman *et al.,* 2010; Brandt *et al.,* 2013)

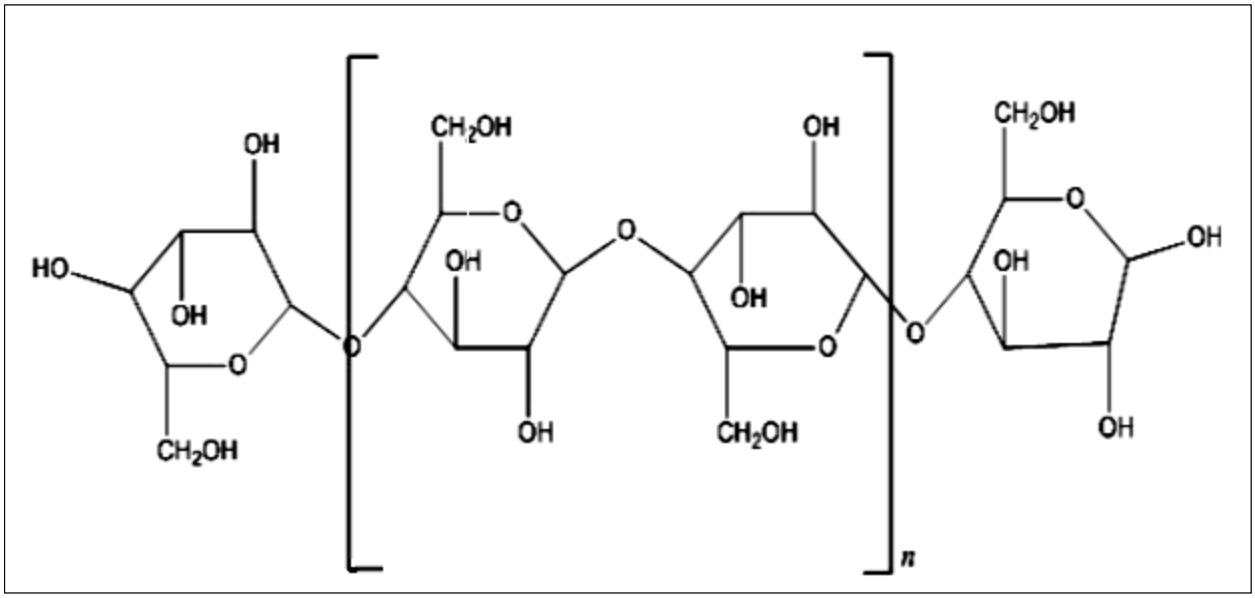


Figure 1.5 A cellulose polymer chain, *n* is typically 400-1000 (Swatloski *et al.,* 2002)

1.2.2.2. Hemicellulose

Hemicellulose is a heterogeneous complex of polysaccharides that makes up approximately 35 % wt. of the biomass (Cheng *et al.,* 2010). Hemicellulose is composed of 5 carbon monosaccharides including D-xylose and D-arabinose, and 6-carbon monosaccharides including D-mannose, D-galactose, and D-glucose and has a lower molecular weight compared to cellulose with a molecular formular of $(C_5H_8O_4)_n$. The structure of the different sugars is shown in figure 1.6 (Brandt *et al.,* 2013).

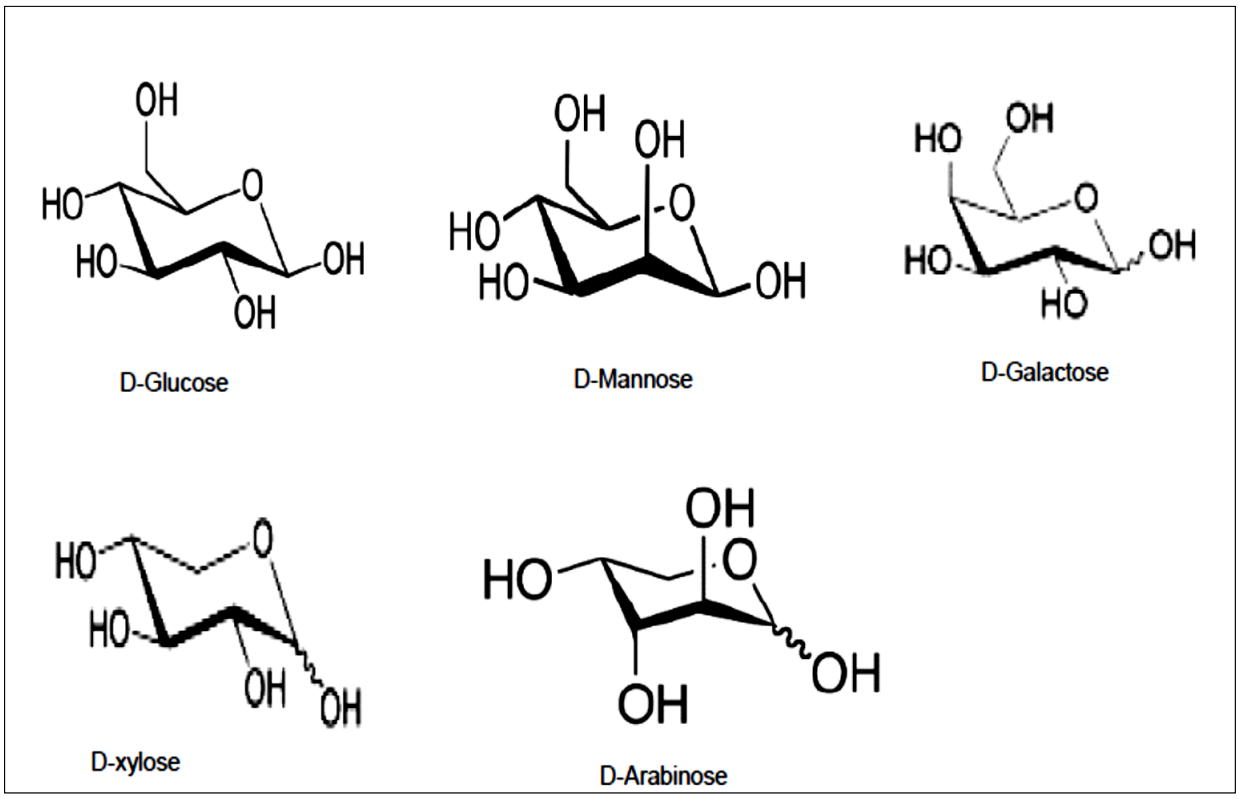


Figure 1.6 The C_6 and C_5 sugars typically found in hemicellulose (Brandt *et. al.,* 2013)

The structure of this polysaccharide is random and amorphous with short side branches of various saccharides that can be easily hydrolysed (Espinoza-Acosta *et al.,* 2014).

Hemicellulose monosaccharides bind to the surface of the cellulose fibrils non-covalently (Brandt *et al.,* 2013).

1.2.2.3. Lignin

Lignin makes up the cell wall of all vascular plants. Lignin is an aromatic, water-insoluble complex network polymer that provides waterproofing, structural reinforcement and resistance to attack (either biological or physical) on the cell walls of plant tissues (Chen and Dixon, 2007; Simmons *et al*., 2010). It is composed of three monomers: coniferyl, sinapyl and *p*-courmarly alcohols (Cheng *et al.,* 2010), in order of abundance and the structures are shown in figure 1.7.

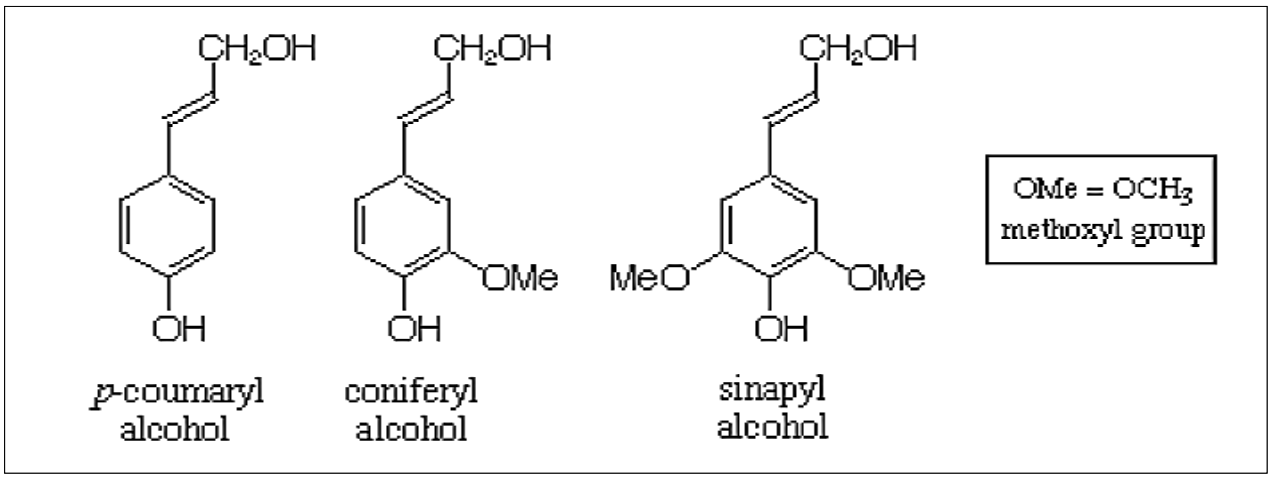


Figure 1.7 The three monolignols from which lignin is synthesised.

The monomers vary in substitution at the C-3 and the C-5 ring positions (Brandt *et al,* 2013). The lignin component in biomass is mainly used in direct combustion; however, lignin can be a promising source for chemicals such as phenols and aromatics (Cheng *et al.,* 2010). Most chemical deconstruction methods change lignin by modifying its ether bonds, but some remove it from the pulp (e.g. Organosolv pulping, for example in base treatments, Kraft pulping). The removal of lignin is a combination of chemical fragmentation and the ability of the liquor to solvate the modified lignin fragments (Brandt *et al.,* 2013).

1.3. Advantages of using lignocellulosic biomass

The most significant advantage with lignocellulosic biomass is the fact that it is renewable; unlike fossil fuels the latter being a diminishing resource. Biomass utilizes waste. The production of biomass energy is environmentally safer since it significantly lessens the emissions of greenhouse gases when compared to fossil fuels.

1.4. Industrial delignification methods

Modern pulp and paper industries have evolved from ancient times when the Chinese developed paper in ca. 105 A.D. Wood is the main source of cellulose in the pulp and paper industry, however other lignocellulosic biomass sources are processed in the same manner as wood with different operational conditions (Klemm *et al*., 1998). The cellulose fibers in biomass are bound together by lignin. The lignin produced from the pulping process is separated and burnt to produce energy or used to make useful chemical products such as sulfonates. The main purpose of the pulping process is to extract cellulose from other components in the biomass including lignin without destroying the fibre strength. Pulping can be done either by mechanical, chemical or semi-chemical methods (Sun and Cheng, 2005).

1.4.1. Mechanical Pulping

Mechanical pulping is a more important pulping method compared to the others mainly because if converts all of the biomass used in the process into paper. However, lignin is not removed during mechanical pulping process. The presence of lignin in the pulp causes low durability and yellowing with age. Further processing the pulp thermo-mechanically by exposing it to steam at elevated temperature and pressure results in softening of lignin and easier fibre separation (Sharman *et al*., 1994).

1.4.2. Chemical Pulping

Chemical pulping method is the most important as it results in the removal of almost all the lignin and other non-fibrous material in the biomass. It produces the highest quality papers such as printing and writing papers. One major disadvantage of this method is its low fibrous yield which is generally between 50 - 55 % which is low compared to other pulping methods. Chemical pulping involves the extraction of cellulose from biomass by dissolving the lignin that ties the cellulose filaments together. The three techniques chiefly utilized as a part of chemical pulping are Kraft in which aqueous sulfide and sodium hydroxide solutions are used, acid sulfite in which sulfuric acid is used and neutral sulfite semi-chemical in which a neutral solution of sodium sulfite and sodium carbonate is used (Someshwar and Pinkerton, 1992; U.S. Environmental Protection Agency, 1983 & 1976; Hendrickson, 1970).

1.5. Pre-treatment of lignocellulosic biomass

Pre-treatment is a crucial step in the production of biofuels and or chemicals from lignocellulosic biomass. Pre-treatment leads to the alteration of the biomass structure in order to make cellulose more accessible to hydrolysis. It also removes lignin and hemicellulose and reduces cellulose crystallinity as illustrated in figure 1.8 (Kumar *et al*., 2009).

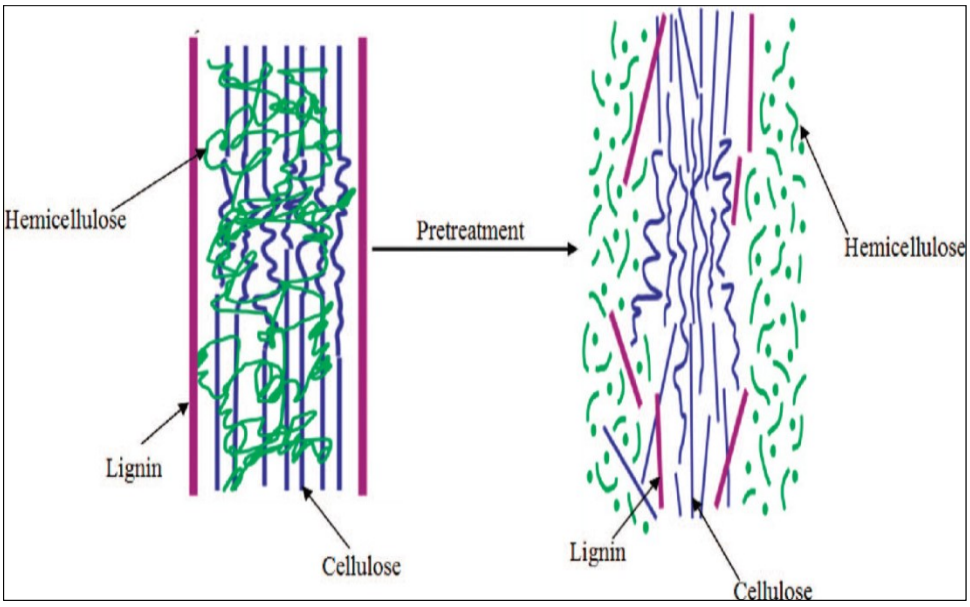


Figure 1.8 Alteration of biomass components after pre-treatment (Hsu *et al.*, 1980, Kumar *et al*., 2009)

Lignocellulosic pre-treatments are commonly used to increase the accessibility of cell wall polysaccharides to enzymes, thus making the conversions of cellulose to monosaccharide more efficient (Wyman *et al*., 2005). Physical, chemical, physicochemical, and biological treatments have been proposed in the past as effective ways to decrease cell wall barrier to cellulose hydrolysis (Alvira *et al*., 2010). Physical pre-treatment is divided into mechanical and pyrolysis. In mechanical technique waste materials can be reduced to small particles by chipping, pounding and processing to reduce cellulose crystallinity (Millet *et al*., 1976, Cadoche and Lopez, 1989). In the pyrolysis technique the materials are dealt with at temperatures higher than 300 ^{0}C, and the cellulose quickly breaks down to create vaporous items and remains singed (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979; Fan *et al*., 1987; Shafizadeh and Lai, 1975).

Physicochemical pre-treatment is divided into steam explosion: In this technique, chipped biomass is treated with high-weight immersed steam to reduce the mass which makes the materials experience an explosive decompression (McMillan, 1994; Grous *et*

al., 1986; Duff and Murray, 1996; Wright, 1998; Morjanoff and Gray, 1987; Holtzapple *et al*., 1989; Clark and Mackie, 1987; Mackie *et al*., 1985; Mes-Hartree *et al*., 1988), ammonia fibre explosion (AFEX): In this technique lignocellulosic materials are treated with alkali at high temperature (Mes-Hartree *et al*., 1988; Vlasenko *et al*., 1997; Holtzapple *et al*., 1991; Mosier *et al*., 2004; Mes-Hartree *et al*., 1988; McMillan, 1994) and carbon dioxide explosion: This technique is like steam and AFEX explosion pre-treatments, CO_2 blast is utilized for pre-treatment of lignocellulosic materials (Dale and Moreira, 1982; Zheng *et al*., 1998).

In biological/ natural pre-treatment methods, microorganisms, for example, brown, white and delicate rot fungi are utilized to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rot fungi attacks cellulose, while white and delicate rot fungus attack both cellulose and lignin. White decay parasites are the best basidiomycetes for natural pre-treatment of lignocellulosic materials (Fan *et al*., 1987; Hatakka, 1983; Ander and Eriksson, 1977; Akin *et al*., 1995; Boominathan and Reddy, 1992; Kirk and Farrell, 1987; Waldner *et al*., 1988; Blanchette, 1991; Shill *et al*., 2011; Zhao *et al*., 2009Lee *et al*., 2009).

Chemical pre-treatment is divided into ozonolysis: In this technique ozone is utilized to degrade lignin and hemicellulose in lignocellulosic materials (Ben-Ghedalia and Miron, 1981; Neely, 1984; Vidal and Molinier, 1988), acid hydrolysis: In ths technique concentrated acids, for example, sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) are utilized to treat lignocellulosic materials (Sivers and Zacchi, 1995; Esteghlalian *et al*., 1997; McMillan, 1994; Hinman *et al*., 1992; Cahela *et al*., 1983; Esteghlalian *et al*., 1997), alkaline hydrolysis: In this technique alkaline solutions such as sodium hydroxide, lime or ammonia are applied to remove lignin and a part of the hemicelluloses, and efficiently increase the accessibility of enzyme to the cellulose (Fan *et al*., 1987; McMillan, 1994; Tarkow and Feist, 1969; (Millet *et al*., 1976; Bjerre *et al*., 1996; Chosdu *et al*., 1993; Iyer *et al*., 1996), oxidative delignification: In this technique lignin biodegradation is catalyzed by the peroxidase enzyme chemical in the presence of hydrogen peroxide (H_2O_2). The pre-treatment of sugarcane bagasse with hydrogen

peroxide significantly improved its susceptibility to enzymatic hydrolysis (Azzam, 1989; Bjerre *et al*., 1996) organosolv process. In this technique a natural or fluid natural dissolvable blend with inorganic acid catalysts (HCl or H_2SO_4) is utilized to break the lignin and hemicellulose bonds. The natural solvents utilized as a part of the procedure incorporate methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl liquor (Thring *et al*., 1990; Sarkanen, 1980; Aziz and Sarkanen, 1989) and ionic liquids (ILs). ILs are promising solvents for the pre-treatment of lignocellulose as they are thermally stable, environmentally friendly, recyclable, and have low volatility. Amongst chemical pre-treatment methods, ionic liquid (IL) pre-treatment has received great interest and has been shown to be a promising biomass pre-treatment technology, enabling fast breakdown of cellulose through the disruption of lignin and the decrystallization of cellulose (Singh *et al*., 2009; Mosier *et al*., 2005).

1.6. Ionic Liquids (ILs)

ILs are a class of salts that are liquids at very low temperatures (below 100 ˚C). They consist purely of cations and anions (Hermanutz *et al*., 2008; Welton, 1999) and the most common anions and cations are shown in figure 1.9. Because of the attractive properties of ILs such as high thermal stability, lack of flammability, low volatility, chemical stability and excellent solubility with many organic compounds ILs have been attracting interest as greener solvents (Hermanutz *et al*., 2008; Yue *et al*., 2012; Magdi *et al*., 2012). ILs appears to be highly polar due to their ionic character, resulting in their enhanced biopolymer dissolving capacity (Pinkert *et al*., 2009). Due to environmental apprehensions, biomass/ILs technology is considered an eco-friendly approach and in the near future it may replace the traditional cellulose dissolution processes to produce fibre by the green processes (Yue *et al*., 2012). This greener approach however is facing some obstacles for example increasing the efficiency of the dissolution (high solid content with short dissolution time), and increasing the recovery of ILs (Feng and Chen, 2008).

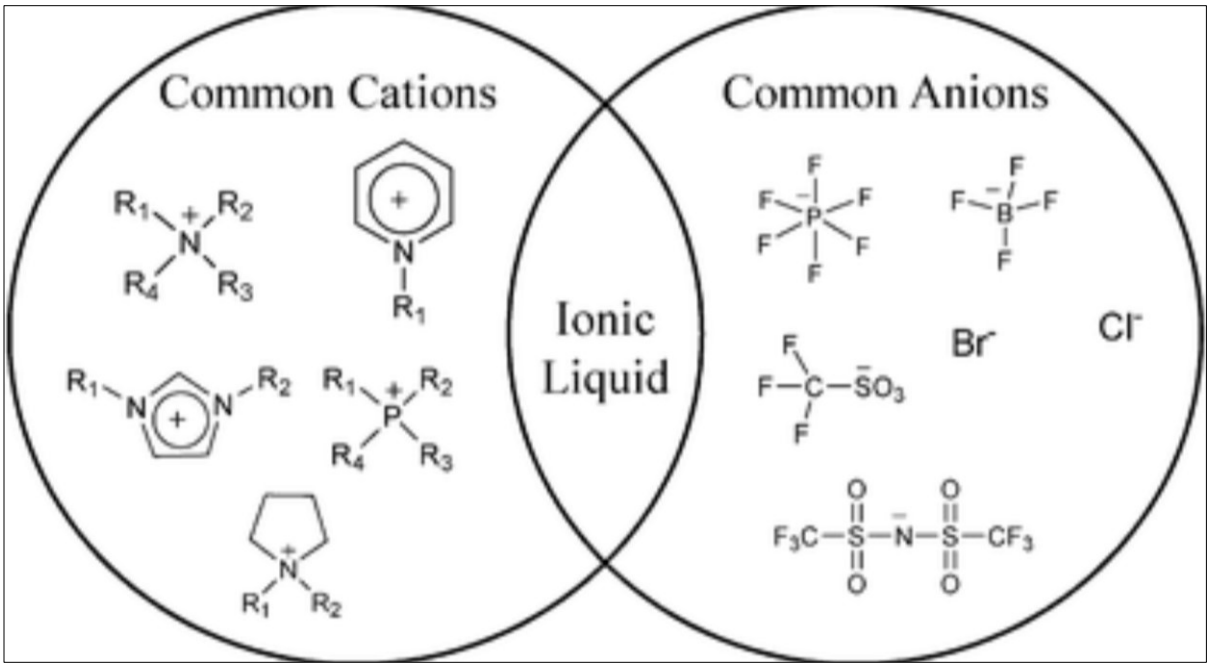


Figure 1.9 Common cations and anions of ionic liquids (RSC adv., 2012, 2)

Recently, ILs have been perceived as revolutionary “green” solvents by the chemical industry which would dissolve cellulose (Swatloski *et al*., 2002; Pinkert *et al*. , 2009; Zhu , 2008; Liebert and Heinze , 2008), owing to their unique properties and a wide variety of structural variations. ILs display fabulous physical qualities including the capacity to break down polar and non-polar natural, inorganic and polymeric mixes. Furthermore, ILs have the upsides of having low instability, being non-combustible low vapour pressure, low electrical conductivity, high electro-elasticity, high heat capacity and effectively recyclable (Swatloski *et al*., 2002; Pinkert *et al*., 2009; Zhu 2008; Liebert and Heinze, 2008). Pre-treatment with ILs can decrease the crystallinity of cellulose and halfway evacuate hemicellulose and lignin while not creating debasement items which are inhibitory to proteins or maturing microorganisms.

Pre-treatment with ILs is less energy demanding, simpler to handle and more environmentally friendly than other pre-treatment methods, for example, mechanical processing, steam blasting, acid, base, or natural dissolvable methodologies (Qiu *et al*.,

2012). Acetic acid derived ILs are less viscous than chloride based ILs and are more thermally stable than formate based ILs (Fukaya *et al*., 2008; Zhao *et al*., 2008). The acetic acid derivation based IL 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) was chosen in this study for the pre-treatment of sugarcane bagasse because of its low dissolving temperature (-20 ^{0}C), low consistency/ viscosity, non-toxicity and non-destructiveness (Samayam and Schall, 2010).

1.7. Statement of the problem

Globally there is an increasing concern reagarding alternative and sustainable energy sources such as biofuels and/ high-end value chemicals. The current methods for producing chemicals from crude oil refining is not economically viable and leads to global warming effects. The abundance of lignocellulosic biomass makes it an attractive renewable energy source for biofuel or fine chemical production, that can potentially substitute a large portion of the fossil fuels that are used today. Sugarcane bagasse is one of the most abundant agro-industrial by-products that addresses the sustainability issue of chemical production from a source other than that generated from crude oil refining. Pre-treatment is the most important step because it enables the disruption of the naturally resistant structure of lignocellulosic biomass to make reactive intermediates such as fermentable sugars to alcohols, acids and to make cellulose accessible to hydrolysis for conversion to biofuels. Many pre-treatment methods such as mechanical, biological, acid, steam explosion, ammonia and ionic liquid have been proposed for lignocellulosic biomass and they have been found to change the physical and chemical structure of lignocellulosic biomass and improve hydrolysis rate. However some of the obstacles currently existing in the pre-treatment process include (1) insufficient separation of cellulose and lignin, (2) formation of by-products that inhibit ethanol fermentation and (3) high use of chemicals and or energy and considerable waste production. Ionic liquids are deemed to be the suitable pre-treatment method because they are potential green solvents and it has been reported that they are able to dissolve cellulose.

Objectives of the study

i The main objective of the present study was to fractionate sugarcane bagasse (SB) through the application of processes utilizing the ionic liquids 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) and triethylammonium hydrogen sulfate ($[HNEt_3][HSO_4]$) for the determination lignin and carbohydrates content.

ii Enzymatic saccharification of regenerated biomass after:
   - ✓ Triethylammonium hydrogen sulfate ($[HNEt_3][HSO_4]$) pre-treatment using *Novozymes* (NS22201).
   - ✓ Acid/alkali pre-treatment with cellulose enzyme (from *Trichoderma* species RCKC65) to determine:
     - Optimum conditions for enzyme dose
     - Surfactant concentration and
     - Substrate consistency for its efficient hydrolysis

iii Characterization of untreated and pre-treated sugarcane bagasse

CHAPTER **2**

LITERATURE REVIEW

2.1. History of ILs for biomass processing

In the past decade many research investigations were done on the application of ILs in biomass fractionation, cellulose dissolution and modification. Many reviews have been published in order to explain this technology in detail (Zhu *et al*., 2006; Cao *et al*., 2009; Pinkert *et al*., 2009; Feng and Chen, 2008; Yue *et al*., 2012). The dissolution of cellulose was of great interest than that of the other components of biomass because of the potential to transform it into bioethanol. The interactions of imidazolium ILs with pure cellulose is a subject of interest, however there needs to be further investigations into the use of ILs for processing cellulose.

ILs are perceived as a revolutionary "green" solvent by the chemical industry since it can dissolve cellulose (Swatloski *et al*. , 2002; Pinkert *et al*., 2009; Zhu, 2008; Liebert and Heinze , 2008). Owing to their unique properties and a wide variety of structural variations, ILs have been exploited for shaping of cellulose into fibres, films, sponges, beads and other cellulosic materials (Kosan *et al*., 2008). In addition, ILs are studied intensively for bio-refinery applications, which includes fractionation of lignocellulosic biomass, IL pre-treatment of cellulose for improving enzymatic hydrolysis, and conversion of cellulose dissolved in ILs into mono/ disaccharides, and biofuels. Graenacher, in the 1930's, first discovered that cellulose could be dissolved in molten N-ethylpyridinium chloride salt (Graenacher, 1934). Little attention was paid into this discovery mainly because of the high melting point of this molten salt. In 2002, Swatloski and co-worker's research on ILs showed that some imidazolium-based ILs could dissolve cellulose efficiently at low temperatures (< 100 ^{0}C) without the formation of any derivatives (Swatloski *et al*., 2002).

2.2. Literature review on ILs for biomass processing

Studies performed on different feedstock's showed that ILs changed enzymatic hydrolysis of cellulose after IL pre-treatment with [Emim][OAc] when contrasted with other pre-treatment methods Li *et al*., (2010); Singh *et al*., (2009). Table 2.1 shows the common cations and anions of ionic liquids. It was observed that anions having high hydrogen bond formation ability strongly interact with enzymes causing the conformational change in the cellulose structure (Zhao, 2010).

Table 2.1 Common cations and anions of ILs

Common IL cations		**Common IL anions**	
$[Emim]^+$	$[OH\text{-}emim]^+$	$[BF_4]^-$	$[PF_6]^-$
$[Me_4N]^+$	$[Et_4N]^+$	$[Tf_2N]^-$	$[Dmp]^-$
$[Pr_4N]^+$	$[Bu_4N]^+$	$[MDEGSO_4]^-$	$[MeSO_3]^-$
$[Me_3NH]^+$	$[Et_3NH]^+$	$[EtSO_4]^-$	$[CF_3OO]^-$
$[Bu_3NH]^+$	$[MTOA]^+$	$[Dca]^-$	$[Dhp]_2^-$
$[Btma]^+$	$[Chol]^+$	$[OTf]^-$	$[MeSO_4]^-$
$[Bmim]^+$	$[Hmim]^+$	$[TMA]^-$	$[CF_3OO]^-$
$[Mmim]^+$	$[Bzmim]^+$		
$[Omim]^+$	$[Pmim]^+$		
$[Bbim]^+$	$[BmPyr]^+$		
$[Gua]^+$			

Vitz *et al*., (2009) investigated cellulose dissolution in a variety of imidazolium based ionic liquids. Their findings revealed that imidazolium based ionic liquids with chloride as the counter ion possessing even numbered alkyl chains dissolved cellulose better than those having odd numbered alkyl chains. However, this effect could not be observed in those having bromide as the counter ion. The water content of the ionic liquids prior to dissolution was measured since water uptake of an ionic liquid has been a critical factor that adversely affected cellulose dissolution. Although [Emim][OAc] was the most

hygroscopic IL, it gave cellulose dissolution of 8 % (w/w) at 100 ^{0}C within one hour. Imidazolium based ILs with chloride and acetate were effective in cellulose dissolution, however the colour change during their interaction with cellulose (from transparent to dark brown) was associated with cellulose degradation.

Sun *et al*., (2009) studied the complete dissolution and delignification of softwood (southern yellow pine) and hardwood (red oak) in 1-ethyl-3-methylimidazolium acetate [Emim][OAc] after mild grinding. Red oak showed higher and faster dissolution than southern yellow pine. The regenerated cellulose-rich materials and lignin fractions were characterized and compared with the original wood samples and biopolymer standards. For pine, 59 % holocellulose (cellulose + hemicellulose) could be recovered in the reconstituted material, whereas 31 % and 38 % of the original lignin was recovered. Thus, partial separation of wood components is possible with [Emim][OAc].

Shill *et al*., (2010) described the use of aqueous kosmotropic salt solutions to form a three-phase system that precipitates the biomass, forming an IL-rich and salt-rich phases. The three phase system consisting of [Emim][OAc], water and cellulose was studied. The process was reported to partially separate the lignin from the cellulose in Miscanthus, and enhanced the rate of hydrolysis of the precipitated cellulose. They then concluded that this process was more rapid and gave higher yields of cellulose conversion to glucose as compared to the cellulose obtained from biomass pre-treated with IL and precipitated with water. Addition of kosmotropic salt during the precipitation resulted in partial delignification of the biomass, which makes the substrate more accessible, enhancing the enzymatic hydrolysis.

In the study conducted by da Silva *et al*., (2011), sugarcane bagasse was pre-treated using six ionic liquids (ILs) at an IL/biomass loading of 20:1 (wt. %). On using 1-ethyl-3-methylimidazolium acetate [Emim][OAc] at 120 ^{0}C for 120 min, 20.7 % of the bagasse components remained dissolved and enzymatic saccharification experiments resulted on 80 % glucose yield within 6 h, which evolved to over 90 % within 24 h. Moreover,

SEM analysis of the precipitated material indicated a drastic lignin extraction and the exposure of nano-scopic cellulose micro-fibrils with widths of less than 100 nm. The ability of [Emim][OAc] to simultaneously increase the surface area and to decrease the biomass crystallinity was deemed responsible for the improved bagasse enzymatic saccharification rates.

Yoon *et al*., (2011) evaluated and compared three pre-treatment methods for sugarcane bagasse namely ionic liquid, acid and alkali pre-treatments. [Emim][OAc] was selected as the most suitable IL for SCB pre-treatment. Although a lower yield of reducing sugar was obtained [Emim][OAc] required the least energy to pre-treat 1 kg of SCB. Moreover the percentage of SCB loss during [Emim][OAc] pre-treatment was the lowest and the regenerated bagasse had the lowest crystallinity index with the most amorphous structure when compared to other pre-treatment methods.

The separation procedure developed for the biomass components presented by Muhammad *et al*., (2011) showed a possible greener alternative to extract useful biomass components for various applications. Zhang and Zhao, (2010) studied the production of 5-hydroxymethylfurfural (HMF) and furfural from lignocellulosic biomass in [Bmim][Cl] and [Bmim][Br] in the presence of $CrCl_3$ under microwave irradiation. Corn stalk, rice straw and pine wood treated under typical reaction conditions were reported to have produced HMF and furfural in yields of (45 - 52 %) and (23 - 31 %) respectively within 3 minutes. This method should be valuable to facilitate energy efficient and cost effective conversion of biomass into biofuels and platform chemicals.

Muhammad *et al*., (2012) studied the dissolution of bamboo biomass using 1-ethyl-3-methylimidazoilum glycinate [Emim][Gly], 1-ethyl-3-methylimidazolium trifluoroacetate [Emim][TFA] and choline propionate synthesized in their laboratory. They observed that [Emim][Gly] was capable of dissolving the biomass completely. They regenerated the dissolved biomass using acetone + water reconstitute solvent and characterized with FTIR spectroscopy, X-ray diffraction and SEM, and they compared the results to

untreated bamboo biomass. They found that the regenerated biomass has more macrostructure and the structure of its cellulose changed from type I to type II during the dissolution and regeneration process.

The study conducted by Qiu *et al.,* (2012) evaluated the effect of 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) for the pre-treatment of energy cane bagasse in terms of biomass composition, structural changes and enzymatic digestibility. Energy cane bagasse was pre-treated with [Emim][OAc] 5 % (w/w) at 120 ^{0}C for 30 min followed by hydrolysis with commercially available enzymes, *Spezyme CP* and *Novozyme 188*. IL-treated energy cane bagasse resulted in significant lignin removal (32.0 %) with slight glucan and xylan losses (8.8 % and 14.0 %, respectively), and exhibited a much higher enzymatic digestibility (87.0 % and 64.3 %) than untreated (5.5 % and 2.8 %) or water-treated (4.0 % and 2.1 %) energy cane bagasse in terms of both cellulose and hemicellulose digestibility, respectively. The enhanced digestibility of IL treated biomass was attributed to delignification and reduction of cellulose crystallinity.

The study conducted by Yoon *et al*., (2012) examined the effect of temperature, time and solid loading on the reducing sugar (RS) yield from [Emim][OAc] pre-treated bagasse. Good correlations between the experimental and predicted RS yields were found. The optimum pre-treatment condition of 145 ^{0}C, 15 min and 14 wt. % solid loading gave 69.7 % of RS. Although changes in the chemical composition of [Emim][OAc] pre-treated sugarcane bagasse (SCB) was not apparent, the pre-treated SCB appeared to have a more porous and less crystalline structure which is a desired feature for subsequent solid state fermentation and enzymatic hydrolysis step. The content of amorphous cellulose increased significantly after [Emim][OAc] pre-treatment which was confirmed by FTIR analysis. Good performance of [Emim][OAc] in enhancing the RS yield and modifying the structure and morphology of the pre-treated SCB rendered this pre-treatment process feasible. Due to the non-corrosive property of [Emim][OAc], cost saving can also be achieved in the process without the usage of expensive corrosion resistant equipment.

Karatzos *et al*., (2012) studied three ILs, [Bmim][Cl], [Emim][Cl] and [Emim][OAc] which were used to dissolve/ pre-treat and fractionate sugarcane bagasse at temperatures greater than 130 ^{0}C and then precipitated by the addition of an anti -solvent. In all the three ILs studied, [Emim][OAc] gave the best saccharification yield, material recovery and delignification. The effects of [Emim][OAc] pre-treatment showed a resemblance to aqueous alkali pre-treatments, while those of [Emim][Cl] and [Bmim][Cl] showed a resemblance to aqueous acid pre-treatments. The use of imidazolium IL solvents with shorter alkyl chains resulted in accelerated dissolution, pre-treatment and degradation.

Ding *et al*., (2012) performed density functional theory (DFT) calculations and atoms in molecules (AIM) theory to investigate the mechanism of cellulose dissolution and regeneration in [Emim][OAc] and 1,4-dimethoxy-β-D-glucose (Glc). The theoretical results showed that [Emim][OAc] forms strong hydrogen (H-bonds) with hydroxyl groups of the cellulose system which showed that the interaction of IL + Glc is stronger than the Glc with Glc interaction. Further research suggested that the H-bonds between cellulose and IL were weakened or destroyed by the addition of water. The experimental results prove that cellulose can be readily reconstituted from the [Emim][OAc] based cellulose solution by the addition of water, and the crystalline structure of cellulose is changed from cellulose I to cellulose II. The theoretical results agree well with the experimental results.

Keskar *et al*., (2012) undertook an analysis of Attenuated Total Reflectance (ATR)-FTIR data obtained from dissolution of sugarcane bagasse in two phosphonium ILs, i.e. tetradecyl (trihexyl) phosphonium chloride [P66614][Cl] and tributyl (methyl) phosphonium methylsulfate [P4441][$MeSO_4$]. Absorption bands related to cellulose, lignin, and hemicellulose dissolution monitored in situ in biomass-IL mixtures indicated that lignin dissolved in both ILs. The quantitative measurement of lignin dissolution in phosphonium ILs based on absorbance at 1510 cm^{-1} which demonstrated utility in understanding the chemical reactions taking place in biomass-IL mixture.

Casas *et al*., (2012a) studied the dissolution of *Pinus radiata* and *Eucalyptus globulus* woods in imidazolium-based ILs: [Bmim][OAc], [Emim][OAc], [Emim][Cl], [Bmim][Cl] and [Amim][Cl] using microwave radiation. All ILs were reported to be able to dissolve wood. Lignin was regenerated successfully from the wood solutions in the ILs with chloride anions. FTIR and NMR analysis of regenerated lignin were reported to have shown the absence of residual sugars.

Qiu and Aita, (2013) studied the effect of multiple recycled ILs on the pre-treatment of ECB for enzymatic hydrolysis. The enzymatic digestibility of [Emim][OAc] treated biomass decreased with increasing numbers of IL recycles. Decreasing pre-treatment temperature from 120 ^{0}C to 100 ^{0}C and extending the reaction time from 0.5 h to 2 h resulted in significant improvements in the pre-treatment efficiency of recycled [Emim][OAc] on energy cane, which resulted in better glucan and xylan recoveries (> 90 %), and less ionic liquid decomposition at a lower temperature. This study demonstrated that the recycling of [Emim][OAc] for energy cane bagasse pre-treatment has potential in future industry application.

Haykir *et al*., (2013) studied the pre-treatment of cotton stalk with several ionic liquids: 2-hydroxy ethyl ammonium formate [HEAF], [Amim][Cl], [Bmim][Cl], [Emim][Cl] and [Emim][OAc] in order to enhance the enzymatic accessibility of the lignocellulosic feedstock. [Emim][OAc] was the most effective among all the ILs, regarding digestibility and structural changes in cotton stalk samples. Digestibility was 65 % for [Emim][OAc] pre-treated cotton stalk after 72 hours of enzymatic hydrolysis, which was 9-fold higher compared to untreated cotton stalk. HEAF pre-treatment resulted in enzymatic digestibility of 26 %, which was similar to the digestibility attained by [Amim][Cl] and [Emim][Cl] pre-treatments. [Emim][OAc] was reported to have maintained its effectiveness as a pre-treatment agent upon recycling. It was recycled 3 times and showed no change in terms of hydrolysis of pre-treated samples.

In the study conducted by Jiang *et al*., (2013), a combination of dilute acid and ionic liquid pre-treatment of sugarcane bagasse for glucose by enzymatic saccharification was investigated. Dilute sulfuric acid was able to remove most of the hemicellulose which were successfully used for the production of 2.3-butanediol with a relatively high yield (36.1 %). The regenerated solid residue was after acid pre-treatment was further pre-treated using ILs to decrease its crystallinity for subsequent enzymatic saccharification. The combination of dilute acid and IL resulted in significant higher glucose yield (95.5 %) in enzymatic saccharification, which was more effective than using dilute acid or IL alone.

Bian *et al*., (2014) studied the effect of [Emim][OAc] pre-treatment enzymatic hydrolysis of sugarcane bagasse cellulose in terms of the changes in physical and chemical structure. They carried out the pre-treatment at mild temperatures (90 ^{0}C). It was found that the regenerated cellulose experienced increased glucose content from (80 - 83 %) to (91.6 - 92.8 %), an increase in the degree of polymerization from (974 - 1039) to (511 - 521), a crystal formation from cellulose I to cellulose II and an increase of surface area during pre-treatment. Their results suggested that the pre-treatment led to an effective disruption of cellulose for subsequent enzyme hydrolysis as evidenced by a high glucose yield of 95.2 %.

Ogura *et al*., (2014) investigated the novel ionic liquid pre-treatment by changing the pre-treatment solvent and the anti-solvent. A mixture of IL, acid and ferric oxide (Fe^{3+}) ion was most effective for pre-treatment, and an acetone-water mixture was also most effective for the precipitation of biomass. These optimized pre-treatment combinations attained a higher degree of glucose released from the pre-treated biomass. The amount of cellulose was concentrated from 36 % up to a level of 84 % of the insoluble fraction by the optimized pre-treatment. Based on this result, it was assumed that the extraction of the lignin fraction from the biomass into an anti-solvent solution was attained. The optimized pre-treatment was applied to the enzymatic hydrolysis of Japanese cedar at high-solid biomass loading, and 110 g/L of glucose production was attained. In addition,

the ethanol fermentation with this hydrolysed solution by Saccharomyces cerevisiae achieved 50 g/L ethanol production, and this yield reached 90 % of the theoretical yield.

Wang *et al*., (2014) investigated the dissolution of lignin in dialkylimidazolium based ionic liquid (IL) water mixtures (40 wt. % -100 wt. % IL content) at 60 ^{0}C. It was found that for all the IL-water mixtures except 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4C_1im]BF_4$), the maximum lignin solubility could be achieved at 70 wt. % IL content. Lignin solubility in IL-water mixtures with different cations followed the order 1-butyl-3-methylimidazolium $(C_4C_1im)^+$ > 1-hexyl-3-methylimidazolium $(C_6C_1im)^+$ > 1-ethyl-3-methylimidazolium $(C_2C_1im)^+$ > 1-octyl-3-methylimidazolium $(C_8C_1im)^+$ > 1-butyl-3-ethylimidazolium $(C_4C_2im)^+$ >1-butyl-3-propylimidazolium $(C_4C_3im)^+$. For IL mixtures with different anions, lignin solubility decreased in the following order: methane-sulfonate $(MeSO_3)^-$ > acetate $(MeCO_2)^-$ > bromide $(Br)^-$ > dibutylphosphate $(BP)^-$.

In the study conducted by George *et al*., (2015), a number of ionic liquids were synthesized with the goal of optimizing solvent cost and stability whilst demonstrating promising processing potential. They used inexpensive feed stocks such as sulfuric acid and simple amines which were combined into a range of protic ionic liquids containing the hydrogen sulfate $[HSO_4]^-$ anion. The performance of these ionic liquids was compared to a benchmark system containing the IL 1-ethyl-3-methylimidazolium acetate [Emim][OAc]. The highest saccharification yields were observed for triethylammonium hydrogen sulfate $[HNEt_3][HSO_4]$, which was 75 % as effective as the benchmark system.

Teh *et al*., (2015) studied the effect of ionic liquids upon the mechanical and biochemical integrity of macadamia nut shells (from Macadamia integrifolia). Generally whole macadamia nuts in shell are notoriously difficult to crack, and the Australian macadamia nut shells used in this study required 2240 ± 430 N of force to crack. Ionic liquids were screened for their solubility values, with 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) able to dissolve 5.5 ± 0.5 wt. % macadamia nut shells. Treatment

with small quantities of [Emim][OAc] resulted in weakened whole nut in shells that could be cracked with only ca. 46 % of the displacement (0.67 ± 0.16 mm), ca. 34 % of the force (760 ± 240 N) and ca. 15 % of the energy (0.25 ± 0.10 J per shell) relative to no treatment. Further treatment by dissolution and precipitation of macadamia nut shell, followed by enzymatic hydrolysis with cellulase, resulted in the release of 80 ± 15 % of the expected glucose content, relative to 1.3 ± 1.0 % before any pre-treatment.

Lau *et al*., (2015) evaluated aqueous solutions of tetrabutylphosphonium hydroxide as pre-treatment media for rice husks, prior to sulfuric acid hydrolysis or cellulase enzymatic hydrolysis at varied water, tetrabutylphosphonium hydroxide, rate of delignification, silica, lignin and cellulose solubility ratios. Pre-treatment with 60 wt. % hydroxide dissolved the rice husk and the regenerated material was heavily disrupted. Sulfuric acid hydrolysis of 60 wt. % treated samples yielded the highest amount of glucose per gram of rice husk. Solutions with good lignin and silica solubility but only moderate to negligible cellulose solubility (10 to 40 wt. % hydroxide) were equally effective as pre-treatment media for both acid and enzymatic hydrolysis. However, pre-treatment with 60 wt. % hydroxide solutions was incompatible with downstream enzymatic hydrolysis. This was due to significant incorporation of phosphonium species in the regenerated biomass, which significantly inhibited the activity of the cellulase enzymes.

2.3. Dissolution of lignin in ILs

One major advantage that ionic liquids exhibit with respect to lignocellulosic biomass processing is their capability to dissolve lignin. In a study reported by Pu *et al*., (2007), several ionic liquids were screened with respect to their effect on solubility of Kraft pulp lignin isolated from softwood. It was shown that anion nature affected lignin solubility; [BMIM] based ionic liquids exhibited lignin solubility in the decreasing order, $MeSO_4$ > Cl > Br > PF_6. [Bmim][$MeSO_4$] was capable of dissolving 344 g/L of lignin at 50 ^{0}C. It was also reported that ionic liquids possessing bulky anions were poor in dissolving lignin.

Pu *et al*., (2007) used the imidazoilum based ILs for the study of dissolution of residual softwood lignin isolated from a southern pine kraft pulp. Table 2.2 shows the solubility of the residual softwood lignin was obtained (Pu *et al*., 2007). They reported a strong temperature reliance of the solvency for methyl sulphate and triflate ILs. The solvency was 6 wt. % in both $[C_1C_1im][MeSO_4]$ and $[C_4C_1im][MeSO_4]$ at 25 ^{0}C, however when the temperature was increased to 50 ^{0}C, the dissolution of lignin increased to 26 wt. % with a similar temperature effect obtained for $[C_6C_1im][OTf]$. It was demonstrated that the methyl sulfate anions are receptive at raised temperatures, especially in the presence of acids, which stimulates ester hydrolysis in the presence of water and/or transesterification reactions with alcohols (Brandt *et al*, 2011; Himmler *et al*., 2006). Therefore, heating lignin in these ionic liquids is most likely to cause condensation reactions between the ionic liquid and lignin and could explain the increment of lignin dissolution with temperature in these ionic liquids.

Table 2.2 Solubility of residual softwood kraft pulp lignin in ionic liquids

Ionic liquid	Temperature (^{0}C)	Solubility (g/L)
$[Mmim][MeSO_4]$	50	344.00
	25	74.20
$[Hmim][CF_3SO_4]$	70	275.00
	50	<10.00
$[Bmim][MeSO_4]$	50	312.0
	25	61.80
[Bmim][Cl]	75	13.90
[Bmim][Br]	75	17.50
$[Bmim][PF_6]$	70-120	Insoluble
$[Bm_2im][BF_4]$	70-100	14.5
$[Bmpy][PF_6]$	70-120	Insoluble

The capacity of $[C_4C_1im]Cl$ to break down lignin was studied using optical lignin evaluation (Kline *et al*., 2010). Generally, higher solubility is possible for lignin than

cellulose and a larger number of ionic liquids can dissolve lignin than cellulose. The solubility of lignin preparations in dialkylimidazolium ionic liquids is recorded in Table 2.3. Similarly as with cellulose, the dissolvability is influenced by the anion, however the hydrogen bond basicity does not have to be as high compared to lignin. Solubility of lignin in ionic liquids not only offers benefits with respect to enhancement of the enzymatic accessibility of cellulose in biomass but also conversion of lignin to high value added products. Binder *et al*., (2009) investigated lignin depolymerization in ionic liquids with catalysts. Cleavage of aryl-alkyl and aryl ether linkages was reported to result with the release of valuable aromatic compounds. Eugenol (phenylpropene), which was used as a model compound instead of an isolated lignin molecule, was depolymerized to guaiacol with 11.6 % of yield and 100 % of conversion in EMIMOTf (1-ethyl-3-methylimidazolium triflate) in the presence of Nafion as catalyst. Depolymerization of eugenol to guaiacol was also found to depend on the anion type. Imidazolium based ionic liquids containing the less basic anions such as triflate performed better than those containing anions with moderate basicity such as chloride, acetate and bromide. Besides, ionic liquids were shown to extract significant amounts of lignin upon pre-treatment of various lignocellulosic feedstocks. The dissolution of pine wood in [Bmim][Cl] at 100 ^{0}C resulted in lignin extraction. Recovered biomass, which was obtained upon acetonitrile addition to the ionic liquid biomass slurry, resembled the pure cellulose samples subjected to dissolution at identical dissolution conditions (Fort *et al*., 2007).

1

[1] Nafion composition: fluorosulfonic acid Nafion polymer on amorphous silica, 10 – 20 % (porous nanocomposite)

Table 2.3 Lignin solubility in immidazolium based ionic liquids. Solubility data were converted from g/L to wt. % where necessary

Ionic Liquid	**Preparation**	**Solubility (wt. / wt. %)**	**Temperature (^{0} C)**	**Reference**
$[C_2C_1im][MeCO_2]$	Kraft lignin (Indulin AT)	30	90	(Fu *et al*., 2010)
$[C_1C_1im][MeSO_4]$	Residual softwood lignin	26	50	(Pu *et al*., 2007)
$[C_4C_1im][MeSO_4]$	Residual softwood lignin	26	50	
$[C_6C_1im][OTf]$	Residual softwood lignin	22	70	
$[C_4C_1im][Cl]$	Residual softwood lignin	13	75	
$[C_4C_1im][Cl]$	Kraft lignin (Indulin AT)	10	90	(Lee *et al*., 2009)
$[C_4C_1im][Br]$	Residual softwood lignin	14	75	(Pu *et al*., 2007)
$[C_4C_1C1im][BF_4]$	Residual softwood lignin	12	100	
$[C_4C_1im][BF_4]$	Kraft lignin (Indulin AT)	4	90	(Lee *et al*., 2009)
$[C_4C_1im][PF_6]$	Kraft lignin (Indulin AT)	1	90	
$[C_4C_1im][PF_6]$	Residual softwood lignin	0	120	(Pu *et al*., 2007)
$[C_4C_1pryr][PF_6]$	Residual softwood lignin	0	120	

Lee *et al*., (2009) examined delignification of maple wood flour samples. It was reported that 40 % of delignification by [Emim][OAc] was sufficient for attaining at least 90 % of cellulose digestibility for the maple wood flour. [Emim][OAc] preserved its effectiveness upon reuse for four times with respect to delignification of maple wood flour since similar percentages of lignin extraction were achieved upon each [Emim][OAc] reuse.

Fu *et al*., 2010 compared the effect of BMIMCl and [Emim][OAc] on lignin extraction from triticale straw. [Emim][OAc] resulted in better delignification in which almost 52 % of alkali insoluble lignin was extracted upon triticale straw dissolution at 150 ^{0}C for 90 minutes. Cellulose digestibility of almost 95 % was achieved which was in accordance with the percentage lignin extracted.

The study by Fu *et al*., (2010) reported [Emim][OAc] as an effective solvent for lignin extraction from triticale straw, flaxsheaves, and wheat straw. Lignin extraction was examined by complete dissolution, then multistep processes to isolate lignin using six different ILs. It was observed that [Emim][OAc] displayed better efficiency compared to [Bmim]Cl and four different protic ILs (PILs). Although the ILs [Emim][OAc] and [Bmim]Cl could dissolve cellulose, hemicellulose, and Kraft lignin, the PILs could dissolve only lignin. When testing the actual biomass, the PILs gave a very poor lignin extraction ability, whereas [Emim][OAc] extracted approximately twice more lignin than [Bmim]Cl. A maximum of 52.7 % of the original lignin could be extracted and subsequently isolated from triticale straw using [Emim][OAc] after a 90 min exposure at 150 ^{0}C.

According to Stark *et al*., (2010), Beech lignin was oxidatively cleaved in four ILs, including 1-ethyl-3-methylimidazolium methylsulfonate, ([C_2mim][$MeSO_3$]), 1-ethyl-3-methylimidazolium ethylsulfonate ([C_2mim][$EtSO_3$]), 1-ethyl-3-methylimidazolium trifluoromethylsulfonate ([C_2mim][CF_3SO_3]), and 1-methyl-3-methylimidazolium methyl-sulfate ([C_1mim][$MeSO_4$]), to give phenols, unsaturated propyl-aromatics, and fragrant aldehydes with $Mn(NO_3)_2$ utilized as catalyst, in blend with [C_2mim][CF_3SO_3]. It ended

up being the best reaction framework. By modifying the reaction conditions, the selectivity of the procedure could be moved from syringealdehyde as the primary product to 2, 6-dimethoxy-1, 4-benzoquinone (DMBQ). DMBQ could be confined as a pure substance by a straightforward extraction–crystallization process described by Stark *et al*., (2010). Alcell and pop lignin were disintegrated in 1-ethyl-3-methylimidazolium diethylphosphate ([C_2min][DEP]) and hence oxidized by O_2 in the presence of a few transition metal catalysts Zakzeski *et al*., (2010). $CoCl_2.6H_2O$ in [C_2min][DEP] demonstrated especially compelling results for the oxidation.

Li *et al*., (2011) noted that significantly more rapid dissolution of bio-mass and more effective lignin removal were observed when using [Emim][OAc] and heating the solution above the glass transition temperature of lignin (150 ^{0}C); however, significant IL decomposition was also observed. Similarly, Pinkert *et al*., (2011) found that using the IL [Bmim][OAc], the lignin extraction efficiency increased with higher temperatures and extended contact times, as also reported by Tan *et al*., (2009); Fu *et al*., (2010) and Hou *et al*., (2012) utilizing [Emim][ABS], [Emim][OAc], and [Ch][AA] respectively. However, as the application of high temperature and long extraction time can lead to lignin side chain reactions and IL decomposition and is related to high energy consumption, the alternative approach of applying multistep treatments of wood by repeating the same extraction step several times under milder conditions can also increase the extraction efficiency. Pinkert *et al*., (2011) reported that 39 % lignin was extracted after 4h at 100 ^{0}C in contact with [Bmim][OAc], after two successive treatments of the same pine wood flour for 2h each, the cumulative extraction efficiency was 51 %.

As water present in either the IL or the biomass can interfere with the ability of the IL to form strong hydrogen bonds between cellulose hydroxyl groups and the anions (i.e. Cl^-) of the IL, solubility is dramatically decreased by the presence of water as shown by Tan *et al*., (2009) and Pinkert *et al*., (2009). Similar observations have been made for the selective extraction of lignin and lignin extraction by [Bmim][OAc] ILs decreasing from 38 % with 0.5 wt. % H_2O down to 25 % with15 wt. % H_2O, (Pinkert *et al*., (2011)). It is

noted here that biomass typically contains large quantities of water unless predried, and it is typically predried for virtually all IL studies. Only chemical or high-temperature techniques appear to be relatively immune, such as the chemical [Emim][XS]/Na[XS] system, which required some water for hydrolytic fragmentation, (Tan *et al*., (2009)). According to Brandt *et al*., (2011) the ILs [Bmim][HSO_4] and [Bmim][$MeSO_3$] were effective for the pre-treatment of pine and willow in the presence of 20 % (v/v) water, before enzymatic treatment of the isolated cellulose rich component. However, given that lignin dissolves in the IL/water phase, significant chemical fragmentation of lignin is probable in these systems (George *et al*., 2011). Deidericks *et al*., (2012) obtained similar results for [Bmim][$MeSO_4$]/H_2SO_4 mixtures with bagasse.

Pinkert *et al*., (2011) investigated the selective extraction of lignin from soft and hardwood biomass under mild conditions without dissolving or degrading the cellulose by utilizing food additive derived ILs based on the acesulfamate anion ([Emim][OAc] and [Bmim][OAc). They also studied the effect of various process parameters on lignin extraction and observed that extraction temperature, time, water content of IL,wood load, wood particle size, wood species, solvent composition, and IL cation species all had a significant effect on the extraction efficiency. The addition of a co-solvent such as dimethylsulfoxide (DMSO) was also beneficial, swelling the cellulosic component and increasing lignin extraction from 38 up to 56 % (T = 100 ^{0}C, t = 2 h).

Hou *et al*., (2012) used a similar method by Fu *et al*., (2010) to study selective lignin extraction from rice straw using more environmentally friendly cholinium amino acids ILs ([Ch][AA]). Up to71.4 % lignin could be extracted by contact with a lysinate- based IL for 24 h at 130 ^{0}C. Cellulose hydrolysis could be increased from 20.4 % for untreated material, up to 86.7 % conversion after IL pretreatment. [Emim][OAc] has been found to dissolve rice hulls and precipitation demonstrated 100 % lignin removal (110 ^{0}C, 8h). Interestingly, and incontrast to all other biomass investigations using the ILs [Amim][Cl] and [Hxmim]Cl, these two ILs were unable to remove any lignin from the naturally silica-shielded ricehulls.

Wei *et al*., (2012) reported the enhanced dissolution of legume straw in [Bmim][Cl] mixed with 20 wt. % water (29.1 wt. % dissolved straw) when compared with [Bmim][Cl] alone (9.8 wt. % dissolved straw). However, the chemical and physical properties of the isolated cellulose-lignin residue were significantly changed, and no mechanism for the observed 'cracking' has been proposed yet.

Deidericks *et al*., (2012a) conducted a study on the fractionation of sugarcane bagasse using a combined process of dilute acid and ionic liquid namely 1-ethyl-3-methylimizazolium acetate [Emim][OAc] and/ 1-butyl-3-methylimidazolium methyl sulfate [Bmim][$MeSO_4$]. The isolation of the constituents from the reaction mixture was done using anti-solvents acetone and sodium hydroxide water mixtures. Delignification was enhanced by NaOH, although resulting in impure product streams. Xylose pre-extraction (75 % w/w) by dilute acid pretreatment prior to IL pretreatment improved the lignin purity after anti-solvent separation. Fractionation efficiency of the combined process was maximised (84 %) by IL treatment at 125 ^{0}C for 120 min, resulting in 80.2 % (w/v) lignin removal and 76.5 % (w/w) lignin recovery. Ionic liquids achievd similar degrees of delignification, although fully digestible cellulose rich solids were produced only by [Emim][OAc] treatment.

Grasvik *et al*., (2014) evaluated four imidazolium-based ILs ([$C{=}C_2C_1$im][$MeCO_2$], [C_4C_1im][$MeCO_2$], [C_4C_1im][Cl], and [C_4C_1im][HSO_4]) who are well known for their capability to dissolve lignocellulosic species. The ILs were synthesized and used for pre-treatment of seven cellulosic, hemicellulosic and lignocellulosic substrates (crystalline and amorphous) which included hybrid aspen and Norway spruce prior to enzymatic hydrolysis. The best results based on the saccharification efficiency, were achieved with [C_4C_1im][Cl] for cellulosic substrates and with the acetate-based ILs for hybrid aspen and Norway spruce. After pre-treatment with acetate-based ILs, the conversion of glucan to glucose in recalcitrant softwood lignocellulose reached similar levels as obtained with pure crystalline and amorphous cellulosic substrates. IL pre-treatment of lignocellulose resulted in sugar yields comparable with that obtained with

acidic pre-treatment. Heterogeneous dissolution with $[C_4C_1im][HSO_4]$ gave promising results with aspen, the less recalcitrant of the two types of lignocellulose included in the investigation.

Mohtar *et al*., (2015) studied the dissolution of oil palm biomass (OPB) in 1-butyl-3-methylimidazolium chloride ([bmim][Cl]), followed by the lignin extraction through the CO_2 gas purging prior to addition of aluminium potassium sulfate dodecahydrate $[AlK(SO_4)_2 \cdot 12H_2O)]$. The lignin yield, YL (% wt.) was found to be dependent of the types of OPB observed for all precipitation methods used. The lignin recovery, RL (% wt.) obtained from CO_2-AlK $(SO_4)_2 \cdot 12H_2O$ precipitation was, however dependent on the types of OPB, which contradicted that of the acidified H_2SO_4 and HCl solutions of pH 0.7 and 2 precipitations. Only about 54% of lignin was recovered from the OPB.

Wei *et al*., (2015) studied the pulping process of wheat straw in acid N - methyl imidazole hydrosulfate. The pulp yield was stabilized at 50 % under optimum conditions (temperature 130 ^{0}C, liquid-solid ratio 8:1, time 30 min). The ionic liquid was recycled and gave 89.2 % recovery. XRD diffraction analysis of cooked wheat straw pulp fibres indicates that the cooking process is mild without causing damage to the cellulose. Delignification reaction kinetics of the ionic liquid N - methyl imidazole hydrosulfate cooking wheat straw pulping was found to be first order with an activation energy of 10.645 $kJ \cdot mol^{-1}$.

A study by Hart *et al*., (2015) developed a technique for the quantification of lignin solubility in ionic liquids (ranging from 0 - 100 % wt. lignin in ionic liquid). This was used to examine the effect of the anionic and cationic components of ionic liquids on lignin solubility. The cation was shown to have a much more subtle effect on lignin solubility, with planar cations being most effective. The anion was required to have minimum hydrogen bonding basicity value for lignin to dissolve, but after this point the anion effect on the overall lignin solubility was negligible relative to the cation. NMR spectra

suggested an interaction between the solute (likely the planar aromatic portions) and the cation.

Wang *et al*., (2015) studied the thermal decomposition of IL/water pre-treated bamboo (Phyllostachys edulis). [Bmim][Cl] (1-Butyl-3-methylimidazolium chloride) - water and [Bmim][BF_4] (1-Butyl-3-methylimidazolium tetrafluoroborate) - water were used in the pre-treatment process. The ILs + water pre-treatment process affected the composition and the cellulose crystalline structure in bamboo samples. The lignin content and ethanol/benzene extractive was conducive to dissolved in ILs + water mixture. The pre-treated bamboo with [BMIM]Cl and water mixture produced more gaseous products, which were associated with the decomposition rate. The behaviour of more gaseous products and less tar in the thermal decomposition products were attributed to ILs and water pre-treatment process.

Dong *et al*., (2015) carried out the pre-treatment of corn stalk with ILs including 1-butyl-3-methylimidazolium chloride [Bmim][Cl], 1-H-3-methylimidazolium chloride [Hmim][Cl], and 1-(1-propylsulfonic)-3-imidazolium chloride [HSO_3-pmim][Cl] at 70 ^{0}C for 2 h. Pre-treatment by ionic liquids with and without the addition of co-solvent (water) was studied. ILs provided the ideal environment for lignin extraction and enzymatic hydrolysis for corn stalk. [HMIM]Cl and [HSO_3-pmim][Cl] were found to be the most efficient ILs to remove lignin and give the recovery biomass for enzymatic hydrolysis.

2.4. Enzymatic hydrolysis

The major factors that affect the enzymatic hydrolysis of lignocellulosic biomass include substrate (solid) loading, enzyme loading and operational conditions such as temperature and pH (Sun and Cheng, 2002, Alvira *et al*., 2010). Substrate loading determines the amount of sugar released upon enzymatic hydrolysis. The higher the substrate loading the higher the concentration of fermentable sugars released. High fermentable sugar concentration will provide high ethanol concentration during fermentation. Higher ethanol concentration obtained upon fermentation facilitates the product recovery during distillation and reduces the process costs since less energy will be utilized to attain the final ethanol concentration during downstream processing (Wingren *et al*., 2003, Jorgensen *et al*., 2007). Utilization of high biomass loadings can be challenging owing to the mass transfer limitations and presence of high inhibitor concentration during enzymatic hydrolysis (Kristensen *et al*., 2009).

Lu *et al*., (2010) reported elevated glucose concentration and lower cellulose conversions owing to the aforementioned challenges. For instance, the substrate loading for the enzymatic hydrolysis of steam exploded wheat straw varied from 10 to 30 % (w/w). The cellulose conversion was found to decrease much more with an increase in substrate loading for the pre-treated biomass subjected to hydrolysis without being washed prior to enzymatic hydrolysis compared to that of washed steam exploded wheat straw which was found to possess lower concentrations of inhibitors (acetic acid and furfural) than the former one. Besides, the initial viscosity of the hydrolysis solution containing 30 % of substrate (unwashed) loading was found to be 10000 cP, whereas it was 4000 cP for the hydrolysis solution containing 10 % of substrate loading. This viscosity difference could have also created mass transfer problems during enzymatic hydrolysis. In another study, the negative impact of increased substrate loadings on cellulose digestibility up to 20 % (w/w) was linked to the presence of inhibitors, whereas the decrease in cellulose digestibility was related to mass transfer limitations at substrate loadings higher than 20 % (w/w) (Hodge *et al*., 2008).

Enzymes contribute significantly to the process costs in cellulosic ethanol production therefore the loading during hydrolysis of biomass is an important factor (Kumar and Wyman, 2009a). The presence of residual lignin at high fractions in biomass structure will require higher enzyme loadings owing to the non-productive adsorption of cellulases on lignin (Chang and Holtzapple, 2000, Van Dyk and Pletschke, 2012). In this regard, the utilization of surfactants during the enzymatic reaction has been offered and been very effective to minimize the interaction of cellulases with lignin, thus, lowering the enzyme loadings (Yang and Wyman, 2006). Though cellulose crystallinity plays a significant role in determination of initial hydrolysis rates of cellulose (Hall *et al*., 2010), no data related to any decisive effect of cellulose crystallinity on enzyme loading has been reported. In addition to lignin content of the biomass, the cellulose surface area accessible to enzymatic attack was also found to be a decisive factor for enzyme loading (Sathitsuksanoh *et al*., 2010, Rollin *et al*., 2011). Bamboo, which was subjected to COSLIF (cellulose solvent-and organic solvent-based lignocellulose fractionation) pre-treatment, was investigated in terms of cellulose accessibility to cellulase (m^2/g). Pre-treated bamboo, which holds 33 fold higher cellulose accessibility to cellulase compared to its native structure, was hydrolysed at very low cellulase loadings; as low as 1 FPU/ g glucan and resulted with almost identical cellulose digestibility (88 % at t = 72 h) with the samples hydrolysed at higher enzyme loadings (Sathitsuksanoh *et al*., 2010).

A study by Rollin *et al*., (2011) compared COSLIF and aqueous ammonia pre-treatments with respect to their effects on the structural changes in switch grass and enzymatic accessibility of the biomass. COSLIF pre-treated switch grass possessed higher cellulose accessibility to cellulase (16 fold higher than that of untreated biomass) compared to the biomass subjected to aqueous ammonia pre-treatment. Thus, it was possible to hydrolyse COSLIF pre-treated biomass at low enzyme loadings such that 3 FPU/g glucan was sufficient to attain cellulose digestibility of switch-grass over 80 % within 24 hours of enzymatic hydrolysis. Cellulose digestibility of aqueous ammonia pre-treated switch grass at 3 FPU/ g glucan of enzyme loading was only 58 % even in the presence of BSA (bovine serum albumin) blocking.

2.4.1. Cellulose degrading enzymes

Cellulase is one of the major enzymes which play a significant role in the conversion of lignocellulosic biomass to ethanol. The potential producer microorganisms of cellulose degrading enzymes are the fungi namely, *Trichoderma reseei* and *Aspergillus niger* (Zhang and Lynd, 2004). *Trichoderma reseei* has been reported to secrete the endoglucanases; Cel7B, Cel5A, Cel12A, Cel61A and Cel45A, exoglucanases; Cel7A, Cel6A as well as a few xylanases and β-xylosidases. Additionally, β-glucosidase produced by *Aspergillus niger* has been utilized as a substantial supplementary to the celluloytic enzymes specified above.

Cellulases are composed of a variety of enzymes, endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4- β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and β-glucosidases or β -glucoside glucohydrolases (EC 3.2.1.21) (Lynd *et al*., 2002). Each enzyme acts on different regions of the cellulose with a synergy that facilitates the degradation or hydrolysis of cellulose (Lynd *et al*., 2002, Van Dyk and Pletschke, 2012). Endoglucanases generate different lengths of oligosaccharides by acting on the random internal amorphous regions of the cellulose chain. Exoglucanases, which target on the crystalline regions of the cellulose, hydrolyse the reducing and non-reducing ends of the polymer and liberate either glucose (cellodextrinases) or cellobiose (cellobiohydrolases). Furthermore, β–glucosidases hydrolyse soluble cellobiose to generate glucose (Lynd *et al*., 2002, Andric *et al*., 2010).

2.4.2. Literature review on enzymatic hydrolysis of IL pre-treated biomass

Bena-Marie *et al*., (2008) studied the impact of IL anions on lipase movement in the enzymatic response of flavonoids with long chain unsaturated fats, and found that IL containing the Tf_2N^-, PF_6^-, and BF_4^- anions were good response media. Wolski *et al*.,

(2011) reported *Trichoderma reesei cellulase* and *β-glycosidase* held action in ILs, [mmim][dmp] and [emim][lactate].

Tavares *et al*., (2008) reported the action of laccase in ILs, [emim][$MDEGSO_4$], [emim][$EtSO_4$], and [emim][$MeSO_3$], and discovered that [emim][$MDEGSO_4$] gives high action. Nonetheless, in another study higher chemical movement was seen in the event of IL having high nucleophilic anion, which repudiates the prior perceptions. These opposing results may be on the grounds that the different elements administer the enzymatic responses.

Baker *et al*., (2010) showed that the use of [bmim][Cl] as a medium to the reaction mixture reduces the thermal stability of green fluorescent protein (GFP) permitting the protein to loosen at lower temperatures than in aqueous solutions. Noritomi *et al*., (2011) investigated the thermal stability and action of lysozyme in the presence of [emim][BF_4], [emim][Tf_2N], and [emim][Cl], and discovered very low activity in the case of [emim][Cl] than that of [emim][BF_4] and [emim][Tf_2N]. Weingartner *et al*. (2010) reported a destabilization impact of [chol][Cl] and [bmim][Br] on the model protein *RNase A*. Dabirmanesh *et al*. (2011) observed that [bmim][Cl] and [hmim][Cl] reduced both enzymatic stabilities and activities of α-amylase from *B. amyloliquefaciens* and *B. licheniformis.*

The study by Çetinkol *et al*., (2010) on the impact of ionic liquid pre-treatment on *E. globulus* showed structural changes at the molecular level in the cell wall, providing new insight into the mechanism by which an ionic liquid acts on the biomass. The aim of that study was to evaluate the effect of compositional differences of three mutants of *E. globulus* characterized by high (H-SG), moderate (M-SG) and low (L-SG) on the lignin S/G ratio), and cellulose hydrolyzability before and after ionic liquid treatment.

Nancharaiah and Francis (2015) studied the effect of 1-ethyl-3-methylimidazolium acetate on bacteria (anaerobic *Clostridium sp*. and aerobic *Pseudomonas putida*). Bacterial growth was stimulated at up to 2.5 g L^{-1} and inhibited at > 2.5 g 25 L^{-1} of [EMIM][OAc]. The growth of *Clostridium sp*. and *P. putida* were higher by 0.4 and 4-fold respectively, in the presence of 0.5 g L^{-1} [EMIM][Ac]. Assessment of the effect of [EMIM][OAc] under different growth conditions showed that the hormesis of [EMIM][OAc] was mediated *via* regulation of medium pH. Hormetic effect of [EMIM][OAc] was evident only in mediam with poor buffering capacity and in the presence of a fermentable substrate as the carbon source. The hormetic effect of [EMIM][OAc] on bacterial growth is most likely associated with the buffering capacity of acetate anion.

2

[2] Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by a low dose stimulant or beneficial effect and a high dose inhibitory or toxic effect, Mattson (2008)

CHAPTER **3**

EXPERIMENTAL METHODS

3.1. Compositional Analysis

3.1.1. Theory

Lignocellulosic materials do not contain readily accessible monosaccharides and chemicals but rather polymers which need to be hydrolysed to release the desired compounds. Also, lignocellulosic material is composed of cellulose, lignin and hemicellulose that need to be separated before processing of the biomass. Characterization of the composition of lignocellulosic materials is critical in providing information for power, fuels and other product research using biomass. Established methods for the characterization of biomass were originally based on gravimetric and/ or colorimetric analysis. Martens (2000, 2002) indicated that more appropriate methods should be developed for the characterization of biomass. TAPPI Technical Association of Pulp and Paper Industry, http: www.tappi.org/s_tappi/index.asp methods were developed for characterizing wood for use in the pulp and paper industry. It has been shown that these methods are not necessarily applicable for other lignocellulosic residues especially when dealing with herbaceous and grass feed-stocks such as bagasse (Hatfield and Fukushima, 2005).

The exact compositional analysis of biomass feedstocks enables for the determination of reaction yields and changes that occur during the reactions. The approximate carbohydrate content in the biomass is crucial as it is directly proportional to the ethanol yield in biochemical conversion processes (Aden *et al*., 2002). Proteins, ash, natural acids and other non-auxiliary materials are present in small quantities in biomass;

however their presence could be noteworthy in the running of a modern scale bio-refinery.

The composition of different feedstock's can change extraordinarily because of the intricate and heterogeneous nature of biomass. The composition of corn Stover, for instance, has been indicated to be variable, depending on for example, harvest year, environment, and mixture gives glucan, lignin, or xylan amounts of up to 10 % (Templeton, *et al*., 2009; Decker, *et al*., 2007). Biomass feedstock variability influences process costs; therefore powerful, reliable, accurate and high throughput methods are critical in determining the composition of different feed stocks. Table 3.1 – 3.4 illustrates the literature procedures for the compositional analysis of various lignocellulosic biomass feed stocks.

Table 3.1 Procedures for cellulose determination in lignocellulosic biomass

Cellulose		
Method	**Reference**	**Comments**
Mono-ethanolamine	Nelson and Leming (1957)	Gives total cellulose by gravimetric method (lignin and the hemicellulose dissolved and cellulose filtered off and weighed)
Sulfuric acid (H_2SO_4)	Saeman *et al* (1944)	72 % H_2SO_4 hydrolysis for 3 hrs at ambient temperature, dilution to 4 % and 1 hr at 100 ^{0}C. The released glucose measured by UV/Vis.
	Grohmann *et al* (1984)	Based on 64 % H_2SO_4 and 2 hr hydrolysis at ambient temperature. A 2 step dilution and heating was used for complete dissolution. Glucose was measured enzymatically (colorimetric/ fluorometric detection using glucose assay kit)

Source: Walford (2008)

Table 3.2 Procedures for hemicellulose determination in lignocellulosic biomass

Hemicellulose		
Method	**Reference**	**Comments**
Trifluoroacetic acid (TFA)	Fengel and Wenger (1979)	Diluted TFA, otherwise similar to cellulose method (Mono-ethanolamine method)
Hydrochloric acid (HCl)	Moore and Johnson (1967)	Hydrolysis with 30 % HCl followed by conversion of pentosans to furfural and measured by UV/Vis

Source: Walford (2008)

Table 3.3 Procedures for total carbohydrate determination in lignocellulosic biomass

Total Carbohydrate		
Method	**Reference**	**Comments**
Trifluoroacetic acid (TFA)	Fengel and Wenger (1979)	Undiluted TFA used. The reaction times of the method modified for differing levels of lignin. All sugars hydrolysed and measured by HPLC.
Sulfuric acid (H_2SO_4)	Foyle *et al* (2007)	Based on the Grohmann cellulose method (1984) with modification of hydrolysis times. Sugars measured by HPLC.

Source: Walford (2008)

Table 3.4 Procedures for lignin determination in lignocellulosic biomass

Lignin		
Method	**Reference**	**Comments**
Sulfuric acid (H_2SO_4)	Klason (1923)	Gravimetric method in which lignin is isolated as the insoluble material from acid hydrolysis.
	Saeman *et al* (1954)	Gravimetric method based on 74 % acid cellulose hydrolysis method (72 % H_2SO_4 hydrolysis for 3 hrs at ambient temperature, dilution to 4 % and 1 hr at 100 ^{0}C. The released glucose measured by UV/Vis)
Acid detergent Neutral detergent	Van Soest (1963)	A gravimetric method developed for fibre and lignin content in forage samples. Lignin is quantified by solubilizing with H_2SO_4 after extraction with acid or neutral detergent.
Nitrobenzene Acidolysis Thiocidolysis	Lapierre *et al* (1989)	A gravimetric method in which oxidation of lignin using these chemicals gives different degradation products leading to structural analysis. The acetyl bromide method is also known as the DFRC method.
Acetyl bromide	Lu and Ralph (1997)	
Permanganate	Tasman and Berzins (1957)	Based on the oxidation of lignin by the addition of excess $KMnO_4$. Basis of the Kappa number used in the paper industry.
Thioglycolate	Hatfield and Fukushimma (2005)	Relies on complete solubilisation of lignin followed by spectroscopic measurement in solution.

Source: Walford (2008)

3.1.2. Experimental methods for determining compositional analysis of biomass feed-stocks

The compositional analysis of the biomass components was carried out according to the methods described by National Renewable Energy Laboratory (NREL 2008).

3.1.2.1. Moisture and total solids

Biomass contains vast and varying amounts of moisture, which can change immediately when exposed to air therefore the aggregate solids and moisture content of the test material is required for use in all calculations. To be significant, the results of biomass analyses are reported on a dry weight basis. A standard convection oven set at 105 ^{0}C is utilized to determine the moisture content of the material and the total solids are obtained by difference. This procedure is intended for determining the amount of total solids and moisture remaining after oven drying of the biomass sample (known as the dry sample). The results are reported using equation 3.1 and 3.2.

$$\% \text{ Total Solids} = \frac{\text{Weight (dry pan + dry sample)} - \text{Weight (dry pan)}}{\text{Weight sample as recieved}} \text{ x } 100 \quad (3.1)$$

$$\% \text{ Moisture} = 100 - (\% \text{ Total Solids}) \quad (3.2)$$

3.1.2.2. Ash content

The measure of inorganic material (either auxiliary or extractable) in biomass ought to be measured as a major aspect of the total composition. This test method covers the determination of ash, expressed as the percentage of residue remaining after dry

oxidation at 550 - 600 ^{0}C in a furnace. All results are reported relative to the 105 ^{0}C oven dry weight (ODW) of the dry sample using the equations 3.3 and 3.4.

$$\text{ODW} = \frac{\text{Weight (air dry sample) x \% Total Solids}}{100} \tag{3.3}$$

$$\% \text{ Ash} = \frac{\text{Weight (crucible + ash)} - \text{Weight (crucible)}}{\text{Oven dry weight (ODW)}} \text{ x } 100 \tag{3.4}$$

3.1.2.3. Lignin

The procedure uses a two-step acid hydrolysis to fractionate the biomass into lignin simpler compounds that are more easily identified and quantified. The lignin fractionates into acid insoluble lignin (AIL) and acid soluble lignin (ASL). The acid insoluble lignin may also include ash and protein, which must be accounted for during gravimetric analysis. The acid soluble lignin is measured by UV-Vis spectroscopy at 240 nm which is the maximum wavelength for lignin. The weight percent of AIL; ASL and total lignin is calculated using equation 3.5, 3.6 and 3.7 respectively.

$$\% \text{ AIL} = \frac{\text{Weight (crucible + acid insoluble residue)} - \text{Weight (crucible)}}{\text{Oven dry weight (ODW)}} \text{ x } 100 \tag{3.5}$$

$$\% \text{ ASL} = \frac{\text{UV (absorbance) x Volume (filtrate) x Dilution factor}}{\text{Ɛ(absorptivity) x ODW (sample)x Pathlength}} \text{ x } 100 \tag{3.6}$$

$$\% \text{ Total Lignin} = (\% \text{ AIL} + \% \text{ ASL}) \tag{3.7}$$

3.1.2.4. UV/ Vis Spectroscopy

Retention of visible and ultraviolet (UV) radiation is connected with the excitation of electrons from lower to higher energy levels in both molecules and atoms. Since the energy levels of matter are quantized, just light with the exact measure of energy can cause transitions starting with one level then onto the next will be retained. Ultraviolet/visible spectroscopy involves the retention of bright light by a particle creating the advancement of an electron from a ground electronic state to an excited electronic state. Figure 3.1 demonstrates the schematic representation of a normal UV spectrophotometer.

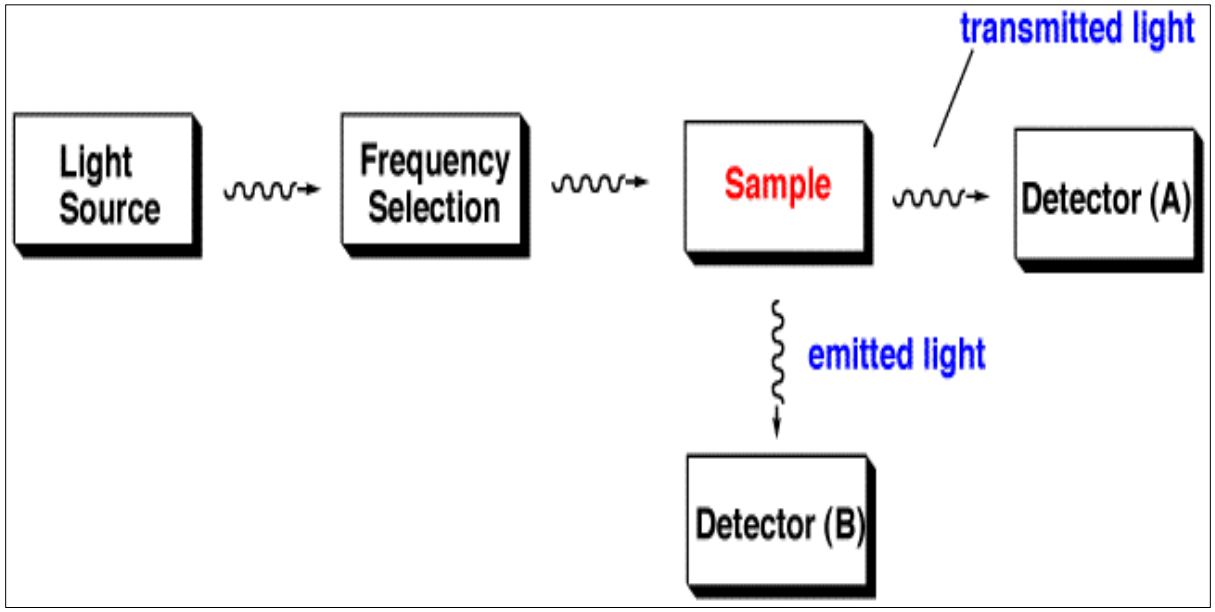


Figure 3.1 Schematic representation of a UV/Vis spectrophotometer

3.1.2.5. High Performance Liquid Chromatography (HPLC)

Liquid chromatography is a separation technique that involves separation, identification and quantification of components that are dissolved in a solution or mixture. It consists of a mobile phase, pump, injector, column and a detector. Figure 3.2 shows a schematic representation of a typical HPLC system. Sugar standards are prepared including D-glucose, D-xylose, D-galactose, L-arabinose and D-mannose etc. The sugar concentrations in the standard are chosen to resemble the ones in lignocellulosic biomass. The sugar standards and sample are then measured by HPLC. A graph of retention time *vs* concentration is plotted for the standards and the concentration of the sample is determined using the equation of the graph.

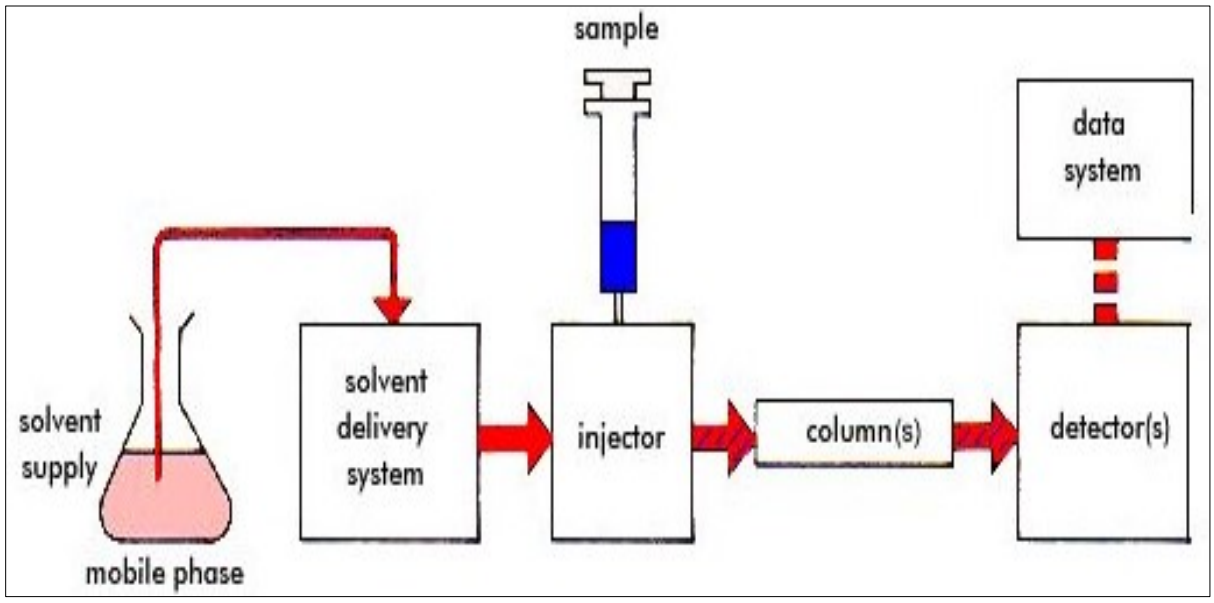


Figure 3.2 Schematic representation of an HPLC system

3.2. Pre-treatment of sugarcane bagasse

The efficient conversion of cellulosic biomass to sugars heavily depends on the effectiveness of the pre-treatment step. During pre-treatment, the blocking layers (lignin and hemicellulose) in the biomass are removed/ disrupted, and the cellulose becomes accessible to enzymes for hydrolysis. Different pre-treatment methods have been developed, varying fundamentally from each other in terms of reaction conditions, process proficiency, complexity and impact on subsequent enzyme and microbial processes. The separation of the three main components of lignocellulosic biomass is rigorously limited by many factors, such as lignin content, cellulose crystallinity, water content and available surface area which additionally impacts the future exploitation of the pre-treated materials, for conversion to ethanol or for fine chemicals blends.

There are a number of key features for the effective pre-treatment of lignocellulosic biomass. The pre-treatment process should have a low capital and operational cost. It should be effective on a variety of biomasses and loading of lignocellulosic material and should result in the recovery of most of the lignocellulosic components in a useable form in separate fractions. The need for preparation/handling or preconditioning steps prior to pre-treatment such as size reduction should be minimized. It should produce no or limited amounts of sugar and lignin degradation products that inhibit the growth of fermentative microorganisms or the action of hydrolytic enzymes, and it should have a low energy demand or be performed in a manner that energy invested could be used for other purposes such as secondary heating (Chandra *et al*. 2007). Other features such as regeneration/cost of catalyst, generation of higher-value lignin co-products, and obtaining hemicellulose sugars in the liquid phase to reduce the need for the use of hemicellulases in subsequent enzymatic hydrolysis forms the basis of comparison of different pre-treatment options (Galbe and Zacchi 2007; Mosier *et al*., 2005b). All these features are considered so that pre-treatment results balance against their impact cost on downstream processing steps and the trade-off with operational cost, capital cost

and biomass cost (Lynd *et al*. 1996; Mosier *et al*. 2005b). Many pre-treatment methods have been studied, and are still in development.

In theory, the ideal pre-treatment process produces a disrupted, hydrated substrate that is easily hydrolysed but avoids the formation of sugar degradation products and fermentation inhibitors. Assessment of biomass pre-treatment processes depends on a parameter called the "severity factor", which is defined as the combined effect of temperature, acidity, and duration of pre-treatment. Studies on biomass pre-treatment have used the severity factor for comparing pre-treatment results even though it does not provide an accurate measure of the severity. In other words it is used for rough estimates (Galbe and Zacchi 2007). Pre-treatment assessment is conducted by analysing the sugars (both monomers and oligomers) released in the liquid and the carbohydrate content of the water insoluble solids (WIS) after pre-treatment, this gives the total amount of recoverable carbohydrates; enzymatic hydrolysis of either the washed or unwashed WIS; fermentation of the pre-treated liquid to assess its fermentibility directly or diluted to target concentration and inhibition of fermenting microorganisms with respect to growth; fermentation of either the slurry or washed WIS for biofuels production and assessment of additional biotechnological potential such as value added products of the pre-treated fractions .

3.2.1. Pre-treatment with ionic liquids

The most commonly used ionic liquids in biomass fractionation are imidazolium salts. The studies by Zhang and Lynd (2006) and Zhu *et al*., (2006) show that 1-allyl-3-methylimidazolium chloride (AMIMCl) and 1-butyl-3-ethylimidazolium chloride (BMIMCl) can be used effectively as a non-derivatising solvent for the dissolution of cellulose at temperatures below 100 ^{0}C. A possible dissolution mechanism suggests that these ionic liquids compete with lignocellulosic components for hydrogen bonding, thus disrupting its three dimensions network (Moultrop *et al*., 2005). The cellulose fraction can be

recovered by the addition of water, ethanol or acetone. The solvent can be recovered and reused by various methods such as pervaporation, reverse osmosis, salting out and ionic exchange. Ionic liquids are being screened for their potential to enhance the digestibility of lignocellulosic biomass (Zavrel *et al*., 2009). In general ILs are considered to be environmentally friendly (Pu *et al*., 2007), non-derivatising, non-volatile, thermostable, single component solvent for cellulose with potential applications in cellulose fractionation and dissolution. However, details of processing lignocellulosic biomass with ionic liquids are still under investigation.

In the study reported by Prado *et al*., (2015), two ILs, butylimidazolium hydrogen sulfate $[HC_4im][HSO_4]$ and triethylammonium hydrogen sulfate $[Et_3NH][HSO_4]$ were used for the delignification of *Miscanthus Giganteus* and subsequent lignin depolymerization by oxidation with H_2O_2 directly applied to the black liquors. Due to imidazolium derived ILs toxicity they compared the obtained results with the ammonium derived IL in order to get a greener processes. The $[Et_3NH][HSO_4]$ was found to be less reactive and gave lower lignin yields during pre-treatment. However, the lignin did not show evidence of IL contamination. It was observed that $[Et_3NH][HSO_4]$ was not affected by H_2O_2 which increases the possibility of its reutilization. Figure 3.3 shows the interaction of ILs with cellulose/ lignin. In this study the pre-treatment of sugarcane bagasse was carried out using two ionic liquids namely 1-ethyl-3-methylimidazolium acetate [Emim][OAc] and triethylammonium hydrogen sulfate $[HNEt_3][HSO_4]$.

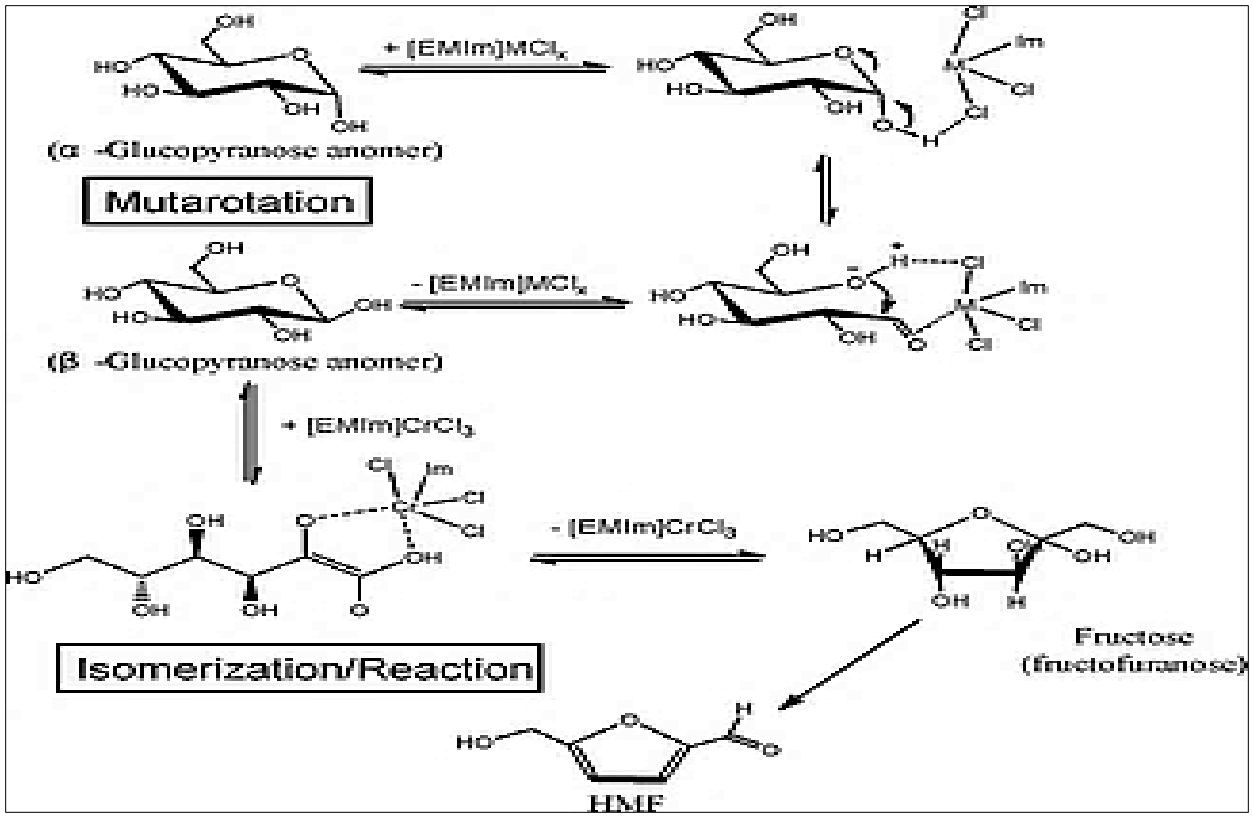


Figure 3.3 IL interactions with cellulose - The proposed metal halide interaction with glucose in [EMIM]Cl. $CuCl_2$ and $CrCl_2$ catalyze the mutarotation leading to interconversion of α- and β-glucopyranose anomers. $CrCl_2$ leads to the isomerization of glucopyranose to fructofuranose, followed by dehydration to HMF (Zhao *et al*., 2007).

3.2.2. Chemical Pre-treatment (Acid / alkali)

3.2.2.1. Acid pre-treatment

In this type of pre-treatment an aqueous solution of biomass is warmed to the required temperature and pre-treated using dilute sulfuric acid with concentrations of 4 % (wt.) or less in a stainless-steel reactor (Esteghalian *et al*., 1997; Torget *et al*., 1990). Dilute acid pre-treatment is done at temperatures ranging from 120 to 215 ^{0}C. The reaction

time ranges from a few seconds to minutes depending on the temperature of the pre-treatment. In another method, preconditioned and physically pre-treated biomass in a wire cross section is submerged in a circling shower of dilute sulfuric acid. The shower is then warmed to desired temperatures to impact pre-treatment at diverse severities. A two stage dilute acid pre-treatment to boost sugar recovery and upgrade biomass edibility was accounted for by Nguyen (2000).

Dilute acid (H_2SO_4) pre-treatment releases oligomers and monomeric sugars by influencing the reactivity of the biomass sugar polymers. Depending on the severity of the pre-treatment conditions, the sugars can be converted to aldehydes, for example, furfural and HMF. Mok and Antal (1992) pre-treated biomass with dilute acid at concentrations less than 0.1 % to hydrolyse hemicellulose in comparison to the normal 0.6 – 3.0 % that is usually used for dilute acid. A glucose yield of more than 80 % at an acid concentration of 0.05 % was reported.

Advantages of dilute acid pre-treatment include accomplishment of high reaction rates and enhancement of hemicellulose and cellulose hydrolysis yields (Sun and Cheng 2002). The drawbacks of acid pre-treatment include the fact that the feedstock subjected to acid pre-treatment may be somewhat harder to ferment with regards to the presence of fermentation inhibitors (Palmqvist and Hahn-Hägerdal 2000a; b); It is more expensive compared to most other physicochemical pre-treatment methods, for example, SP and AFEX, particularly the two-stage DA pre-treatment. Erosion brought on by dilute acid pre-treatment with sulfuric acid requires development material and the counter cost of nitric acid discredits the likelihood of utilizing it as a less destructive substitution for sulfuric acid (Brink 1994; Mosier *et al*., 2005b). The application of dilute sulfuric acid to cellulosic material has been utilized to produce furfural which is decontaminated by refining and promoted (Zeitsch 2000). Dilute acid has been utilised in the pre-treatment of corn Stover, woody and herbaceous crops (Torget *et al*., 1990; Torget *et al*. 1992).

3.2.2.2. Alkali pre-treatment

Alkaline pre-treatment is a minimal effort physicochemical pre-treatment that has been used to improve the absorbability of lignocellulosic biomass (Chang *et al*., 1998). The pre-treatment uses aqueous calcium hydroxide [Ca $(OH)_2$] at low temperature and pressure as a pre-treatment medium to dissolve hemicellulose and lignin (Chang *et al*., 1997). The adequacy of alkaline pre-treatment has been attributed to opening of 'acetyl valves' and 'lignin valves', i.e. de-acetylation and fractional delignification (Chang and Holtzapple 2000). Oxidative elements become an integral factor when oxygen is present at high pressures to upgrade the pre-treatment. Alkaline pre-treatment of wheat straw at 85 ^{0}C for 3 h (Chang *et al*., 1998) and poplar wood at 150 ^{0}C for 6 h utilizing 14 atm oxygen (Chang *et al*., 2001b) gave a high yield compared to the one without oxygen.

As an alkaline pre-treatment, lime (calcium hydroxide) has the extra advantages of low reagent cost, security, and simplicity of recovery as calcium carbonate compared to sodium, potassium and ammonium hydroxide, however sodium hydroxide (NaOH) is mostly used (Sharmas *et al*., 2002). Pre-treatment performed at temperatures below 100 ^{0}C uses less energy needed to keep the vessels warm and enormous heaps of biomass could essentially be pre-treated without the requirement for an extraordinary vessel or utilizing a basic configuration for pilot plants (Chang *et al.,* 1997; Wyman *et al*., 2005). Lime pre-treatment has a few disadvantages which include its inability to extract lignin in high lignin biomass for example softwood (Chang *et al*., 2001a). The activity of lime is slower than ammonia and regularly the oxidizing agent is not very specific therefore losses in hemicellulose and cellulose may happen. The utilization of huge volumes of water in the washing step (5 - 10 g H_2O/g biomass) and the potential requirement for balance makes downstream handling difficult furthermore increasing the expense of scaling up. The oxidation of lignin to other soluble aromatic compounds is a danger because of the likelihood of the development of inhibitors (Hendricks and Zeeman 2009).

3.3. Characterization of raw and pre-treated biomass

Characterization is used to determine the structure and interactions of the biomass components. Scientific and modern groups need both quicker and more extensive screening methods and additionally molecular and imaging procedures fit for the thorough examination of the biomass. The National Renewable Energy Laboratory (NREL) strategies for biomass characterization have been the major contributor in this field, despite the fact that it requires numerous grams of test sample (Sluiter *et al*., 2008; Selig and Weiss 2008). Advancements have been made that computerize and decrease the sample size requirements to milligrams. Pre-treatment and enzymatic hydrolysis steps can now be performed in a 96-well plate (Studer *et al*., 2011; Decker *et al*., 2009; Studer *et al.*, 2010; Chundawat *et al*., 2008 and Santoro *et al.*, 2010). Santoro *et al*., (2010) fused crushing into their mechanized sample preparation to enhance edibility for screening under various conditions. These techniques are applicable to a variety of biomass samples.

CHAPTER **4**

EXPERIMENTAL

4.1. Materials

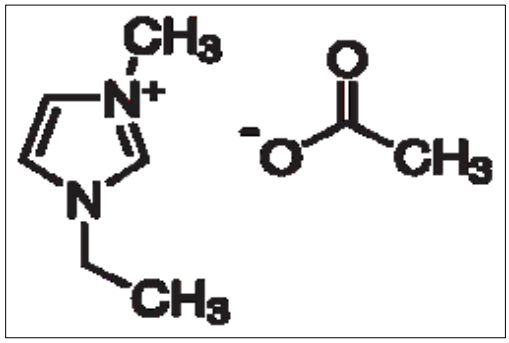
The sugarcane bagasse samples used in this study were obtained from a local mill in Kwa-Zulu Natal, South Africa. The photograph of the bagasse sample is shown in figure 4.1.

Figure 4.1 Photograph of the sugarcane bagasse sample

Bagasse samples were washed with deionised water to remove sand and dried in an oven at 100 °C for 6 hours to remove the water. The samples were then milled using a lab Retsch Muhle laboratory mill at a particle size of 20-60 mesh. The photograph of the mill is shown in figure 4.2.

Figure 4.2 Retsch Muhle laboratory mill used in this study

The chemicals used in this study were purchased from Sigma Aldrich, Titan Biotec and some were synthesised in house. Table 4.1 gives the list of chemicals used, the suppliers and the purity. The structures of the ionic liquids 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) and triethylammonium hydrogen sulfate ([$HNEt_3$][HSO_4]) are shown in figures 4.3 and 4.4.



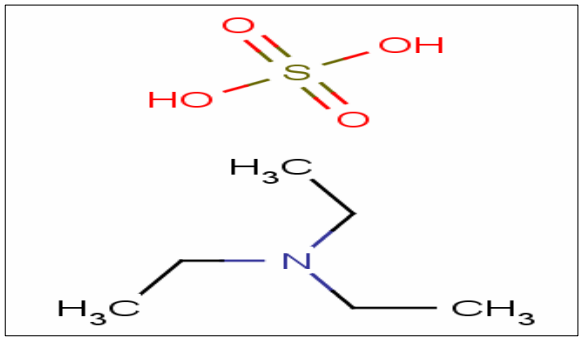


Figure 4.3 Structure of ([Emim][OAc])

Figure 4.4 Structure of ([$HNEt_3$][HSO_4])

Table 4.1 Summary of chemicals used and suppliers

Name of chemical	Supplier	Purity
[Emim][OAc	Sigma Aldrich	> 95 wt.%
Triethylamine		99.5 % (w/v)
H_2SO_4		5 M
H_2SO_4		72 % v/v
H_2SO_4	Titan Biotech, India	98 %
Sodium hydroxide		-
Tween 80		-
DNSA		-
Citric Acid buffer		-
Cellulase	Synthesized in-house using RCKC65 at University of Delhi, South Campus	-
$[HNEt_3][HSO_4]$	Synthesized in-house using triethylamine and sulfuric acid at Imperial College, London	$\geq$ 99.5 % and 5 M

4.2. Experimental

4.2.1. Compositional Analysis

Prior to undertaking compositional analysis, untreated SB was analysed using NREL-TP-510-42619 method (Sluiter *et al*., 2008). A 3-5 g of untreated biomass was accurately weighed into a cellulose thimble and refluxed in deionized water (250 ml, 24 h) and in ethanol (250 ml, 24 h) in a two-step soxhlet extraction to remove non-structural material from biomass prior to analysis to prevent interference with later

analytical steps. Moisture content of the untreated SB was determined following NREL/TP-510-42618 method. The chemical composition determination of untreated extractives-free biomass was carried out following NREL/TP-510-42618 method. Compositional analysis was carried out in triplicate. Deionised water was used for all parts of the experiment.

4.2.1.1. Moisture content

A 0.1 g of the sample was weighed into a pre-dried weighing dish and placed into a convection oven at 105 ^{0}C overnight. The sample was removed from the oven and allowed to cool to room temperature in a desiccator. The dish containing the oven-dried sample was weighed to the nearest 0.1 g and the mass recorded. The above steps were repeated until the constant weight was achieved. The percent total solids were calculated on a dry weight basis according to equations 3.1 and 3.2.

4.2.1.2. Ash content

A 1.0 g of sugarcane bagasse was weighed into the tared crucible. The crucible with sample was placed in a Naberthen programmable muffle furnace (photograph shown in figure 4.5) at 575 ^{0}C for 6 hours, thereafter removed from the furnace to the desiccator for cooling. The crucibles with samples were weighed and the mass recorded. The samples were placed back into the muffle furnace and ashed to constant weight which symbolised by a (less than ± 0.3 mg change in the weight upon one hour of re-heating the crucible). The percent ash was calculated according to equations 3.3 and 3.4. The analysis was done in triplicate. The analysis was done in triplicate.

Figure 4.5 Photograph of Nabertherm Controller P330 LT 5/13, Nabertherm GmbH, Germany muffle furnace

4.2.1.3. Acid soluble lignin

A 3 g sample of SCB was hydrolysed with 3 ml of 72 % sulfuric acid (H_2SO_4) for 1 hour at 80 ^{0}C and thereafter diluted to 4 % concentration by adding deionised water to determine the percentage of acid insoluble lignin. The acid soluble lignin (ASL) content of the sample was determined by measuring the UV absorption of the acid hydrolysis supernatant at 240 nm wavelength. The solids remaining after a two stage acid hydrolysis was dried at 105 ^{0}C overnight (photograph shown in figure 4.6). The total lignin was calculated as the sum of acid soluble and acid insoluble lignin. All results were recorded on a weight percent basis using equations 3.5, 3.6 and 3.7 respectively.

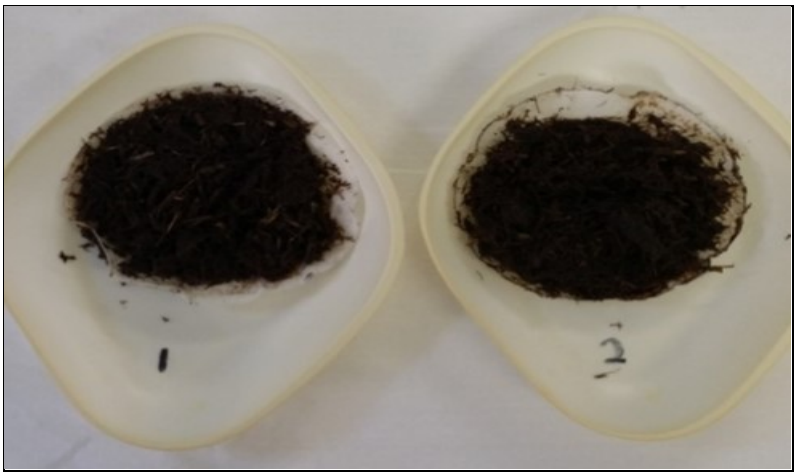


Figure 4.6 Sugarcane bagasse sample after acid hydrolysis

4.2.1.4. Klason lignin

Pulp was treated with 72 % H_2SO_4 for 2 hours. The material is then diluted to 3 % H_2SO_4 and then boiled for 4 hours. These two steps dissolve the carbohydrates leaving chunks of lignin floating in the acid. The lignin is filtered, washed and weighed.

4.2.2. Ionic Liquid Properties

4.2.2.1. Karl Fischer measurements on ILs

The water content was of the IL was determined to ascertain its water uptake during the experimental procedure. A Mettler - Toledo Karl-Fischer coulometer (photograph shown in figure 4.7) was used to check the water content of [Emim][OAc] and [$HNEt_3$][HSO_4]. Water can be readily absorbed in IL from the atmosphere during storage or experiments; therefore dryness of IL is essential for optimum dissolution of biomass components. As minimal as possible water content is desired as water has been reported to be an impurity that is present in most ILs. Trace amounts of water have been shown to significantly affect IL physical properties. Properties of ILs including solubility, polarity, viscosity, and conductivity, are not only changed but are also dependent on the amount of absorbed water. As a result absorbed water may alter

rates of chemical reactions and efficiencies of various processes in ILs (Welton, 1999; Fletcher *et al.*, 2002; Aki *et al.*, 2001; Kazarian *et al.*, 2000; Cammarata *et al.*, 2001). The water content of the IL as reported by the supplier Sigma Aldrich was 0.5 % (5000 ppm).

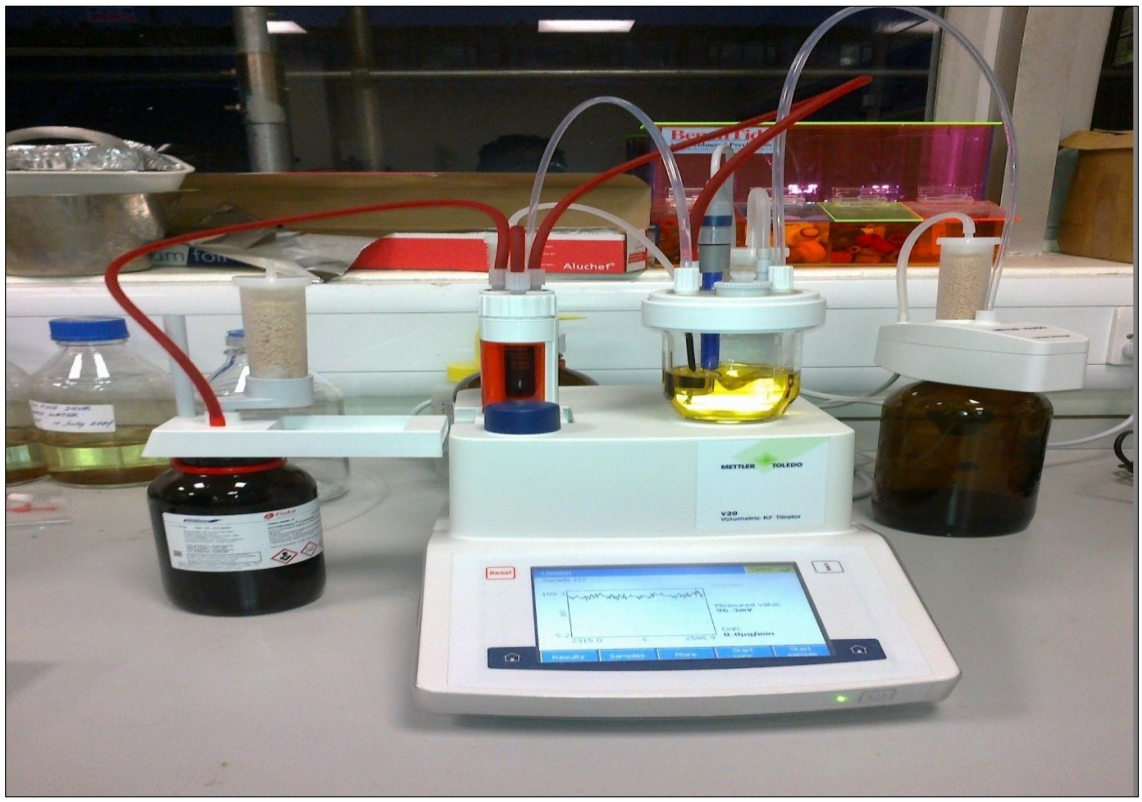


Figure 4.7 Photograph of Mettler-Toledo V20 Karl-Fischer coulometer

4.2.2.2. Speed of sound, density and refractive index

In this study, the thermo-physical properties (speed of sound, density and refractive index) of [Emim][OAc] was measured as a function of temperature using the Anton Paar DSA 5000M densitometer (photograph shown in figure 4.8). It has an automatic sampler X452 and is equipped with a refractive index RXA 156/170. The pure IL physical properties as shown in table 4.2 were obtained as an added tool to assess the purity of the IL.

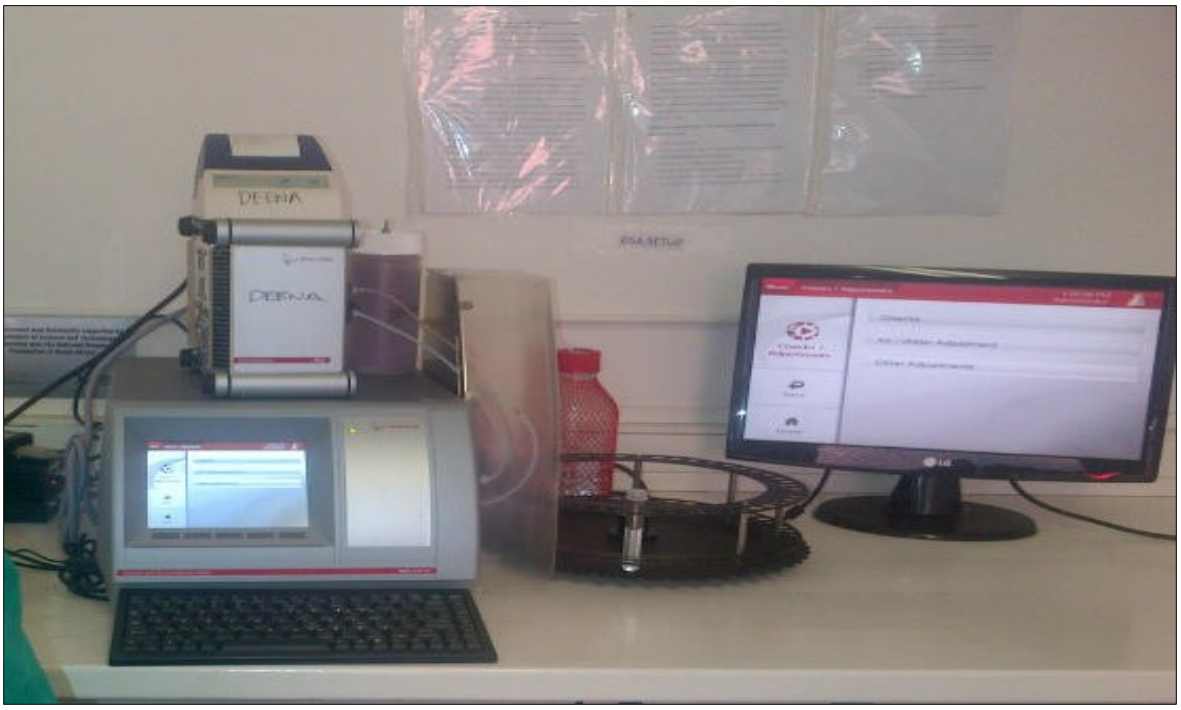


Figure 4.8 Photograph of the Anton Paar DSA 5000M densitometer that has an automatic sampler X452, and equipped with a refractive index RXA 156/170

Table 4.2 Densities, speed of sound and refractive index values of [Emim][OAc] measured and compared to literature at 298.15 K

	Density ($g.cm^{-3}$)	**Speed of sound ($m.s^{-1}$)**	**Refractive Index (n_D)**
Experimental	1.098	1734.76	1.499
	1.097	1730.11	1.498
	1.097	1729.59	1.499
Literature	1.098[1]	1329.10[1]	1.429[1]

*[1] Quijada-Maldonado *et.al.* (2012:55)

Since ($[HNEt_3][HSO_4]$) was synthesized in house, its acid/base ratio was verified by determining the density of 80.0 wt. % solution in distilled water. The density of 1910 ± 0.0004 g cm^{-3} was recorded. The mixture of 80 wt. % $[HNEt_3][HSO_4]$ in distilled water was used for all pretreatment experiments with this IL.

4.2.3. Pre-treatment of sugarcane bagasse with 1-ethyl-3-methylimidazolium acetate ([Emim][OAc])

The pre-treatment of sugarcane bagasse was carried out as per the fractionation process below in figure 4.9 in a water bath.

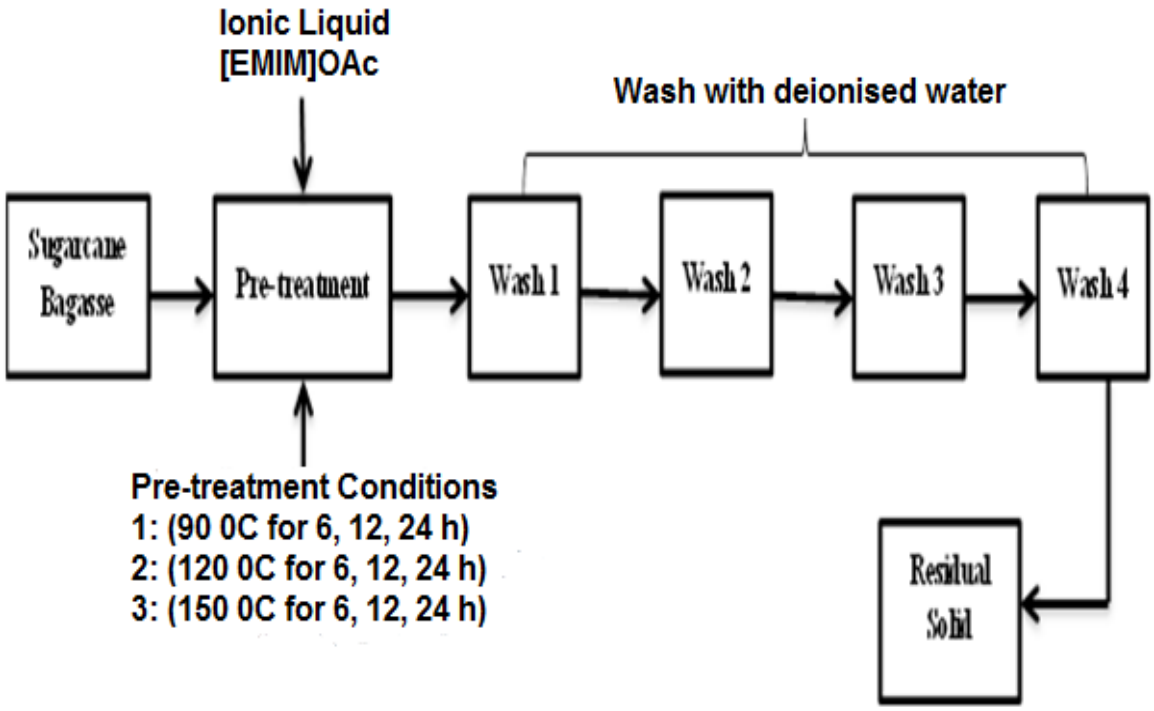


Figure 4.9 IL Fractionation Process

The samples were prepared as follows: 0.50 g previously dried and grounded sugarcane bagasse sample was placed in a 10 ml glass vial with a cap and weighed on an analytical mass balance, 5 g of [Emim][OAc] was added and the mixture was stirred and heated in a water bath for 6, 12 and 24 hours at varying temperatures of 90, 120 and 150 ^{0}C. After dissolution was complete, the samples were cooled to room temperature. The photograph of the samples after pre-treatment is shown in figure 4.10.

Figure 4.10 Photograph of the sugarcane bagasse samples after IL pre-treatment

4.2.3.1. Pulp recovery

The cooled solution was washed with 40 ml of water. The washings were repeated three times to remove the IL from the pulp and were collected in a centrifuge tube. The solution was let to stand for 24 hours thereafter a brownish lignin material was observed settled at the bottom of the centrifuge tube (photograph shown in figure 4.11). The mixture was vacuum filtered and air dried for 24 hour or until dry. The dry weight was recorded. After 24 hours the air dried pulp was oven dried to determine the percentage of dry content pulp and total dry pulp using equations 4.1 and 4.2. The yield of regenerated biomass (% pulp recovery) was calculated using equation 4.3.

$$\%\ \text{Dry pulp}\ \frac{\text{Mass after oven drying} - \text{Mass foil}}{\text{Mass before oven drying} - \text{Mass foil}}\ x\ 100 \qquad (4.1)$$

$$\% \text{ Total dry pulp } \frac{\text{Total air dry pulp x dry content pulp}}{100}$$

(4.2)

$$\% \text{ Pulp recovery} = \frac{\text{Mass of regenerated biomass}}{\text{Mass sample for dissolution}} x\ 100$$

(4.3)

Figure 4.11 Photograph of lignin material observed at the bottom of the tube after centrifugation

4.2.3.2. Lignin recovery

The pulp was washed with deionised water after which the mixture was cooled and filtered. The lignin was dried and weighed.

4.2.3.3. Sugars analysis

HPLC was used for separation and detection of sugars. The composition of the liquid phase obtained after pre-treatment was determined using the HPLC system consisting of Perkin Elmer 250 pump, a Bio-Grad Organic Acid Column Aminex HPX-87P, an LC 235 diode array detector as shown in figure 4.12. HPLC was used for both qualitative and quantitative analysis of sugars. The following HPLC conditions were used:

Parameters

Mobile phase	: 5 mM sulfuric acid/ Deionised Water
Sample volume	: 0.005 mL (5 µL)
Column Temperature	: 80 - 85 ^{0}C
Flow rate	: 0.6 mL/min
Column type	: Aminex HPX-87P (300 x 7.8 mm internal diameter)

Figure 4.12 Photograph of a Perkin Elmer – HPLC system

4.2.4. Pre-treatment of sugarcane bagasse with triethylammonium hydrogen sulfate ([$HNEt_3$][HSO_4])

4.2.4.1. Synthesis of ([$HNEt_3$][HSO_4]

The synthesis of triethylammonium hydrogen sulfate ([$HNEt_3$][HSO_4]) was achieved by combining triethylamine (≥ 99.5%) and sulfuric acid (5 M) in stoichiometrically equal amounts as described previously.(George *et al*., 2015; Wang *et al*., 2006) H_2SO_4 was added dropwise into triethylamine at 4 ^{0}C. Careful dosing of sulfuric acid and triethylamine, based on the purity stated by the manufacturer, was used to endeavor to control the acid/base mole ratio (1:1). The mixtures were magnetically stirred in an ice bath for several hours. Water was then removed *in vacuo* at 40 ^{0}C until the water content was reduced to 20 wt. %. The final water content was verified by Karl Fischer (METTLER TOLEDO (V20) volumetric titrator) measurements in triplicate.

4.2.4.2. Preparation of samples

The whole sugarcane bagasse sample was separated into five fractions as shown in figure 4.13. The samples were prepared as follows: 1.0 g of previously dried and grounded sugarcane bagasse sample was weighed into a 15 ml pre-weighed pressure tube with Teflon cap and silicone O-ring. A mass (10 g) of thoroughly dried ([$HNEt_3$][HSO_4]) was added to the biomass and the mixture was stirred in a vortex (photograph shown in figure 4.14) until a homogenous solution was obtained. The tube was then placed in a fan assisted oven for 2, 4, 8, 12, 16, 24 hours at 120 ^{0}C (photograph of a Heratherm fan assisted oven shown in figure 4.15). The weight of the tube before and after treatment was recorded.

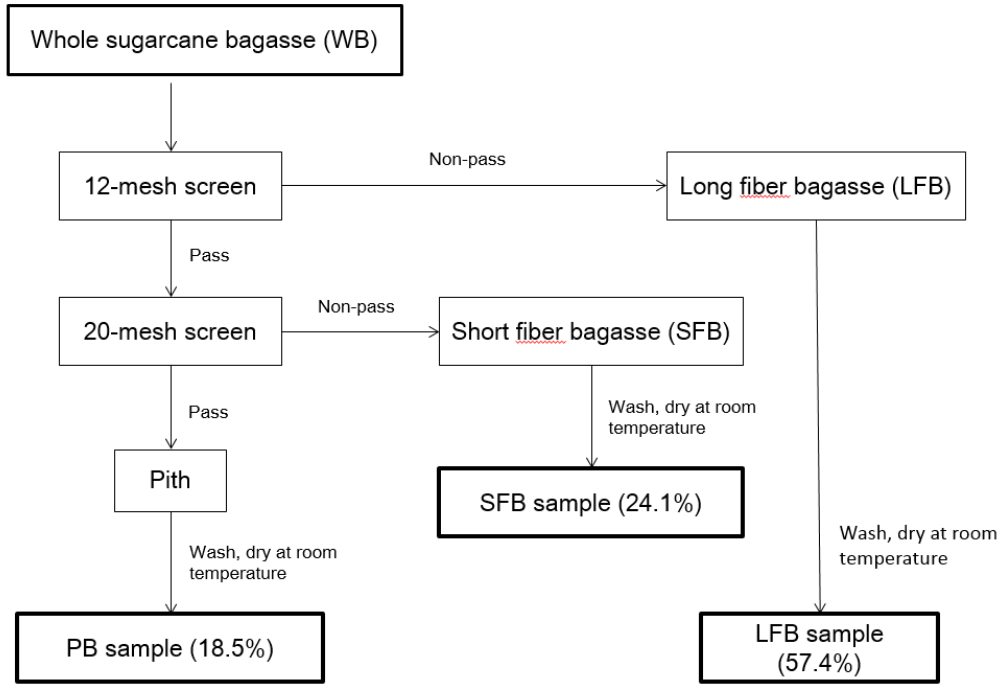


Figure 4.13 Schematic representation of the five bagasse samples

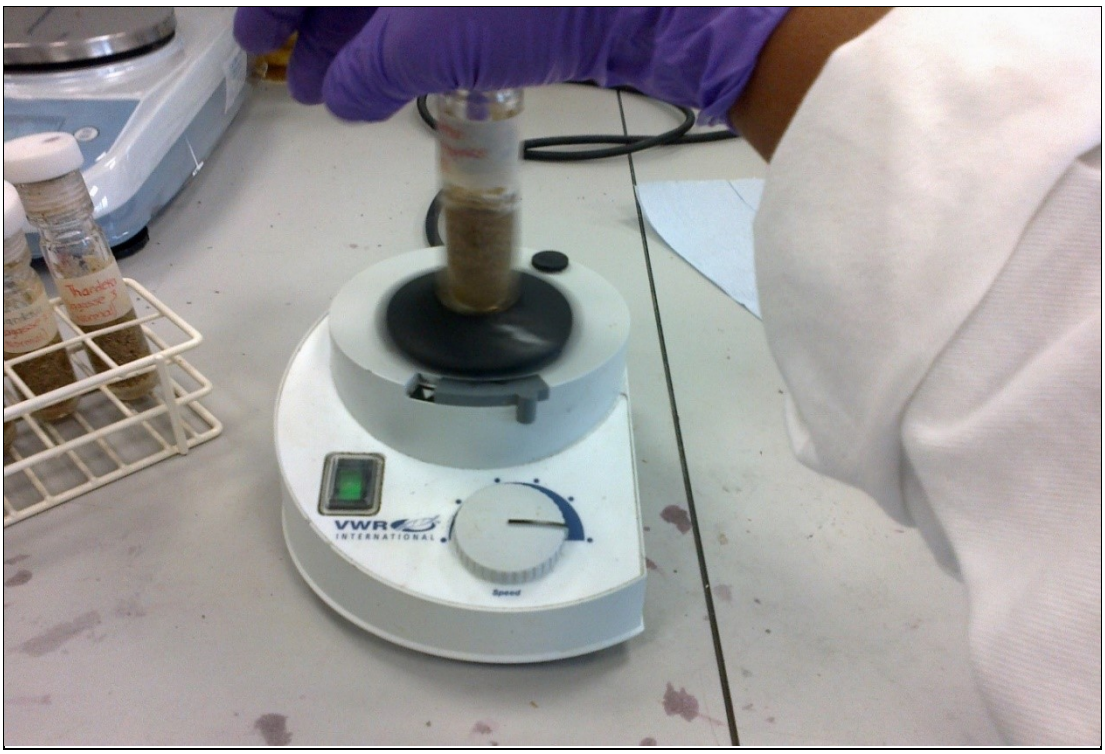


Figure 4.14 Photograph of a VWR vortex

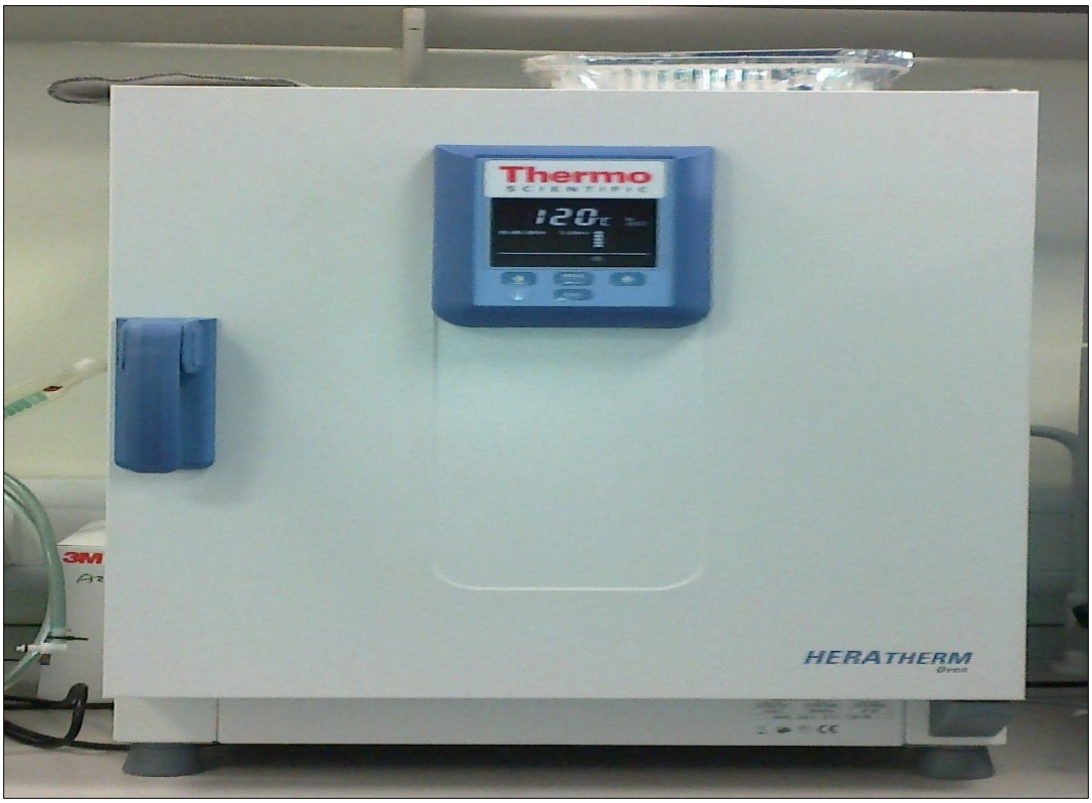


Figure 4.15 Photograph of a Heratherm Thermo Scientific fan assisted oven

4.2.4.3. Pulp Wash

The cooled solution was washed with 40 ml of absolute ethanol, transferred into a 50 mL centrifuge tube and shaken for 1 minute to mix well. The tube was left at room temperature for 1 hour. The solution was then centrifuged in a Boeco C-28 centrifuge at 4000 rpm for 50 minutes (photograph of the centrifuge is shown in figure 4.16). The liquid and solid was separated by careful decantation. The liquid was collected in a 250 ml round bottom flask (photograph shown in figure 4.17). The washings were repeated three times to remove the IL from the pulp. The ethanol from the biomass washes was removed with a Buchi R-210 rotovap or parallel evaporator (photograph shown in figure 4.18). The mixture was then filtered using a membrane filter paper 10 µm, then dried in an oven at 80 ^{0}C for one hour or until dry. The dried weight was recorded. The yield of regenerated biomass (% Recovery) was calculated using equation 4.3.

Figure 4.16 Photograph of Boeco C-28 centrifuge

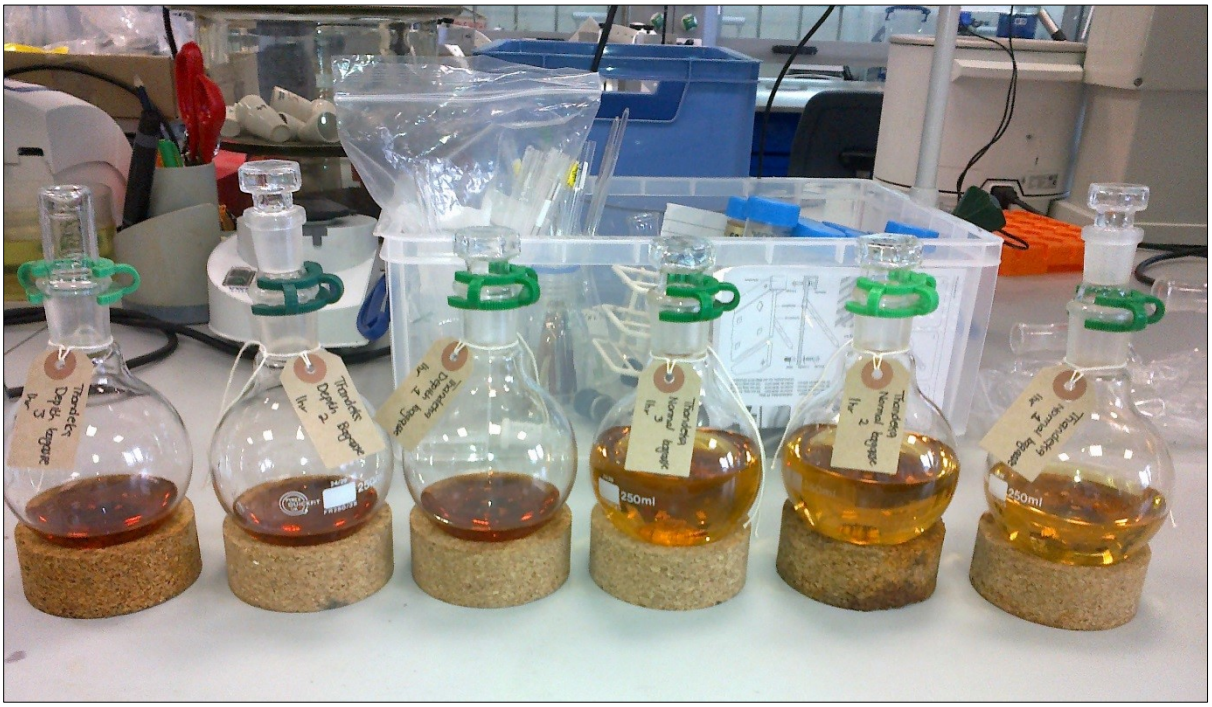


Figure 4.17 Photograph of the collected liquid after ethanol washing of pulp

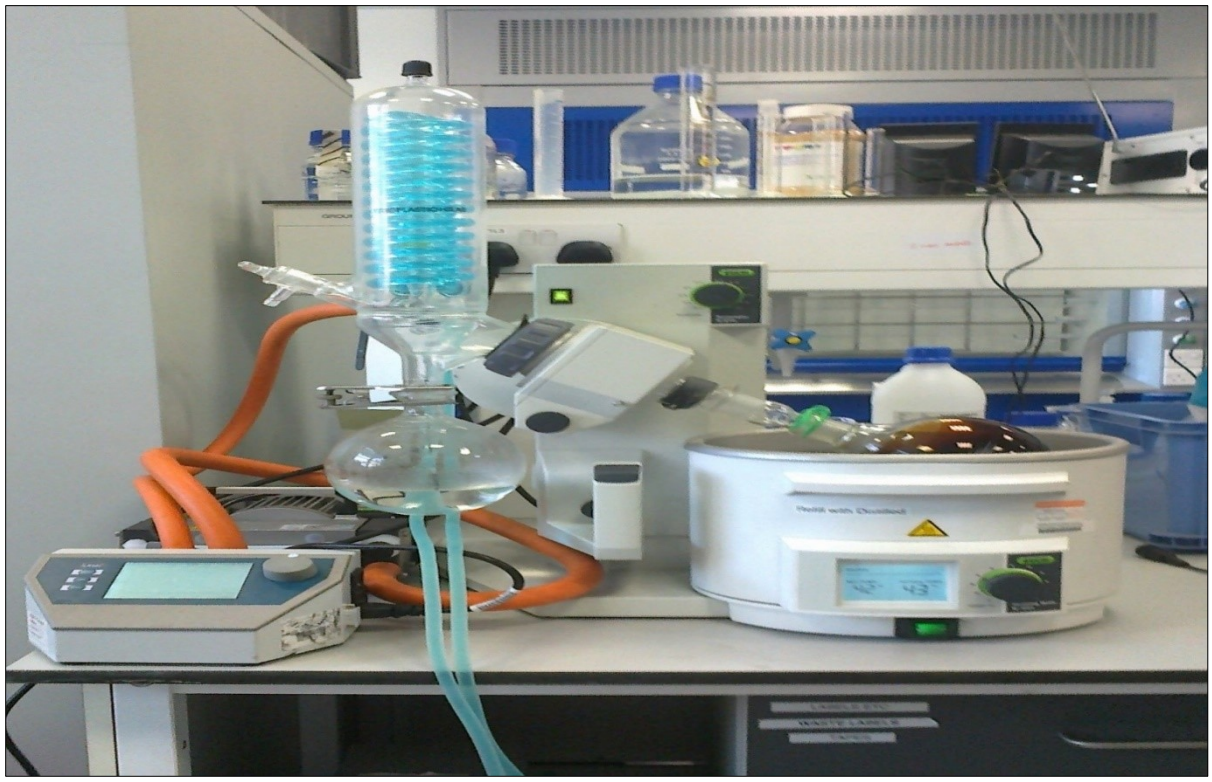


Figure 4.18 Photograph of a rotovap (Buchi R-210) used for ethanol evaporation

4.2.4.4. Soxhlet extraction of pulp

The pulp was transferred into cellulose thimbles. Into a clean 250 ml round bottom flask, 150 mL of absolute ethanol was filled with oval REM stir bar. Soxhlet extractor, sample containing thimble and condenser were added to the Starfish parallel extractor work station (photograph shown in figure 4.19) and the pulp extracted for 20 hours at 135 ^{0}C. The wet pulp in the thimble was air dried overnight and transferred into a tarred aluminium foil and weighed. The moisture content of the pulp was determined immediately to calculate the oven dry yield.

Figure 4.19 Photograph of a Starfish parallel Soxhlet extraction setup

4.2.4.5. Lignin precipitation and wash

The ethanol from the biomass wash and soxhlet extraction was evaporated with a rotovap. Distilled water was added to the IL, mixed and left for approximately 1 hour. This was transferred into a centrifuge tube and centrifuged for 40 minutes. The liquid was separated from the solid by decanting. The lignin washes were repeated 3 times.

4.2.4.6. Lignin drying

The precipitated lignin was dried in a vacuum oven (photo shown in figure 4.20) at 45 ^{0}C overnight with the trap cooled with ice. The lignin was then transferred straight from the oven into a tarred aluminium foil, weighed and then stored in the fridge.

Figure 4.20 Photograph of a Binder vacuum oven

4.2.4.7. IL recovery

The ionic liquid liquor (filtrate) obtained after washing was transferred into a round bottom flask attached to a rotary evaporator and distilled to remove any water and ethanol that remained in the mixture. The ($[HNEt_3][HSO_4]$) recovered was then dried under vacuum line at 40 ^{0}C until no further evaporation could be observed. The flask containing the recovered IL was weighed to determine the wet IL recovery (by subtracting the empty flask weight from the weight of the flask containing the recovered IL). The water content was measured after drying using a coulometric Karl Fischer titration. The dry IL recovery was calculated and the IL characterized by FTIR and ^{1}H NMR and compared with the neat IL spectrum before dissolution process.

4.2.4.8. High performance liquid chromatography (HPLC)

Saccharide content in the reaction mixture was measured by Shimadzu HPLC system (photograph shown in figure 4.21). The mobile phase was deionised water containing 0.02 % trifluoroacetate (v/v) at a flow rate of 1 mL/min with the temperature of column at 40 ^{0}C. The sample volume injected was 0.005 mL (5 µL).

Figure 4.21 Photograph of Shimadzu HPLC-10AT VP Scheme 1 setup with a Honenpak C18 column (75 mm x 4.6 mm internal diameter)

4.2.5. Acid / alkali pre-treatment of sugarcane bagasse

SCB was pre-treated with 3 % (v/ v) sulfuric acid for 30 minutes in an autoclave at a temperature of 121 ^{0}C afterwhich it was cooled, filtered and washed to neutral pH and dried at 50 ^{0}C in an oven overnight. The filtrate was analysed for reducing sugars. The dried substrate from the acid pre-treatment was further pre-treated with 4 % (w/v) sodium hydroxide for 15 minutes in an autoclave at 121 ^{0}C. This was allowed to cool, filtered and washed to neutral pH and dried at 50 ^{0}C in an oven overnight. This was stored for further processing. Figure 4.22 shows the steps followed for acid/ alkali pre-treatment followed by enzymatic saccharification.

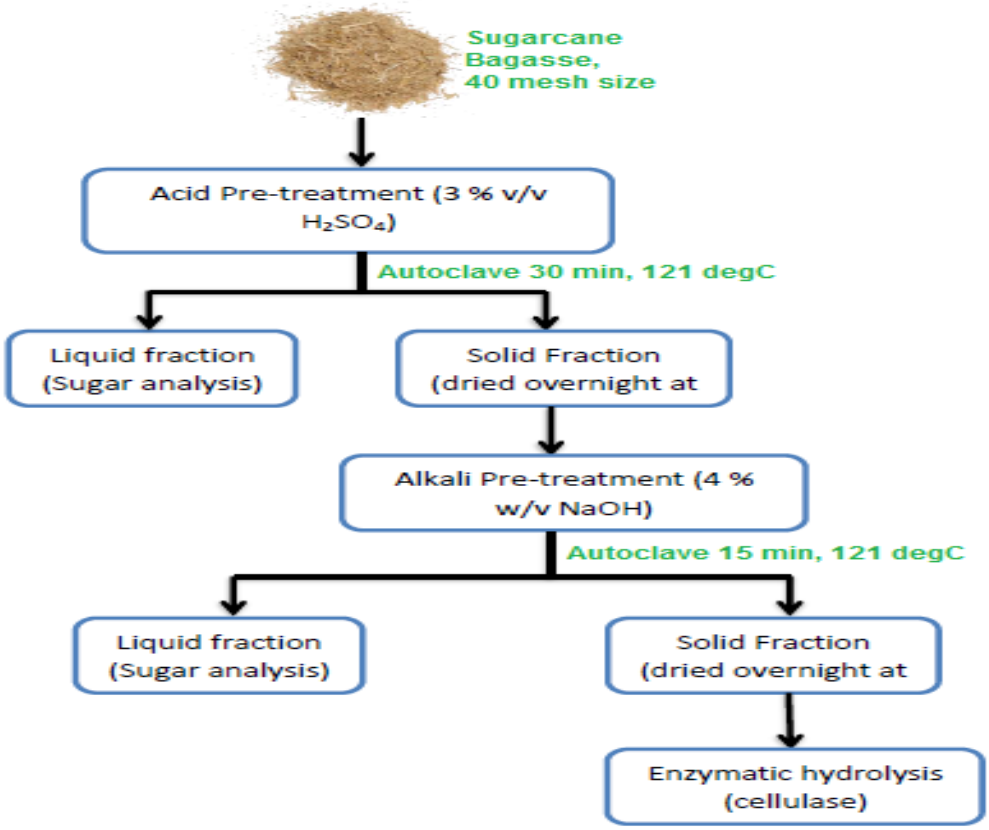


Figure 4.22 Flow diagram of the acid/ alkali pre-treatment followed by enzymatic saccharification

4.2.5.1. Enzymatic saccharification of acid / alkali pre-treated sugarcane bagasse

The acid-alkali pre-treated sugarcane bagasse was saccharified using crude enzyme extract produced by fungal RBT strain (8 FPU/mL). Enzymatic hydrolysis of pre-treated material (1.0 g each) was carried out at 10 % (w/v) substrate consistency in 50 mM citrate phosphate buffer (pH 5.0). The substrate with buffer was pre-incubated at 50 ^{0}C on a rotatory shaker (picture shown in figure 4.23) at 150 rpm for 1 h. Thereafter, to obtain the optimized enzyme doses for the saccharification of pre-treated biomass, varied doses of exoglucanase (5 - 40 U/g) were added to the pre-incubated slurry. The reaction continued up to 24 h. Samples were withdrawn at regular intervals of 8 h, centrifuged at 12000 rpm for 10 min in a centrifuge (Sigma, Germany) and the supernatant was analysed for total reducing sugars released and saccharification yield. Figure 4.24 shows the steps followed for the saccharification. The pre-treated and enzyme hydrolysed samples are shown in figure 4.25.

Figure 4.23 Innova-44, New Brunswick Scientific, incubator

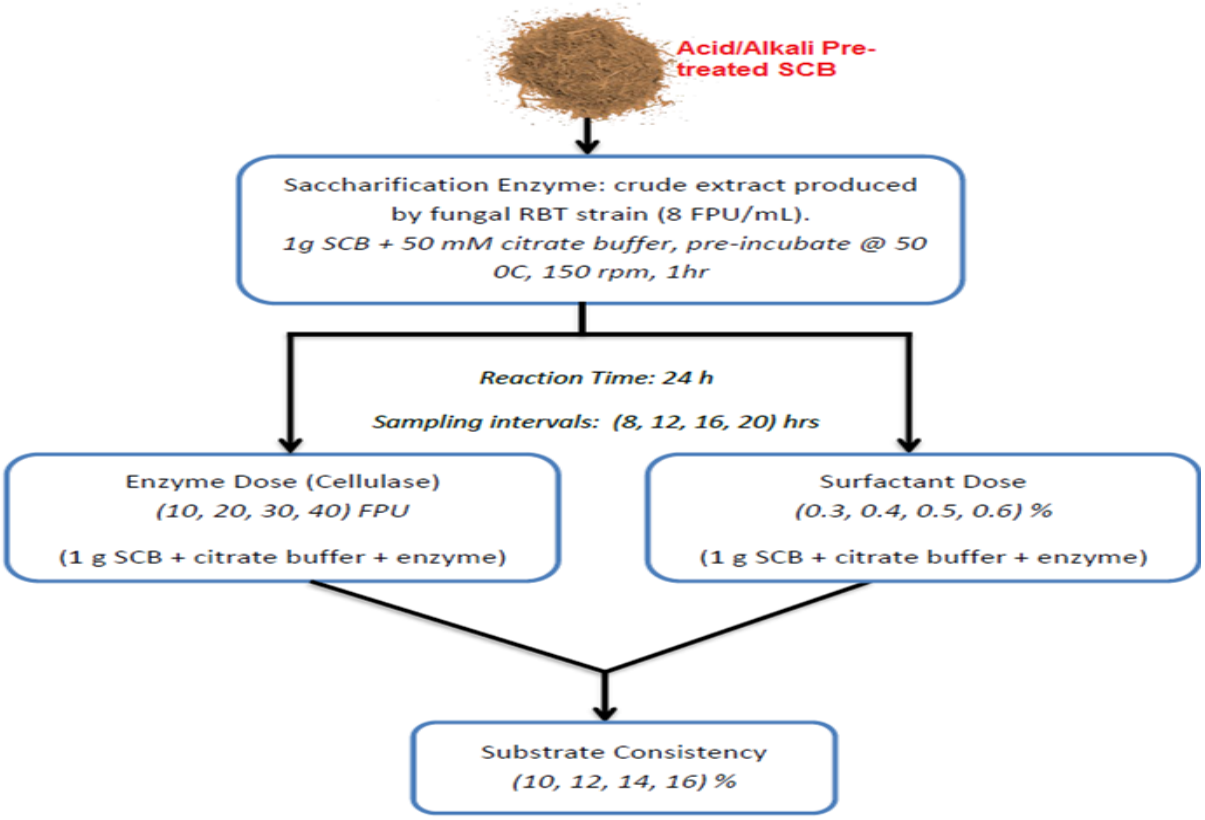


Figure 4.24 Flow-diagram of the saccharification steps

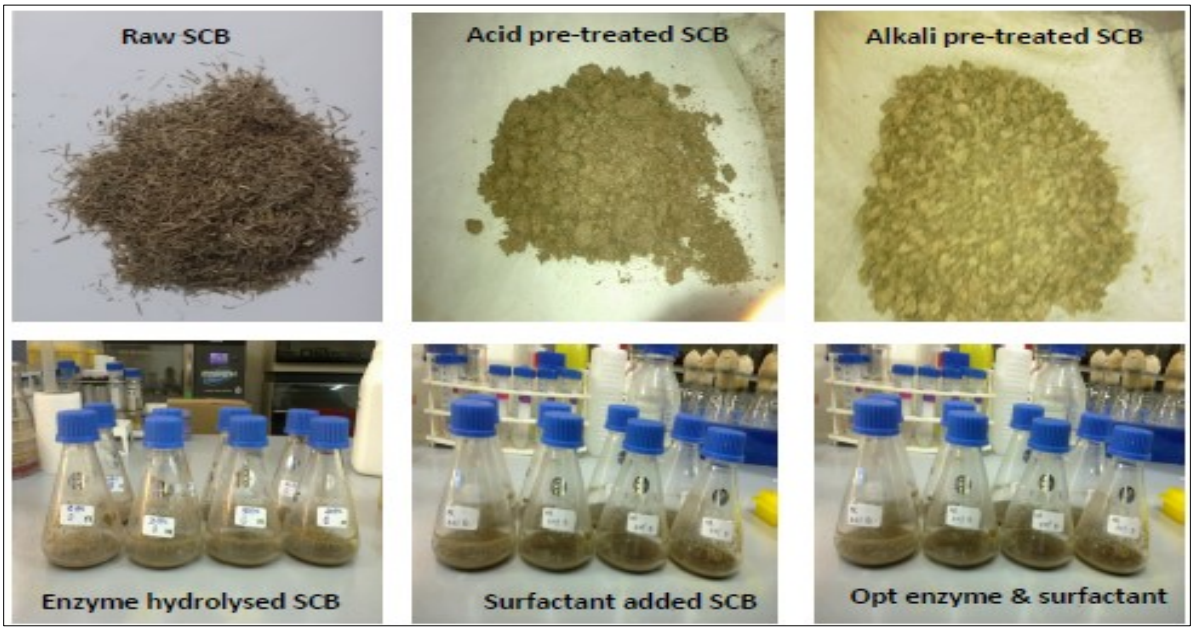


Figure 4.25 Pre-treated and enzyme hydrolysed sugarcane bagasse (SCB) samples

4.3. Characterization of regenerated biomass

4.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

The aim of using FTIR analysis in the present study was to study characteristic bands and the structural changes of the raw and regenerated biomass samples in [Emim][OAc]. The scans for the regenerated biomass were compared with the FTIR spectrum of raw sugarcane bagasse sample using a Varian 800 FTIR spectrometer - Scimitar Series (photograph is shown in figure 4.26) and spectra were obtained in the range of 380 – 4000 cm^{-1}.

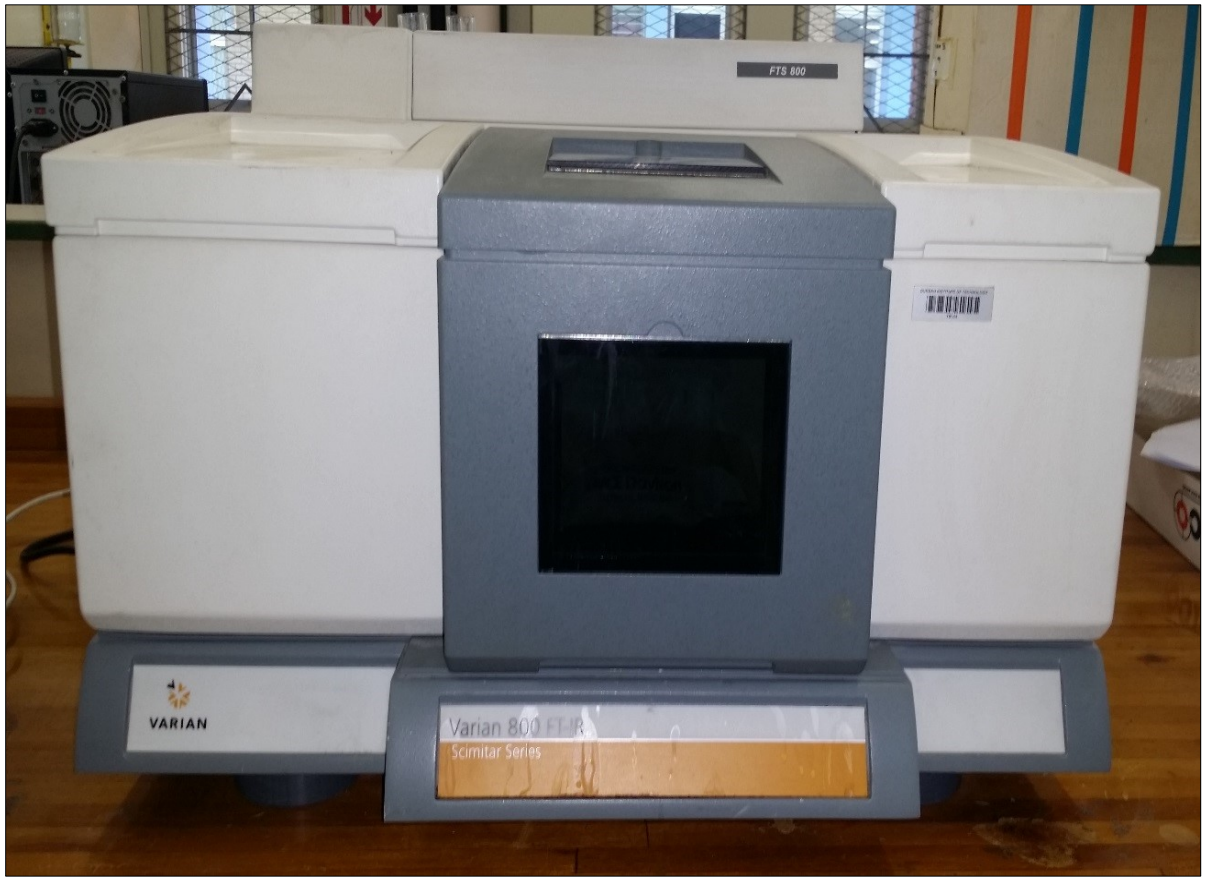


Figure 4.26 Photograph of a Varian 800 – FTIR

4.3.1.1. FTIR Protocol

Infrared spectrophotometry involves detection of the twisting, bending, rotating, and vibrational motions of atoms in a molecule. Upon interaction with infrared radiation, portions of the incident radiation are absorbed at particular wavelengths. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum, which is uniquely characteristic of the functional groups comprising the molecule and of the overall configuration of atoms as well. The infrared spectrum of a compound is essentially the superposition of absorption bands of specific functional groups, yet subtle interactions with the surrounding atoms of the molecule impose the stamp of individuality on the spectrum of each compound. For qualitative analysis, one of the best features of an infrared spectrum is that the absorption or the lack of absorption in specific frequency regions can be correlated with specific stretching and bending motions and, in some cases, with the relationship of these groups to the remainder of the molecule. Thus, by interpretation of the spectrum, it is possible to state that certain functional groups are present in the material and that certain others are absent. With this one datum, the possibilities for the unknown can be sometimes narrowed so sharply that comparison with a library of pure spectra permits identification (Willard *et.al*., 1986).

4.3.2. Scanning Electron Microscopy (SEM)

SEM images of the regenerated biomass samples were taken at 300 x magnification using a Zeiss Supra 35 Variable Pressure SEM with a field emission source, an EDAX x-ray fluorescence detector and HKL electron backscatter diffraction (EBSD) detector. The photograph is shown in figure 4.27. Prior to imaging, the samples were sputter-coated with gold to make the fibres electrically conductive, avoiding degradation and build-up of charge on them. The samples were mounted onto the SEM aluminium stub with a carbon tape and then Au (gold) coated. They were cooked for 10 minutes with a Gold Polaron SC 5000 sputter coater to make the samples more electrically conducting.

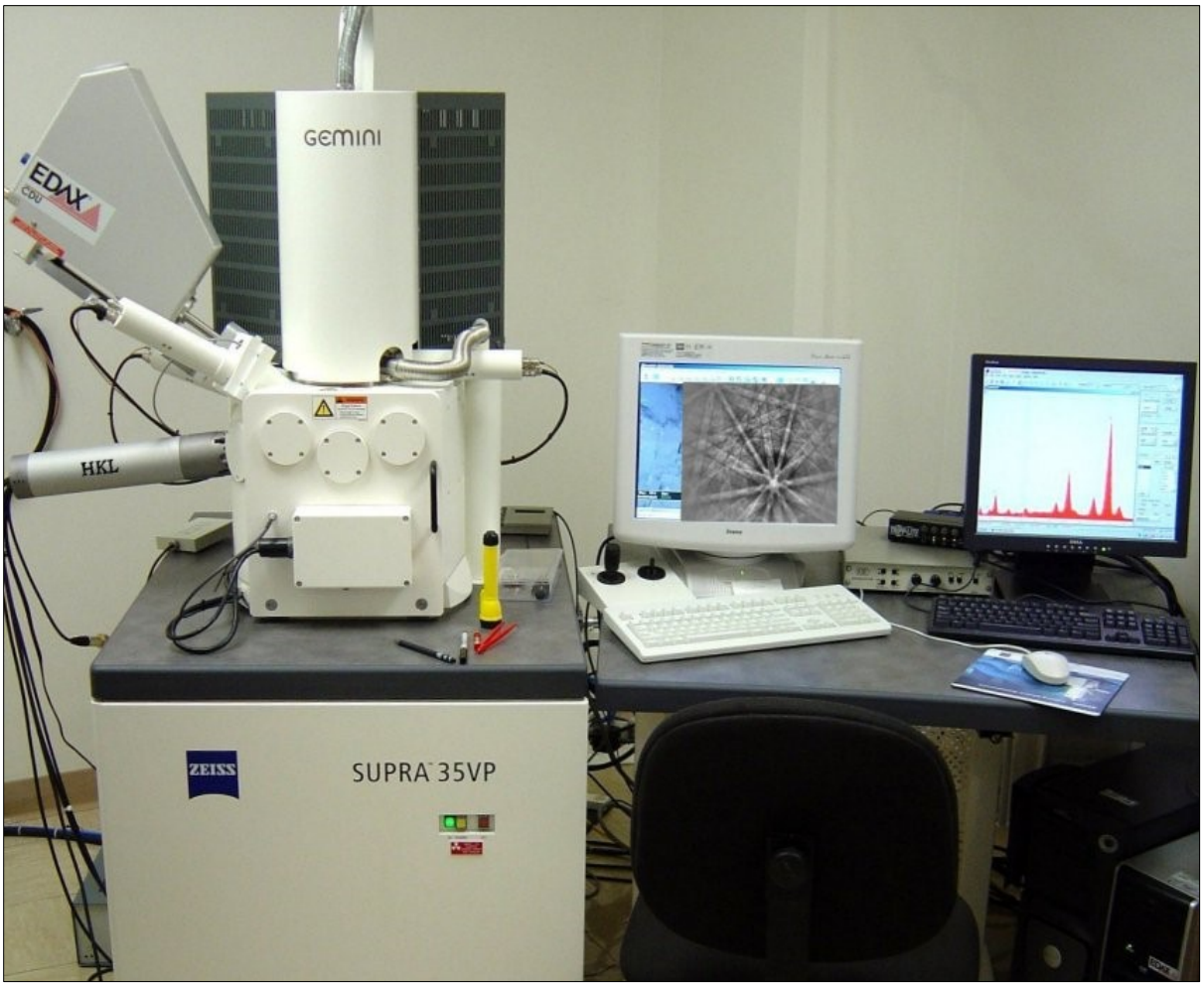


Figure 4.27 Photograph of a Zeiss Supra 35 Variable pressure SEM

4.3.2.1. SEM Protocol

The basic principle of SEM is that a beam of electrons is generated by a suitable source, typically a tungsten filament or a field emission gun, the electron beam is accelerated through a high voltage and passed through a system of apertures and electromagnetic lenses to produce a thin beam of electrons, and then the beam scans the surface of the specimen by means of scan coils. Electrons are emitted from the specimen by the action of the scanning beam and collected by a suitably-positioned detector. The beam scanning the specimen surface is exactly synchronized with the spot in the screen that the operator watches. The electron detector controls the

brightness of the spot on the screen. The magnification of the image is the ratio of the size of the screen to the size of the area scanned on the specimen. There are different types of electron images and the two most common types are: (I) The secondary electron image (SEI) used mainly to image fracture surfaces and gives high resolution images, and (II) The backscattered electron image (BEI) used typically to image a polished section. Brightness of BEI is dependent on the atomic number of the specimen or the average atomic number for compounds. All SEM images are in black and white (Willard *et.al.,* 1986).

4.3.3. Thermo-Gravimetric Analysis

Thermal analysis was recorded with a TA Instrument Q50 TGA (shown in figure 4.28) and a TA Instrument Q2000 DSC (shown in Figure 4.29). Approximately 10 mg of the regenerated raw and regenerated samples (previously dissolved in [Emim][OAc]) were carried out by the Thermal Analyser at a heating rate of 5 ^{0}C /min under continuous nitrogen flow at a temperature range from 25 ^{0}C to 600 ^{0}C.

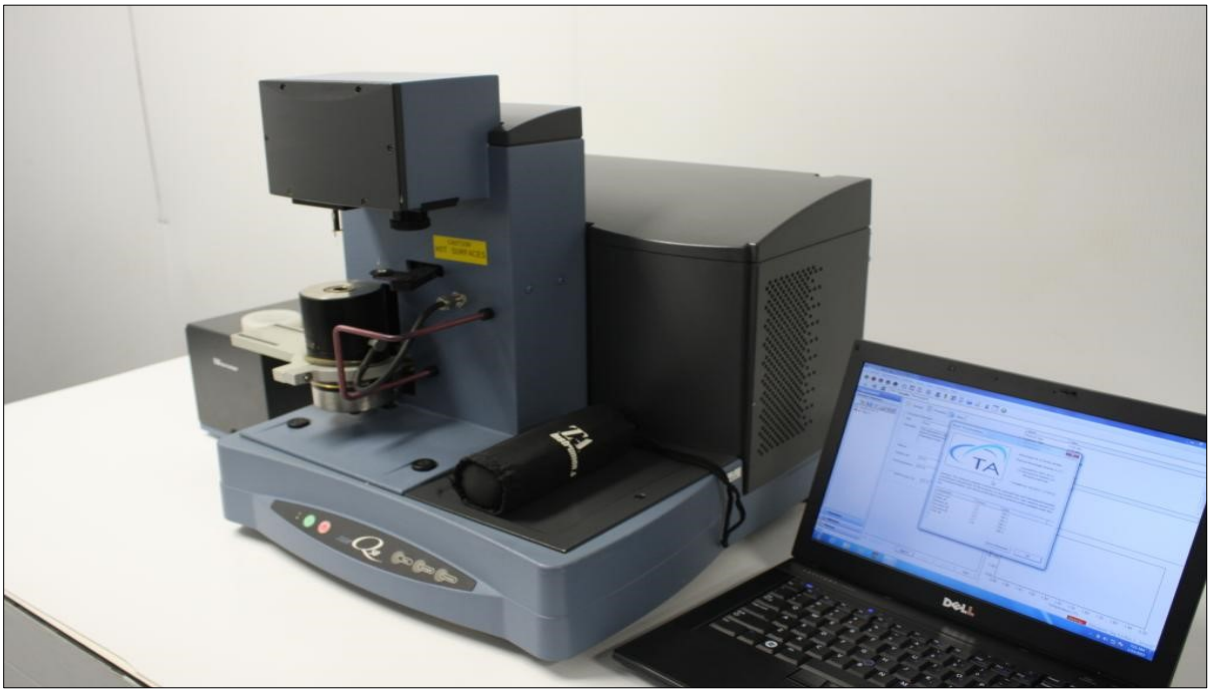


Figure 4.28 Photograph of a Q50 TGA (TA Instrument)

Figure 4.29 Photograph of a Q2000 DSC (TA Instrument)

4.3.3.1. TGA Protocol

Thermal analysis includes a group of techniques in which a physical property of a substance is measured as a function of temperature while the substance is subjected to a controlled temperature program. A complete modern thermal analysis instrument measures temperatures of transitions, weight losses in materials, energies of transitions, dimensional changes, and viscoelastic properties. TGA provides the analyst with a quantitative measurement of any weight change associated with a transition. TG curves are characteristic for a given compound or system because of the unique sequence of physicochemical reactions which occur over definite temperature ranges and at rates that are a function of the molecular structure. Changes in weight are a result of the rupture and/or formation of various physical and chemical bonds at elevated temperatures that lead to the evolution of volatile products or the formation of heavier reaction products. From the thermos-grams, data obtained is usually concerning

the thermodynamics and kinetics of the various chemical reactions, reaction mechanisms, and the intermediate and final reaction products. The usual temperature range is from ambient to 1200 ^{0}C with inert or reactive atmospheres. The derivative in the TG is often used to pinpoint completion of the weight-loss steps or to increase resolution of overlapping weight-loss occurrences (Willard *et.al.,* 1986:316).

4.3.3.2. DSC Protocol

Differential scanning calorimetry (DSC) is an experimental technique that measures the heat energy uptake that takes place in a sample during controlled increases (or decrease) in temperature. At the simplest level it may be used to determine thermal transition ("melting") temperatures for samples in solution, solid, or mixed phases (e.g. suspensions).

CHAPTER **5**

RESULTS

5.1. Compositional analysis

(A) Moisture and lignin content of whole SCB

5.1.1. Moisture content of whole bagasse

The moisture content of whole sugarcane bagasse was determined according to the NREL procedure. The water content is shown in Table 5.1, the results are reported on a dry weight basis (% wt.). All the experiments were repeated 3 times. The average moisture content was found to be 9.25 %. Water reduces not only cellulose solubility in ionic liquids, (Mazza *et al*., 2009) but also the effectiveness of ionic liquid pre-treatment (Doherty *et al.,* 2010, Wo. Pat. 2008). Biomass contains significant quantities of water, 2 - 300 % relative to the oven-dried weight. In addition, ionic liquids are hygroscopic and will absorb significant quantities of moisture when exposed to air (Cammarata *et al.,* 2001). The drying of ionic liquids requires heat and vacuum, particularly when the ILs are strongly hydrogen bonded. Therefore, an IL pre-treatment that tolerates moisture would be beneficial to the overall energy and cost of a lignocellulose processing system using ILs.

Table 5.1 Moisture content of sugarcane bagasse sample

Sample No	Sample air dry (g)	Mass of Al foil (g)	Total before (g)	Total after (g)	Water content (wt. %)
1	1.05	0.47	1.52	1.42	9.02
2	1.03	0.55	1.58	1.49	8.97
3	1.05	0.65	1.70	1.60	9.76
Average	1.04	0.55	1.60	1.50	9.25

5.1.2. Lignin content

Sulfuric acid was used for the hydrolysis of sugarcane bagasse to determine the soluble and insoluble lignin content. The reaction was carried out in a water bath at 30 ^{0}C for 1 hour and 4 replicates were done. The absorbance values are shown in table 5.2. The acid soluble lignin was found to be 31.2 %, acid insoluble lignin was found to be 1.29 % and the total lignin 32.5 %. All calculations are shown below and all results are reported in percentage (%).

Table 5.2 Absorbance at 240 nm, acid soluble lignin, acid insoluble residue, acid insoluble lignin and total lignin

Sample Number	Absorbance	Acid Soluble Lignin (ASL) %	Acid Insoluble Residue (AIR) %	Acid Insoluble Lignin (AIL) %	Total Lignin %
1	0.81	31.30	1.15	1.27	32.57
2	0.80	30.76	1.19	1.31	32.08
3	0.82	31.68	1.16	1.28	32.96
4	0.81	31.22	1.17	1.29	32.52
Average	0.81	31.24	1.17	1.29	32.53

$$\% \text{ ASL} = \frac{\text{UV Absorbance x Filtrate Volume x Dilution factor}}{\text{Absorptivity (e) x ODW (sample)x Pathlenghth}} \text{ x } 100$$

$$= \frac{0.86 \text{ x } 86.73 \text{ x } 10}{25 \text{ x } 90.65 \text{ x } 1} \text{ x } 100$$

$$= 32.92 \%$$

$$\% \text{ AIL} = \frac{\text{Weight (crucible + acid insoluble residue (AIR)} - \text{Weight (crucible)}}{\text{ODW}} \text{ x } 100$$

$$= \frac{(2.1111 + 1.1438) - 2.1111}{90.65} \text{ x } 100$$

$$= 1.26\ \%$$

$$\% \text{ Total lignin} = \% \text{ AIL} + \% \text{ ASL}$$

$$= 32.922 + 1.2711$$

$$= 34.19\ \%$$

(B) Cellulose, hemicellulose, lignin, ash and extractives in whole bagasse

The compositional analysis of the main components (cellulose, hemicellulose, lignin, ash and extractives) that make up the raw whole sugarcane bagasse was repeated and the values are shown in table 5.3. The hemicellulose content was found to be 28.4 %, total lignin 22.6 %, cellulose 42.3 %, extractives 5.6 % and ash 1.2 %. All results are reported in percentage and normalised.

Table 5.3 Hemicellulose, total lignin, cellulose, extractives and ash content of whole sugarcane bagasse

Component	**Whole bagasse (WB)** **%**
Hemicellulose	28.40
Total lignin	22.60
Cellulose	42.30
Extractives	5.60
Ash	1.20

(C) Composition of pith and long fiber bagasse ash

A sample of whole bagasse which was separated into pith and long fiber bagasse was analysed for elemental composition. The elemental composition of pith bagasse and long fiber bagasse ash was obtained by wavelength-dispersive X-ray fluorescence (WD-XRF) and presented in Table 5.4.

Table 5.4 Composition of pith ash and long fiber bagasse ash by WD-XRF

Compound	**Long fiber bagasse ash (wt. %)**	**Pith ash (wt. %)**	**Trace elements**	**Long fiber bagasse ash (ppm)**	**Pith ash (ppm)**
SiO_2	54.57	70.06	Cu	136	<60
Al_2O_3	0.47	1.11	Cr	185	96
Fe_2O_3	0.74	1.35	Hf	<95	<95
Mn_3O_4	0.31	0.11	Ni	<60	<60
SO_3	2.17	1.76	Pb	<90	<90
CaO	11.15	13.07	V	<60	62
MgO	10.23	5.22	Zr	<90	<90
ZnO	0.08	0.06			
Na_2O	4.37	4.99			
K_2O	9.60	1.00			
P_2O_5	5.96	1.66			
TiO_2	0.09	0.17			
SrO	0.05	0.05			

5.2. Pre-treatment of sugarcane bagasse with ILs

Many studies have reported the influence of IL treatment on the composition of the pre-treated biomass with the most data reported for $[C_2C_1im][MeCO_2]$ as a dissolution medium for biomass treatment. Table 5.5 as adapted from literature (Brandt *et al*., 2011) shows the lignin and hemicellulose removal by $[C_2C_1im][MeCO_2]$ at optimum conditions for different biomass substrates.

Table 5.5 Lignin and hemicellulose removal by $[C_2C_1im][MeCO_2]$ treatment of lignocellulosic biomass at optimum conditions

Feedstock	**Temp.**	**Time**	**Glucose yield (%)**	**Lignin yield (%)**	**Hemicellulose yield (%)**
Corn Stover	125 ^{0}C	1 h	83	44	34
Energy cane	120 ^{0}C	30 min	87	32	14
Switchgrass	120 ^{0}C	3 h	97	34	22
	160 ^{0}C	3 h	79	65	83
	160 ^{0}C	5 h	85	69	77
Bagasse	165 ^{0}C	10 min	not measured	34	not measured
Maple wood	130 ^{0}C	1.5 h	84	52	26
Oak	110 ^{0}C	16 h	not measured	35	not measured
Pine	110 ^{0}C	16 h	not measured	26	not measured
Miscanthus (20 % water)	120 ^{0}C	22 h	96	56	37
Willow (20 % water)	120 ^{0}C	22 h	79	18	44
Pine (20 % water)	120 ^{0}C	22 h	88	17	0
Triticale straw	150 ^{0}C	1.5 h	86	64	76
Triticale straw (50 % water)	150 ^{0}C	1.5 h	95	29	62

Several other ionic liquids besides $[C_2C_1im][MeCO_2]$ have also been reported in literature to have the capability of removing lignin in lignocellulosic biomass. Chloride derived ILs have been mostly used for the dissolution of wood. Table 5.6 below shows the delignification and hemicellulose removal by different ILs.

Table 5.6 Delignification and hemicellulose removal by different ILs on different biomasses at optimum temperature

Ionic liquid	**Biomass**	**Temp.**	**Time**	**Lignin (%)**	**Hemicellulose (%)**	**Reference**
$[C_4C_1im][MeSO_4]$ (20 % water)	*Miscanthus*	120 ^{0}C	2 h	27	0	Brandt *et al*., (2011)
$[C_4C_1im][HSO_4]$ (20 % water)	*Miscanthus*	120 ^{0}C	2 h & 22 h	44 & 93	51 & 82	
	Willow		22 h	85	79	
	Pine		22 h	65	66	
$[C_4C_1im][MeSO_3]$ (20 % water)	*Miscanthus*	120 ^{0}C	22 h	68	73	
$[C_4Him][HSO_4]$ (20 % water)	*Miscanthus*	120 ^{0}C	4 h & 20 h	81 & 80	84 & 92	
$[C_4C_1im][Cl]$ (20 % water)	*Miscanthus*	120 ^{0}C	22 h	15	6	
$[C_4C_1im][Cl]$ (40 % water)	Legume straw	150 ^{0}C	2 h	30	9	Wei *et al*., (2012)
$[C_4C_1im][Cl]$	Triticale straw	90 ^{0}C	24 h	15	11	Fu *et al*., (2010)
$[C_4C_1im][MeSO_4]$ (7% H_2SO_4)	Sugarcane bagasse	125 ^{0}C	2 h	26	88	Deidericks *et al*., (2012)

For $[C_4C_1im][HSO_4]$ and its acid–base IL equivalent ($[C_4Him][HSO_4]$), the delignification yield is more compared to $[C_2C_1im][MeCO_2]$. Sulfonate and sulfate based ionic liquids seems to be the more effective ILs for cellulose and lignin separation.

5.3. Pre-treatment of sugarcane bagasse with 1-ethyl-3-methylimidazolium acetate ([Emim][OAc])

An effective pre-treatment should be able to deconstruct the three-dimensional structure of lignocellulose, and break down the semi-crystalline cellulose and hemicellulose; give high yield of sugars and/ or chemicals (or give highly digestible pre-treated solid); avoid carbohydrate degradation and in particular preserve the utility of the pentosane (hemicellulose) fraction; avoid the formation of inhibitory toxic by-products; allow lignin recovery and exploitation to give valuable by-products and be cost–effective. Table 5.7 shows the SCB pre-treatment conditions and mixture preparations used in this study.

Table 5.7 Sugarcane bagasse ([Emim][OAc]) pre-treatment conditions

Sample Name	Temp. (^{0}C)	Time (h)	IL added (g)	Biomass (g)	Dry biomass (g)	Mass before pre-treatment (g)	Mass after pre-treatment (g)
1	90	6	6.05	0.60	0.56	23.22	23.22
2	90	12	6.02	0.61	0.57	22.78	22.78
3	90	24	6.03	0.60	0.55	22.80	22.80
4	120	6	5.02	0.51	0.47	21.66	21.57
5	120	12	5.01	0.52	0.48	22.19	22.10
6	120	24	5.01	0.52	0.48	21.74	21.65
7	150	6	5.03	0.50	0.46	21.68	21.62
8	150	12	5.04	0.51	0.47	22.14	22.06
9	150	24	5.05	0.50	0.46	21.68	21.60

5.3.1. Pulp recovery after ([Emim][OAc]) pre-treatment

After IL pre-treatment the pulp was recovered by washing the reaction mixture with deionised water to remove the IL and released sugars. Pre-treatment at 90 ^{0}C for 6 hours gave the highest pulp recovery (73.86 %) whereas 120 ^{0}C for 6 hours gave the lowest pulp recovery (70.94 %). Table 5.8 shows the pulp recoveries after pre-treatment and indicates that at a temperature of 90 ^{0}C for 6 hours less extraction occurred (27 %). At 90 ^{0}C for 6 hours more extraction occurred (29.0 %). The graph of pre-treatment time *vs* pulp yield is shown in figure 5.1.

Table 5.8 SCB pulp recoveries after ([Emim][OAc]) pre-treatment

Sample No	Temp (^{0}C)	Time (h)	Total air dry pulp (g)	Air dry pulp sample (g)	Weight Al foil (g)	Total before drying (g)	Total after drying (g)	Dry content pulp (%)	Total dry pulp (g)	Total Pulp recovery (%)
1	90	6	0.48	0.105	0.18	0.28	0.27	86.13	0.41	73.86
2	90	12	0.39	0.104	0.13	0.24	0.22	88.92	0.35	61.38
3	90	24	0.47	0.101	0.16	0.26	0.25	83.44	0.40	71.50
4	120	6	0.41	0.102	0.15	0.25	0.24	81.73	0.34	70.95
5	120	12	0.34	0.104	0.19	0.29	0.28	80.01	0.28	57.97
6	120	24	0.41	0.105	0.18	0.29	0.27	79.34	0.32	68.20
7	150	6	0.47	0.105	0.24	0.34	0.31	71.64	0.34	72.63
8	150	12	0.42	0.103	0.12	0.23	0.20	67.36	0.28	59.05
9	150	24	0.43	0.105	0.18	0.28	0.26	76.52	0.33	71.43

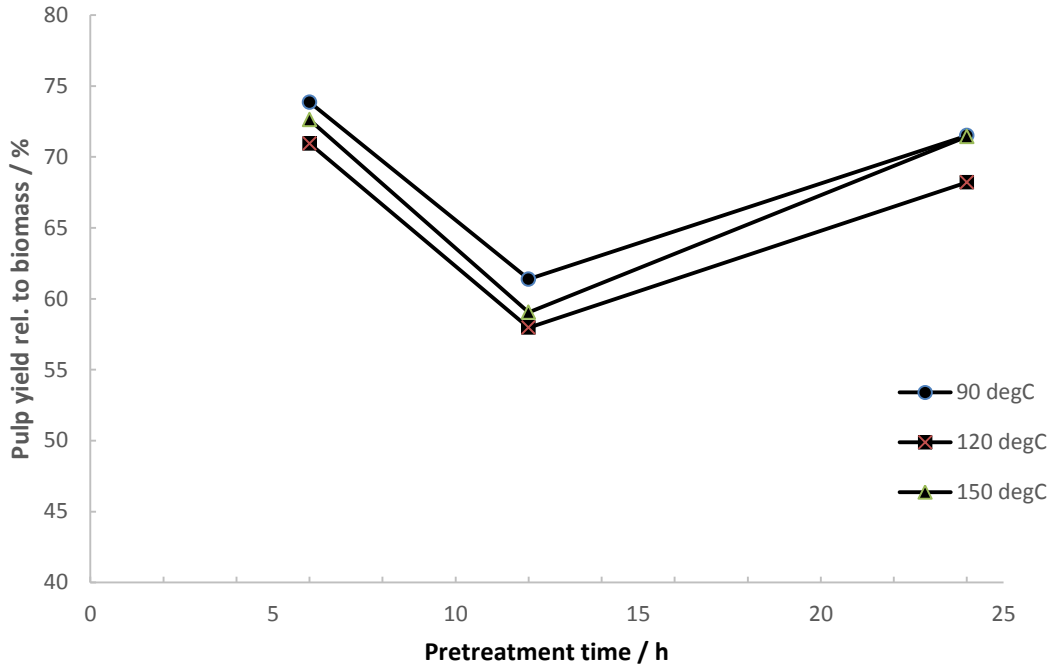


Figure 5.1 Pulp recovered after ([Emim][OAc]) pre-treatment of SCB at varied times and temperatures

5.3.2. Lignin recovery after ([Emim][OAc]) pre-treatment

To estimate the lignin content of the pre-treatment, the pulps were washed with deionized water, filtered and the solids air dried. Table 5.9 shows the lignin recoveries. The highest lignin recovery (28.8 %) was observed at 120 ^{0}C after 12 hours of pre-treatment and the lowest (2.1 %) was observed at 90 ^{0}C after 24 hours of pre-treatment. The graphs of mass loss, lignin recovery and pulp recovery at 90 ^{0}C, 120 ^{0}and 150 ^{0}C respectively relative to biomass are shown in figures 5.2, 5.3 and 5.4 respectively.

Table 5.9 Lignin recoveries after ([Emim][OAc]) pre-treatment

Sample name	Temp (^{0}C)	Time (h)	Lignin recovered (g)	Lignin yield relative to dry biomass (%)
1	90	6	0.48	9.53
2	90	12	0.39	4.50
3	90	24	0.48	2.13
4	120	6	0.41	22.13
5	120	12	0.35	28.82
6	120	24	0.41	17.32
7	150	6	0.48	16.35
8	150	12	0.42	11.65
9	150	24	0.44	4.40

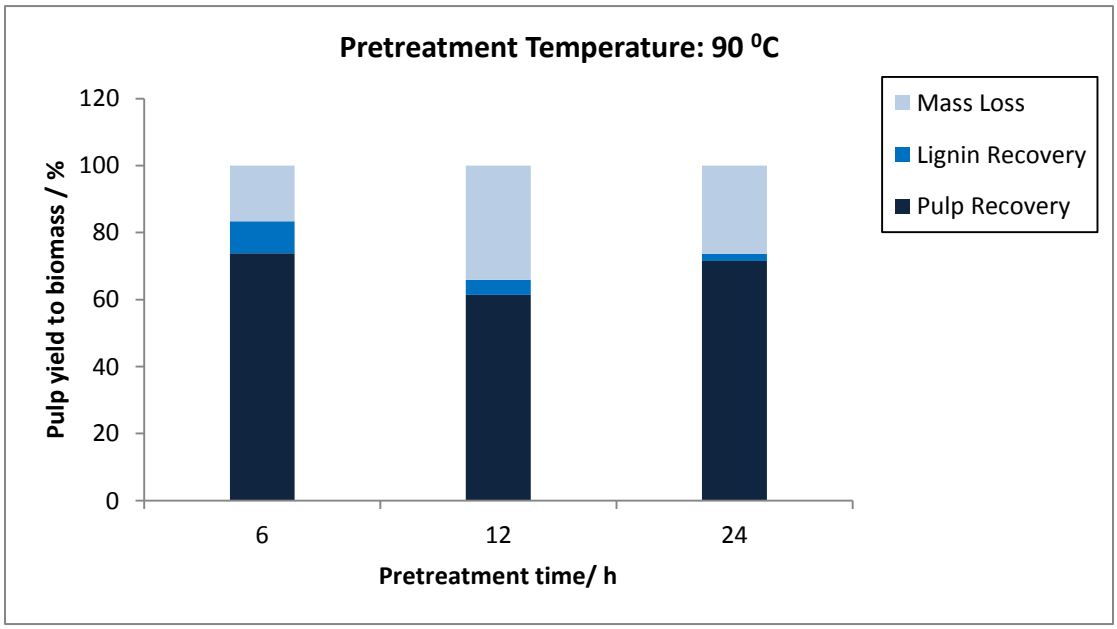


Figure 5.2 Mass loss, lignin recovery and pulp yield relative to biomass at 90 ^{0}C

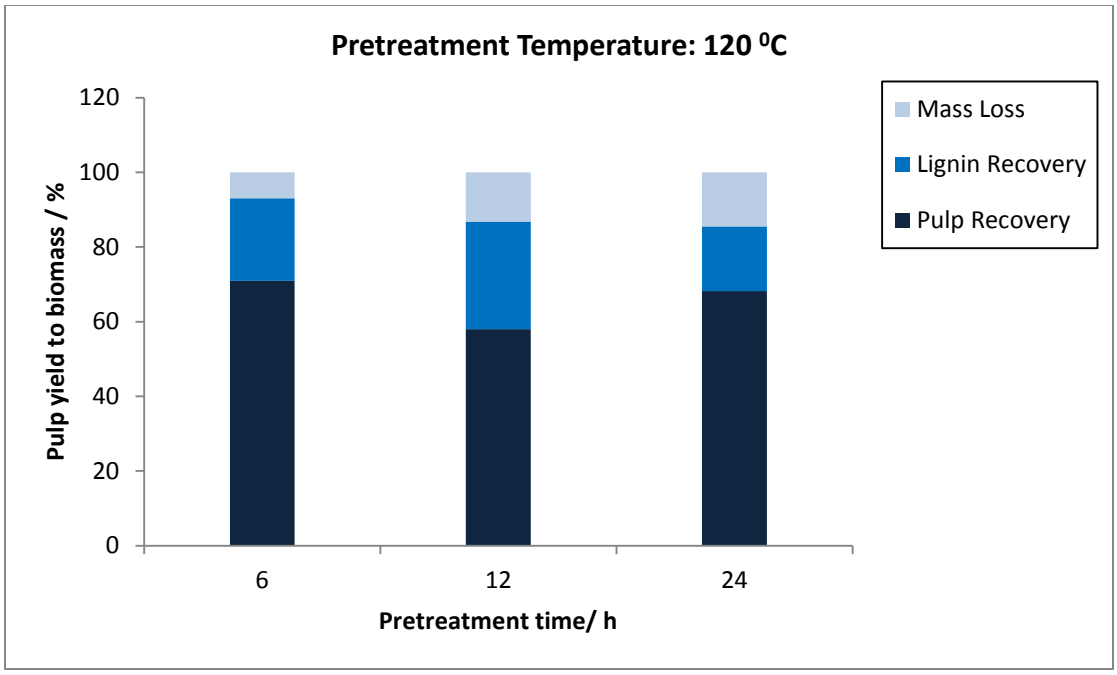


Figure 5.3 Mass loss, lignin recovery and pulp yield relative to biomass at 120 ^{0}C

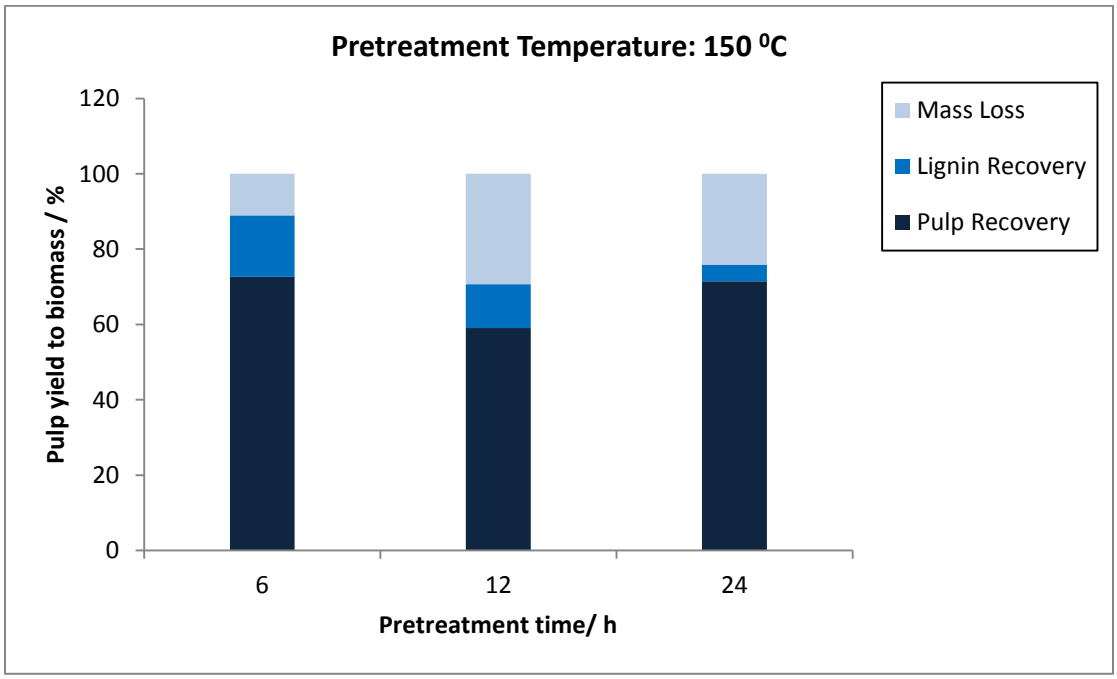


Figure 5.4 Mass loss, lignin recovery and pulp yield relative to biomass at 150 ^{0}C

5.3.3. Sugars released yield after ([Emim][OAc]) pre-treatment

A series of mixed sugar standards ranging from 0.025 – 0.1 % were prepared. These standards are arabinose, fructose, galactose, glucose, mannose and sucrose. The order of elution for standards was as follows: sucrose, glucose, galactose, arabinose, mannose, and fructose. A calibration curve was plotted to determine the concentration of the sugars in the pre-treatment liquor at optimum conditions (120 ^{0}C, 12 h). Figure 5.5 shows the chromatogram of the mixed standards, figure 5.6 shows the chromatogram of the sample at optimum conditions and figure 5.7 shows the calibration plot for the determination of the sample concentration. Table 5.10 shows the respective retention times.

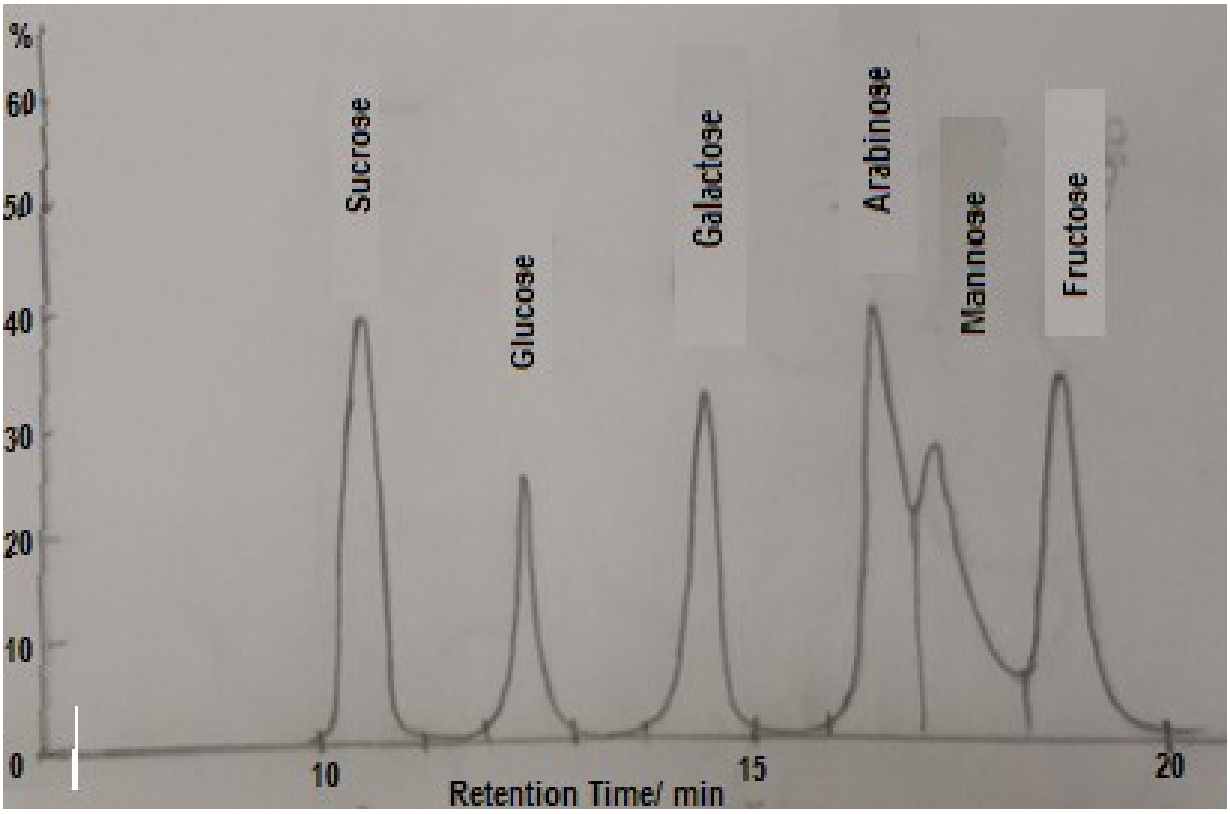


Figure 5.5 HPLC chromatogram of the mixed standard

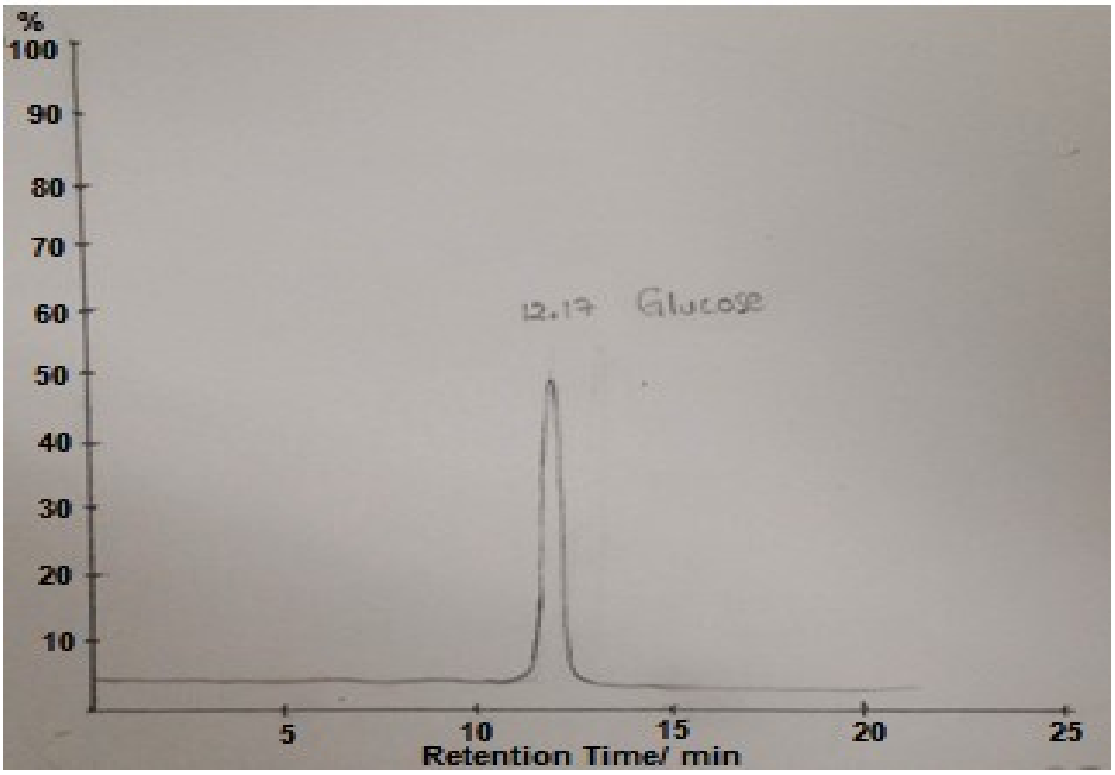


Figure 5.6 HPLC chromatogram of the sample at optimum conditions

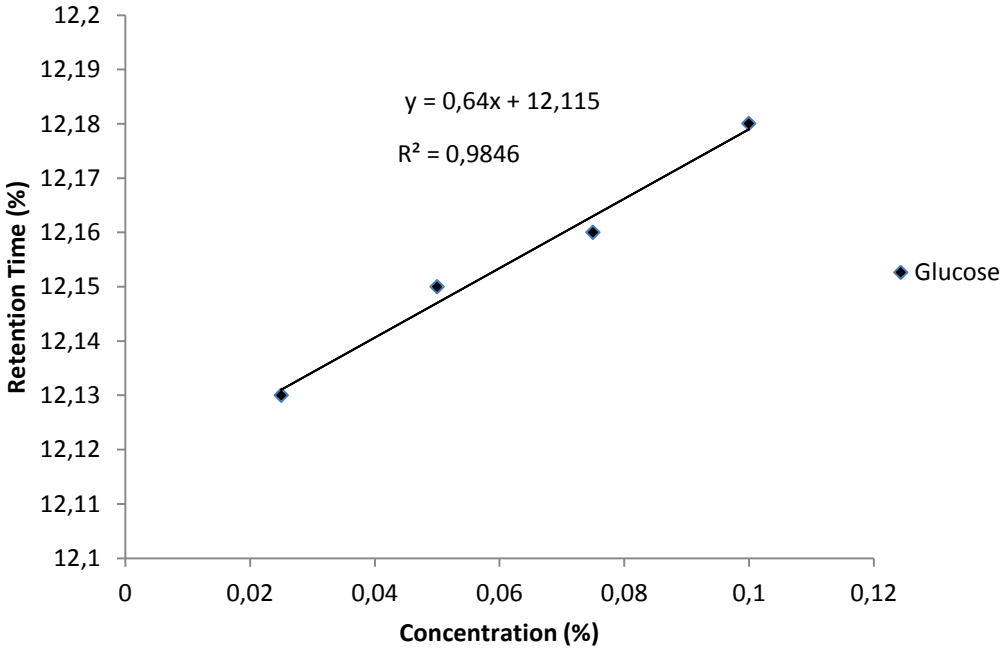


Figure 5.7 Calibration plot of glucose standard at different concentrations

Table 5.10 Retention times of the mixed standards and sample in the order of elution

Concentration (%)	Sucrose	Glucose	Galactose	Arabinose	Mannose	Fructose
0.025	10.01	12.13	14.40	16.10	16.84	18.16
0.050	10.02	12.15	14.41	16.12	16.86	18.17
0.075	10.04	12.16	14.42	16.12	16.87	18.17
0.100	10.07	12.18	14.42	16.11	16.86	18.19
Sample	-	12.17	-	-	-	-

Amongst the sugars tested only glucose was detected in the sample with a retention time of 12.17 min. From the calibration plot the concentration of the sample was extrapolated using the equation of the graph as shown in the calculation below:

$$y = 0.64\,x + 12.115$$

$$12.17 = 0.64\,x + 12.115$$

$$x = \frac{12.17 - 12.115}{0.64}$$

$$= 0.0860\ \%$$

5.4. Pre-treatment of sugarcane bagasse with triethylammonium hydrogen sulfate ([$HNEt_3$][HSO_4])

Ionic liquid pre-treatment of whole bagasse (WB) and industrially depithed bagasse (DB) was performed using triethylammonium hydrogen sulfate [$HNEt_3$][HSO_4] containing 20 wt. % water, an inexpensive solvent selected for its potential application in a commercial bio-refinery. Pre-treatment was carried out at 120 ^{0}C (optimum temperature for SCB pre-treatment using this IL). Table 5.11 shows the SCB pre-treatment conditions and mixture preparations used in this study.

Table 5.11 Sugarcane bagasse ([$HNEt_3$][HSO_4]) pre-treatment conditions at 120 ^{0}C

Sample Name	IL added (g)	Biomass (g)	Dry biomass (g)	Mass before pre-treat. (g)	Mass after pre-treat. (g)	Mass loss during exp. (%)
1 h Dep	10,05	1,04	0,99	109,42	109,42	-0,0050
1 h NB	10,05	1,01	0,94	109,18	109,18	-0,0030
2 hr Dep	10,26	1,02	0,97	109,91	109,91	0,0027
2 h NB	10,05	1,01	0,95	109,05	109,05	0,0012
4 h Dep	10,10	1,03	0,97	109,65	109,64	-0,0060
4 h NB	10,12	1,03	0,96	109,12	109,11	-0,0090
8 h Dep	10,09	1,03	0,98	111,19	111,19	-0,0010
8 h NB	10,18	1,02	0,95	109,77	109,77	-0,0010
16 h Dep	10,38	1,05	0,99	110,65	110,63	-0,0190
16 h NB	10,36	1,02	0,95	109,08	109,07	-0,0130
24 h Dep	10,14	1,05	0,99	109,64	109,61	-0,0190
24 h NB	10,06	1,02	0,95	111,77	111,42	-0,3090

*Dep = depithed bagasse, NB = normal mill run bagasse

5.4.1. Pulp recovery after ([$HNEt_3$][HSO_4]) pre-treatment

After IL pre-treatment the pulp was recovered by washing the reaction mixture with ethanol to remove the IL and released sugars. Figure 5.8 shows the pulp and lignin recoveries after pre-treatment at 120 ^{0}C for between 1 and 24 h. WB and DB were compared and seen to give very similar mass recoveries within experimental error. Pulp yields were minimized at 48 - 51 % of original biomass weight at 16 h, suggesting maximum lignin and/ or hemicellulose removal occurs after 16 h of pre-treatment, confirmed by a maximum in lignin recovery at 17 - 18 % within 16 h. Table 5.12 shows the pulp recoveries after pre-treatment.

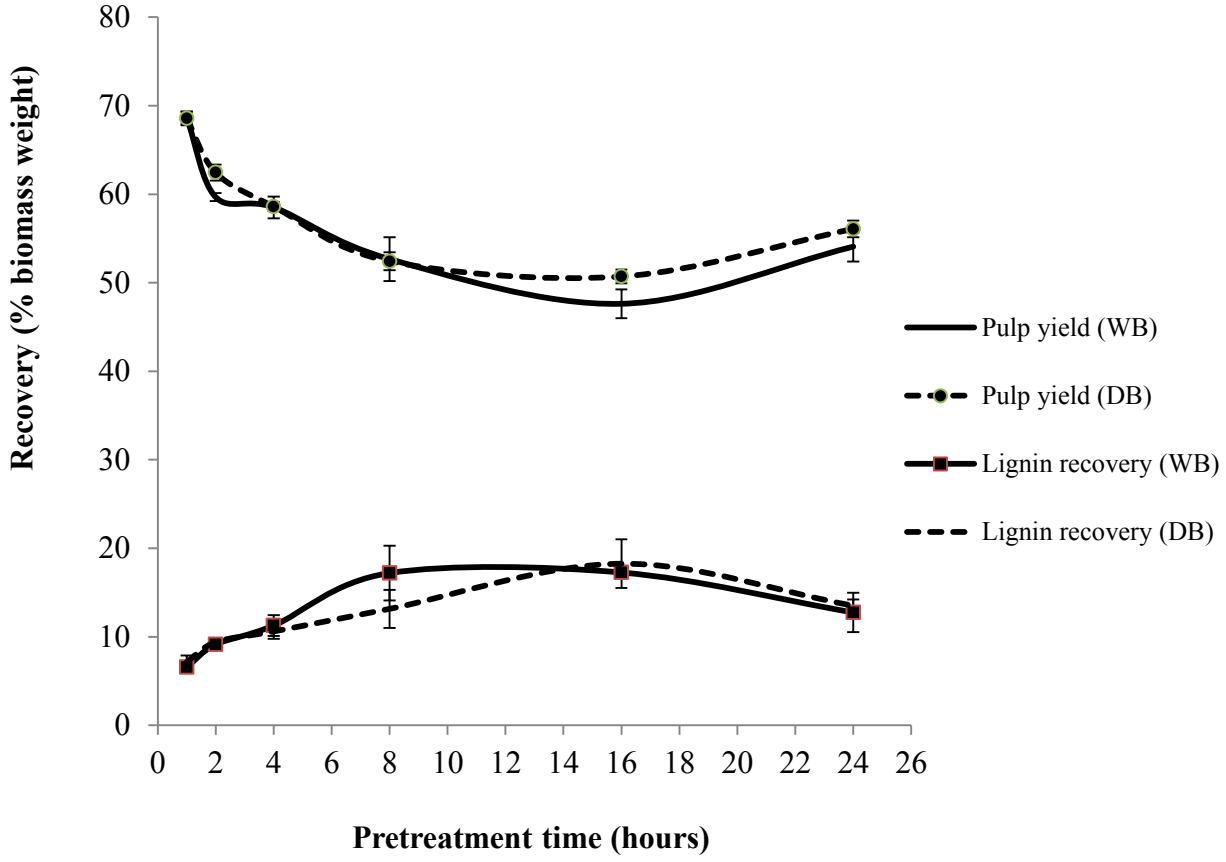


Figure 5.8 Pulp and lignin recoveries after ([$HNEt_3$][HSO_4]) pre-treatment at 120 ^{0}C

Table 5.12 SCB pulp recoveries after ([$HNEt_3$][HSO_4]) pre-treatment

Sample Name	Total air dry pulp (g)	Air dry pulp sample (g)	Weight Al foil (g)	Total before drying (g)	Total after drying (g)	Dry content pulp (%)	Total Pulp recovery (%)
1 h Dep	0.66	0.10	0.14	0.24	0.23	91.54	61.37
1 h NB	0.60	0.10	0.14	0.25	0.24	91.26	57.91
2 hr Dep	0.57	0.11	0.17	0.27	0.26	92.11	54.75
2 h NB	0.53	0.10	0.17	0.27	0.26	92.65	52.24
4 h Dep	0.84	0.12	0.14	0.25	0.24	92.46	79.88
4 h NB	0.79	0.40	0.15	0.26	0.25	92.70	76.61
8 h Dep	0.69	0.13	0.16	0.28	0.27	92.42	65.61
8 h NB	0.66	0.11	0.14	0.25	0.24	92.67	64.75
16 h Dep	0.55	0.09	0.18	0.27	0.26	91.23	50.70
16 h NB	0.50	0.10	0.16	0.26	0.26	91.63	47.62
24 h Dep	0.60	0.40	0.14	0.24	0.24	92.87	56.08
24 h NB	0.56	0.11	0.17	0.28	0.27	90.29	54.08

5.4.2. Lignin recovered after ([$HNEt_3$][HSO_4]) pre-treatment

The amount of Klason lignin found in the pre-treated pulp material was found to decrease with increasing pre-treatment time to reach a minimum after 4 h, increasing slightly between 4 and 16 h before reaching a level significantly higher than the native lignin content after 24 h. Delignification was calculated using Klason lignin and was calculated relative to the percentage of lignin present in untreated biomass on an ODW basis (i.e. 19 %). Figure 5.9 shows the Klason lignin yield in the pre-treated SCB pulp. Table 5.13 shows the pulp recoveries after pre-treatment.

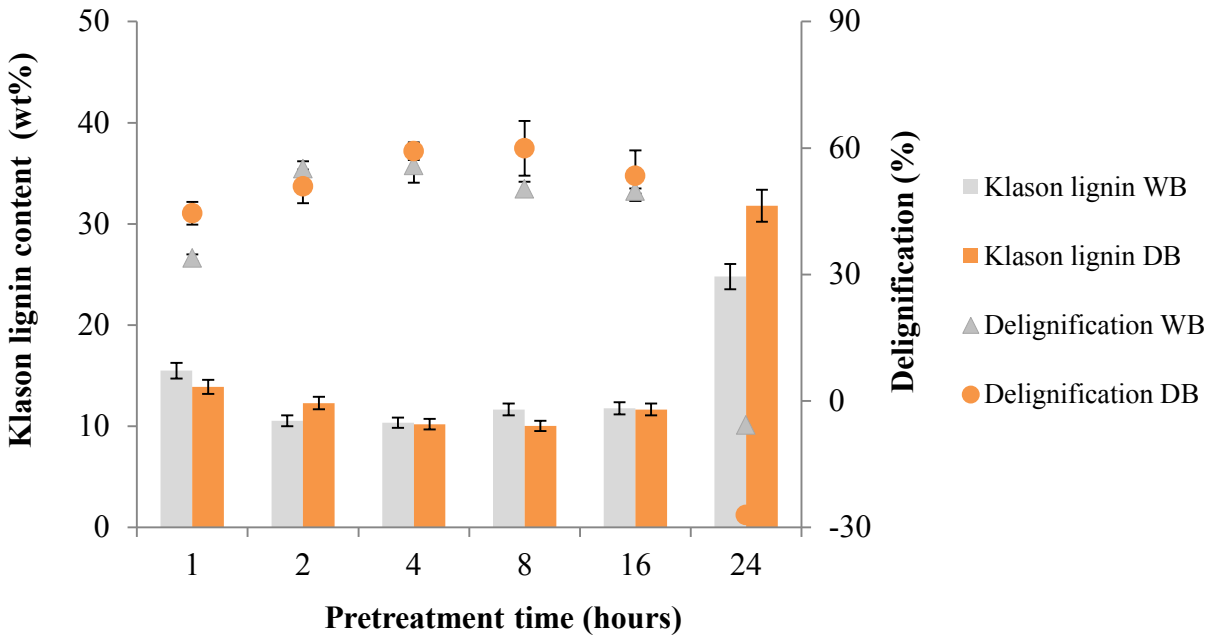


Figure 5.9 Klason lignin in the ([$HNEt_3$][HSO_4]) pre-treated substrate

Table 5.13 SCB lignin recoveries after ([$HNEt_3$][HSO_4]) pre-treatment

Sample Name	Lignin recovered (g)	Lignin yield relative to dry biomass (%)
1 h Dep	0.10	9.97
1 h NB	0.08	7.92
2 hr Dep	0.08	8.36
2 h NB	0.12	12.28
4 h Dep	0.05	4.66
4 h NB	0.07	7.21
8 h Dep	0.08	8.51
8 h NB	0.06	5.88
16 h Dep	0.16	16.48
16 h NB	0.15	15.98
24 h Dep	0.13	13.52
24 h NB	0.12	12.76

5.4.3. Saccharification after ([$HNEt_3$][HSO_4]) pre-treatment

A high fermentable glucose yield is an important factor that indicates the efficiency of pre-treatment. Sugarcane bagasse pulp samples were treated with a non-commercial *Novozyme* enzyme preparation. Figure 5.10 is the saccharification yields obtained for the samples.

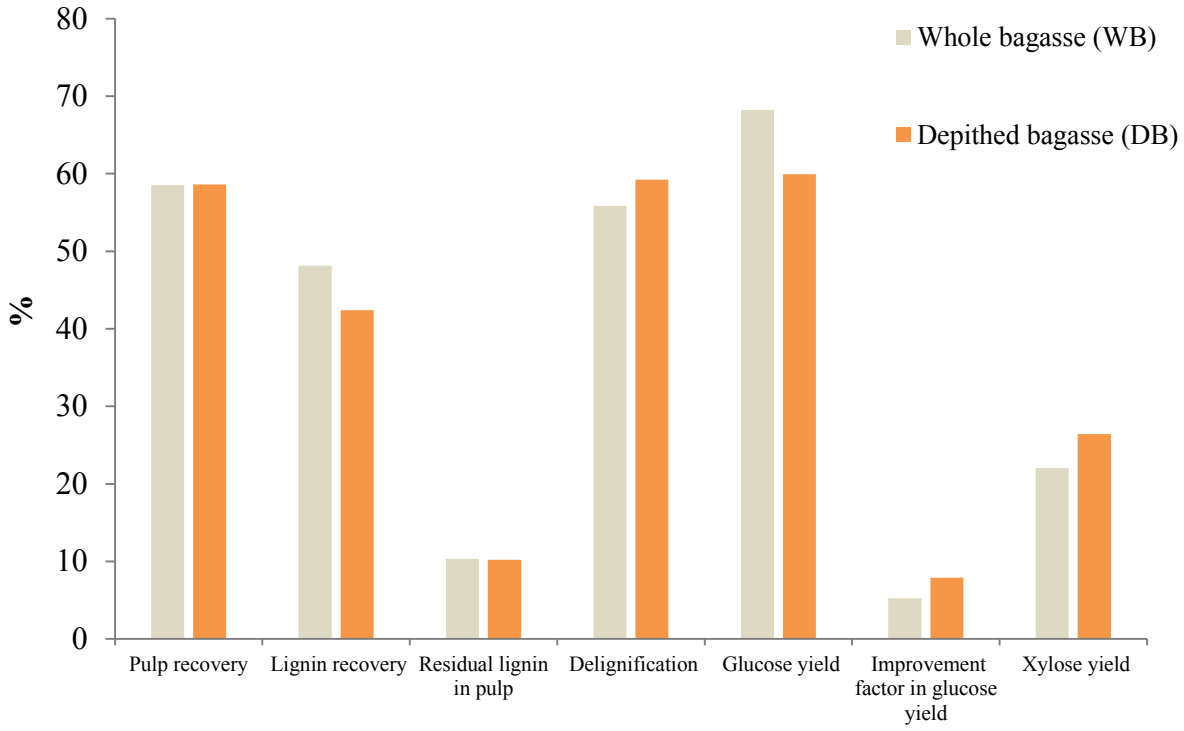


Figure 5.10 Saccharification yield of the ([$HNEt_3$][HSO_4]) pre-treated pulp

In figure 5.11 the xylose yields are given for WB and DB, they were optimized within 1 - 2 h and a maximum release of 30 % for WB and 35 % for DB was obtained following enzymatic saccharification for 7 days.

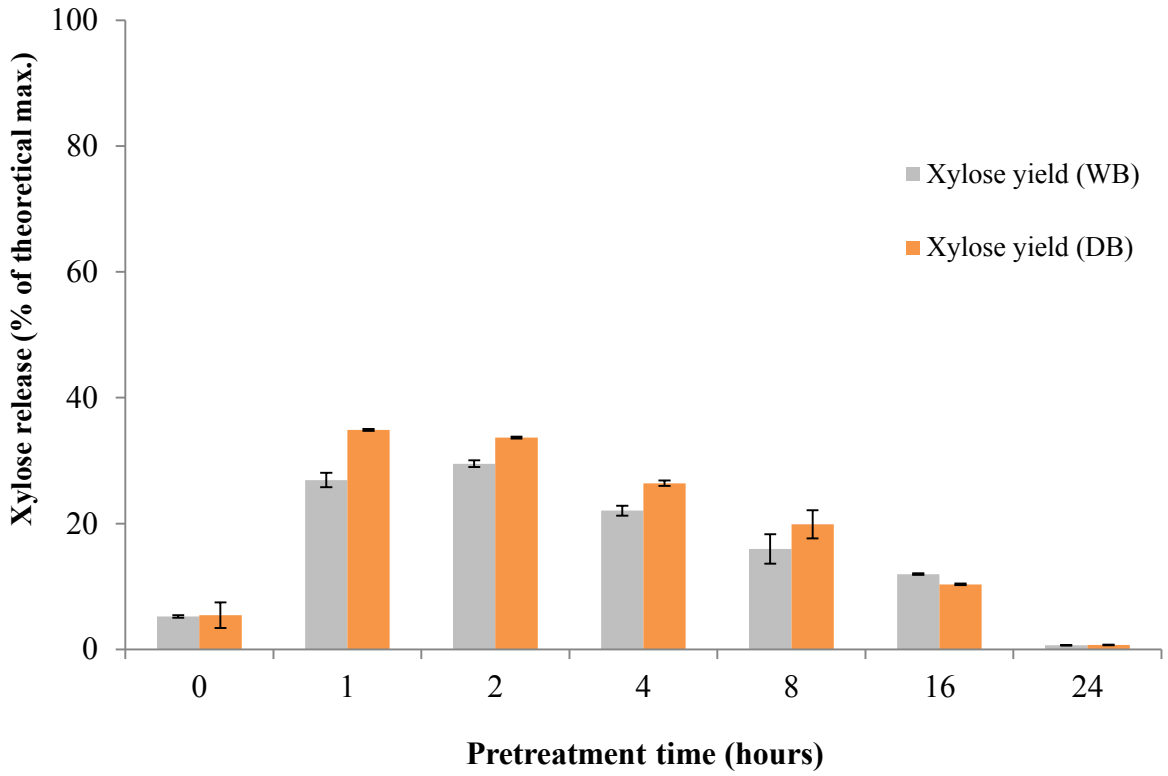


Figure 5.11 Xylose yield of the ($[HNEt_3][HSO_4]$) pre-treated pulp

5.5. Acid/ Alkali pre-treatment of SCB

5.5.1. Optimization of enzyme dose and time for saccharification of pre-treated sugarcane bagasse

Figure 5.12 illustrates the action of cellulase enzyme on alkali pre-treated mill-run sugarcane bagasse samples. The sugarcane bagasse samples when hydrolysed with 40 FPU/g, released maximum amount of sugars (536 mg/g), after 20 h of incubation. 10 FPU/g gave the lowest glucose yield.

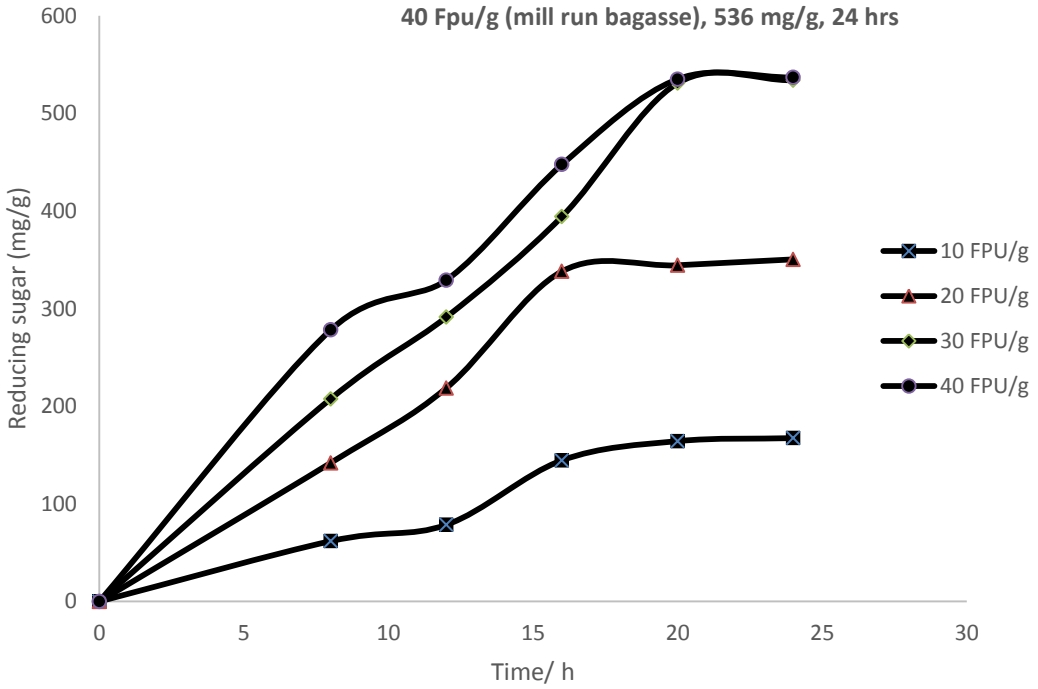


Figure 5.12 Effect of different enzyme dosages on the hydrolysis of mill-run sugarcane bagasse

5.5.2. Optimization of surfactant (Tween 80) dose and time for the saccharification of pre-treated sugarcane bagasse

Amongst various surfactant dosages studied, Tween 80 at a dose of 0.4 % (v/v) caused maximum increase in the saccharification efficiency with a release of 608 and 605 mg sugars/g substrate after 20 h of incubation as shown in figure 5.13.

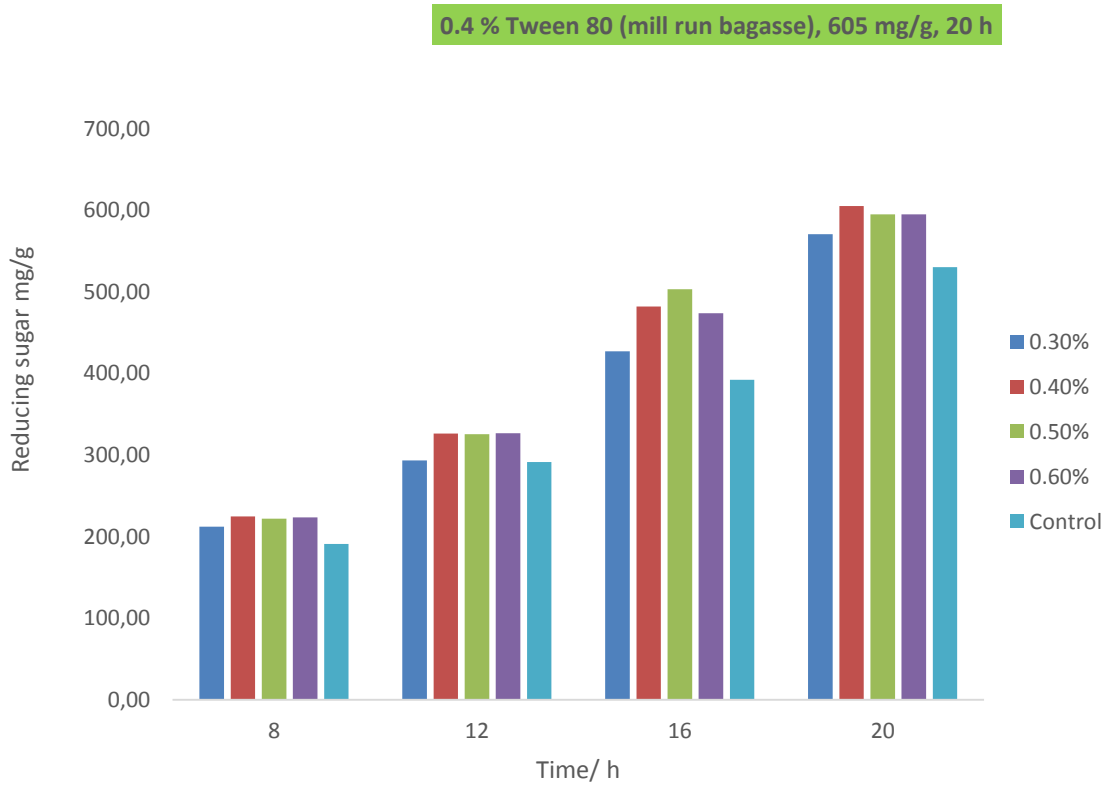


Figure 5.13 Effect of different surfactant dosages on the hydrolysis of mill-run sugarcane bagasse

5.5.3. Optimization of substrate consistency

The optimum conditions for enzyme dose (30 FPU/ g) and surfactant dose (0.4 %) were used to optimize the substrate consistency. The maximum glucose yield was obtained at a 10 % substrate consistency with a sugar release yield of 604 mg/ g as shown in figure 5.14. A 16 % substrate consistency gave the lowest glucose yield. This shows that as the substrate consistency increases the glucose yield decreases.

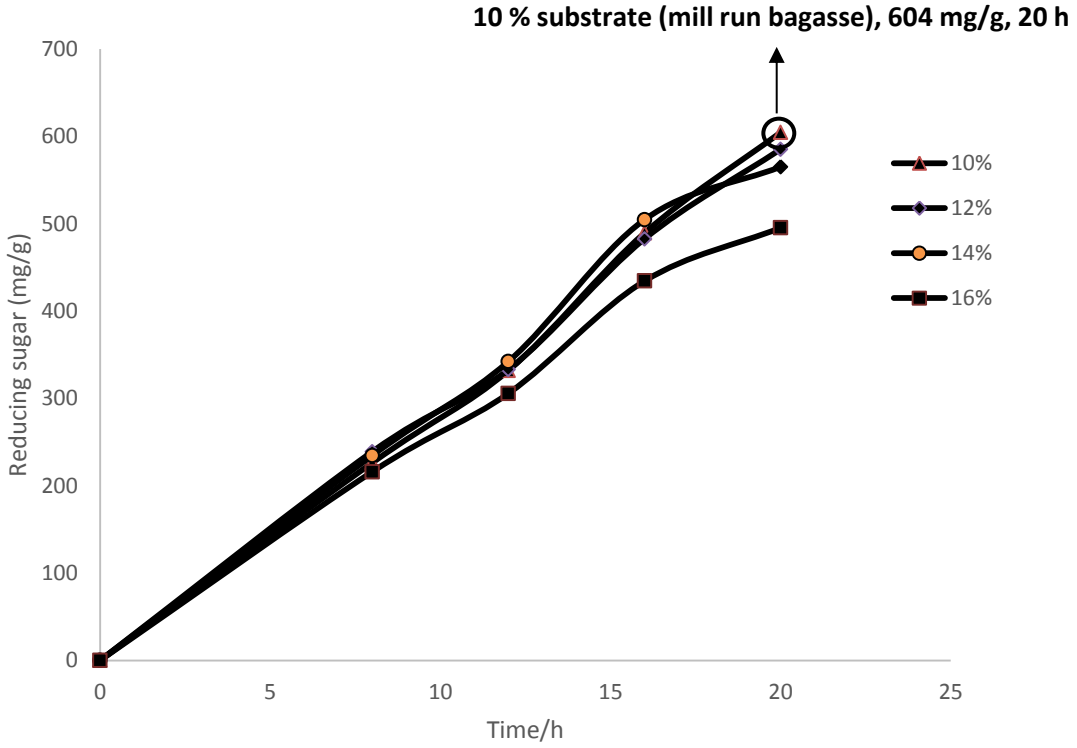


Figure 5.14 Effect of different substrate consistencies on the hydrolysis of mill-run sugarcane bagasse

5.6. Characterization of raw and pre-treated SCB pulp

5.6.1. SEM

The images below (figure 5.15 – 5.19) shows the SEM images of raw and pre-treated SCB pulps ([Emim][OAc], $[HNEt_3][HSO_4]$ and acid/ alkali). Both IL pre-treatment SEM images were recorded at optimum conditions (highest lignin content). The SEM images were recorded at different magnifications.

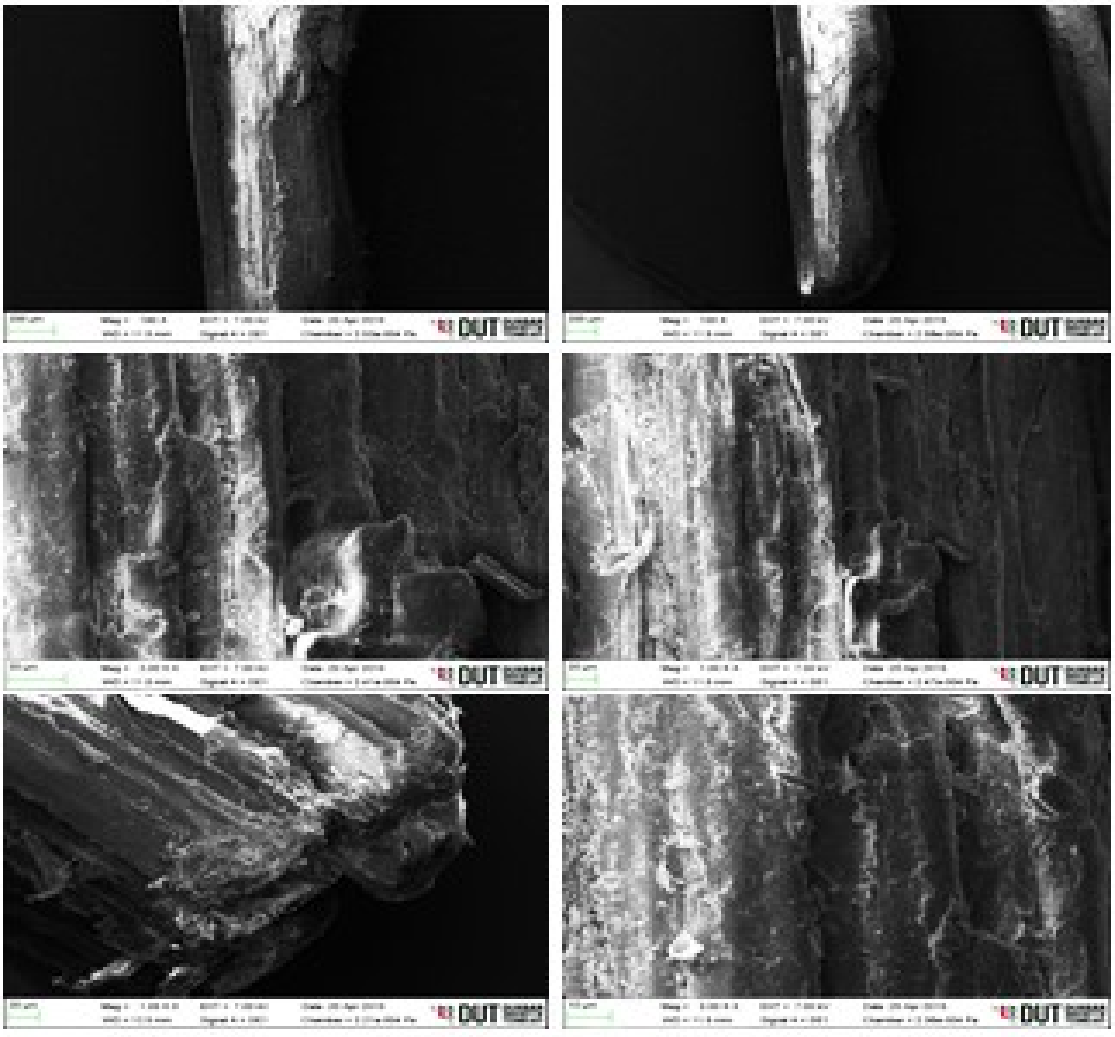


Figure 5.15 SEM images of raw SCB at different magnifications (100 X, 160 X, 1.00 kX, 1.05 kX, 2.00 kX and 3.00 kX)

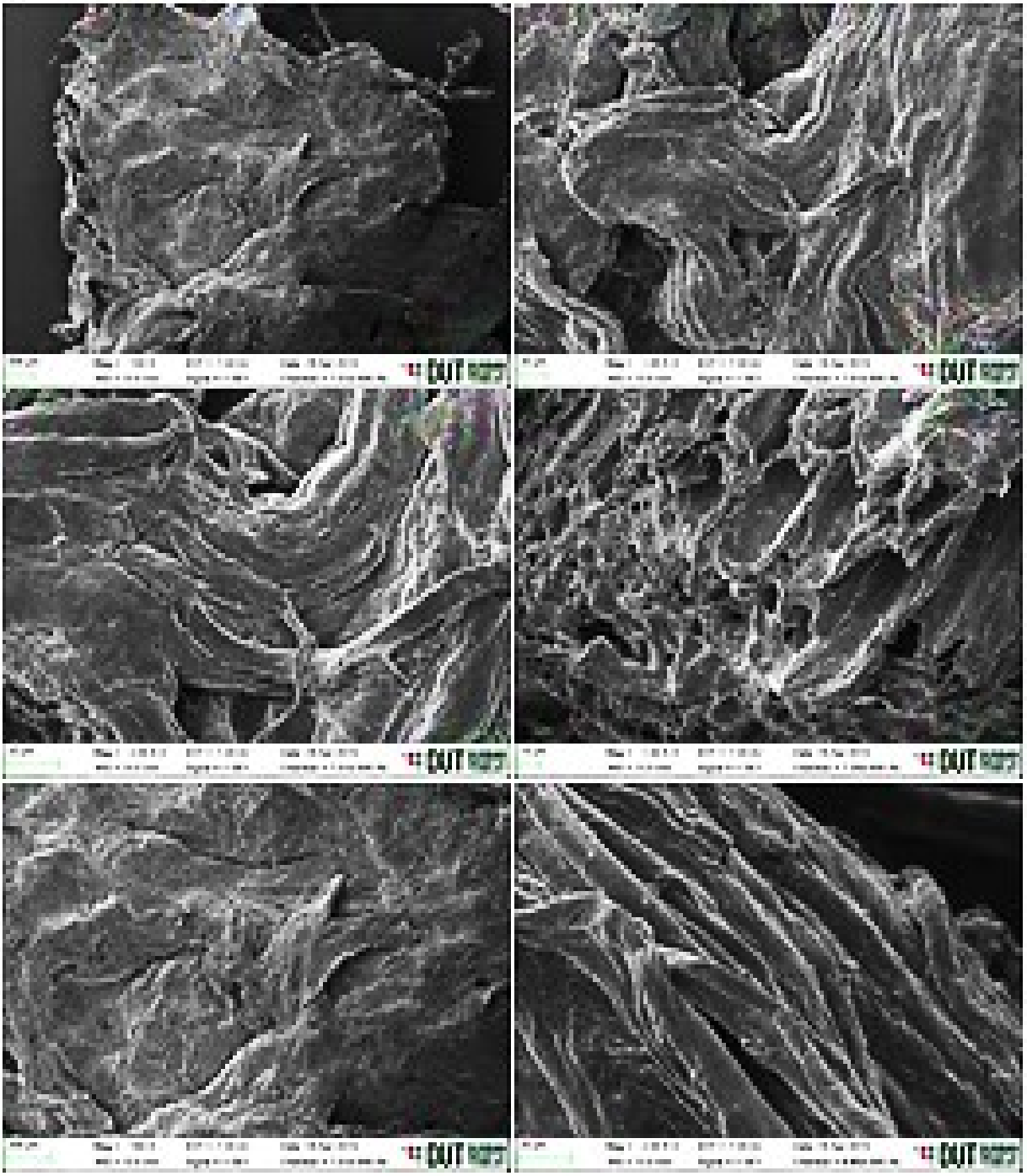


Figure 5.16 SEM images of ([Emim][OAc]) pre-treated SCB pulp at optimum conditions (120 ^{0}C, 12 h) at different magnifications (100 X, 160 X, 1.00 kX, 1.05 kX, 2.00 kX and 3.00 kX)

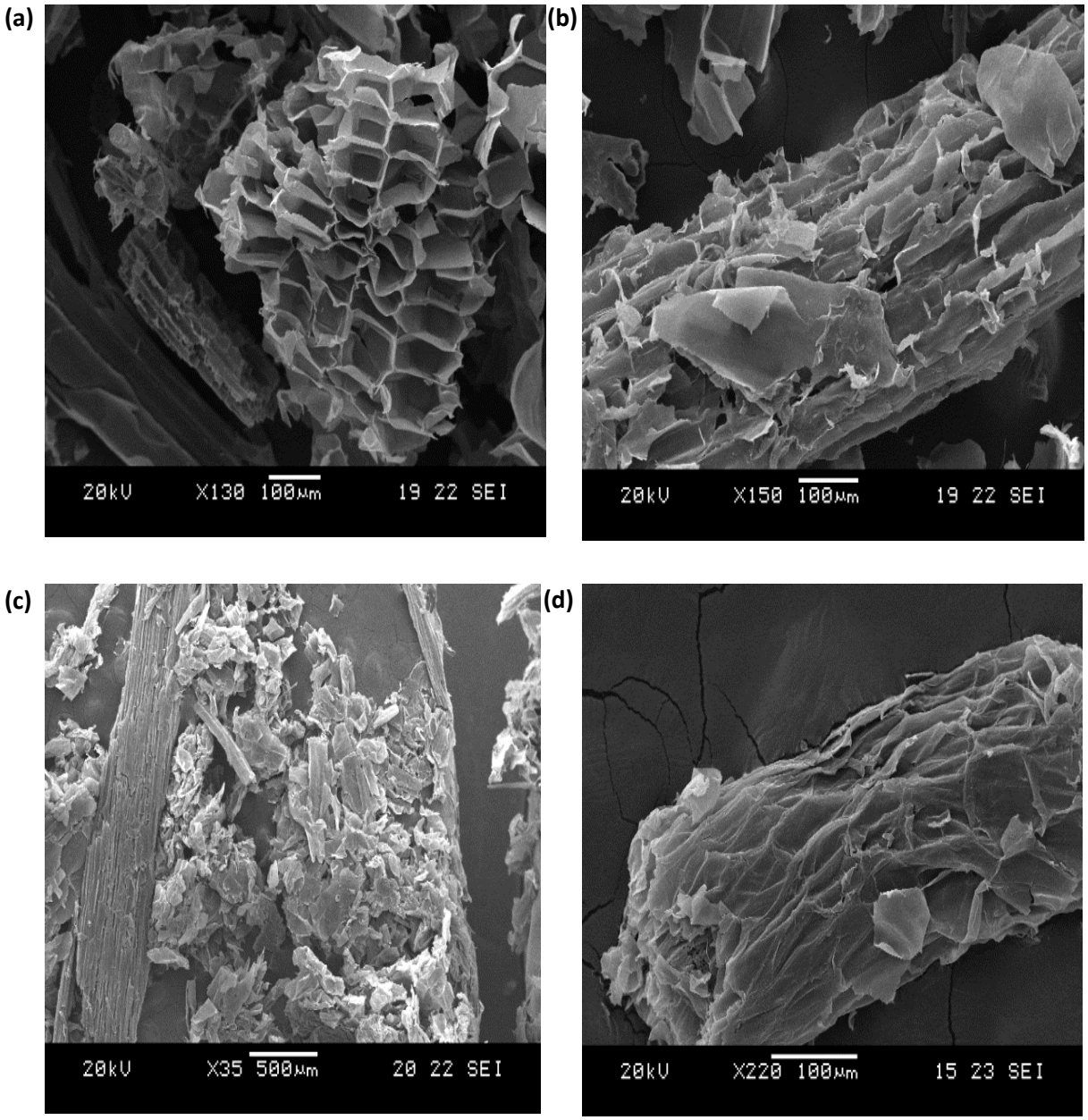


Figure 5.17 SEM images of ([$HNEt_3$][HSO_4]) pre-treated SCB pulp at optimum conditions (a) cross-section and (b) longitudinal view of pith bagasse before pre-treatment; (c) cross-section and (d) longitudinal view of pith bagasse after pre-treatment in [$HNEt_3$][HSO_4] solution at 120 ^{0}C and 4h.

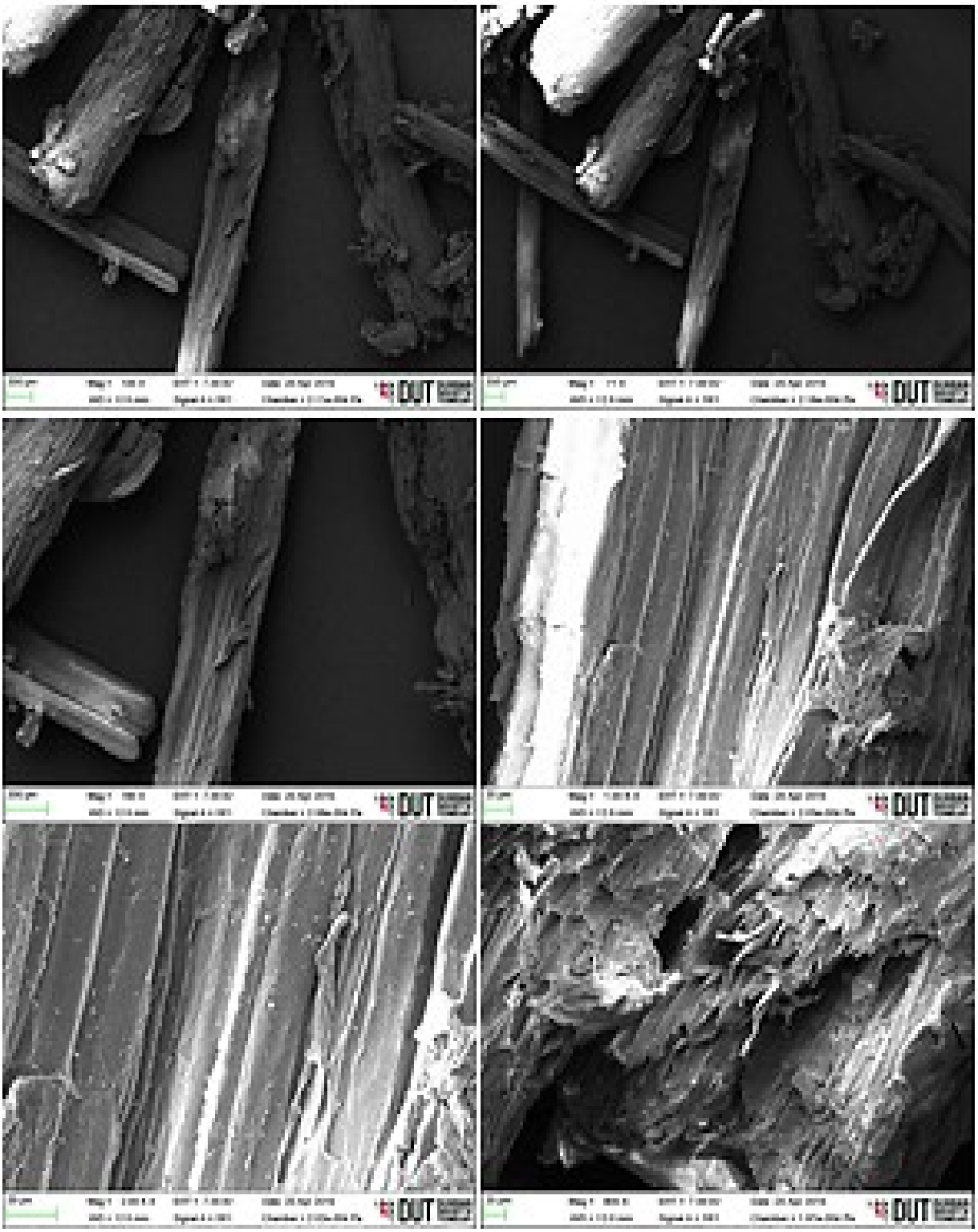


Figure 5.18 SEM images of acid pre-treated SCB pulp at different magnifications (100 X, 160 X, 1.00 kX, 1.05 kX, 2.00 kX and 3.00 kX)

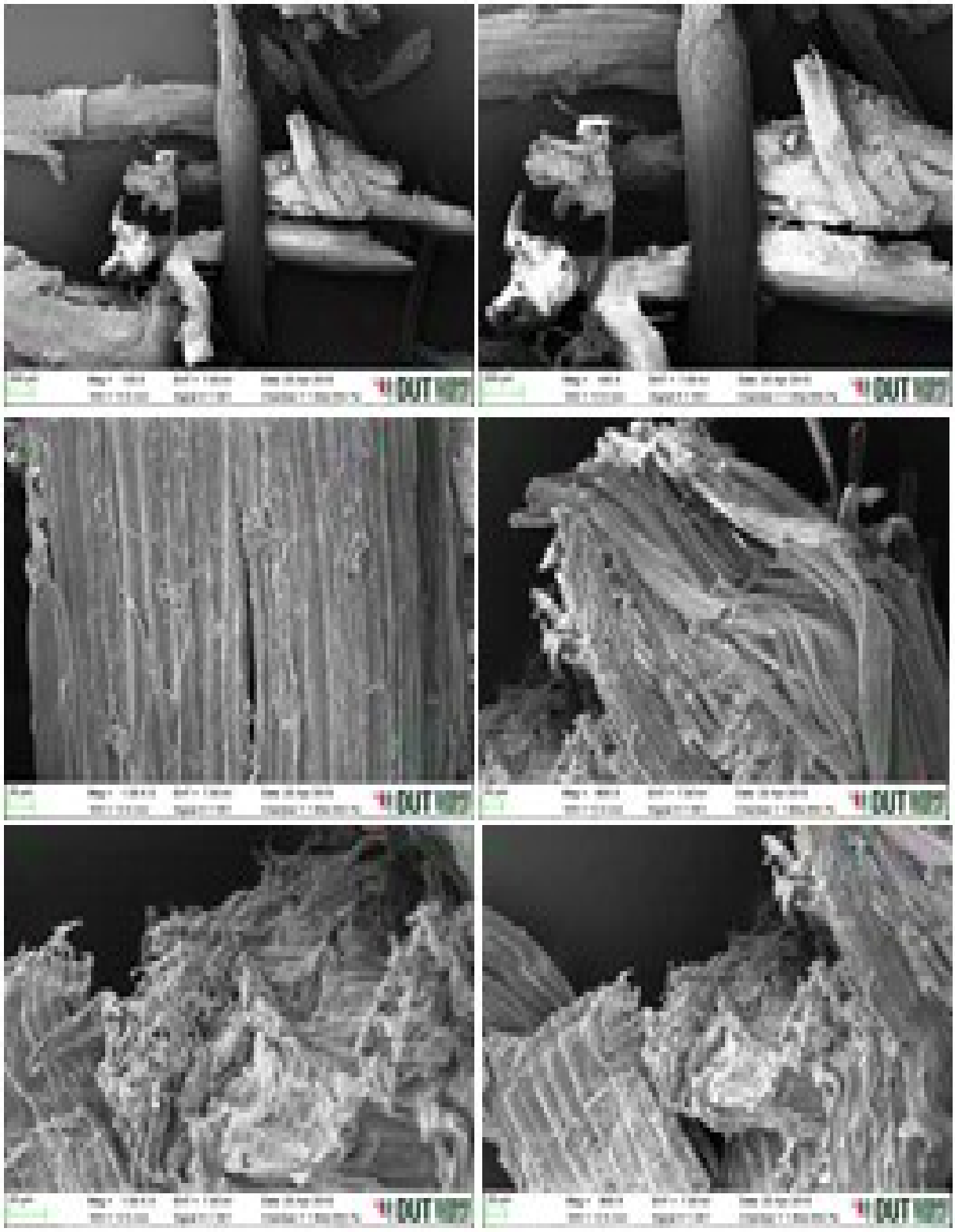


Figure 5.19 SEM images of acid/ alkali pre-treated SCB pulp at different magnifications (100 X, 160 X, 1.00 kX, 1.05 kX, 2.00 kX and 3.00 kX)

5.6.2. Thermal Analysis

5.6.2.1. DSC

The scans below (figure 5.20 and 5.21) shows the DSC profiles of raw and pre-treated SCB pulps ([Emim][OAc], $[HNEt_3][HSO_4]$ and acid/ alkali). The profiles were recorded at a temperature range of 0 – 600 ^{0}C at a rate of 5 ^{0}C per minute in a nitrogen atmosphere. Table 5.14 shows the degradation temperatures and heat flows of the pre-treated bagasse pulps.

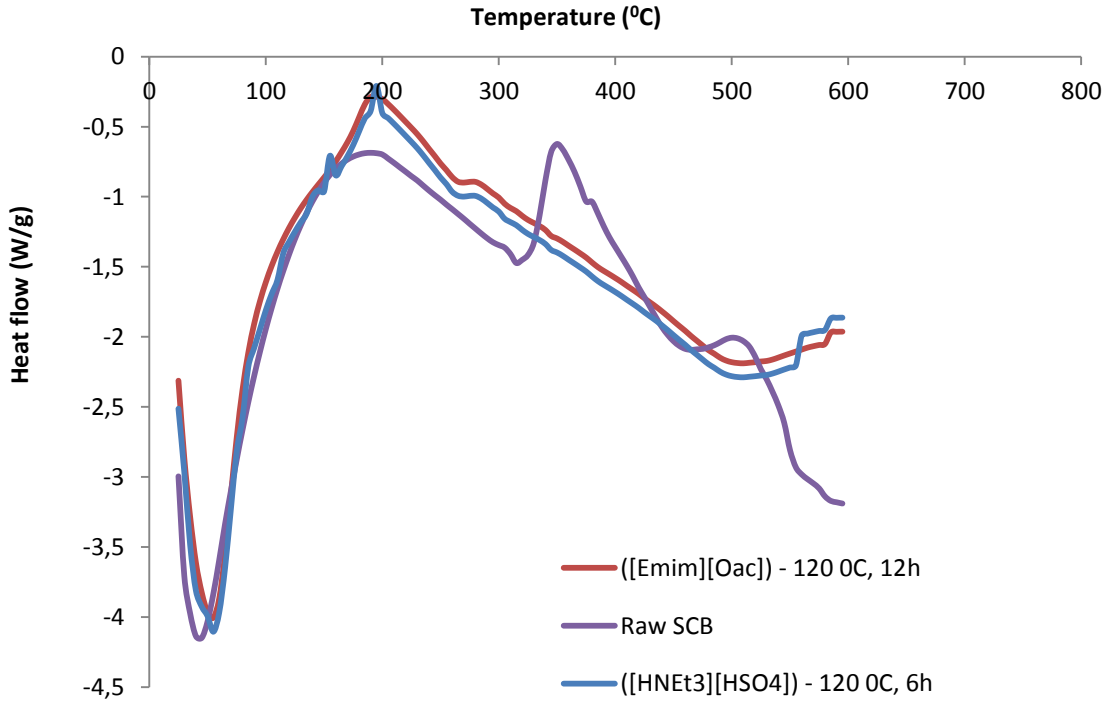


Figure 5.20 DSC profile of raw SCB, ([Emim][OAc]) and ($[HNEt_3][HSO_4]$) pre-treated SCB pulp at optimum conditions

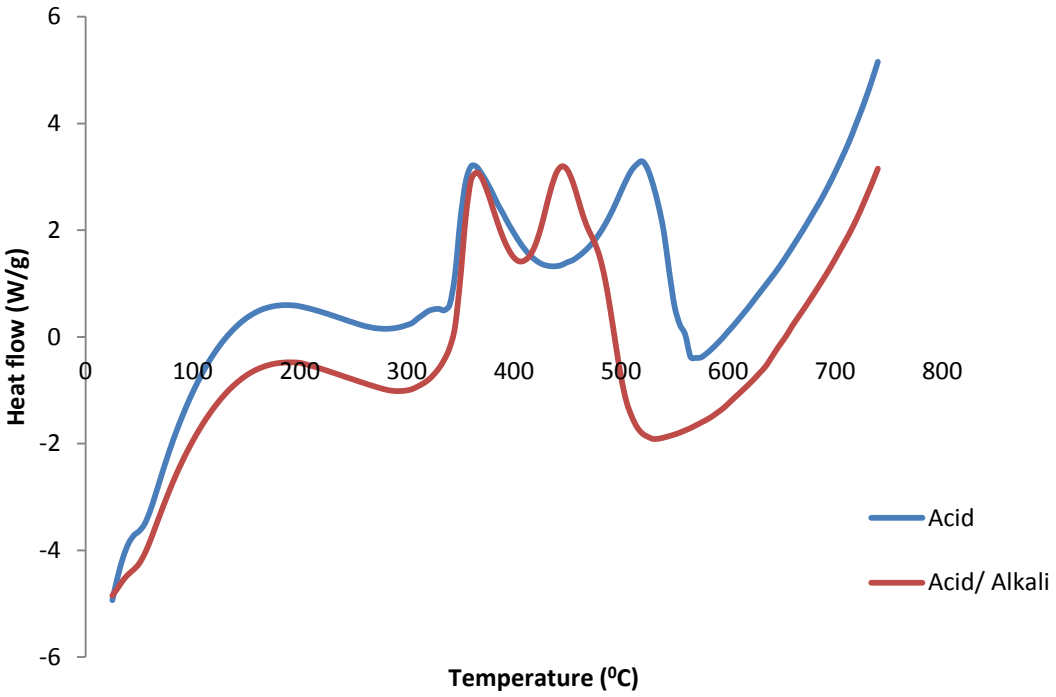


Figure 5.21 DSC profile of acid and acid/ alkali pre-treated SCB pulp

Table 5.14 Thermal properties of bagasse pulps: heat flow

Bagasse sample	Degradation Temperature (^{0}C)		
	65 ^{0}C	200 ^{0}C	350 ^{0}C
Raw	3.34	0.69	0.62
([Emim][OAc])	3.55	0.30	1.29
($[HNEt_3][HSO_4]$)	3.36	0.40	1.39
Acid	3.84	0.57	1.09
Alkali/ Alkali	357	0.57	1.09

5.6.2.2. TGA

The scans below (figure 5.22 and 5.23) shows the DSC profiles of raw and pre-treated SCB pulps ([Emim][OAc], $[HNEt_3][HSO_4]$ and acid/ alkali). The profiles were recorded at a temperature range of 0 – 600 ^{0}C at a rate of 5 ^{0}C per minute in a nitrogen atmosphere. Table 5.15 shows the degradation temperatures and weight losses of the pre-treated bagasse pulps.

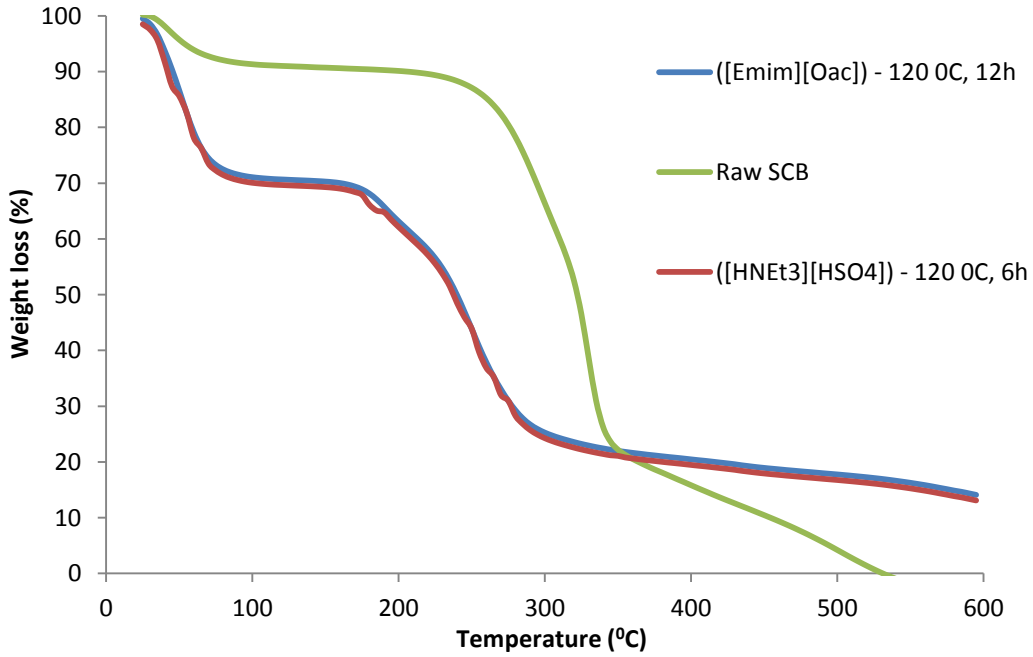


Figure 5.22 TGA profile of raw SCB, ([Emim][OAc]) and ($[HNEt_3][HSO_4]$) pre-treated SCB pulp at optimum conditions

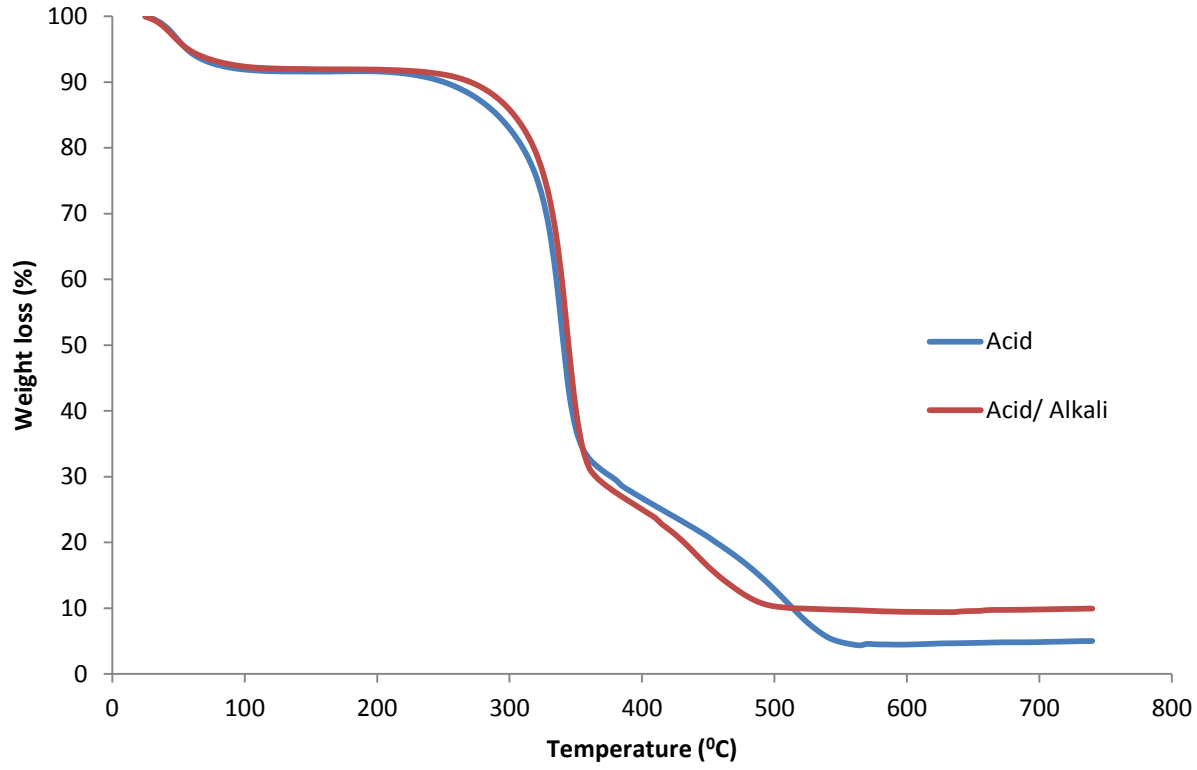


Figure 5.23 TGA profile of acid and acid/ alkali pre-treated SCB pulp

Table 5.15 Thermal properties of bagasse pulps: weight loss

Bagasse sample	Weight Loss (%)		
	65 ^{0}C	**200 ^{0}C**	**350 ^{0}C**
Raw	93.25	90.13	22.22
([Emim][OAc])	76.34	63.15	22.01
($[HNEt_3][HSO_4]$)	76.29	62.15	21.10
Acid	93.75	91.62	37.33
Acid/ Alkali	94.66	91.90	40.25

5.6.3. FTIR

FTIR spectroscopy has been widely used in biomass research since it presents a relatively easy method for obtaining direct information on chemical changes that occur during various treatment stages (Ristolainen *et al.,* 2002). The scans below (figure 5.24 – 5.28) shows the FTIR spectra of raw and pre-treated SCB pulps ([Emim][OAc], $[HNEt_3][HSO_4]$ and acid/ alkali pre-treated). The spectrums were recorded in the range of 600 – 3000 cm. Table 5.16 shows the relative intensity bands of different groups in the untreated and pre-treated SCB pulps.

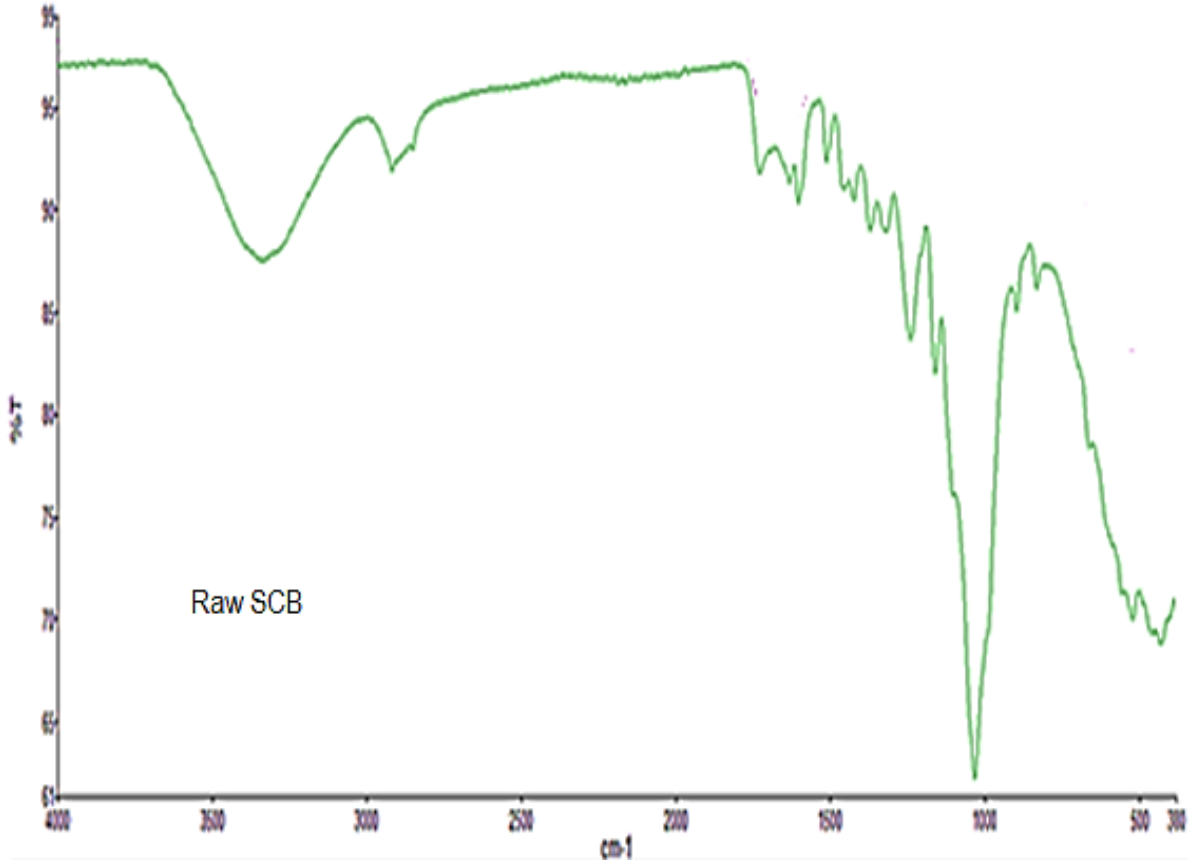


Figure 5.24 FTIR spectrum of raw SCB

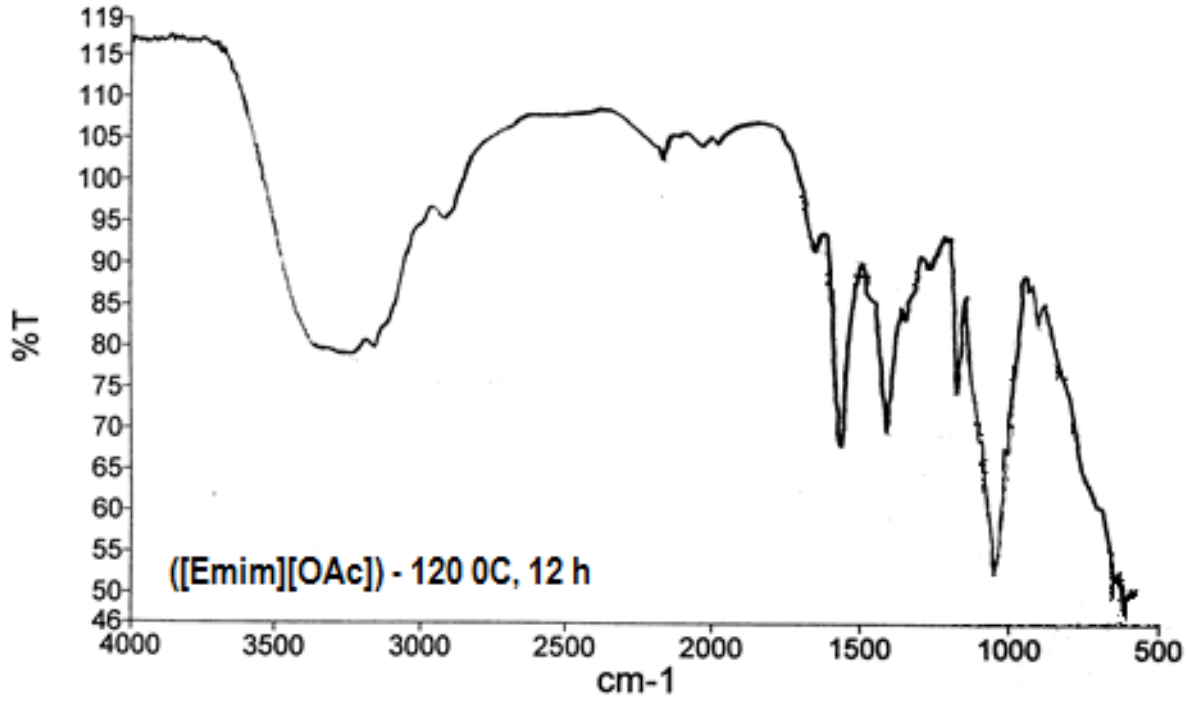


Figure 5.25 FTIR spectrum of ([Emim][OAc]) pre-treated SCB pulp at optimum conditions (120 ^{0}C, 12 h)

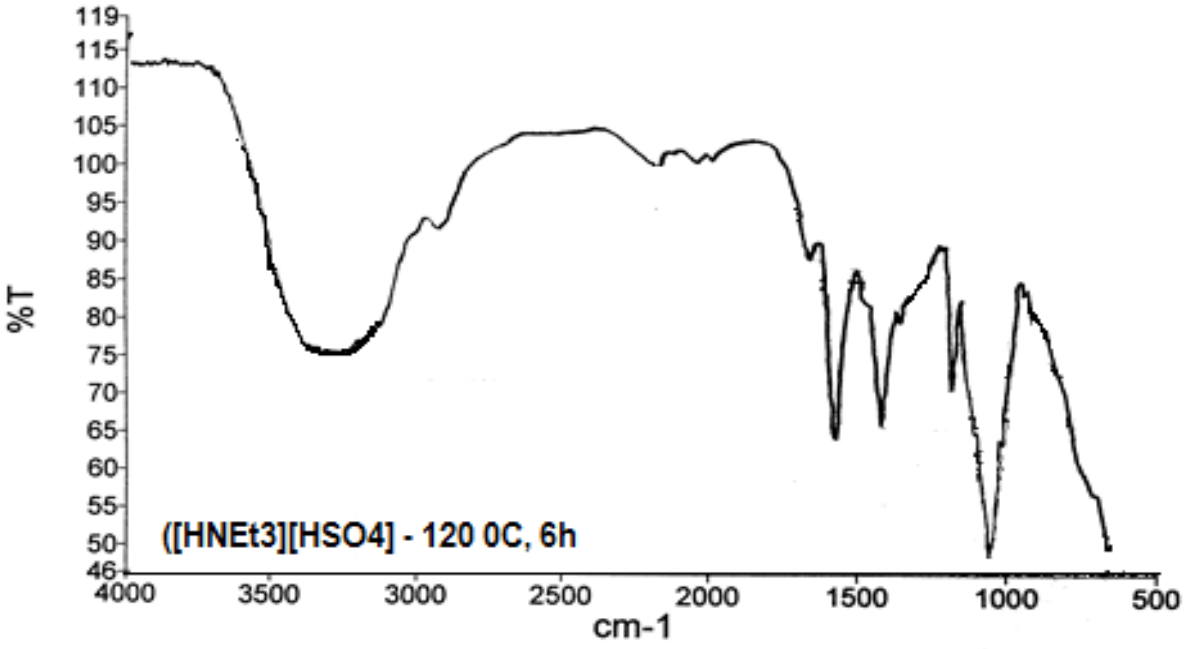


Figure 5.26 FTIR spectrum of ([$HNEt_3$][HSO_4]) pre-treated SCB pulp at optimum conditions (120 ^{0}C, 6 h)

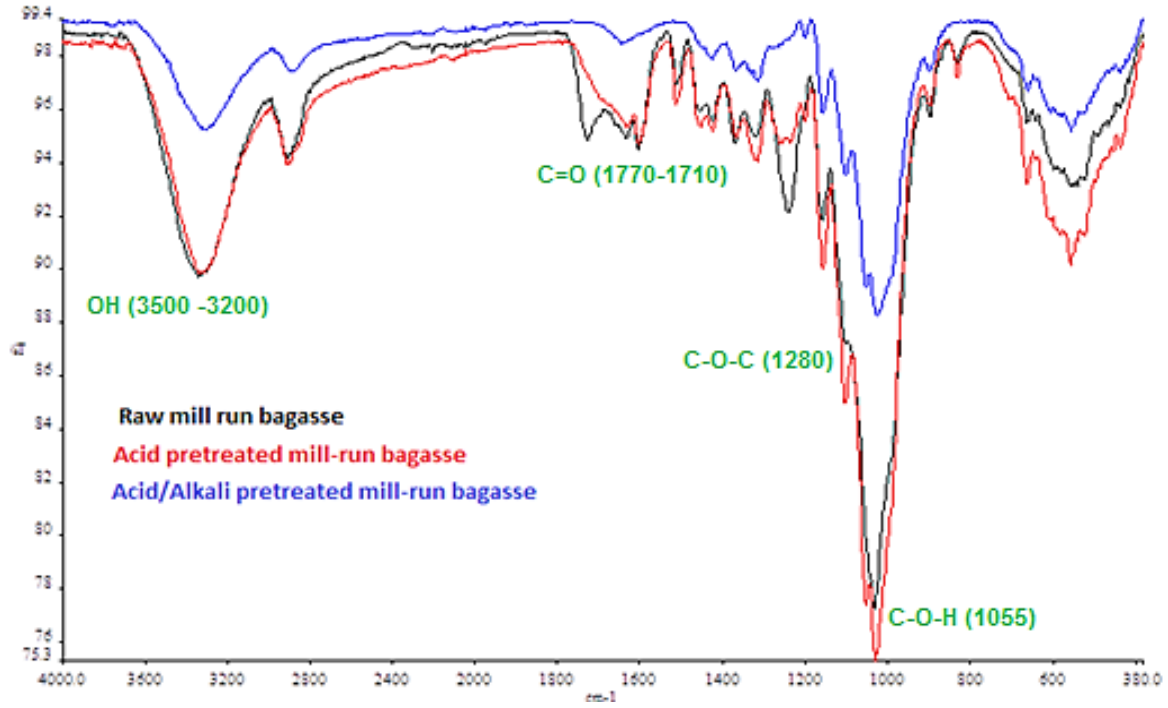


Figure 5.27 FTIR spectrums of acid/ alkali pre-treated SCB pulp

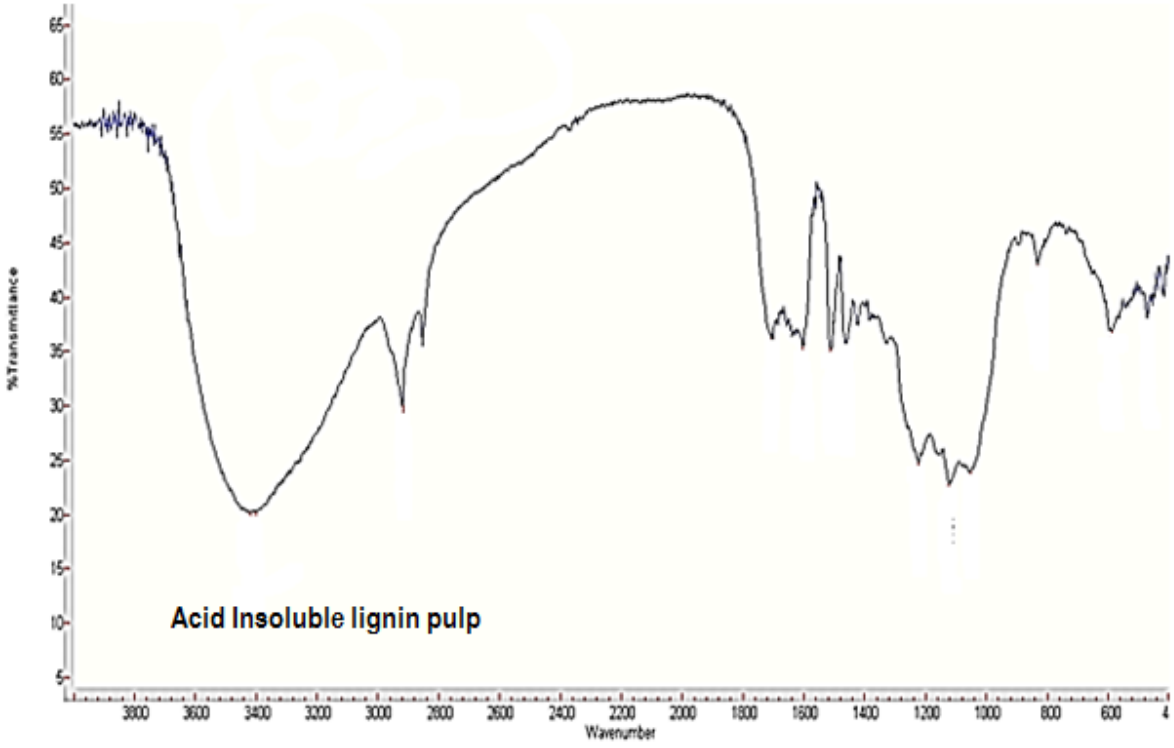


Figure 5.28 FTIR spectra of pulp from acid insoluble lignin determination

Table 5.16 Relative intensity bands in the FTIR spectra of different groups in the untreated and pre-treated SCB (Sain and Panthapulakkal, 2006).

FTIR absorption band	Raw SCB	IL pre-treat pulp (opt. cond.)	Acid pre-treat pulp	Alkali pre-treat pulp	Acid Insoluble Lignin (AIL)
O-H stretching (H bonded)	3408	3454	3429	3434	3423
C-H Aliphatic axial deformation	2918	2911	2909	2921	2919
C=O stretching	1734	1737	1740	1732	1707
C=C Aromatic skeletal vibration	1638	1634	1636	-	1604
1595-very strong aromatic ring stretch, aromatic C-O stretch; 1,595- phenylpropanoid polymer; 1,595-aromatic skeletal vibrations plus C = O stretch	1605	1573	-	-	-
1,510-semicircle ring stretching (aromatic lignin), 1,510-phenylpropanoid polymer; 1,510-very strong aromatic ring stretch, aromatic C-O stretch; 1,513-aromatic C = C stretch; 1,514-semi-circle stretch of para-substitute benzene rings; 1,550-protein	1513.6	1514	1513	1510	-
C-H Angular deformation of methoxy (methyl and methylene) group	1458	1464	1461	1430	1463
C-H bending vibration & C–O bond in the polysaccharide aromatic ring	1382	1383	1390	1375	
C-O stretching of phenols	1254	1256	1259	-	1223
1,160-glycosidic linkage; 1,162-C-O-C ring vibrational stretching	1164	1168	1167	1155	1124
C-O-C stretching	1109	1110	1112	1108	1053
1,078-β (1-3) polysaccharide; 1,098-weak absorbance	1048	1063	1050	1030	
B-glycosidic linkages	898	838	899	895	833

CHAPTER **6**

DISCUSSION

6.1. Compositional analysis

Figure 6.1 depicts the chemical composition of five untreated bagasse fractions, namely whole bagasse (WB), depithed bagasse (DB) obtained after industrial depithing, and a further three fractions obtained by a laboratory depithing process to produce long fiber bagasse (LFB), short fiber bagasse (SFB) and pith bagasse (PB). Pith bagasse (1.4 %) has the highest ash content, though not significantly higher than whole bagasse (1.2 %). This may be because pith particles and other inorganic material adhering to the pulp were removed during the washing process, effectively reducing the 'ash' (i.e. inorganics) content of WB compared with unwashed WB, which typically has ash contents of up to 5-6 wt. % (Anzaldo *et al*., 2001).

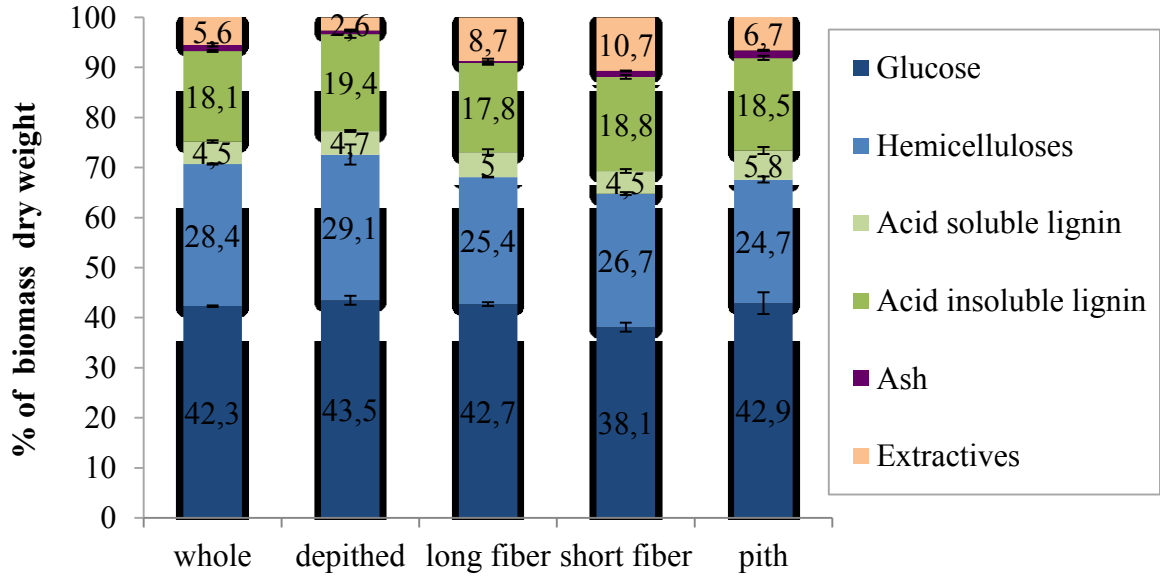


Figure 6.1 Composition of untreated bagasse fractions reported as a percentage of native biomass on an oven dry weight basis.

6.1.1. Ash analysis

At present, about half of the bagasse produced in the sugarcane industry is burned, producing significant quantities of fly ash. A more efficient use of the energy in biomass may be to pretreat bagasse to isolate carbohydrates and pyrolyse the lignin produced, enabling transformation of bagasse into high-density renewable fuels. The fate of the inorganic material ('ash') in biomass following pre-treatment is not widely documented; however it is suspected to partition predominantly in the pulp whilst some ash is recovered in the lignin precipitate. In evidence of this, the ash content of pre-treated bagasse pulps was determined by Klason analysis to be between 1.0 - 3.5 wt. % for all samples investigated (results not shown), accounting for nearly all of the ash in untreated biomass which was found to be 1.2 % as shown in Table 5.3. The chemical composition of the ash is of interest for bagasse pyrolysis. Previous work has documented that the inorganic compounds found within the ash promote the formation of gaseous species and char at the expense of bio-oil yield (Patwardhan *et al*., 2010). Ash also has a significant role in catalyzing polymerization reactions in the resulting bio-oils, increasing their viscosity. This instability may be attributed to the presence of alkali metals in the ash *(*Das *et al*., 2004). Selective removal of ash components is therefore desirable to produce more stable bagasse-derived bio-oils.

Silica was found to be the main constituent of the ash (70 and 55 wt. % for PB and LFB, respectively) as shown in Table 5.4. Other major constituents were calcium, magnesium, sodium, potassium and phosphorous, respectively. Pith ash had a larger fraction of silica and calcium oxide while long fiber bagasse ash had over double the level of alkali metals (Na, K). Silica has been found to influence tar decomposition, notably reducing the concentration of naphthalene found in bio-oil, which causes problems in down-stream processing and so we would expect pith ash to produce more stable oil. The higher concentration of alkali metals in long fiber bagasse ash might be expected to catalyze polymerization reactions and thereby increase the viscosity of LFB-derived bio-oil.

6.2. Pre-treatment of sugarcane bagasse with 1-ethyl-3-methylimidazolium acetate ([Emim][OAc])

6.2.1. Dissolution of SCB

In this study the dissolution of SCB in this IL at different conditions (reaction time and temperature) at a 10 % biomass loading was investigated. It was noted that the reaction mixture was very viscous and hard to stir; this was also reported by Karatzos *et al*., (2012) at a 5 % biomass loading reaction conditions 150 ^{0}C, 90 min. The effect of IL anions on dissolution have been studied extensively comparing especially [Emim]Cl to [Emim]OAc. The acetate anion appears to support dissolution rather than losses. It is likely that by reducing the severity of the treatment conditions, the proportion of dissolution to losses will be made less. Acetic acid derivation anion has higher hydrogen bond basicity than chloride and therefore its capacity to disrupt hydrogen bonds and break down cellulose is higher. This positive connection between the hydrogen bond basicity of the IL anion and the IL's capacity to disintegrate cellulose or lignocellulose is accounted for in the literature (Brandt *et al*., 2010). The predominant dissolving limit of [Emim]OAc has additionally been reported by Sun *et al*., (2009) who measured 93.5 % dissolution degree of southern yellow pine in [Emim]OAc and only 26 % in [Emim]Cl under the same conditions (particle size 0.25 – 0.50 mm, 5 % mass loading, 110 ^{0}C for 16 h.

Water was used to wash the pre-treated SCB in order to remove the IL and lignin leaving behind cellulose rich pulp. Karatzos *et al*., (2012) also used water as a co-solvent to fractionally precipitate IL dissolved SCB to polysaccharide rich and lignin rich fractions. The first water addition used for the partial precipitation of dissolved solids resulted in water: IL ratio of 0.5. Based on visual observations of fractional precipitation with water on preliminary studies it was indicated that this water amount should precipitate all cellulose and keep lignin in solution for water: [C_4mim]Cl mixtures and

possibly for water: [C_2mim]OAc mixtures. Native bagasse lignin dissolved in IL to NaOH dissolved lignin is prone to have different properties. Sun *et al*., (2013) dissolved rice straw in five different ILs ([Bmim]Cl, [Emim]OAc, [Amim]Cl, [Emim]$C_2H_5OSO_3$ and [Emim]DEP) at 120 ^{0}C with 12 h reaction time. Complete dissolution occurred in [Emim]OAc pre-treatment. Water was used as a co-solvent to dissolve the regenerated rice straw pulp and form a precipitate. A change in the outer structure was observed in the regenerated rice straw in comparison to the untreated rice straw.

6.2.2. Effect of pre-treatment on the yield of regenerated biomass (pulp, lignin and mass recovery)

The percent recoveries were obtained after the regeneration process of each biomass pulp by adding deionized water as an anti-solvent, followed by centrifugation/ filtering and drying. The % recovery values ranged between 57 % and 78 % as shown in Table 5.8, this is highly dependent on the source of the biomass, type of ionic liquid and pre-treatment conditions. Pre-treatment at 90 ^{0}C for 6 hours gave the highest percent recovery (72 %) meaning less lignin was extracted. The percent recovery values at 120 and 150 ^{0}C were 70 and 72 % respectively which is slightly different from 90 ^{0}C. Pre-treatment at 120 ^{0}C for 6 hours at 90 ^{0}C gave the lowest percent recovery meaning more lignin was extracted. Since lignin and hemicellulose fractions were partially extracted by ionic liquids in the pre-treatment process (Zhao *et al*., 2008), it is not surprising that the YRB of Eucalyptus is higher than the bagasse value, because Eucalyptus is a hardwood that has a higher density of cells. The colour of the ILs or regenerated water turned brown following the pre-treatment, implying the presence of a soluble lignin fraction in each media. The colour change of the ILs following pre-treatment was also reported for Maple and Spruce woods (Lee *et al*., 2009; Kilpelainen *et al*., 2007). At 90 and 150 ^{0}C as the pre-treatment time increased, the lignin yield increased however at 120 ^{0}C lignin content was highest after 12 hours and lowest after 24 hours as shown in Table 5.9.

6.3. Pre-treatment of sugarcane bagasse with triethylammonium hydrogen sulphate ([$HNEt_3$][HSO_4])

All the five bagasse fractions (WB), (DB), (LFB), (SFB) and (PB) were subjected to pre-treatment and enzymatic saccharification, however only whole and pith bagasse are reported and discussed in this study for comparative studies with another IL ([Emim][OAc]) pre-treatment that is discussed in this thesis.

6.3.1. Pulp, lignin and mass recoveries

Pulp yields were minimized at 48 – 51 % of original biomass weight at 16 h, suggesting maximal lignin and/or hemicellulose removal occurs after 16 h of pre-treatment as shown in Table 5.12. This is confirmed by a maximum in lignin recovery at 17 – 18 % within 16 h as shown in Table 5.13. After 16 h, pulp yields were observed to increase whilst lignin recoveries decreased, which may indicate the formation of less soluble lignin-derived products such as highly condensed lignin's or "pseudo-lignin", a lignin-like material derived from carbohydrate degradation products, which is formed as a consequence of severe pre-treatment conditions (time, temperature, acidity) and appears as a recalcitrant lignin-like product). These undesirable products are known to adhere to the pulp surface and may contribute to reducing pre-treatment efficacy at longer pre-treatment times. The mass recovered from the [$HNEt_3$][HSO_4]:H_2O mixture after pre-treatment, washing and drying varied between 69 – 75 %. Higher mass recoveries may indicate either incomplete lignin removal or the formation of insoluble compounds upon severe pre-treatment conditions. Maximum mass loss was observed after 16 h, indicating lignin removal and/or hemicellulose dissolution.

6.3.2. Klason lignin

The amount of Klason lignin found in the pre-treated material was found to decrease with increasing pre-treatment time to reach a minimum after 4 h, increasing slightly between 4 and 16 h before reaching a level significantly higher than the native lignin content after 24 h as shown in figure 5.9. Errors at longer pre-treatment times (24 h) may be attributed to substrate heterogeneity due to local differences in lignin deposition onto cellulose micro-fibrils as a result of more severe pre-treatment conditions. It should be noted that the Klason method can result in artificially high values for lignin, as sugar degradation products and entrapped low molecular weight phenolics can also be measured as "lignin" (Hu *et al*., 2012). Pseudo-lignin or condensed lignin macromolecules formed during severe pre-treatment conditions have been reported to adhere to the pre-treated pulp surface, limiting lignin recovery and enzyme accessibility. Delignification was found to maximize at 4 – 8 h. It reached negative values at 24 h, indicating the formation of lignin-like material.

6.3.3. Enzymatic hydrolysis of ($[HNEt_3][HSO_4]$) pre-treated SCB pulp

At optimal pre-treatment conditions (120 ^{0}C, 4 – 8 h), glucose released following 7 days of saccharification reached 68 % of the theoretical maximum glucose release as shown in Figure 5.10. This corresponds to a 5.5 and 8.0-fold increase in enzymatic saccharification yields compared with untreated biomass. Similar glucose yields were obtained from WB and DB, with no statistically significant differences being attributable to the type of bagasse used. These results agree with observations by Hernandez-Salas *et al.,* (2009) who used Novozymes enzyme preparations on bagasse following acid hydrolysis, where the amount of glucose liberated following enzymatic hydrolysis was similar for depithed and whole bagasse (Hernandez-Salas *et al.*, 2009). However, the authors report a higher alcohol yield using depithed bagasse following fermentation, despite similar sugar yields. Decreasing glucose release beyond 4 - 8 h seems to

confirm the presence of pseudo-lignin or other carbohydrate degradation products, such as furfural, that are inhibitory to enzymes.

The xylose yields were optimum within 1-2 h with a maximum release of 30 % as shown in figure 5.11. The maximum amount of glucose released from enzymatic hydrolysis of whole bagasse was double the amount of xylose liberated. In contrast to glucose yields, xylose yields are slightly improved at optimum conditions. The enzymatic hydrolysis yields fall short of the near-theoretical fermentable sugar yields achieved using $[C_2C_1im][OAc]$ under similar pre-treatment conditions (120 ^{0}C, 0.5 – 2 h) following only 72 h saccharification (da Silva *et al*., 2013; Diedericks *et al*., 2011). However, these studies use very expensive imidazolium-based ILs which has additionally been shown to be structurally unstable under biomass pre-treatment conditions due to thermal degradation. For instance, de-alkylation reactions have been observed in $[C_2C_1im][OAc]$ at temperatures as low as 120 ^{0}C, raising important concerns that these ILs may not be recoverable for repeated use, making their use in a commercial process unfeasible.(George *et al*., 2015). Moreover, the use of ILs based on the $[HSO_4]^-$ anion enables the pre-treatment of wet feedstocks containing up to 50 % moisture, including raw sugarcane bagasse. [OAc]-based ILs would not be capable of handling such raw feedstocks due to the process requirement of very dry conditions. The saccharification yields obtained using $[HNEt_3][HSO_4]$ are therefore 70 % as high as $[C_2C_1im][OAc]$. This result is highly promising given that this IL is thought to be capable of handling wet feedstocks at a fraction of the processing cost.(George *et al*., 2015).

6.3.4. Impact of depithing on bagasse pre-treatment

Depithing is normally viewed as a key initial step before biomass pre-treatment to remove undesirable pith, sand and other inorganic segments from the exploitable fibrous bit of bagasse. Commercial depithing typically increases the fiber content of bagasse pulp from 60 to 80 %, empowering its utilization for biofuels production

(Anzaldo *et al*., 2001). Bagasse pith has long been viewed as an undesirable crude material for pulping because of processing issues mentioned above; however these issues may be of less significance during bioethanol production and lignin valorization in a bio-refinery. Laboratory sieving of bagasse is exceedingly likely to remove a higher portion of the pith compared to industrial depithing (ca. 40 % of aggregate pith removed compared to ca. 30 % in a commercial depither) (Rainey *et al*., 2013). This study analyzed both commercially depithed and lab depithed material to explore whether a depithing step is fundamental preceding bioethanol production.

Depithing using a room-temperature laboratory separation of pith and fibrous fractions in bagasse seems to be useful where lignin extraction is a key objective. Lignin extraction from depithed sugarcane bagasse, producing a high purity lignin with low sulfur content, (Brandt *et al.,* 2015) may provide a feedstock for further processing and upgrading to value-added aromatic chemicals. Avoiding the high energy input and equipment costs of the depithing operation, estimated to be around $5.50 per ton of bagasse, may also contribute to lowering operating and capital costs for sugarcane bagasse bioconversion, helping to accelerate the development of cost-effective biofuels production from bagasse. In processes where a high yield of fermentable sugars is the priority of bagasse utilization, the depithing operation may be entirely unnecessary, with the potential to improve the economic viability of bagasse biorefining. However, as discussed in detail by Rainey *et al*., (2013), depithing also has implications for the bulk density and compaction of stockpiled bagasse, its permeability and water holding capacity which must be considered prior to recommending using whole non-depithed bagasse as a feedstock for a commercial-scale bio-refinery.

6.3.5. ([$HNEt_3$][HSO_4]) as a low cost ionic liquid for biomass pre-treatment

Ionic liquids have many advantages over conventional solvents for biomass pre-treatment however one major disadvantage is their high cost which makes it hard to upscale to industrial scales. Chen *et al*., (2014) evaluated the economic feasibility of two HSO_4 based ILs that were synthesized by acid base neutralization using conventional and escalation methods. They estimated the price of raw materials triethylammonium hydrogen sulfate and 1-methylimidazolium hydrogen sulfate to be $1.24 and 2.96 – 5.88 per kilogram respectively. In comparison with conventional organic solvents such as acetone or ethyl acetate which is selling at a price of $1.3 – 1.4 kg-1, this contrasts favorably. In addition, raw materials contribute the dominant part of the cost and the strengthened procedure utilizing a compact plate reactor is more efficient because of lower energy demands. These outcomes show that ionic liquids are not as costly as they are deemed to be; therefore upscale IL based production is possible.

6.4. Acid/ Alkali pre-treatment

In this research, acid pre-treatment using sulfuric acid was used to remove hemicellulose and alkaline pre-treatment using sodium hydroxide was used for the purpose of breaking down the chemical bonds of lignin and cellulose of SCB. The high content of cellulose in the biomass natural form (42.3 %) as shown in Table 5.3 indicates its high potential for conversion through the hydrolysis/ saccharification. For bagasse to be hydrolysed with enzymes it is important to pre-treat first to enhance enzymatic conversion of cellulose, mainly because the lignin in the plant cell walls forms a protective barrier against enzymatic attack. An ideal chemical pre-treatment would reduce the lignin content, crystallinity of cellulose and increase the surface area and separation of the structural linkage between lignin and carbohydrate, increasing hydrolysis of cellulose. Pre-treatment is however, crucial for ensuring good ultimate yield of sugars from both polysaccharides i.e. cellulose and hemicellulose of the biomass (Khokhar *et al*., 2010 a, b). Acid pre-treatment using dilute sulfuric acid has been shown to be more effective in terms of improving the enzymatic hydrolysis of cellulose for conversion to bioethanol (Rocha and Silva, 2005). It also minimizes the formation of degradation products and maximizes sugar yields at the end of the process (Lloyd and Wyman, 2005).

A 10 % biomass loading was used in this study with SCB starting mass of 100 g. After acid pre-treatment, the mass decreased considerably to 67.6 g, which means that there was a 32 % mass loss, mostly because of the removal of hemicellulose as reducing sugars. The acid-pre-treated substrate was then subjected to alkali pre-treatment and the amount left was 29.6 g, which means there was a 56 % mass loss, which may be attributed mostly to the loss of lignin, leaving cellulose behind. In a similar study by Candido *et al*., (2012) it was verified there was a decline in the mass recovery yields as a function of reaction time. The higher the reaction time, the higher the removal yield of components. When compared to other chemical pre-treatment methods for example NaOH or Kraft processes whose mass loss yields may reach up to 50 % of the native

biomass; acid pre-treatment mass loss may be considered small. This is indicative of its selectiveness when compared to other chemical pre-treatment methods. The hemicelluloses that are removed in an acid medium have lower molecular weights and their presence in lignocellulosic biomass is less compared to lignin and cellulose, for example their removal does not have a significant effect on the final yield and this might be the reason they result in low weight loss during pre-treatment reactions. It is reported that the relationship between weight loss and reaction time is not linear, mainly because of structural complexity presented by the bagasse in terms of the pattern in which the cell wall components are organised and the fact that their physical structures does not allow that their elimination follow a pattern (on the account of sulfuric acid access to these components).

Alkaline pre-treatment of lignocellulosic materials causes swelling, leading to decreased crystallinity, increased internal surface area, disruption of the lignin structure, and separation of structural linkages between lignin and carbohydrates (Fan and Gharpuray *et al*., 1987). A similar study by Maryana *et al*., (2014) on alkaline pre-treatment of SCB for bioethanol production revealed that delignification by NaOH (1N) for 15 minutes resulted in an increase in cellulose yield (66.16 %) and a decrease of 40 % in the lignin content. After 30 minutes of delignification the cellulose content shifted slightly to 62 % whereas the lignin yield was 7 % (59 % decrease). More-over after 45 minutes of delignification the cellulose content increased by 38 % and the lignin content decreased by 57 %. As the cellulose content increased, the lignin content decreased except for the 15 minute delignification. This could be due to a number of factors such as the chemical bonds between lignin and cellulose being still intact in comparison to other delignification reaction times and the fact that cellulose was still in its crystalline form hence only a small amount of lignin was dissolved. They concluded that delignification by NaOH (1N) for 30 minutes gave the optimum results due to the highest decrease in lignin content. Also using 1 N concentration proved to be more effective compared to higher concentrations in decreasing the lignin content thus increasing cellulose content of SCB after delignification.

In another study by Khuong *et al*., (2014) the lignin yield after 0.8 %, 1.0 %, and 5.0 % (w/v) NaOH pre-treatments were 84.7, 47.9, and 25.5 mg, respectively. The initial lignin weight was 234 mg, implying that 63.8 %, 79.5 %, and 89.1 % of the initial lignin was removed, respectively. The weight losses are corresponding to the amount of dissolved carbohydrates and other components in the hydrolysate. Several studies have reported the hydrolysis of hemicelluloses and the release of oligoxylans (polyoses) of mixed molecular weights after alkali pre-treatment (Himmel *et al*., 2007; Kumar *et al*., 2009). The reduction in the amount of the reducing/ fermentable sugars is considered to be one of the major drawbacks of alkaline pre-treatment; however this is being exploited extensively by the current bio-refineries to recover the high molecular weight oligoxylans/ arabino xylans to produce value added by-products (Moure *et al*., 2006; Persson *et al*., 2009). The reduction of the reducing/ fermentable sugars is one of the major drawbacks of alkaline pre-treatment however bio-refineries are exploiting this in order to be able to recover the high molecular weight hemicelluloses (ie oligoxylans/ arabinoxylans) that may lead to the formation of value added by-products (Moure *et al*., 2006; Persson *et al*., 2009).

6.4.1. Enzymatic saccharification of acid-alkali pre-treated SCB

From the enzymatic hydrolysis results of the acid/ alkali pre-treated SCB pulp, it is noted that the pre-treatment was effective in improving the glucose yield as shown in figure 5.12. The effect of different conditions for enzymatic saccharification such as enzyme dose, reaction time, and amount of surfactant were studied in detail. The pre-treated substrate (10 % w/v) when hydrolysed using 20 FPU/g dry substrate (mL) with 0.4% (v/v) Tween® 80 for 20 h resulted in 604 mg/mL of the sugars. Enzymatic hydrolysis without pre-treatment gives less than 20 % sugar yield whereas pre-treatment raises the sugar yield to above 90 %. The biodegradability of lignocellulosic biomass is limited by several factors like crystallinity of cellulose, available surface area, and lignin content. Dilute sulfuric acid is used as a catalyst for hemicellulose and lignin solubilization at low concentration of 0.05 - 5 % (Hamelik *et al*., 2005; Schmidt and Thomsen, 1998).

The addition of a surfactant to the enzymatic hydrolysis process increased the reducing sugar (glucose) yield by 13 % as shown in figure 5.13. For this study Tween 80 was selected because it is known not to denature the cellulotyic enzymes that bring about the saccharification (Wilke, 1978). The role of a surfactant is to lower the surface tension between two liquids or between a solid and a liquid (in the case of this study between pre-treated bagasse and cellulase enzyme) thereby increasing the spreading and wetting properties (www.britannica.com). Surfactants have both hydrophyllic and hydrophobic properties and enhance hydrophobic substances withdrawal by decreasing the surface tension between the two liquids. Adsorption and orientation of the surfactant molecules at the solid-liquid interface should render the substrate readily wettable by the enzyme solution, bringing the substrate quickly into intimate contact with the enzymes and allowing the enzymes to reach the inaccessible substrate sites. Moreover, interactions between the sorbed surfactant molecules, the substrate, and the adsorbed enzymes might result in a decrease of the force with which the enzyme is held on the substrate (Castanon and Wilke, 1981).

The performance of enzymes on the hydrolysis of biomass/ cellulose is highly dependent on the physical and chemical physical characteristics of the biomass (Zhang and Lynd, 2004), structural and surface modifications introduced by the surfactants during pre-treatment could cause changes in the sequential enzymatic hydrolysis. In a study by Qing *et al*., (2010) the hydrophobicity tests suggested that Tween-80 pre-treatment of corn stover made the surface area much more hydrophilic, in that manner making it easier for enzymes to access the surface. The surfactants properties are also a major contributor to the modification of the solid surface of the biomass resulting in improved enzymatic hydrolysis.

6.5. Factors that affect pre-treatment

6.5.1. Effect of pre-treatment temperature

In this study at low temperature (90 ^{0}C) a high % of pulp and a low lignin content was obtained. At moderate temperature (120 ^{0}C) a high % recovery and a high lignin content was obtained. At high temperature (150 ^{0}C) a low % recovery and a slightly higher lignin content compared to lower temperature was obtained. At high temperatures higher biomass solubility is typically achieved as well as shortened pre-treatment time and reduced biomass resistance; however this means higher energy input. The required pre-treatment temperature for ionic liquid pre-treatment is relatively mild with a typical temperature range of 80 - 180 ^{0}C which makes them advantageous over other pre-treatment methods. Also pyrolysis side reactions are easier to avoid when using ionic liquids as a pre-treatment medium compared with other pre-treatment methods such as steam or hot water pre-treatment (Schultz *et al*., 1983; Kaar *et al*., 1998; Kim and Hong 2001; Yu *et al*., 2010).

6.5.2. Effect of pre-treatment time

In this study at short pre-treatment time (6 h) the % pulp recovered was high and the lignin recovered was high. When pre-treated for 12 h the recovered pulp was low and the lignin yield was low, however when pre-treated for 12 h at 120 ^{0}C the lignin yield increased. At longer pre-treatment time (24 h), the pulp recovered was high and the lignin yield was low. Pre-treatment time is dependant on pre-treatment temperature. Typically, several hours are required for biomass dissolution in ionic liquids but it differs from IL to IL. Swatloski *et al*., (2002) investigated the solubility of dissolving pulp cellulose in different ionic liquids. The highest solubility was obtained using [C_4mim]Cl. Various types of biomass were pre-treated with [C_2mim]Cl at 150 ^{0}C by Pezoa *et al*. (2010) to study how lignin removal made the residual biomass easier to hydrolyse.

Apart from lignin dissolution, some cellulose and hemicellulose were also dissolved. Thus, it was important to control pre-treatment time. They found that for corn stover, wheat straw, and eucalyptus, 1 h pre-treatment time led to significantly higher glucose and xylose yields compared with 0.5 h. However, the opposite trend was observed for Lenga (Nothofagus pumilio). They argued that the Lenga biomass pieces were smaller in size and thus faster for ionic liquid pre-treatment. More cellulose and hemicellulose were lost due to dissolution in $[C_2mim]Cl$ when the pre-treatment time was excessive.

6.6. Characterization of bagasse pulps

6.6.1. SEM

The micro-structural features of untreated biomass and pre-treated pulp material produced after ([Emim][OAc]), ($[HNEt_3][HSO_4]$) and acid/ alkali pre-treatment were studied using scanning electron microscopy (SEM). Visual observance of substrate microstructure following pre-treatment is crucial to supplement evidence for lignin re-deposition and disruption of the lignocellulose matrix obtained from chemical analysis. Observations performed on a number of mounts confirmed that there were no significant differences among different SEM preparations. SEM images of untreated and pretreated SCB were visually observed and are shown at different magnifications. The untreated native bagasse has a complete, compact and smooth surface, consisting of cellulose fibers bound by natural polymers including lignin and pectin as shown in Figure 5.15. After undergoing pre-treatment, the lignocellulosic structure is disrupted, with many smaller fibrous fragments peeling off from the biomass surface. The changes may be due to disruption of hydrogen bonding between the fibers following pretreatment.

For the untreated pith bagasse, a three-dimensional, anisotropic, amorphous, porous structure is observed with a high aspect ratio and a rough surface as shown in figure 5.17. The pith consists of many hollow cavities (pore diameter ca. 100 *μ*m), known as the lumen. The presence of the hollow lumen decreases the bulk density of pith fiber. This is in agreement with reports of the pith having spongy particles and less abundant cellulose fibers (Lee and Mariatti, 2008). In the SEM images of both IL pre-treatment pulps (EmimOAc and $[HNEt_3]HSO_4$), disruption of the lignocellulosic structure occurs, resulting in a collapsed lumen and a lot of debris as shown in figure 5.16. The fiber surface is seen to be smoother following pre-treatment, which may be due to lignin deposition on the pulp surface. The deposition of lignin droplets onto the surface of pretreated biomass has been reported previously,(Selig *et al*., 2007) notably under acidic pre-treatment conditions. These droplets may be detected using Klason analysis and are hypothesized to be composed of lignin and lignin-carbohydrate complexes, which deposit on the pulp surface and inhibit enzymes during enzymatic saccharification.

The SEM images of the acid/ alkali pre-treated SCB pulps as shown in figures 5.18 and 5.19 in comparison with raw SCB shows that the surfaces appear rougher with more porosity. The changes may be attributed to the disruption of the structure and reduction in crystallinity. One of the main objectives of pre-treatment is to increase the surface area and pore size for enzymatic hydrolysis (Alvira *et al.,* 2010); therefore, this objective was met, as an increase in porosity was observed after pre-treatment.

6.6.2. Thermal analysis (DSC and TGA)

The effect of pre-treatment on the thermal behaviour of SCB was studied by differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA). The thermal behaviour of raw and pre-treated SCB pulps were studied in the temperature range of room temperature to 800 ^{0}C at a heating rate of 10 ^{0}C/ min under a nitrogen flow. The

DSC curves as shown in Figures 5.20 and 5.21 show an exothermic peak at approximately 100 ^{0}C which is due to the removal of moisture when the samples were heated. Two exothermic peaks are observed at 310 and 385 ^{0}C and they are attributed to charring (Yang *et al*., 2007). A large endothermic peak at 365 ^{0}C is closely related to the cellulose fraction. This behaviour is related to the full decomposition of cellulose that might be attributed to quick devolatilization reactions leading to very little solid residue. The TGA curves as shown in figures 5.22 and 5.23 exhibited three degradation steps. The initial weight loss was observed between 30 and 100 ^{0}C in all scans. This may be attributed to the evaporation of loosely bound moisture on the surfaces of the samples. The second degradation step is observed between 250 and 350 ^{0}C and the third step between 350 and 500 ^{0}C. The exothermic peaks that appear at 350 and 400 ^{0}C are attributed to charring (Yang *et al*., 2007).

In a study by Perez-Pimienta *et al*., (2015) on agave bagasse the degradation onset temperature was observed to decrease for IL treated samples when compared to the untreated. A similar trend was also observed for thermal decomposition temperature stage, in both cases the lowest value corresponds to IL pre-treated sample at 140 ^{0}C. The results indicated that IL pre-treatment reduced the activation energy that is needed to decompose woody biomass by deconstructing the tight plant cell wall structures. The temperature region between 220 and 300 ^{0}C is mainly attributed to thermal depolymerization of hemicelluloses, while lignin decomposition extends to the whole temperature range, from 200 ^{0}C until 700 ^{0}C, due to different activities of the chemical bonds present on its structure and the degradation of cellulose taken place between 275 and 400 ^{0}C (Deepa *et al*., 2011). The final decomposition of all samples was completed between 600 and 680 ^{0}C, attributed to secondary reactions of carbon containing residues, which Fisher *et al*., (2002) have shown that, at temperatures above 400 ^{0}C, a weight loss due to thermolysis of carbon containing residues does take place.

Yang *et al*., (2007) reported the pyrolysis characteristics of pure hemicellulose, cellulose and lignin at a heating rate of 10 ^{0}C/ min in terms of maximum weight loss. They found that the decomposition temperature of hemicellulose and cellulose were 268 ^{0}C and 355 ^{0}C, respectively. Lignin was the most difficult to decompose, which happened slowly under the whole temperature range from ambient to 900 ^{0}C. Couhert *et al*., (2009) stated that decomposition of pure components differs from real biomass because the pyrolysis reactions are less hindered by interaction with other components. Hypothetically IL treated samples are more thermally stable in terms of decomposition temperatures after pre-treatment that leads to a downshift in degradation temperatures, which makes them suitable for subsequent higher yields during enzymatic saccharification.

6.6.3. FTIR

Based on the FTIR results, a significant difference was observed in the spectra of raw SCB and the pre-treated SCB pulps (IL and acid/alkali). These differences are an indicator of the fact that structural changes occurred during each pre-treatment method. All the spectra consist of a broad band in the region of 3400 – 3500 cm^{-1} which is primarily due to the free OH stretching vibration functional groups in lignin and carbohydrate molecules. The spectra of raw SCB as shown in figure 5.24 contains bands at a wavelength of 2918 cm^{-1} associated with CH_2 stretching vibration band for axial deformation and it shifted to 2911 cm^{-1} on IL pre-treated bagasse pulps shown in Figures 5.25 and 5.26 and 2908 cm^{-1} on acid/ alkali pre-treated bagasse pulp after delignification as shown in Figure 5.27 (Khalil *et al.,* 2001). Increased peak intensity observed between 900 – 1150 cm^{-1} indicates the presence of increased levels of cellulose (Sindhu *et al*., 2010; Maryana *et al*., 2014).

The peak situated in the region of 1730 cm^{-1} is mostly attributed to the C=O stretching vibration of the acetyl and uronic ester groups, from pectin, hemicellulose or the ester

linkage of the carboxylic group of ferrulic and p-coumaric acids of lignin and or hemicellulose (Sain, and Panthapulakkal, 2006; Sun *et al.,* 2005). The characteristic peak in the region of 1638 cm^{-1} is due to C=C aromatic skeletal vibration. The absorption peak at 1513 cm^{-1} is associated with the aromatic C=C in plane symmetrical stretching vibration of aromatic ring present in lignin (Garside and Wyeth, 2003). The vibration peak detected at 1382 cm^{-1} is predominantly related to the bending vibration of the C-H and the C-O bonds in the polysaccharide aromatic rings. The absorption peak at 1254 cm^{-1} represents the C-O out of plane stretching vibration of the aryl/ phenol group in lignin (Troedec *et al*., 2008).

The band detected at 1164 cm^{-1} is due to the O-H stretching of the secondary alcohol. In the region of 1110 cm^{-1} the observed band is due to the C-O-C stretching. The peak observed at 1048 cm^{-1} is due to the O-H stretching of the primary alcohol (Pappas *et al*., 1999). The absorption peak at 898 cm^{-1} represents the β-glycosidic linkages which arise from the polysaccharide component. The absorption peak at 1605 cm^{-1} is associated with the aromatic C-Ph vibration of the aromatic ring present in lignin (Corrales *et al*., 2012).

CHAPTER **7**

CONCLUSION

Sugarcane bagasse samples (whole and depithed) were pre-treated using two ILs and chemical treatment. Chemical pre-treatment is an effective pre-treatment method which is the cheapest out of the three methods studied; however it uses very corrosive and hazardous chemicals which are not environmental friendly. For this reason ILs are preferred pre-treatment methods. ([Emim][OAc]) is very expensive to purchase from suppliers (+/ - R7000, 50 ml), this makes it impossible for upscaling purposes. Moreover it has an undesirable smell. When compared to ([Emim][OAc]), ($[HNEt_3][HSO_4]$) is an inexpensive, moisture-tolerant ionic liquid-water mixture. An 80 wt. % triethylammonium hydrogen sulfate was seen to effectively fractionate a variety of bagasse feedstocks. The optimal pre-treatment condition (120 ^{0}C, 4 h at 10 wt. % biomass loading) was used to compare the pre-treatment efficacy in whole bagasse, laboratory depithed and industrially depithed feedstocks. Pulp yields were similar for all substrates whilst lignin recoveries and delignification of the pulp were significantly improved for bagasse that had been subjected to sieving in the laboratory ('laboratory depithing'). Despite differential delignification of the pulps, enzymatic saccharification yields were similar for all feedstocks and maximized at 68 % of theoretical maximum glucose yields after 7 days.

SEM observation of the produced pulps agreed with the chemical analyses conducted on the pulp and indicated swelling and disruption of the lignocellulose matrix. The most effective pre-treatment using ($[HNEt_3][HSO_4]$) was achieved with long fiber bagasse fractions obtained from laboratory depithing, which produced pulps with 43 % pulp yield, 6.8 % residual lignin and 75 % delignification. This study presents evidence that a depithing step prior to pre-treatment and cellulose saccharification may be unnecessary

for bagasse utilization in a bio-refinery, but remains effective for improved lignin extraction and upgrading. However, industrial depithing was not as effective as laboratory sieving. The results suggest that in the context of commercial-scale bagasse conversion to bioethanol, a depithing step may offer no advantages other than dust prevention and safe stockpiling of bagasse.

REFERENCES

Aden, A., Ruth, M., Ibsen, K., Jechura, J., Neeves, K., Sheehan, J., Wallace, B., Montague, L., Slayton, A., Lukas, J., (2002). Lignocellulosic biomass to ethanol process design and economics utilizing co-current Dilute acid prehydrolysis and enzymatic hydrolysis for corn stover. *National Renewable Energy Laboratory,* NREL/TP-510-32438.

Aki, S. N. V. K., Brennecke, J. F., Samanta, A., (2001). How polar are room temperature ionic liquids?. *Chem. Commun*. 413 – 414.

Akin, D. E., Rigsby, L. L., Sethuraman, A., Morrison, W. H.-III., Gamble, G. R., Eriksson, K. E. L., (1995). Alterations in structure, chemistry, and biodegradability of grass lignocellulose treated with the white rot fungi Ceriporiopsis subvermispora ad Cyathus stercoreus. *Appl. Environ. Microbiol.,* **61**, 1591 – 1598.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M. J., (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.*, **101** (13), 4851 – 4861.

Ander, P., Eriksson, K. E., (1977). Selective degradation of wood components by white-rot fungi. *Physiol. Plant*., **41**, 239 – 248.

Andric, P., Meyer, A. S., Jensen, P. A., Dam-Johansen, K., (2010). Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. *Biotechnol. Adv*., **28** (3), 308 – 324.

Anzaldo, J., Vargas, J., Turrado, J., Patt, R., Sanjua, R., (2001). Morphological and Chemical Composition of Pith and Fibers from Mexican Sugarcane Bagasse. *Holz als Roh- und Werkst*., **59**, 447 – 450.

Armstrong, D. W., He, L., Liu, Y. S., (1999). Examination of Ionic Liquids and Their Interaction with Molecules, When Used as Stationary Phases in Gas Chromatography. *Anal. Chem*., **71** (17), 3873 – 3876.

Atchison, J. E., (1971). Review of Bagasse Depithing, in: Proc. Int. Sec. Sugar Cane Technologists.

Aziz, S., Sarkanen, K., (1989). Organosolv pulping – a review. *Tappi., J.* **72**, 169 -175.

Azzam, A. M., (1989). Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation. *J. Environ. Sci. Health. B.,* **24** (4), 421 – 433.

Baker, G. A., Heller, W. T., O'Neill, H. M., Zhang, Q., (2010). Characterization of the Influence of the Ionic Liquid 1-Butyl-3-methylimidazolium Chloride on the Structure and Thermal Stability of Green Fluorescent Protein. *J. Phys. Chem. B.*, **114** (43), 13866 – 13871.

Bena-Marie, L., Zheng, G., Xuebing, X., (2008). High-perfomance liquid chromatography analysis methods developed for quantifying enzymatic esterification of flavonoids. *J. Chrom. A.*, **1198/1199**, 107 – 114.

Ben-Ghedalia D., Miron, J., (1981). The effect of combined chemical and enzyme treatments on the saccharification and in vitro digestion rate of wheat straw. *Biotechnol. Bioeng.*, **23** (4), 823 – 831.

Bian, J., Peng, F., Peng, X-P., Xia, X., Peng, P., Xu, F., Sun, R-C., (2014). Effect of [Emim]Ac pretreatment on the structure and enzymatic hydrolysis of sugarcane bagasse cellulose. *Carbohydrate Polymers,* **100**, 211 – 217.

Binder, J. B., Gray, M. J., White, J. F., Zhang, Z. C., Holladay, J. E., (2009). Reactions of lignin model compounds in ionic liquids. *Biomass and Bioenergy*, **33**(9), 1122 – 1130.

Bjerre, A. B., Olesen, A. B., Fernqvist, T., (1996). Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnol. Bioeng*., **49**, 568 – 577.

Blanchette, R. A., (1991). Delignification by wood-decay fungi. *Annu. Rev. Phytopathol*., **29**, 381 – 398.

Boominathan, K., Reddy, C. A., (1992). cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete Phanerochaete chrysosporium. *Proc. Natl. Acad. Sci.,* **89** (12), 5586 – 5590.

Bose, S., Armstrong, D. W., Petrich, J. W., (2010). Enzyme-catalysed hydrolysis of cellulose in ionic liquids: A Green approach toward the production of biofuels. *J. Phys. Chem. B.*, **114**, 8221 – 8227.

Botha, T., Blottnitz, H. V., (2006). A comparison of environmental benefits of bagasse-derived electricity and fuel ethanol on a life cycle basis. *Energy Polic.,* **34**, 2654 – 2661.

Brandt, A., Chen, L., Dongen, B.E., Welton, T., Hallett, J.P., (2015). Structural changes in lignins isolated using an acidic ionic liquid water mixture. *Green Chem*. Advance Article - doi:10.1039/C5C01314C.

Brandt, A., Hallett, J. P., Leak, D. J., Murphy, R. J., Welton, T., (2010). The effect of the ionic liquid anion in the pre-treatment of pine wood chips. *Green Chem*, **12** (4), 672 – 679.

Brandt, A., Ray, M. J., To, T. Q., Leak, D. J., Murphy, R. J., Welton, T., (2011). Ionic liquid pretreatment of lignocellulosic biomass with ionic liquid-water mixtures. *Green Chem*., **13**, 2489 – 2499.

Brennan, A. H., Hoagland, W., Schell, D. J., (1986). High temperature acid hydrolysis of biomass using an engineering-scale plug flow reactor: result of low solids testing. *Biotechnol. Bioeng. Symp.*, **17**, 53 – 70.

Brink, D. L., (1994). Method of treating biomass material. *US Patent*, **5**, 366 – 558.

Cadoche, L., Lopez, G. D., (1989). Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. *Biol. Wastes,* **30**, 153 – 157.

Cahela, D. R., Lee, Y. Y., Chambers, R. P., (1983). Modeling of percolation process in hemicellulose hydrolysis. *Biotechnol. Bioeng*., **25**, 3 – 17.

Cammarata, L., Kazarian, S. G., Salter, P. A., Welton, T., (2001). Molecular states of water in room temperature ionic liquids. *Phys. Chem. Chem. Phys*. **3**, 5192 – 5200.

Candido, R. G., Godoy, G. G., Goncalves, A. R., (2012). Study of sugarcane bagasse pre-treatment with sulphuric acid as a step of cellulose obtaining. *Int. J. Biol. Biomol. Agric. Food and Biotechnol Eng*, **6** (1), 101 – 105.

Cardona, C. A., Quintero, J. A., Paz, I. C., (2010). Bioresource Technology Production of bioethanol from sugarcane bagasse : Status and perspectives. *Bioresour. Technol*., **101**, 4754 – 4766.

Carrier, M., Hardie, A. G., Uras, U., Görgens, J., Hansie, J., (2012). Production of char from vacuum pyrolysis of South-African sugar cane bagasse and its characterization as activated carbon and biochar. *J. Anal. Appl. Pyrolysis*, **96**, 24 – 32.

Casas, A., Palomar, J., Alonso, M. V., Oliet, M., Omar, S., Rodriguez, F., (2012). Comparison of lignin and cellulose solubilities in ionic liquids by COSMO-RS analysis and experimental validation. *Ind. Crops and Prod.*, **37** (1), 155 – 163.

Castanon, M., and Wilke, C. R., (1981). Effects of the surfactant Tween 80 on enzymatic hydrolysis of newspaper. *Biotechnol. Bioeng.* **23** (6), 1365 – 1372.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., Saddler, J. N., (2007). Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics. *Adv. Biochem. Eng. Biotechnol.*, **108**, 67 – 93.

Chang, V. S, Kaar, W. E., Burr, B., Holtzapple, M. T., (2001 a) Simultaneous saccharification and fermentation of lime-treated biomass. *Biotechnol. Lett.*, **23**, 1327 – 33.

Chang, V. S., Burr, B., Holtzapple, M. T., (1997). Lime pre-treatment of switch grass. *Appl. Biochem. Biotechnol.*, **63/65**, 3 – 19.

Chang, V. S., Holtzapple, M. T., (2000). Fundamental factors affecting biomass enzymatic reactivity. *Appl. Biochem. Biotechnol* - Part A Enzyme Engineering and Biotechnology, **84/86**, 5 – 37.

Chang, V. S., Nagwani, M., Holtzapple, M. T., (1998). Lime pre-treatment of crop residues bagasse and wheat straw. *Appl. Biochem. Biotechnol*., **74**, 135 – 59.

Chang, V. S., Nagwani, M., Kim, C. H., Holtzapple, M. T., (2001 b). Oxidative lime pretreatment of high lignin biomass - poplar wood and newspaper. *Appl Biochem. Biotechnol.*, **94**, 1 – 28.

Chen, F., Dixon, R. A., (2007). Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.,* **25**, 759 – 761.

Chen, L., Sharifzadeh, M., Dowell, N. M., Welton, T., Shah, N., Hallett, J. P., (2014). Inexpensive ionic liquids: [HSO4] −-based solvent production at bulk scale. *Green Chem*., **16**, 3098 – 3106.

Chosdu, R., Hilmy, N., Erizal, Erlinda, T. B., Abbas, B., (1993). Radiation and chemical pretreatment of cellulosic waste. *Radiat. Phys. Chem*., **42**, 695 – 698.

Chundawat, S. P., Shishir, P. S., Balan, V., Dale, B. E., (2008). High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. *Biotechnol. Bioeng.*, **99**, 1281 – 1294.

Clark, T. A., Mackie, K. L., (1987). Steam explosion of the soft-wood Pinus radiata with sulphur dioxide addition. I. Process optimization. *J. Wood Chem. Technol*., **7**, 373 – 403.

Converse, A. O., Kwarteng, I. K., Grethlein, H. E., Ooshima, H., (1989). Kinetics of thermochemical pretreatment of lignocellulosic materials. *Appl. Biochem. Biotechnol*., **20/21**, 63 – 78.

Corrales, R. C. N. R., Mendes, F. M. T., Perrone, C. C., Anna, C. S., Souza, W., Abud, Y., Bon, E., P., S., Ferreira-Leitao, V., (2012). Structural evaluation of sugarcane bagasse steam pretreated in the presence of CO_2 and SO_2. *Biotechnology for biofuels*, **5**, 36 – 44.

Couhert, C., Commandre, J. M., Salvador, S., (2009). Is it possible to predict gas yields of any biomass after rapid pyrolysis at high temperature from its composition in cellulose, hemicellulose and lignin? *Fuel*, **88** (3), 408 – 417.

da Silva, A. S, Lee, S., Endo, T., Bon, E. P. S., (2011). Major improvement in the rate and yield of enzymatic saccharification of sugarcane bagasse via pre-treatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim] [Ac]). *Bioresource Technol*., **102**, 10505 – 10509.

Da Silva, A. S., Teixeira, R. S. S., Endo, T., Bon, E. P. S., Lee, S.-H., (2013). Continuous pretreatment of sugarcane bagasse at high loading in an ionic liquid using a twin-screw extruder. *Green Chem*., **15**, 1991 – 2001.

Dabirmanesh, B., Daneshjou, S., Sepahi, A. A., Ranjbar, B., Khavari-Nejad, R. A., Gill, P., Heydari, A., Khajeh, K., (2011). Effect of ionic liquids on the structure, stability and activity of two related α-amylases. *Int. J. Biol. Macromol.*, **48** (1), 93 – 97.

Dale, B. E., Moreira, M. J., (1982). A freeze-explosion technique for increasing cellulose hydrolysis. *Biotechnol. Bioeng. Symp*., **12**, 31 – 43.

Das, P., Ganesha, A., Wangikar, P., (2004). Influence of pretreatment for deashing of sugarcane bagasse on pyrolysis products. *Biomass and Bioenergy*, **27**, 445 – 457.

Decker, S. R., Brunecky, R., Tucker, M. P., Himmel, M. E., Selig, M. J., (2009). High-throughput screening techniques for biomass conversion. *Bioenerg. Res*., **2**, 179 – 192.

Decker, S. R., Sheehan, J., Dayton, D. C., Bozell, J. J., Adney, W. S., Hames, B., Thomas, S. R., Bain, R. L., Czernik, S., Zhang, M., Himmel, M. E., (2007). Biomass conversion. In Kent and Riegel's Handbook of *Industr. Chem. and Biotechnol.*, **2** (11), 1449 – 1548.

Deepa, B., Abraham, E., Cherian, B. M., Bismarck, A., Blaker, J. J., Pothan, L. A., (2011). Structure, morphology and thermal characteristics of banana nano fibers obtained by steam explosion. *Bioresour Technol*, **102** (2), 1988 – 1997.

Diedericks, D., van Rensburg, E., Garcia-Aparicio, M. D., Gorgens, J. F., (2012). Enhancing the enzymatic digestibility of sugarcane bagasse through the application of an ionic liquid in combination with an acid catalyst. *Biotechnol. Progr*., **28** (1), 76 – 84.

Diedericks, D., van Rensburg, E., Gorgens, J. F., (2012a). Fractionation of sugarcane bagasse using a combined process of dilute acid and ionic liquid treatments. *Appl Biochem Biotechnol*., **167** (1), 1921 – 1937.

Dong, S. J., Zhang, B. X., Gao, Y. F., Hu, X. M., (2015). An Efficient Process for Pre-treatment of Lignocelluloses in Functional Ionic Liquids. *Int.l J. Pol. Sci.*, (Article ID 978983), 1 – 6.

Duff, S. J. B., Murray, W. D., (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. *Bioresour. Technol*., **55**, 1 – 33.

Dustex Africa, 2015. http://www.dustex.co.za/about/ (accessed 4.23.15).

Environmental Pollution Control, Pulp And Paper Industry, Part I: Air, (1976), EPA-625/7-76-001.

Esteghalian, A., Hashimoto, A. G., Fenske, J. J., Penner, M. H., (1997). Modelling and optimization of dilute sulphuric acid pre-treatment of corn stove, poplar and switch grass. *Bioresour. Technol.*, **59**, 129 – 36.

Fan, L. T., Gharpuray, M. M., Lee, Y. H., (1987). Cellulose Hydrolysis Biotechnology Monographs. Berlin: *Springer*, 3, 57.

Feng, L., Chen, Z., (2008). Research progress on dissolution and functional modification of cellulose in ionic liquids. *J. Molec. Liq.*, **142** (1), 1 – 5.

Fengel, D., and Wegener, G., (1979). Hydrolysis of polysaccharides with trifluoroacetic acid and its applications to rapid wood analysis. In: Brown, R. D., and Jurasek, L., (Eds.),. Hydrolysis of cellulose: mechanisms of enzymatic and acid catalysis. Advances in chemistry series No 181. *American Chemical Society*, 145 – 158.

Fisher, T. Hajaligol, M., Waymack, B., Kellogg, D., (2002). Pyrolysis behaviour and kinetics of biomass derived materials. *J Anal Appl Pyrolysis*, **62** (2), 331 – 349.

Fletcher, K. A., Pandey, S., (2002). Effect of Water on the Solvatochromic Probe Behavior within Room-Temperature Ionic Liquid 1-Butyl-3-Methylimidazolium Hexafluorophosphate. *Applied Spectroscopy*, **56** (2), 266 – 271.

Foyle, T., Jennings, L., Mulcahy, P., (2007). Compositional analysis of lignocellulosic materials: Evaluation of methods used for sugar analysis of waste paper and straw. *Bioresour. Technol.*, **98**, 3026 – 3036.

Fu, D., Mazza, G., Tamaki, Y., (2010). Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the residues. *J. Agric. Food Chem.*, **58** (5), 2915 – 2922.

Fukaya, Y., Hayashi, K., Wada, M., Ohno, H., (2008). Cellulose dissolution with polar ionic liquids under mild conditions: required factors for anions. *Green Chem.*, **10**, 44 – 46.

Galbe, M., Zacchi, G., (2007). Pre-treatment of lignocellulosic materials for efficient bioethanol production. *Biofuels*, **108**, 41 – 65.

Garcia-Perez, M., Chaala, A., Roy, C., (2002). Vacuum pyrolysis of sugarcane bagasse. *Fuel*, **81**, 893 – 907.

Garrote, G., Dominguez, H., Parajo, J. C., (1999). Hydrothermal processing of lignocellulosic materials. *Eur J Wood Wood Prod*, **57**, 191 – 202.

Garside, P., Wyeth, P., (2003). Identification of cellulose fibers by FTIR spectroscopy: Thread and single fiber analysis by attenuated total reflectance. *Studies in Conservation*, **48** (4), 269 – 275.

George, A., Brandt, A., Tran, K., Zahari, S. M. S. N. S., Klein-Marcuschamer, D., Sun, N., Sathitsuksanoh, N., Shi, J., Stavila, V., Parthasarathi, R., Singh, S., Holmes, B. M., Welton, T., Simmons, A., Hallett, J. P., (2015). Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. *Green Chem.*, **17**, 1728 – 1734.

George, A., Tran, K., Morgan, T. J., Benke, P. I., Berrueco, C., Lorente, E., Wu, B. C., Keasling, J. D., Simmons, B. A., Holmes, B. M., (2011). The effect of ionic liquid cation and anion combinations on the macromelecular structure of lignins. *Green Chem.*, **13**, 3375 – 3385.

Gomez, S. M. R., Andrade, R. R., Santander, C. G., Costa, A. C., Maciel, F. R., (2010). Pretreatment of sugar cane bagasse with phosphoric and sulphuric diluted acid for fermentable sugars production by enzymatic hydrolysis. *Chem. Eng. Trans.,* **20**, 321 – 326.

Graenacher, C. (1934). Cellulose Solution. U.S. Patent 1,943,176.

Gräsvik, J., Winestrand, S., Normark, M., Jönsson, L. J., Mikkola, J. P., (2014). Evaluation of four ionic liquids for pretreatment of lignocellulosic biomass. *BMC Biotechnol.*, **14**, 34 – 45.

Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, J., Torget, R., Graboski, M., (1984). Chemical - mechanical methods for the enhanced utilization of straw. *Biotec. Bioeng. Symp.*, **14**, 137 – 157.

Grous, W. R., Converse, A. O., Grethlein, H. E., (1986). Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme Microb. Technol.,* **8**, 274 – 280.

Ha, S. H., Mai, N. L., An, G., Koo, Y. M., (2011). Microwave-assisted pre-treatment of cellulose in ionic liquid for accelerated enzymatic hydrolysis. *Bioresour Technol*, **102**, 1214 – 1219.

Hall, M., Bansal, P., Lee, J. H., Realff, M. J., Bommarius, A. S., (2010). Cellulose crystallinity - A key predictor of the enzymatic hydrolysis rate. *FEBS Journal*, **277** (6), 1571 – 1582.

Hamelinck, C. N., Hooijdonk, G. V., Faaij, A., (2005). 'Cradle-to- grave' assessment of existing lignocellulose pre-treatment technologies. *J. Biomass and Bioenergy*, **28**, 384 – 388.

Hart, W. E. S., Harper, J. B., Aldous, L., (2015). The effect of changing the components of an ionic liquid upon the solubility of lignin. *Green Chem*., **17**, 214 – 218.

Hatakka, A. I., (1983). Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose. *Appl. Microbiol. Biotechnol*., **18**, 350 – 357.

Hatfield, R., Fukushima, R. S., (2005). Can lignin be accurately measured? *Crop Sci*., **45**, 832 – 839.

Haykir, N. I., Bahcegul, E., Bicak, N., Bakir, U., (2013). Pretreatment of cotton stalk with ionic liquids including 2-hydroxy ethyl ammonium formate to enhance biomass digestibility. *Industrial Crops and Products*, **41**, 430 – 436.

Hendricks, A. T., and Zeeman, G., (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.*, **100**, 10 – 18.

Hendrickson, E. R., (1970). Control of Atmospheric Emissions in the Wood Pulping Industry, Volume I, HEW Contract Number CPA-22-69-18, U. S. Environmental Protection Agency.

Hermanutz, F., Gahr, F., Uerdingen, E., Meister, F., Kosan, B., (2008). New developments in dissolving and processing of cellulose in ionic liquids. *Macromolecular Symposia* (Wiley Online Library), 23 – 27.

Hernandez-Salas, J. M., Villa-Ramirez, M. S., Veloz-Rendon, J. S., Rivera-Hernandez, K. N., Gonzalez-Cesar, R. A., Plascencia-Espinosa, M. A., Trejo-Estrada, S. R., (2009). Comparative hydrolysis and fermentation of sugarcane and agave bagasse. *Bioresour. Technol.,* **100**, 1238 – 1245.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Andey, W. S., Nimlos, M. R., Brady, J. W., Foust T. D., (2007). Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Sci.*, **315**, 804 – 807.

Himmler, S., Hormann, S., van Hal, R., Schulz, P. S., Wasserscheid, P., (2006). Transesterification of methylsulfate and ethylsulfate ionic liquids – an environmentally benign way to synthesize long chain and functionalized alkylsulfate ionic liquids. *Green Chem*., **8** (10), 887 – 894.

Hinman, N. D., Schell, D. J., Riley, C. J., Bergeron, P. W., Walter, P. J., (1992). Preliminary estimate of the cost of ethanol production for SSF technology. *Appl. Biochem. Biotechnol.,* **34/35**, 639 – 649.

Hodge, D. B., Karim, M. N., Schell, D. J., McMillan, J. D., (2008). Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.*, **99** (18), 8940 – 8948.

Holtzapple, M. T., Humphrey, A. E., Taylor, J. D., (1989). Energy requirements for the size reduction of poplar and aspen wood. *Biotechnol. Bioeng*., **33**, 207 – 210.

Hou, X. D., Smith, T. J., Li, N., Zong, M. H. (2012). Novel renewable ionic liquids as highly effective solvents for pretreatment of rice straw biomass by selective removal of lignin. *Biotechnol. Bioeng*., **109** (10), 2484 – 2493.

Hsu, T. A., Ladisch, M. R., Tsao, G. T., (1980). Alcohol from cellulose. *Chemical Technol.*, **10** (5), 315 – 319.

http://www.epsilon-econ.gr/en/lignocellulose biomass examples (Accessed on 13/04/2015)

http://www.pinterest.com/biomass sources (Accessed on 22/05/2015)

Hu, F., Jung, S., Ragauskas, A., (2012). Pseudo-lignin formation and its impact on enzymatic hydrolysis. *Bioresour. Technol*., **117**, 7 – 12.

Iyer, P. V., Wu, Z. W., Kim, S. B., Lee, Y. Y., (1996). Ammonia recycled percolation process for pretreatment of herbaceous biomass. *Appl. Biochem. Biotechnol*., **57/58**, 121 – 132.

Janghathaikul, D., Gheewala, S. H., (2004). Bagasse - a sustainable energy resource from the sugar mills. The Joint International Conference on Sustainable Energy and Environment (SEE), 1-3 December, Hua Hin,Thailand.

Jiang, L. Q., Fang, Z., Li, X. K, Luo, J., Fan, S., (2013). Combination of dilute acid and ionic liquid pretreatments of sugarcane bagasse for glucose by enzymatic hydrolysis. *Process Biochem.*, **48** (12), 1942 – 1946.

Jorgensen, H., ibe-Pedersen, J., Larsen, J., Felby, C., (2007). Liquefaction of lignocellulose at high-solids concentrations. *Biotechnol. and Bioeng.,* **96**(5), 862 – 870.

Joshi, M. D., Anderson, J. L., (2012). Recent advances of ionic liquids in separation science and mass spectrometry. *Royal Society of Chemistry*, **2**, 5470 – 5484.

Kaar, W., Gutierrez, C., Kinoshita, C., (1998). Steam explosion of sugarcane bagasse as a pre-treatment for conversion to ethanol. *Biomass Bioenergy*, **14**, 277 – 287.

Karatzos, K. S., Edye, L. A., Doherty, W. O. S., (2012). Sugarcane baggase pretreatment using three imidazolium-based ionic liquids; mass balances and enzyme kinetics. *Biotechnol. for Biofuels*, **5**, 62 – 73.

Karatzos, S. K., Edye, L. A., Doherty, W. O. S., (2012). Sugarcane bagasse pre-treatment using three imidazolium-based ionic liquids; mass balances and enzyme kinetics. *Biotechnol. for Biofuels*, **5**, 62 – 74.

Kazarian, S. G., Briscoe, B. J., Welton, T., (2000). Combining ionic liquids and supercritical fluids for green technology. *Chem. Commun*., 2047 – 2048.

Keskar, S. S., Edye, L. A., Fellows, C. M., Doherty, W. O. S., (2012). ATR-FTIR measurement of biomass components in phosphonium ionic liquids. *Journal of Wood Chemistry and Technology*, **32** (3), 175 – 186.

Khalil, H. P. A., Ismail, H., Rozman, H. D., Ahmad, M. N., (2001). The effect of acetylation on interfacial shear strength between plant fiber and various matrices. *European Polymer Journal*, **37** (5), 1037 – 1045.

Khokhar, U. Z., Syed, Q., Baig, S., Nadeem, M., Irfan, M., Athar, A. M., (2010 a). Study on filtrate obtained from batch-wise pre-treatment of wheat straw at different concentrations of H2SO4 and autoclave time. *World Appl. Sci. J*., **10** (9), 1047 – 1055.

Khokhar, U. Z., Syed, Q., Nadeem, M., Baig, S., Irfan, M., Gull, I., Tipu, I., Aslam, S., Samra, Q. Z., Athar, A. M., (2010 b). Delignification of wheat straw with acid and hydro-steam under pressure. *World Appl. Sci. J*., **11** (12), 1524 – 1530.

Kilpeläinen, I., Xie, H., King, A., Granstrom, M., Heikkinen, S., Argyropoulos, D. S., (2007). Dissolution of wood in ionic liquids. *J Agric Food Chem*, **55** (22), 9142 – 9150.

Kilzer, F. J., Broido, A., (1965). Speculations on the nature of cellulose pyrolysis. *Pyrodynamics*, **2**, 151 – 163.

Kim, K. H., Hong, J., (2001). Supercritical CO2 pre-treatment of lignocellulose enhances enzymatic cellulose hydrolysis. *Bioresour Technol*, **77**, 139 – 144.

Kirk, T. K., Farrell, R. L., (1987). Enzymatic combustion: the microbial degradation of lignin. *Annu. Rev. Microbiol*., **41**, 465 – 505.

Klason, P., (1923). The determination of lignin. Chapter 6, pp 151 In: Brauns, F. E., (Ed.), The Chemistry of Lignin. Academic Press, New York (1952).

Klemm, D., Philipp, B., Heinze, T., Heinze, U., Wagenknecht, W., (1998). Comprehensive Cellulose Chemistry: Fundamentals and Analytical Methods. *Wiley–VCH Verlag: Weinheim, Germany*, 1, Chapter 3, 181

Kline, L. M., Hayes, D. G., Womac, A. R., Labbe, N., (2010). Simplified determination of lignin content in hard and soft woods via UV-spectrophotometric analysis of biomass dissolved in ionic liquids. *BioRes*., **5** (3), 1366 – 1383.

Kosan, B., Michels, C., Meister, F., (2008). Dissolution and forming of cellulose with ionic liquids. *Cellulose*, **15** (1), 59 – 66.

Kristensen, J. B., Börjesson, J., Bruun, M. H., Tjerneld, F., Jorgensen, H., (2007). Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. *Enzyme and Microbial Technol.*, **40** (4), 888 – 895.

Kumar, P., Barret, D. M., Delwiche, M. J., Stroeve, P., (2009 b). Methods for pre-treatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res*., **48**, 3713 – 3729.

Kumar, R., Wyman, C. E., (2009). Cellulase adsorption and relationship to features of corn stover solids produced by leading pretreatments. *Biotechnol. and Bioeng.*, **103**(2), 252 – 267.

Lapierre, C., Jouin, D., Monties, B., (1989). Lignin characterization of wheat straw samples determined by chemical degradation procedures. Pp 118 – 130 in: Cheson, A., and Orskov, E. R., (Eds.), Physico-chemical Characterization of Plant Residues for Insustrial and Feed Use.

Lau, B. B. Y., Luis, E. T., Hossain, M. M., Hart, W. E. S., Cencia-Lay, B., Black, J. J., To, T. Q., Aldous, L., (2015). Facile, room-temperature pre-treatment of rice husks with tetrabutylphosphonium hydroxide: Enhanced enzymatic and acid hydrolysis yields. *Bioresour. Technol.*, **197**, 252 – 259.

Laus, G., Bentivoglio, G., Schottenberger, H., Kahlenberg, V., Kopacka, H., Röder, T., Sixta, H., (2005). Ionic liquids: current developments, potential and drawbacks for industrial applications. *Lenzinger Berichte*, **84**, 71 – 85.

Lee, S. C., Mariatti, M., (2008). The effect of bagasse fibers obtained (from rind and pith component) on the properties of unsaturated polyester composites. *Mater. Lett.*, **62**, 2253 – 2256.

Lee, S. H., Doherty, T.V., Linhardt R., J., Dordick, J., S., (2009). Ionic Liquid-Mediated Selective Extraction of Lignin From Wood Leading to Enhanced Enzymatic Cellulose Hydrolysis. *Biotechnol. and Bioeng.*, **102** (5), 1368 – 1376.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M., Vogel, K. P., Simmons, B. A., Singh, S., (2010). Comparison of dilute acid and ionic liquid

pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. *Bioresour. Technol*., **101**, 4900 – 4906.

Li, C., Wang, Q., Zhao, Z. K., (2008). Acid in ionic liquid: An efficient system for hydrolysis of lignocellulose. *Green Chem*., **10**, 177 – 182.

Li, W. Y. Sun, N., Stoner, B. Jiang, X. Y., Lu, X. M., Rogers, R. D., (2011). Rapid dissolution of lignocellulosic biomass in ionic liquids using temperatures above the glass transition of lignin. *Green Chem*., **13**, 2038 – 2047.

Liebert, T., Heinze, T., (2008). Interaction of ionic liquids with polysaccharides 5. Solvents and reaction media for the modification of cellulose. *BioResources*, **3** (2), 576 – 601.

Lloyd, T. A., Wyman, C. E., (2005). Combined sugar yields for dilute sulfuric acid pre-treatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour. Technol*., **96** 1967 – 1977.

Lois-Correa, J. A., (2012). Depithers for Efficient Preparation of Sugar Cane Bagasse Fibers in Pulp and Paper Industry. *Ing. Investig. y Tecnol*., **13**, 417 – 424.

Lu, F., Ralph, J., (1997). Derivatization followd by reductive cleavage (DFRC method), a new method for lignin analysis: Protocol for analysis of DFRC monomers. *J. Agric. Food Chem*., **45**, 2590 – 2592.

Lu, Y., Wang, Y., Xu, G., Chu, J., Zhuang, Y., Zhang, S., (2010). Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. *Applied Biochem. and Biotechnol.*, **160** (2), 360 – 369.

Lue, B. M., Guo, Z., Xu, X. (2010). Effect of room temperature ionic liquids on the enzymatic acylation of flavonoids. *Process Biochem.*, **45**, 1375 – 1382.

Lynam, J. G., Toufiq, R. M.,Vasquez, V. R., Coronella, C. J., (2012). Pretreatment of rice hulls by ionic liquid dissolution. *Bioresour. Technol*., **114**, 629 – 636.

Lynd, L. R., Elander, R. T., Wyman, C. E., (1996). Likely features and costs of mature biomass ethanol technology. *Appl. Biochem. Biotechnol*., **57/58**, 741 – 761.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., Pretorius, I. S., (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. and Molecular Biol. Rev.*, **66**(3), 506 – 577.

Magdi, E. G., Yue, Z., Li Xin da; Huan, L., Xuan, Z., Feng, L. H., Muhuo, Y., (2012). Current status of applications of ionic liquids for cellulose dissolution and modifications: review. *Int. J. Eng. Sci. and Technol*., **4** (7), 3556 – 3571.

Martens, D. A., Loeffelmann, K. L., (2002). Improved accounting of carbohydrate carbon from plants and soil. *Soil Biol. Biochem*., **34**, 1393 – 1399.

Martens, D. A., (2000). Plant residue biochemistry regulates soil carbon cycling and carbon sequestration. *Soil Biol. Biochem*., **32**, 361 – 369.

Mattson, M. R., (2008). Hormesis defined. *Aging Research Reviews*, **7**(1), 1 – 7.

Maryana, R., Ma'rifatunb, D., Wheni, A. I., Satriyo K. W., Rizala, W. A., (2014). Alkaline Pre-treatment on sugarcane bagasse for bioethanol production. *Energy Procedia*, **47**, 250 – 254.

McMillan, J. D., (1994). Pretreatment of lignocellulosic biomass. In: Himmel, M. E., Baker, J. O., Overend, R. P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. *J. Am. Chem. Soc.*, 292 – 324.

Mes-Hartree, M., Dale, B. E., Craig, W. K., (1988). Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose. *Appl. Microbiol. Biotechnol.,* **29**, 462 – 468.

Millet, M. A., Baker, A. J., Scatter, L. D., (1976). Physical and chemical pretreatment for enhancing cellulose saccharification. *Biotech. Bioeng. Symp*., **6**, 125 – 153.

Mohtar, S. S., Tengku Malim Busu, T. N., Md Noor, A. M., Shaari, N., Yusoff, N. A., Bustam Khalil, M. A., Abdul Mutalib, M. I., Mat, H. B., (2015). Extraction and characterization of lignin from oil palm biomass via ionic liquid dissolution and non-toxic aluminium potassium sulfate dodecahydrate precipitation processes. *Bioresour. Technol.*, **192**, 212 – 218.

Mok, W. S. L., Antal, Jr. M. J., (1992). Uncatalyzed solvolysis of whole biomass hemicellulose by hot compresses liquid water. *Ind. Eng. Chem. Res*., **31**, 1157 – 1161.

Moore, W. E., Johnson, D. B., (1976). Determination of pentosans in pulp. In: Procedures for the Chemical Analysis of Wood Products. United States Department of agriculture, Forest and Service, Forest Products Laboratory.

Morjanoff, P. J., Gray, P. P., (1987). Optimization of steam explosion as method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. *Biotechnol. Bioeng*., **29**, 733 – 741.

Mosier, N., Wyman, C. E., Dale, B. E., Elander, R., Lee, Y. Y., Holtzapple, M. T., Ladisch, M., (2005). Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresour. Technol.*, **96** (6), 673 – 686.

.Moultrop, J. S., Swatloski, R. P., Moyna, G., Rogers, R. D., (2005). High resolution 13C NMR studies of cellulose and cellulose oligomers in ionic liquid solutions. C*hem. Commun.*, 1557 – 1559.

Moure, A., Gullon, P., Dominguez, H., Parajo, J. C., (2006). Advances in the manufacture, purification and applications of xylooligosaccharades as food additives and nutraceuticals. *Process Biochem*, **41**, 1913 – 1923.

Muhammad, N., Man, Z., Khalil, M. A. B., (2012). Ionic liquid - a future solvent for the enhanced uses of wood biomass. *Eur. J. Wood. Prod*., **70**, 125 – 133.

Nancharaiah, Y. V., Francis, A. J., (2015). Hormetic effect of ionic liquid 1-ethyl-3-methylimidazolium acetate on bacteria. *Chemosphere.*, **128**, 178 – 183.

Narayanaswamy, N., (2010). Supercritical carbon dioxide pre-treatment of various lignocellulosic biomasses. MSc Thesis, Ohio University, Athens.

Neely, W.C., (1984). Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnol. Bioeng.*, **20**, 59 – 65.

Nelson, L., Leming, J. A., (1957). Evacuation of monoethanolamine method of cellulose determination for agricultural residues. *Tappi J*, **40**, 846 – 850.

Nguyen, L. M., (2000). Organic matter composition, microbial biomass and microbial activity in gravel-bed constructed wetlands treating farm dairy wastewaters. *Ecol. Eng*., **33** (1), 54 – 67.

Noritomi, H., Minamisawa, K., Kamiya, R., Kato. S., (2011). Thermal stability of proteins in the presence of aprotic ionic liquids. *Journal of Biomed. Sci. and Eng.*, **4** (2), 94 – 99.

Ogura, K., Ninomiya, K., Takahashi, K., Ogino. S., Kondo, A., (2014). Pretreatment of Japanesse cedar by ionic liquid solutions in combination with acid and metal ion and its applications to high solid loading. *Biotechnol. Biofuels.*, **7**, 120 – 130.

Palmqvist, E., Hahn-Hägerdal, B., (2000 a). Fermentation of lignocellulosic hydrolysates I: Inhibition and detoxification. *Bioresour. Technol*., **74**, 17 – 24.

Palmqvist, E., Hahn-Hägerdal, B., (2000 b). Fermentation of lignocellulosic hydrolysates II: Inhibitors and mechanisms of inhibition. *Bioresour. Technol.*, **74**, 25 – 33.

Pandey, A., Soccol, C. R., Nigam, P., Soccol, V. T., (2000). Biotechnological potential of agro-industrial residues . In : sugarcane bagasse. *Bioresour. Technol*., **74**, 69 – 80.

Pappas, C., Tarantillis, P. A., Daliani, I., Mavromoustakos, T., & Polissiou, M., (2002). Comparison of classical and ultrasound – assisted isolation procedure of cellulose from kenaf (Hibiscuss cannabinus L.) and eucalyptus (Eucalyptus rodustrus Sm.). *Ultrasonic Sonochemistry*, **9** (1), 19 – 23.

Patwardhan, P. R., Satrio, J. A., Brown, R. C., Shanks, B. H., (2010). Influence of organic salts on the primary pyrolysis of cellulose. *Bioresour. Technol*., **101**, 4646 – 4655.

Perez-Pimienta, J. A., Lopez-Ortega, M. G., Chavez-Carvayar, J. A., Varanasi, P., Stavila, V., Cheng G., Singh, S., Simmons, B. A., (2015). Characterization of agave bagasse as a function of ionic liquid pre-treatment. *Biomass and bioenergy*, **75**, 180 – 188.

Persson, T., Ren, J. L., Joelsson, E., A. S. J., (2009). Fractionation of wheat and barley straw to access high molecular mass hemicelluloses prior to ethanol production. *Biores Technol*, **100**, 3906 – 3913.

Pezoa, R., Cortinez, V., Hyvarinen, S., Reunanen, J., Linenqueo, M. E., Salazar, O., Carmona, R., Garcia, A., Murzin, D. Y., Mikkola, J-P., (2010). Use of ionic liquids in the pretreatment of forest and agricultural residues for the production of bioethanol. *Cellul Chem Technol*, **44**, 165 – 172.

Pinkert, A., Goeke D. F., Marsh, K. N., Pang, S., (2011) Extracting Wood Lignin without Dissolving or Degrading Cellulose: Investigations on the Use of Food Additive-Derived Ionic Liquids. *Green Chem.*, **13**, 3124 – 3136.

Pinkert, A., Marsh, K. N., Pang, S. S., Staiger, M. P., (2009). Ionic liquids and Their Interaction with cellulose. *Chem. Rev*., **109**, 6712 – 6728.

Prado, R., Brandt, A., Erdocia, X., Hallet, J., Welton, T., Labidic, J., (2015). Lignin oxidation and depolymerisation in ionic liquids. *Green Chem.*, Advance Article.

Pu, Y. Q., Jiang, N., Ragauskas, A. J., (2007). Ionic liquids as a green solvent for lignin. *J. Wood Chem. Technol*., **27**, 23 – 33.

Qing, Q., Yang, B., Wyman, C. E., (2010). Impact of surfactants on pre-treatment of corn stover. *Biores Technol*, **101**, 5941 – 5951.

Qiu, Z, Aita, G. M., Walker, M. S., (2012) Effect of ionic liquid pretreatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. *Bioresour Technol*., **44** (4), 1 – 11.

Qiu, Z., Aita, G. M., (2013). Pre-treatment of energy cane bagasse with recycled ionic liquid for enzymatic hydrolysis. *Bioresour Technol*., **129**, 532 – 537.

Qiu, Z., Aita, G. M., Walker, M. S., (2012). Effect of ionic liquid pre-treatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. *Bioresour. Technol.*, **117**, 251 – 256.

Rainey, T. J., O'Hara, I. M., Mann, A. P., Bakir, C. H., Plaza, F., (2013). Effect of depithing on the safety and environmental aspects of bagasse stockpiling. *Process Saf. Environ. Prot*., **91** (5), 378 – 385.

Ramos, L. P., (2003). The chemistry involved in the steam treatment of lignocellulosic materials. *Quim. Nova*, **26**, 863 – 871.

Rasul, M. G., Rudolph, V., Carsky, M., (1999). Physical properties of bagasse. *Fuel,* **78**, 905 – 910.

Review of new source performance standards for kraft pulp mills, (1983), EPA-450/ 3-83-017, U. S. Environmental Protection Agency, Research Triangle Park, NC.

Ristolainen, M., Alen, R., Malkavaara, P., Pere, J., (2002). Reflectance FTIR microspectroscopy for studying effect of xylan removal on unbleached and bleached birch Kraft pulps. *Holzforschung*, **56** (5), 513 – 521.

Rocha, G. J.M., Martin, C., Souto-Maior, A. M., Baudel, H. M., de Abreu C. A. M., (2011). Dilute mixed-acid pre-treatment of sugarcane bagasse for ethanol production. *Biomass and Bioenergy,* **35** (1), 663 – 670.

Rollin, J. A., Zhu, Z., Sathitsuksanoh, N., & Zhang, Y. P., (2011). Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnol. and Bioeng.*, **108** (1), 22 – 30.

Saeman, J. F., Bulb, J. L., Harris, E. E., (1944). Quantitative saccharification of wood and cellulose. USDA Forest Service, Forest Products Laboratory Bulletin R1458, Madison, Wisconsim, USA.

Sain, M., Panthapulakkal, S., (2006). Bioprocess preparation of wheat straw fibers and their characterization. *Industrial Crops and Products*, **23**, 1 – 8.

Samayam, I. P., Schall, C. A., (2010). Saccharification of ionic liquid pre-treated biomass with commercial enzyme mixtures. *Bioresour. Technol*., **101**, 3561 – 3566.

Santoro, N., Cantu, S. L., Tornqvist, C. E., Falbel, T., Bolivar, J. L., Pattersen, S. E., Pauly, M., Walton, J. D., (2010). A high-throughput platform for screening milligram quantities of plant biomass for lignocellulose digestibility. *Bioenerg. Res.,* **3** (1), 93 – 102.

Sarkanen, K. V., (1980). Acid-catalyzed delignification of lignocellulosics in organic solvents. *Prog. Biomass Convers*., **2**, 127 – 144.

Sathitsuksanoh, N., Zhu, Z., Ho, T., Bai, M., Zhang, Y. P., (2010). Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings. *Bioresour. Technol.*, **101** (13), 4926 – 4929.

Schmidt, A. S., Thomsen, A. B., (1998). Optimization of wet oxidation pre-treatment of wheat straw. *J. Bioresour Technol*., **64**, 139 – 151.

Schultz, T. P., Biermann, C. J., McGinnis, G. D., (1983). Steam explosion of mixed hardwood chips as a biomass pre-treatment. *Ind Eng Chem Prod Res Dev*, **22**, 344 – 348.

Schurz, J., (1978). In: Ghose, T. K. (Ed.), Bioconversion of Cellulosic Substances into Energy Chemicals and Microbial Protein Symposium Proceedings, IIT, New Delhi, 37.

Selig, M., Weiss, N., Ji, Y., (2008). Enzymatic saccharification of lignocellulosic biomass. National Renewable Energy Laboratory: Technical Report - NREL/TP-510-412629.

Selig, M. J., Viamajala, S., Decker, S. R., Tucker, M. P., Himmel, M. E., Vinzant, T. B., (2007). Deposition of Lignin Droplets Produced During Dilute Acid Pre-treatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. *Biotechnol. Prog*. **23**, 1333 – 1339.

Shafizadeh, F., Bradbury, A. G. W., (1979). Thermal degradation of cellulose in air and nitrogen at low temperatures. *J. Appl. Poly. Sci*., **23**, 1431 – 1442.

Shafizadeh, F., Lai, Y. Z., (1975). Thermal degradation of 2-deoxy-Darabino- hexonic acid and 3-deoxy-D-ribo-hexono-1,4-lactone. *Carbohyd. Res*., **42**, 39 – 53.

Sharman, P., Harris, G., (1994). High yield pulping. Mill product news, 31.

Sharmas, S. K., Kalra, K. L., and Grewal, H. S., (2002). Enzymatic saccharification of pre-treated sun flower stalks. *Biomass Bioenerg.*, **23**, 237 – 43.

Shill, K., Padmanabhan, S., Xin, Q., Prausnitz, J. M., Clark, D. S., Blanch, H. W., (2011). Ionic liquid pretreatment of cellulosic biomass: enzymatic hydrolysis and ionic liquid recycle. *Biotechnol. Bioeng*., **108**, 511 – 520.

Simmons, B. A., Loqué, D., Ralph, J., (2010). Advances in modifying lignin for enhanced biofuel production. *Curr. Opin. Plant Biol*., **13**, 313 – 320.

Sindhu, R., Binod, P., Satyanagalakshmi, K., Janu, K. U., Sajna, K. V., Kurien, N., Sukumaran, R. K., Pandey, A., (2010). Formic acid as a potential pre-treatment agent for the conversion of sugarcane bagasse to bioethanol. *Appl Biochem Biotechnol*, **162**, 2313 – 2323.

Singh, S., Simmons, B. A., Vogel, K. P., (2009). Visualization of biomass solubilization and cellulose regeneration during ionic liquid pre-treatment of switchgrass. *Biotechnol. Bioeng*., **104**, 68 – 75.

Sivers, M. V., Zacchi, G., (1995). A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresour. Technol*., **51**, 43 – 52.

Sluiter, A., Hames, B. R., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., (2008). Determination of sugars, byproducts, and degradation products in liquid fraction process samples. National Renewable Energy Laboratory: Technical Report - NREL/TP-510-42623.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D Wolfe, J., (2008). Determination of total solids in biomass and total dissolved solids in liquid process samples. National Renewable Energy Laboratory: Technical Report - NREL/TP-510-42621.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., (2008). Determination of ash in biomass. National Renewable Energy Laboratory: Technical Report - NREL/TP-510-42622.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., (2008). Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory: Technical Report - NREL/TP-510-42618.

Someshwar, A. V., Pinkerton, J. E., (1992). Wood processing industry: Air Pollution Engineering Manual, Air and Waste Management Association, New York, 835 – 849.

Stark, K., Taccardi, N., Bosmann, A., Wasserscheid, P., (2010). Oxidative depolymerization of lignin in ionic liquids. *ChemSusChem.*, **3** (6), 719 – 727.

Studer, M. H., DeMartini, J. D., Brethauer, S., McKenzie, H. L., Wyman, C. E., (2010). Engineering of a high-throughput screening system to identify cellulosic biomass pre-treatments and enzyme formulations that enhance sugar release. *Biotechnol. Bioeng*., **105** (2), 231 – 238.

Studer, M. H., DeMartini, J. D., Davis, M. F., Sykes, R. W., Davison, B., Keller, M., Tuksan, G. A., Wyman, C. E., (2011). Lignin content in natural Populus variants affects sugar release. *Proc. Natl. Acad. Sci.*, **108** (15), 6300 – 6305.

Sun, N., Rodríguez, H., Rahmana, M., Rogers, R. D., (2011). Where are ionic liquid strategies most suited in the pursuit of chemicals and energy from lignocellulosic biomass? *Chem. Commun*., **47**, 1405 – 1421.

Sun, N., Mustafizur, R., Qin, Y., Maxim, M. L., Rodríguez, H., Rogers, R. D., (2009). Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chem*, **11** (5), 646 – 655.

Sun, N., Rahman, M., Qin, Y., Maxim, M. L., Rodríguez, H., Rogers R. D., (2009). Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chem*., **11**, 646 – 655.

Sun, W. L., Ye, W. F., Tao, W. Y., (2013). Improving Enzymatic Hydrolysis of Cellulose from Rice Straw Using an Ionic Liquid [EMIM]OAc Pre-treatment. *Energy Sources (Part A)*, **35**, 2042 – 2050.

Sun, X. F., Xu, F., Sun, R. C., Fowler, P., Baird, M. S., (2005). Characteristics of degraded cellulose obtained from steam exploded wheat straw. *Carbohydrate Research*, **340**, 97 – 106.

Sun, Y., Cheng, J., (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol*., **83**, 1 – 11.

Sun, Y., Cheng, J., (2005). Dilute acid pre-treatment of Rye straw and Bermuda grass for ethanol production. *Bioresour. Technol.*, **96**, 1599 – 1606.

Sunday times business times. Sugar looks to ethanol to help pick up speed. Loni Prinsloo, 27 April 2014

Swatloski, R. P., Spear, S. K., Holbrey, J. D., Rogers, R. D., (2002). Dissolution of cellulose with ionic liquids. *J. Am. Chem. Soc.*, **124** (18), 4974 – 4975.

Tan, S. S. Y., MacFarlane, D. R., Upfal, J., Edye, L. A., Doherty, W. O. S., Patti, A. F., Scott, J. L., (2009). Extraction of lignin from lignocellulose at atmospheric pressure using alkylbenzenesulfonate ionic liquid. *Green Chem.*, **11** (3), 339 – 345.

Tarkow, H., Feist, W. C., (1969). In: A Mechanism for Improving the Digestibility of Lignocellulosic Materials with Dilute Alkali and Liquid NH_3. Advance Chemistry Series 95. *J. Am. Chem. Soc.*, 197 – 218.

Tasman, J., Berzins, V., (1957). The permanganate consumption of pulp materials. In: Development of basic procedure. *Tappi J*. **40**, 691 – 694.

Tavares, A. P. M., Rodriguez, O., Macedo, E. A., (2008). Ionic liquids as alternative co-solvents for laccase: study of enzyme activity and stability. *Biotechnol. and Bioeng*, **101**, 201 – 207.

Teh, W. X., Hossain, Md. M., To, T. Q., Aldous, L., (2015). Pre-treatment of Macadamia Nut Shells with Ionic Liquids Facilitates Both Mechanical Cracking and Enzymatic Hydrolysis. *ACS Sust. Chem. Eng*., **3** (5), 992 – 999.

Templeton, D. W., Sluiter, A. D., Hayward, T. K., Hames, B. R., Thomas, S. R., (2009). Assessing corn stover composition and sources of variability via NIRS. *Cellulose*, **16** (4), 621 – 639.

Thring, R. W., Chorent, E., Overend, R., (1990). Recovery of a solvolytic lignin: effects of spent liquor/acid volume ration, acid concentration and temperature. *Biomass*, **23**, 289 – 305.

Torget, R. W., Werdene, P., Grohmann, K., (1990). Dilute acid pretreatment of two short-rotation herbaceous crops. *Appl. Biochem. Biotechnol*., **24/25**, 115 – 126.

Troedec, M., Sedan, D., Peyratout, C., Bonnet, J. P., Smith, A., Guinebretiere, R., Gloaguen, V., Krausz, P., (2008). Influence of various chemical treatments on the composition and structure of hemp fibers. *Composites Part A - Applied Science and manufacturing*, **39** (3), 514 – 522.

UNICA, 2013. Session 5: Global approaches to support for advanced biofuels - how the need for a comprehensive policy framework is being addressed, in: Fifth Stakeholder Plenary Meeting of EBTP.

Van Dyk, J. S., Pletschke, B. I., (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.*, **30**(6), 1458 – 1480.

Van Soest, P. J., (1963). Use of detergents in the analysis of fibrous feeds. II: A rapid method for the determination of fibre and lignin. *J. Ass. of Agric. Chem*., **46**, 829 – 835.

Vidal, P. F., Molinier, J., (1988). Ozonolysis of lignin - Improvement of in vitro digestibility of poplar sawdust. *Biomass*, **16** (1), 1 – 17.

Vitz, J., Erdmenger, T., Haensch, C., Schubert, U. S., (2009). Extended dissolution studies of cellulose in imidazolium based ionic liquids. *Green Chem.,*, **11** (3), 417 – 424.

Vlasenko, E. Y., Ding, H., Labavitch, J. M., Shoemaker, S. P., (1997). Enzymatic hydrolysis of pretreated rice straw. *Bioresour. Technol*., **59**, 109 – 119.

Waldner, R., Leisola, M. S. A., Fiechter, A., (1988). Comparison of ligninolytic activities of selected fungi. *Appl. Microbiol. Biotechnol*., **29**, 400 – 407.

Walford, S., (2008). Sugarcane bagasse: how easy is it to measure its constituents? Proceedings of the South African Sugar Technologists Association, **81**, 266 – 273.

Wang, C., Guo, L., Li, H., Wang, Y., Weng, J., Wu, L., (2006). Preparation of simple ammonium ionic liquids and their application in the cracking of dialkoxypropanes. *Green Chem*. **8**, 603 – 607.

Wang, H., Gurau, G., Rogers, R. D., (2012). Ionic liquid processing of cellulose. *Chem Soc Rev*, **41**, 1519 – 1537.

Wang, K., Yang, H. Y., Xu, F., Sun R., (2011). Structural comparison and enhanced enzymatic hydrolysis of the cellulosic preparation from Populus tomentosa Carr. by different cellulose-soluble solvent systems. *Biores. Technol*., **102**, 4524 – 4529.

Wang, Q. Y., Chen, Q. Y., Endo, T., (2015) Thermal Decomposition of Bamboo Phyllostachys Edulis Pre-treated with Ionic Liquids-Water Mixtures. *Green and Sust. Chem.*, **5**, 55 – 62.

Wang, Y., Wei, L., Li, K., Ma, Y., Ma, N., Ding, S., Wang, L., Zhao, D., Yan, B., Wan, W., Zhang, Q., Wang, X., Wang, J., Li, H., (2014). Lignin dissolution in dialkylimidazolium-based ionic liquid-water mixtures. *Bioresour. Technol*., **170**, 499 – 505.

Wei, L., Li, K., Ma, Y., Hou, X., (2012). Dissolving lignocellulose biomas in a 1-butyl-3-methylimidazolium chloride – water mixture. *Ind. Crops and Prod*., **37** (1), 227 – 234.

Wei, S., Zongyu, L., Hong, Z., (2015). Ionic liquid cooking wheat straw pulping and delignification reaction kinetics. *J. Chem. and Pharma. Res.*, **7** (5), 831 – 835.

Weingartner, H., Constantinescu, D., Herrmann, C., (2010). Patterns of protein unfolding and protein aggregation in ionic liquids. *Phys. Chem. Chem. Phys.*, **12** (8), 1756 – 1763.

Welton, T., (1999). Room Temperature Ionic Liquids - Solvents for Synthesis and Catalysis. *Chem. Rev*., **99** (8), 2071 – 2084.

Wilke, C. R., (1978). Process development studies on the bioconversion of cellulose and production of ethanol. Lawrence Berkeley Laboratory Report LBL-6881.

Willard, H. H., Merritt, L. L., Dean, J. A., Settle, F. A., (1986). Instrumental methods of analysis. CBS Publishers, Indian Edition, Delhi, 7th ed.

Wingren, A., Galbe, M., Zacchi, G., (2003). Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. *Biotechnol. Progr.*, **19** (4), 1109 – 1117.

Wolski, P. W., Clark, D. S., Blanch, H. W., (2011). Green fluorescent protein as a screen for enzymatic activity in ionic liquid-aqueous systems for in situ hydrolysis of lignocellulose. *Green Chem.*, **13** (11), 3107 – 3110.

Wright, J. D., (1998). Ethanol from biomass by enzymatic hydrolysis. *Chem. Eng*. *Prog*., **84** (8), 62 – 74.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M. T., Ladisch, M. R., Lee, Y. Y., (2005). Coordinated development of leading biomass pretreatment technologies. *Bioresour. Technol.*, **96**, 1959 – 1966.

Yang, B., Wyman, C. E., (2006). BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. *Biotechnol and Bioeng.*, **94** (4), 611 – 617.

Yang, H., Yan, R., Chen, H., Lee, D. H., Zheng, C., (2007). Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel*, **86** (12), 1781 – 1788.

Yoon, L. W., Ang, T. N., Ngoh, G. C., Chua, A. S. M., (2012). Regression analysis on ionic liquid pre-treatment of sugarcane bagasse and assessment of structural changes. *Biomass and Bioenerg.*, **36**, 160 – 169.

Yoon, L. W., Ngoh, G. C., Chua, A. S. M., Hashim, M. A., (2011). Comparison of ionic liquid, acid and alkali pretreatments of sugarcane bagasse enzymatic saccharification. *Chem Technol and Biotechnol.*, **86**, 1342 – 1348.

Yu, G., Yano, S., Inoue, H., Inoue, S., Endo, T., Sawayama, S., (2010). Pretreatment of rice straw by a hot compressed water process for enzymatic hydrolysis. *Appl Biochem Biotechnol*, **160**, 539 – 551.

Zakzeski, J., Jongerius A. L., Weckhuysen, B. M., (2010). Transition metal catalyzed oxidation of alcell lignin, soda lignin and lignin model compounds in ionic liquids. *Green Chem.*, **12**, 1225 – 1236.

Zavrel, M., Bross, D., Funke, M., Buchs, J., Spiess, A. C., (2009). High-throughput screening for ionic liquids dissolving (ligno-) cellulose. *Bioresour. Technol.*, **100** (9), 2580 - 2587.

Zeitsch, K. J., (2000). In: The chemistry and technology of furfural and its many by-products, sugar series. 13, Elsevier, New York.

Zhang, Y-H. P., Lynd, L. R., (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. *Biotechnol and Bioeng.*, **88** (7), 797 – 824.

Zhang, Y-H. P., Lynd, L. R., (2006). A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnol. Bioeng.*, **94**, 888 – 898.

Zhang, Y-H. P., Lynd, L. R., (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: non-complexed cellulase systems. *Biotechnology and Bioengineering,* **88**, 797 – 824.

Zhao, H., Baker, G. A., Song, Z., Olubajo, O., Crittle, T., Peters, D., (2008). Designing enzyme-compatible ionic liquids that can dissolve carbohydrates. *Green Chem*., **10** (6), 696 – 705.

Zhao, H., Holladay, J. E., Brown, H., Zhang, Z. C., (2007). Metal chlorides in ionic liquid solvents convert sugars to 5-hydroxymethylfurfural. Science, **316**, 1597 – 1600.

Zhao, H., Jones, C. L., Baker, G. A., Xia, S., Olubajo, O., Person, V. N., (2009). Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. *Biotechnol.*, **139**, 47 – 54.

Zhao. H., (2010). Methods for stabilizing and activating enzyme in ionic liquids – a review. *J. of Chem. Techno.l and Biotechnol.*, **85** (7), 891 – 907.

Zheng, Y. Z., Lin, H. M., Tsao, G. T., (1998). Pretreatment for cellulose hydrolysis by carbon dioxide explosion. *Biotechnol. Prog*., **14**, 890 – 896.

Zhu, S. D., Wu, Y. X., Chen. Q. M., Yu, N., Wang, C. W., Jin, S. W., Ding, Y. G., Wu, G., (2006). Dissolution of cellulose with ionic liquids and its application: a mini-review. *Green Chem.*, **8**, 325 – 327.

Zhu, S., (2008). Use of ionic liquids for the efficient utilization of lignocellulosic materials. *J. Chem. Tech. and Biotechnol*, **83** (6), 777 – 779.

Zhu, S., Wu, Y., Chen, Q., Yu, Z., Wang, C., Jin, S., Ding, Y., Wu, G., (2006). Dissolution of cellulose with ionic liquids and its application: a mini-review. *Green Chem*., **8** (4), 325 – 327.

Enzymatic Saccharification of Acid/Alkali Pre-treated, Mill-run, and Depithed Sugarcane Bagasse

Thandeka Mkhize,[a] Lethiwe Debra Mthembu,[a,*] Rishi Gupta,[b,c] Amandeep Kaur,[c] Ramesh Chander Kuhad,[b,c] Prashant Reddy,[a] and Nirmala Deenadayalu [a]

In South Africa, approximately 3 × 10^6 tons of sugarcane bagasse is produced annually by 14 factories located on the north coast of KwaZulu-Natal. It is one of the most readily available lignocellulosic materials for ethanol production through enzymatic saccharification and hydrolysis. Pre-treatment enables disruption of the naturally resistant structure of lignocellulosic biomass to make the cellulose accessible to hydrolysis for conversion to biofuels. In this study, pre-treatment of depithed bagasse and mill-run bagasse was done using acid (3% H_2SO_4 v/v) followed by alkali (4% NaOH w/v), and the pre-treated solid was subjected to enzymatic hydrolysis. The effects of different conditions for enzymatic saccharification such as enzyme dose, reaction time, and amount of surfactant were studied in detail. The pre-treated substrate (10% w/v) when hydrolysed using 30 FPU/gds/40 FPU/g dry substrate (gds) with 0.4% (v/v) Tween® 80 for 20 h resulted in 608 mg/gds (depithed bagasse) and 604 mg/gds (mill-run bagasse) total reducing sugars.

Keywords: Bagasse; Pre-treatment; Total reducing sugars; Saccharification

Contact information: a: Department of Chemistry, Durban University of Technology, P. O. Box 1334, Durban, 4000, South Africa; b: Central University of Haryana, Mahendergarh, Haryana, India; c: Department of Microbiology, University of Delhi, India;
** Corresponding author: 21451030@dut4life.ac.za*

INTRODUCTION

Globally, there is a drive to use renewable materials for the production of biofuels due to several sustainability concerns, such as the scarcity of fossil fuels, mitigation of green-house gas emissions, economic issues, and environmental concerns (Alam *et al*. 2013). Lignocellulosic materials are renewable feedstock comprising a wide variety of agricultural residues, forest products, and dedicated crops.

Sugarcane bagasse, an agriculture residue, possesses great potential for conversion to biofuels and is one of the most important agro-industrial crops produced in South Africa. Sugarcane bagasse is an extremely nonhomogeneous material made up of around 30% to 40% pith fibre, which is derived from the core of the plant and is mainly parenchyma material. The sugarcane bagasse in which the pith is removed is called depithed bagasse. Sugarcane bagasse consists of 38% to 50% cellulose, 22% to 28% hemicellulose, and 19% to 33% lignin, as illustrated in Table 1. These components of lignocellulosic biomass form a very complex structure that makes it resistant to chemical and/or microbial degradation (Gupta *et al.* 2011). By virtue of this strong packaging of the biomass structural polymers, an effective pre-treatment method is required to break the lignin and hemicellulose seal to expose the cellulose for hydrolysis (Gupta *et al*. 2009, 2011; Manzoor *et al*. 2012).

Table 1. Chemical Composition of Raw Sugarcane Bagasse (% w/w, dry basis) from the Literature

Components (%)	References					
	Rainey (2009)	da Silva *et al*. (2010)	Rocha *et al*. (2011)	Rabelo *et al*. (2011)	Canilha *et al*. (2012)	Chandel *et al*. (2014)
Cellulose	47.0	38.8	45.5	38.4	45.0	39.53
Hemicellulose	27.0	26.0	27.0	23.2	25.8	25.63
Lignin	23.0	32.4	21.1	25	19.1	30.36
Ash	1.0	2.8	2.2	1.5	1.0	1.44
Extractives	-	-	4.6	-	9.1	2.90

Pre-treatment is a critical step in cellulosic bioethanol technology because it has a huge impact on the quality of the carbohydrate-containing streams and the cost of the carbohydrates (Balat *et al*. 2008). Pre-treatment methods can be classified into different categories: physical, physiochemical, chemical, biological, and electrical, and they differ in their mechanisms of action (Kumar *et al*. 2009; Kuhad *et al*. 2011a). The most commonly used acid for pretreatment is sulphuric acid, where its reaction with biomass promotes hemicellulose breakdown to xylose and other sugars (Mosier *et al*. 2005; Kuhad *et al*. 2010). Acid pretreatment is preferred because the operation process takes place at low and medium temperatures and consequently decreases energy costs (Girio *et al*. 2010). However, when there are high concentrations of acid, problems can occur with equipment corrosion and result in expensive maintenance costs (Alvira *et al*. 2010). This is the reason why dilute acid is widely used for acid treatment. After acid pretreatment on the sugarcane bagasse is completed, the solid fraction obtained consists of cellulose and lignin and the liquid fraction contains hemicellulose.

To remove lignin from the solid fraction, alkali pretreatment is used, which causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerisation and crystallinity, a separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Zheng *et al*. 2009; Gupta *et al.* 2011), making cellulose available for the enzymatic degradation (Gupta *et al.* 2011; Sarkar *et al*. 2012). Hydrolysis using enzymes (cellulases) is an ideal approach to degrade cellulose into reducing sugars because mild reaction conditions (pH between 4.8 to 5.0 and temperatures between 45 to 50 °C) can be used; it does not present corrosion problems in the reactors and results in negligible by-product formation with high sugar yields. However, enzymatic hydrolysis depends on optimised conditions for maximal efficiency (hydrolysis temperature, reaction time, pH, enzyme loading, and substrate concentration) and suffers from end-product inhibition and biomass structural restraints (Canilha *et al.* 2012).

Cellulases are composed of domains or modules, which are structurally and functionally discrete units, hence the term "cellulases module" (Kuhad *et al*. 2011b). Cellulases are inducible enzymes synthesised by a large diversity of microorganisms, including both fungi and bacteria, during their growth on cellulosic materials (Kuhad *et al*. 2011b). Cellulases break down the cellulose molecule into monosaccharides (simple sugars), such as β-glucose, or shorter polysaccharides and oligosaccharides. Enzymatic hydrolysis of cellulosic fractions requires three classes of cellulolytic enzymes (cellulases): (1) endo-β-1,4-glucanases (EG.E.C.3.2.1.4), which attack regions of low crystallinity in the cellulose fibre, creating free chain ends; (2) cellobiohydrolases or exoglucanase (CBH.E.C.3.2.1.91) which degrade the molecule further by removing cellobiose units the

free chains-ends; and (3) β-glucosidases (E.C.3.2.1.21) which hydrolyse cellobiose to produce glucose (Kuhad *et al.* 2011a; Canilha *et al.* 2012).

Due to the high cost of enzymes, the addition of surfactant after biomass pretreatment is another approach used for the conversion of cellulose to reducing sugars to improve enzymatic hydrolysis in a way that the enzymes required may be reduced. Surfactants have been said to enhance enzymatic digestion because surfactants: (i) alter the substrate structure and make it more accessible to enzymes. (ii) stabilize enzymes and prevent their denaturation during hydrolysis. (iii) increase positive interactions between substrates and enzymes, and (iv) reduce non-productive adsorption of enzymes (Qing *et al*. 2010). Another two primary mechanisms of surfactant effects that have been suggested are that surfactants: (i) adsorb to the lignin surface and reduce unproductive enzyme binding and (ii) positively affect cellulase activity and enzymes stabilities (Yang *et al*. 2015). Surfactants consist of both hydrophobic and hydrophilic properties which can decrease the surface tension between two liquid phases to improve the removal of hydrophobic compounds (Qing *et al*. 2010; Cao 2012). Tween and PEG are the mostly-used non-ionic surfactants to enhance cellulose conversion and that is due to their ability to block non-productive adsorption of cellulase enzymes on lignin matrix hence efficiently increasing the enzymatic hydrolysis of pretreated lignocellulosic biomass (Zhou *et al*. 2015; Borjesson *et al*. 2007). Tween 80 (polysorbate 80) is derived from polyethoxylated sorbitan and oleic acid. The hydrophilic-lipophilic balance (HLB) of Tween 80 is 15, which means it is highly water soluble. Surfactants with high HLB values are useful in extracting hydrophobic degradation products from lignin and hemicellulose (Kurakake *et al*. 1994; Cao. 2012). PEGs are expensive when compared to polysorbates; the market prize for PEGs is about U.S. $1.51 kg, where for polysorbates it is about $0.25 kg (Tu and Saddler. 2010). The addition of Tween 80 during enzymatic hydrolysis of biomass can significantly reduce enzyme concentrations and assist with the recycling of enzymes (Tu and Saddler. 2010). For this work Tween 80 was used to study the effect of surfactant on the saccharification of pre-treated sugarcane bagasse.

In this study, the chemical pretreatment of depithed (DP) and mill-run (MR) sugarcane bagasse with dilute sulphuric acid followed by dilute alkali was performed. The pre-treated substrate was subsequently hydrolysed using cellulases. The final yield of enzymatic hydrolysis was highly dependent on the following factors: composition of biomass, pre-treatment type, enzyme dosage, and efficiency (Alvira *et al*. 2010). Therefore, in the present study, enzymatic hydrolysis of the pre-treated bagasse was done using cellulase from *Trichoderma* species RCKC65 to determine the optimisation of enzyme load, surfactant dosage, and high solid loading for efficient hydrolysis.

EXPERIMENTAL

Materials

Carboxymethyl cellulose, *p*-nitrophenol β-D glucopyranoside, and 3,5-dinitrosalicylic acid (DNSA) were purchased from Sigma (St. Louis, USA), and media components were purchased from Himedia, USA. Other chemicals, *viz.* sulphuric acid (98%), sodium hydroxide, Tween® 80, citric acid, and sodium dihydrogen orthophosphate, of analytical grade, were purchased locally in India. Cellulase was synthesised in-house using *Trichoderma* sp. RCKC65 at the University of Delhi, South Campus, New Delhi, India. Sugarcane bagasse was obtained from a local mill (KwaZulu-Natal, South Africa).

Methods

Preparation of sugarcane bagasse

Depithed and mill-run sugarcane bagasse was dried at 105 °C, milled, and sieved to 40-mesh particle size. The cellulose, hemicellulose, lignin, moisture, and ash contents of raw and pre-treated biomass were determined according to the method described by TAPPI (TAPPI T203 om-83 (1983); TAPPI T222 om-83 (1983); TAPPI T223 hm-84 (1984); TAPPI T208 om-84 (1984); and TAPPI T211om-93 (1996).

Procedure

One gram of sugarcane bagasse and (72%) sulphuric acid (15 mL) were added in a 100 mL beaker for 1 h while stirring at room temperature for lignin determination. The resulted mixture was placed in a 1 L flask, and then 360 mL of water was added. The mixture was boiled at 100 °C for 4 h, filtered, and weighed. For holocellulose determination, in a 500 mL flask, 5 g of sugarcane bagasse and water were added, heated at 100 °C covered with a 100 mL flask for refluxing; 1.5 g of $NaClO_2$ and 0.5 mL of glacial acetic acid were added. The system was set up for refluxing at 100 °C; $NaClO_2$ and acetic acid were added until the bagasse became white. The solids were filtered and washed with water, dried at (100±5 °C) and weighed until the weight was constant. For ash content determination, 1.0 g of sugarcane bagasse was weighed into the silica crucible; it was kept in the muffle furnace for 4 h at 500 °C and weighed. For cellulose content determination, 2.0 g of sugarcane bagasse, 5.0 g of $NaClO_2$, and 100 mL of water were mixed, autoclaved for 30 min, cooled, and then filtered and weighed. Hemicellulose was calculated as a difference of holocellulose and cellulose. All these analyses were done in triplicate.

Depithed and mill-run sugarcane bagasse pre-treatment

Acid: To remove hemicellulose, the substrate was pre-treated with 3% (v/v) sulphuric acid for 30 min in an autoclave at 121 °C. The solution was allowed to cool, and was filtered and washed to neutral pH, and overnight dried at 60 °C in an oven.

Alkali: The dried substrate from acid pre-treatment was further pre-treated with 4% (w/v) sodium hydroxide for 15 min in an autoclave at 121 °C. The solution was allowed to cool, filtered to remove the lignin, washed to a neutral pH, and dried at 60 °C in an oven overnight. The residual pre-treated substrate was stored for further processing.

Production of cellulase

The cellulase was produced by *Trichoderma* sp. RCK65, which was cultivated in solid state fermentation, where wheat bran was used as a substrate. The culture conditions were: pH of 4.5, moisture ratio 1:1 at 30 °C for 3 days (Ghose 1987)

Saccharification of pre-treated biomass

The acid-alkali pre-treated depithed and mill-run sugarcane bagasse was saccharified using crude enzyme extract produced by *Trichoderma* sp. RCKC65 (8 FPU/mL). Enzymatic hydrolysis of the pre-treated material (1.0 g each) was carried out at 10% (w/v) solid loading in 50 mM citrate phosphate buffer (pH 5.0). The substrate with buffer was pre-incubated at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 150 rpm for 1 h in a 100 mL Erlenmeyer flask. The temperature and agitation were the same as the pre-incubation until optimized. Thereafter, to obtain efficient saccharification of the pre-treated biomass, optimisation of cellulase doses (10-40 FPU/g), surfactant, Tween® 80 (0.3% to 0.6%), and solid loading (10% to 16%) was

carried out using One Factor at A Time (OFAT) method (Wahid and Nadir 2013). The reaction continued up to 24 h and the samples were withdrawn at regular intervals, centrifuged at 12,000 rpm for 10 min in a centrifuge (Sigma, Germany), and the supernatant was analysed for total reducing sugars released and saccharification yield.

Analysis of reducing sugars

The DNS method (Miller 1959) was employed in this work, which detects the whole range of reducing sugars. The method consists of reading the absorption at 540 nm in a solution composed of the sample that contains reducing sugars and DNS reagent. The intensity of the color was transformed to mg of sugars using a standard curve. Yao *et al*. (2007); Nasirpour *et al*. (2014); Pandey and Negi. (2015); Das *et al*. (2015); Li *et al*. (2016); Aswathy *et al*. (2010) and Han *et al*. (2012) used DNS method to determine the total reducing sugars. The saccharification yield was calculated using Eq. 1.

$$\text{Saccharification yield} = \frac{\text{total sugar concentration (mg/ml) x volume of saccharified product (ml)}}{\text{Substrate (g)}} \quad (1)$$

Statistical Analysis

All experiments were carried out in triplicate and the data reported were expressed as mean values. Experimental errors, which were calculated as the relative standard deviation, were shown by the error bars in the figures.

Characterization of Untreated and Pre-treated Sugarcane Bagasse

FTIR analysis

The FTIR spectra of the untreated, acid treated and acid/alkali treated sugarcane bagasse samples were recorded on an instrument (Shimadzu FTIR 8400) in the range of 380–4500 cm^{-1} with a resolution of 4 cm^{-1}. This method provides information about the chemical bonds and molecular structure of the material.

TGA/ DSC analysis

A Simultaneous Differential Scanning Calorimeter and Thermo-Gravimetric Analyser (SDT) manufactured by TA instruments, model SDT Q600 were used to characterize the thermal stability of the untreated, acid treated and acid/alkali treated sugarcane bagasse samples. Approximately 3.0 mg of each sample was heated from 20 to 1500 °C at a heating rate of up to 50 °C/min.

SEM analysis

A scanning electron microscope manufactured by Carl Zeiss, model EVO 15 HD was used to visualize the difference between untreated, acid and acid/alkali treated bagasse. The samples were coated with gold to avoid charging effects (sputter-coating).

RESULTS AND DISCUSSION

Pretreatment

The compositional analysis of raw samples revealed that the sugarcane bagasse contains cellulose (52.5% depithed, 46.1% mill-run); hemicellulose (20.6% depithed, 23.2% mill-run), and lignin (25.0% depithed, 28.7% mill-run). When comparing these

values with the literature values (Rainey 2009; da Silva *et al*. 2010; Rocha *et al*. 2011; Rabelo *et al*. 2011; Canilha *et al*. 2012 and Chandel *et al*. 2014), it can be observed that there is not much difference. The pre-treatment of substrate with 3% H_2SO_4 decreased the proportion of hemicellulose and lignin and increased the cellulose content for both depithed and mill-run bagasse, which was predictable since the acid pretreatment was used to remove hemicellulose. The pre-treatment of substrate with 4% NaOH increased the proportion of cellulose for both depithed and mill-run bagasse; and reduced the lignin content and hemicellulose for both depithed and mill-run bagasse, which was also expected because alkali pre-treatment was used to remove lignin (Table 2). The compositional analysis of raw, acid pre-treated, and acid/alkali pre-treated bagasse shows that the acid/alkali pre-treatment was able to disrupt the sugarcane bagasse and extract cellulose, which was the compound of interest.

A 10% biomass loading was used in this study; the starting mass was 100 g before pre-treatment. After acid pre-treatment, the mass decreased considerably to 67.6 g, which means that there was a 32% mass loss, mostly because of the removal of hemicellulose as reducing sugars. The acid-pre-treated substrate was then subjected to alkali pre-treatment and the amount left was 29.6 g, which means there was a 56% mass loss, which may be attributed mostly to the loss of lignin, leaving cellulose behind.

Table 2. Compositional Analysis of Raw and Pre-treated Sugarcane Bagasse

Bagasse Sample		Components (wt. %)			
		Cellulose	Hemicellulose	Lignin	Ash
Depithed	Raw	52.5	20.6	25.0	1.9
	Acid Pre-treated	65.7	8.1	24.6	1.6
	Acid/Alkali Pre-treated	83.1	2.7	12.7	1.2
MR	Raw	46.1	23.2	28.7	2.0
	Acid Pre-treated	60.8	10.0	27.0	1.5
	Acid/Alkali Pre-treated	80.6	2.4	14.6	1.4

Depithed = Depithed sugarcane bagasse
MR = Mill-run sugarcane bagasse

Optimisation of enzyme load and time for saccharification of pre-treated depithed and MR sugarcane bagasse

Figures 1 and 2 illustrate the action of cellulase enzyme on acid-alkali-pre-treated, depithed, and mill-run sugarcane bagasse samples, respectively. In order to determine the effect of enzyme load and time on both acid/alkali pretreated mill-run and depithed bagasse, the pretreatment were carried out for 24 h, sampled after 4 h at 50 °C.

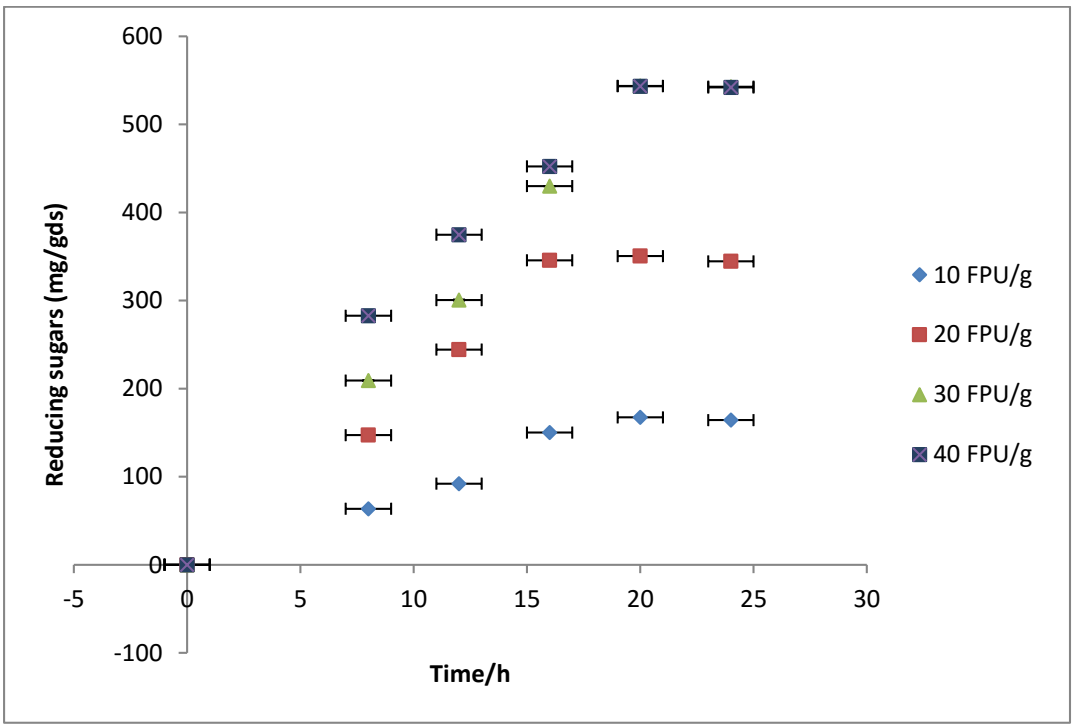


Fig. 1. Optimisation of enzyme load on the hydrolysis of depithed sugarcane bagasse

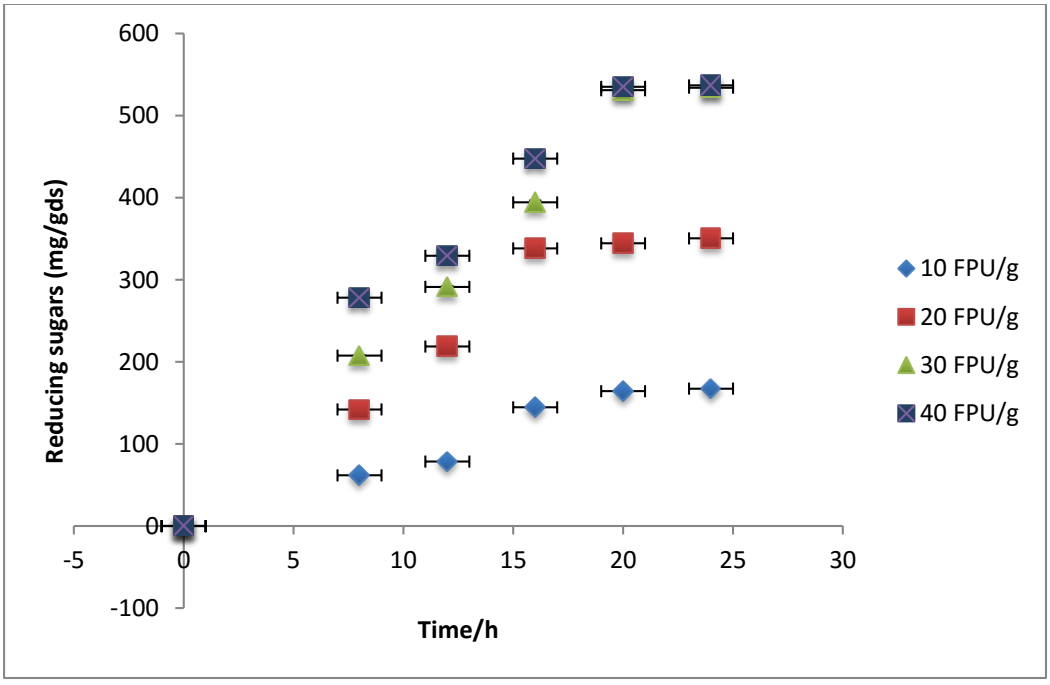


Fig. 2. Optimisation of enzyme load on the hydrolysis of mill-run sugarcane bagasse

The samples were hydrolyzed with enzyme load of 10 FPU/gds (grams dry substrate), 20 FPU/gds, 30 FPU/gds, and 40 FPU/gds. It was found that when the mill-run bagasse was hydrolysed with 40 FPU/gds (cellulase) for 24 h, the maximum amount of total reducing sugars was released (536 mg/gds), whereas the depithed bagasse released a maximum amount of total reducing sugars when it was hydrolysed with 30 FPU/gds (cellulase) for 20 h. 10 FPU/gds gave the lowest total reducing sugars for both pretreated depithed and mill-run bagasse. It was observed that as the enzyme load increases and time increases, more reducing sugars are released from the pretreated bagasse.

Ramachandriyaa *et al*. (2013) performed experiments using 46 FPU/g of Accelerase® 1500/g glucan (0.5 mL/g glucan) to study the effect of enzyme dosage on glucose yield. Various enzyme loadings of 46 FPU/g glucan (0.5 19 mL/g glucan), 34.5 FPU/g glucan (0.375 mL/g glucan), 23 FPU/g glucan (0.25 mL/g glucan), and 11.5 FPU/g glucan (0.125 mL/g glucan) were used to hydrolyse pretreated biomass. Their findings also showed that increasing the enzyme dosage also increased the glucose concentration or total reducing sugars and that is because the low enzyme dosage lowers enzyme binding to cellulose which decreases the glucose yield or total reducing sugars. 46 FPU/g glucan gave the maximum yield of glucose (82.7 g/L) at 672 h using 16% solid loading.

Optimisation of surfactant dose and time for the saccharification of pre-treated depithed and MR sugarcane bagasse

Zhou *et al*. (2015) reported that the enzymatic hydrolysis of pure cellulose was not consistently improved by surfactants, meaning that surfactants can increase or decrease the enzymatic hydrolysis reaction. Wen *et al*. (2013) showed that one of the reasons that can cause surfactant to decrease the enzymatic hydrolysis reaction is the application of a high concentration surfactant (Tween 80), which could inhibit the enzyme activity and thus hinder the enzymatic hydrolysis process.

Figures 3 and 4 illustrate the action of Tween 80 surfactant on acid/alkali-pre-treated depithed and mill-run sugarcane bagasse samples, respectively. The acid/alkali-pre-treated bagasse was used as a substrate with the enzyme dose that released the maximum amount of total reducing sugars in Figs. 1 and 2. The samples were hydrolysed with an enzyme dosage of 30 FPU/gds (depithed bagasse)/ 40 FPU/gds (mill-run bagasse) with the addition of surfactant (Tween 80) from 0.3 to 0.6% for 20 h. Among the various dosages of Tween 80 tested, 0.4% (v/v) after 20 h of incubation resulted in the maximum increase in saccharification efficiency with a release of 608 and 605 mg/gds total reducing sugars from depithed and mill-run sugarcane bagasse samples, respectively. The addition of a surfactant to the enzymatic hydrolysis process increased the total reducing sugars yield by 12% for depithed bagasse and 13% for mill-run bagasse. For this study, Tween® 80 was selected because it does not denature the cellulolytic enzyme that brings about saccharification (Nystrom and Allen 1976; Wilke 1978). Wen *et al*. (2013) studied the effects surfactant on enzymatic hydrolysis using a different type of biomass called sweet sorghum bagasse (SSB), which showed that Tween 80 can increase the enzymatic hydrolytic efficiency of SSB by 21.3% but they also discovered that high concentration of Tween 80 would inhibit the enzyme activity, and thus hinder the enzymatic hydrolysing process. This effect was also observed in the present work; when the concentration of surfactants increased from 0.5% to 0.6%, the total reducing sugars produced decreased. Wen *et al*. (2013) also studied adsorption, which showed that there was limited adsorption of Tween 80 and cellulase on SSB and that Tween 80 could reduce the adsorption of cellulase on SSB, interfering with the reaction between the substrate and enzyme.

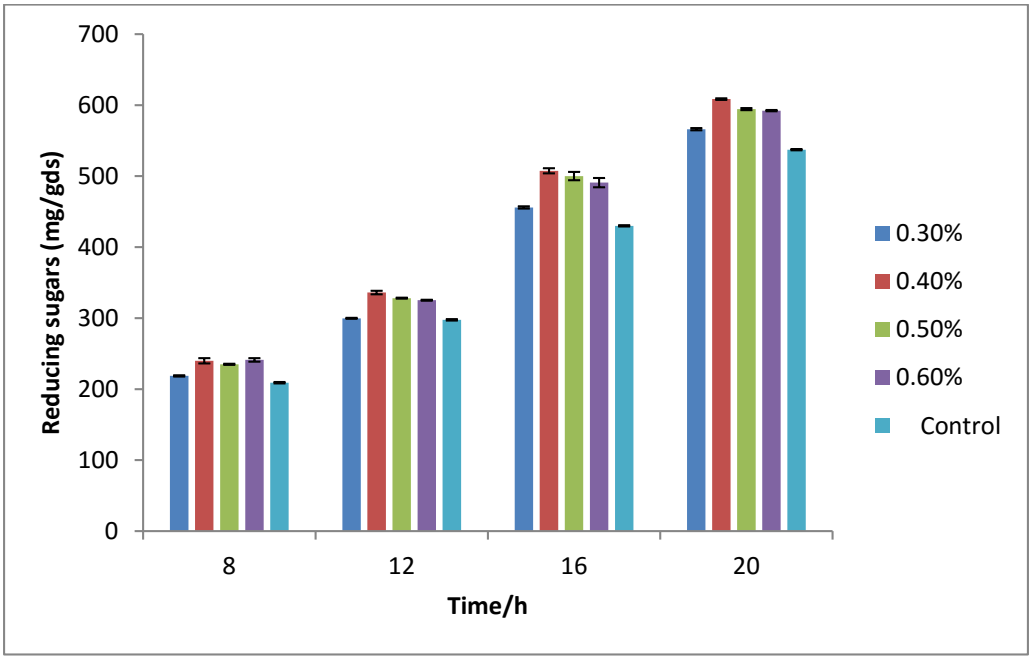


Fig. 3. Optimisation of surfactant dosages on the hydrolysis of depithed sugarcane bagasse

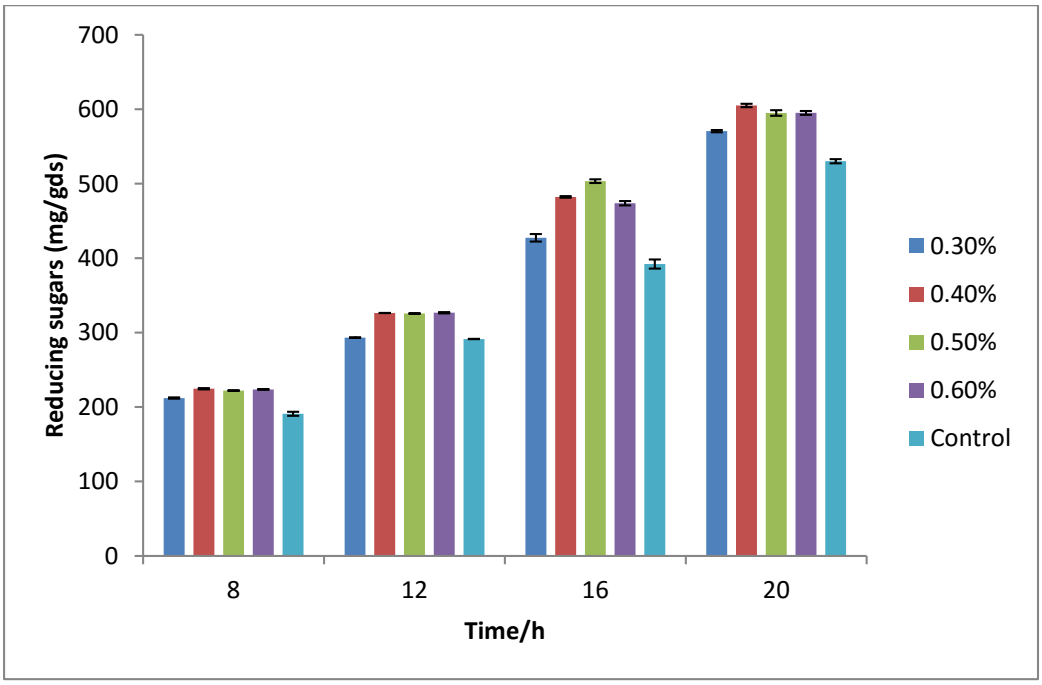


Fig. 4. Optimisation of surfactant dosages on the hydrolysis of mill-run sugarcane bagasse

Optimisation of high solid loading for the saccharification of pre-treated depith and MR sugarcane bagasse

Figures 5 and 6 illustrate the high solid loading effect on acid/alkali pre-treated depithed and mill-run sugarcane bagasse samples, respectively. Various amounts of substrate were used, ranging from 10 to 16%, hydrolysed with enzyme dosage of 30 FPU/gds (depithed bagasse)/ 40 FPU/gds (mill-run bagasse), 0.4% of Tween 80 for 20 h. The maximum total reducing sugars was obtained at 10% solid loading for both depithed and mill-run bagasse, with a sugar release of 609 and 604 mg/gds, respectively. A solid loading of 16% gave the lowest total reducing sugars; this shows that as the solid loading increases, the total reducing sugars released decreases. Also, previous studies have shown that as the substrate concentration increases, the feedback inhibition by cellobiose and glucose is enhanced, leading to the reduced production of reducing sugars in the enzymatic reaction (Han *et al*. 2012).

Han *et al*. (2012) studied the effect of substrate concentration on the enzymatic hydrolysis by using a different type of biomass in a range of 1% to 10% of wheat straw pretreated with alkaline solution (1% sodium hydroxide); they discovered that 3% of wheat straw gave maximum total reducing sugars of 350.18 mg/g. Whereas in this work 10% to 16% of sugarcane bagasse was used and 10% gave the maximum total reducing sugars of 609 mg/gds, 10% in their work gave a low reducing sugars less than 300 mg/g. The huge difference observed in our results may be due to the difference in biomass, organisms for cellulose, and also the enzyme loading. Han *et al*. (2012) used a wheat straw, cellulase produce by *Penicillium* and enzyme loading of 25 FPU/gds, whereas in this work sugarcane pretreated with both acid and alkaline was used, cellulase produced by *Trichoderma sp.* RCKC 65 and enzyme loading of 30 FPU/gds was used. The difference in conditions and the type of biomass may have cause the difference in total reducing sugars produced by 10% substrate.

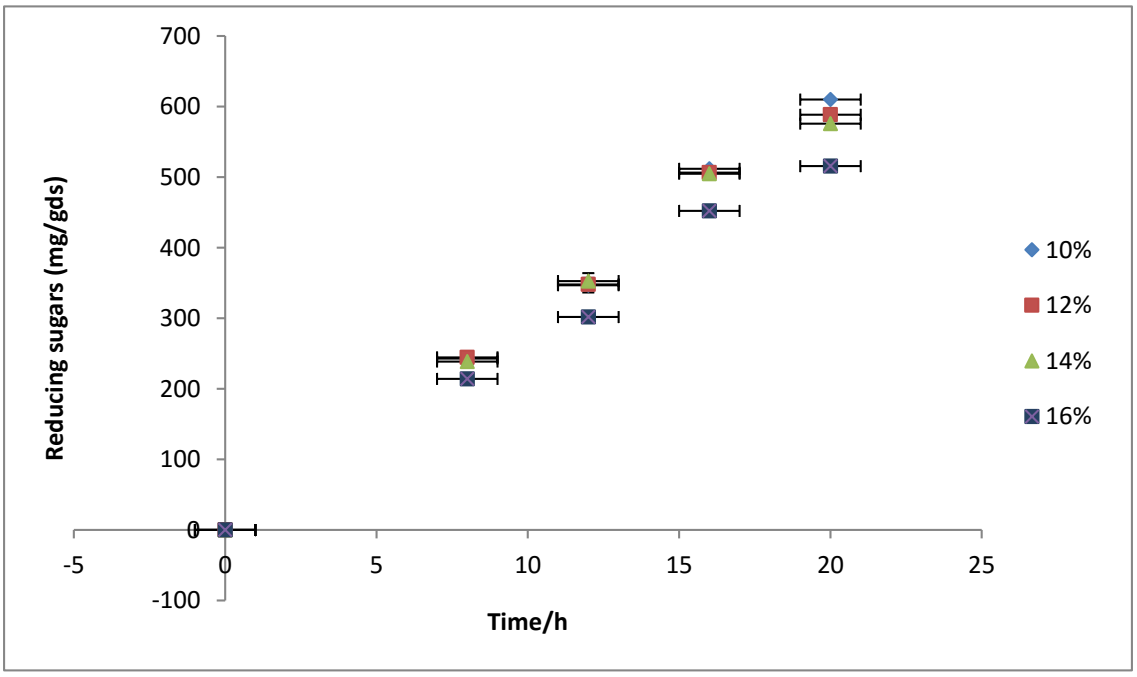


Fig. 5. Optimisation of high solid loading on the hydrolysis of depithed sugarcane bagasse

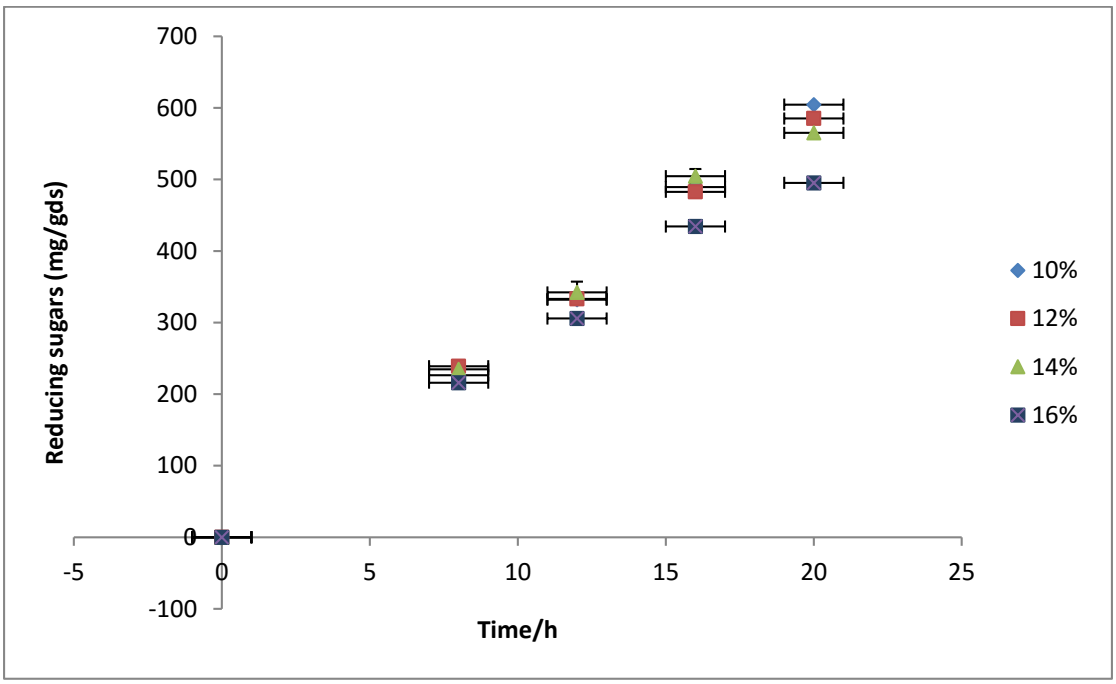


Fig. 6. Optimisation of high solid loading on the hydrolysis of mill-run sugarcane bagasse

Characterisation of Raw and Pre-treated Bagasse

Fourier transform infrared spectroscopy (FTIR)

FTIR was performed to detect changes in functional groups and molecular conformation that were caused by the pre-treatment process. Figures 7 and 8 illustrate the FTIR spectra of the raw and regenerated depithed and mill-run material after acid and alkali pre-treatment. The main functional groups of the biomass components are cellulose, hemicellulose, and lignin. It is notable that these components mostly consist of alkenes, esters, aromatics, ketone, and alcohol, with different oxygen-containing functional groups: for example, OH (3500 to 3200 cm^{-1}), C=O (1770 to 1710 cm^{-1}), C-O-C (1280 cm^{-1}), and C-O-H (1055 cm^{-1}) (Demibras 2000). In both mill-run and depithed sugarcane bagasse, acid/alkali-pre-treated bagasse showed the lowest OH absorption and raw bagasse showed the highest OH absorption. In comparison with raw and acid pre-treated bagasse, a big difference is noticeable in the fingerprint region (1830 to 730 cm^{-1}) for acid/alkali-pre-treated bagasse. This revealed that the pre-treatment specifically removed hemicellulose and lignin fractions and the residual pre-treated material was rich in cellulose. The band at 898 cm^{-1} is characteristic of the glycosidic bond β-(1 → 4) cellulose, corresponding to the C-H deformation in cellulose (Pandey and Pitman 2003; Oh *et al*. 2005; Chandel *et al*. 2014). The region between 1,200 and 1,100 cm^{-1} is a large contribution of hemicellulose and cellulose, which exhibits a maximum value around 1,035 cm^{-1} due to C-O stretching and 1,164 cm^{-1} for the asymmetrical stretching of C-O-C (Pandey 1999; Colom *et al*. 2003; Pandey 2005; Chandel *et al*. 2014). The region around 1,247 cm^{-1} was due to the stretching of C-O, which is characteristic of hemicellulose and lignin (Pandey and Pitman 2003; Chandel *et al*. 2014). A band around 1,458 cm^{-1} is reported to be a deformation of lignin CH_2 and CH_3, and 1,604 cm^{-1} is reported to be stretching of the C=C and C=O lignin aromatic ring. The band around 1,515 cm^{-1} is because of the C=C stretching of the aromatic ring in lignin (Colom *et al*. 2003; Pandey 2005; Chandel *et al*. 2014).

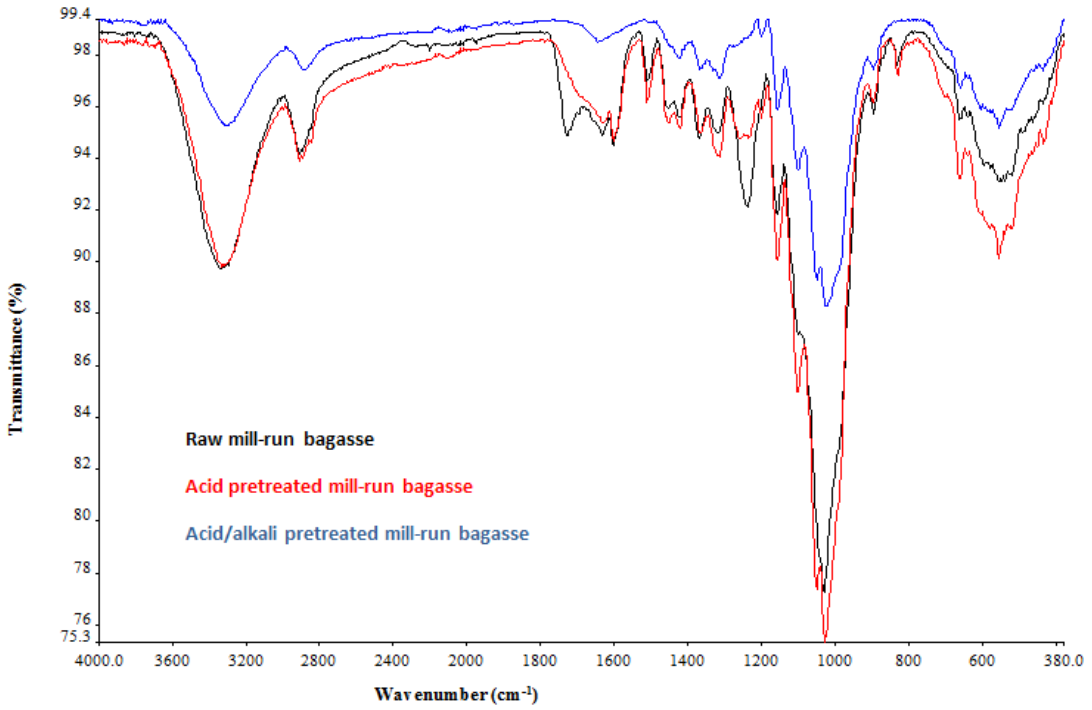


Fig. 7. FTIR spectra of mill-run bagasse (raw, acid-pre-treated, and acid-alkali-pre-treated)

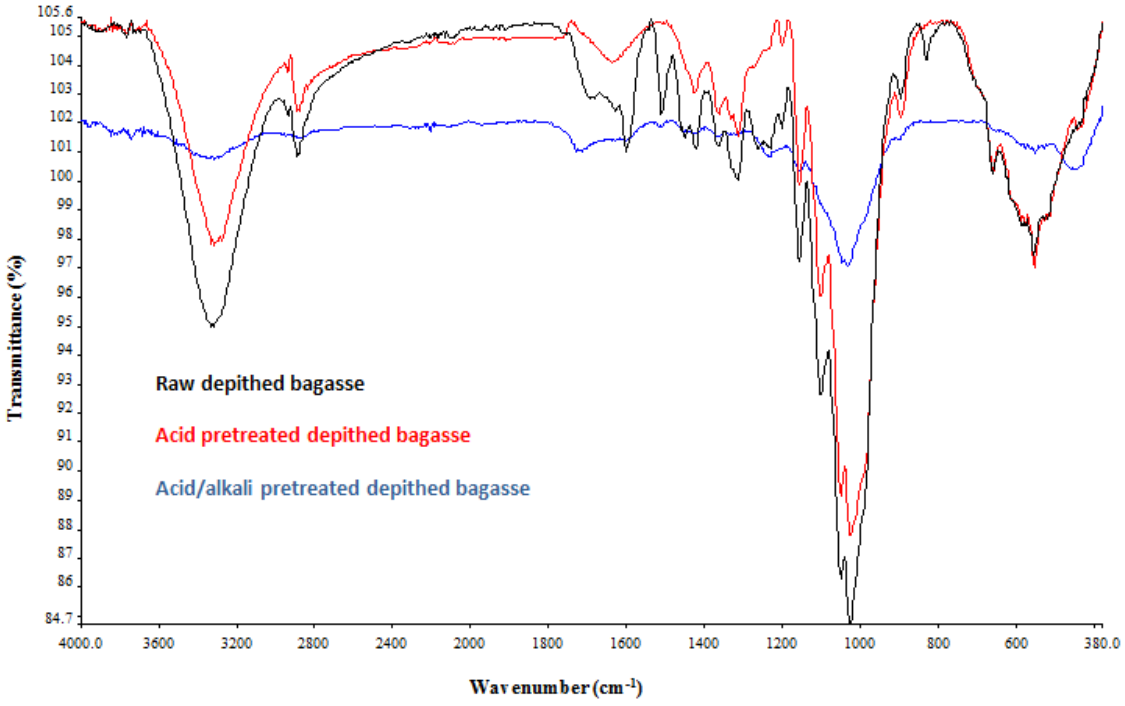


Fig. 8. FTIR spectra of depithed bagasse (raw, acid-pre-treated, and acid/alkali-pre-treated)

A band around 1,733 cm^{-1} is characteristic of C=O stretching of unconjugated hemicellulose. The peak around 2,850 cm^{-1} is reportedly due to the symmetric stretch of

CH and CH_2, while the peak at 2,918 cm^{-1} is due to asymmetrical stretching of CH_2 and CH. Both denote the characteristics of cellulose (Ivanova and Korolok 1989; Chandel *et al*. 2014). The region between 3,800 and 3,000 cm^{-1} covers the related crystalline structure of cellulose. This region represents the sum of the vibration of valence bands of the hydrogen bond of the OH group and the bands of intra-molecular and intermolecular hydrogen bonds (Hinterstoisser and Salmen 1999; Chandel *et al*. 2014).

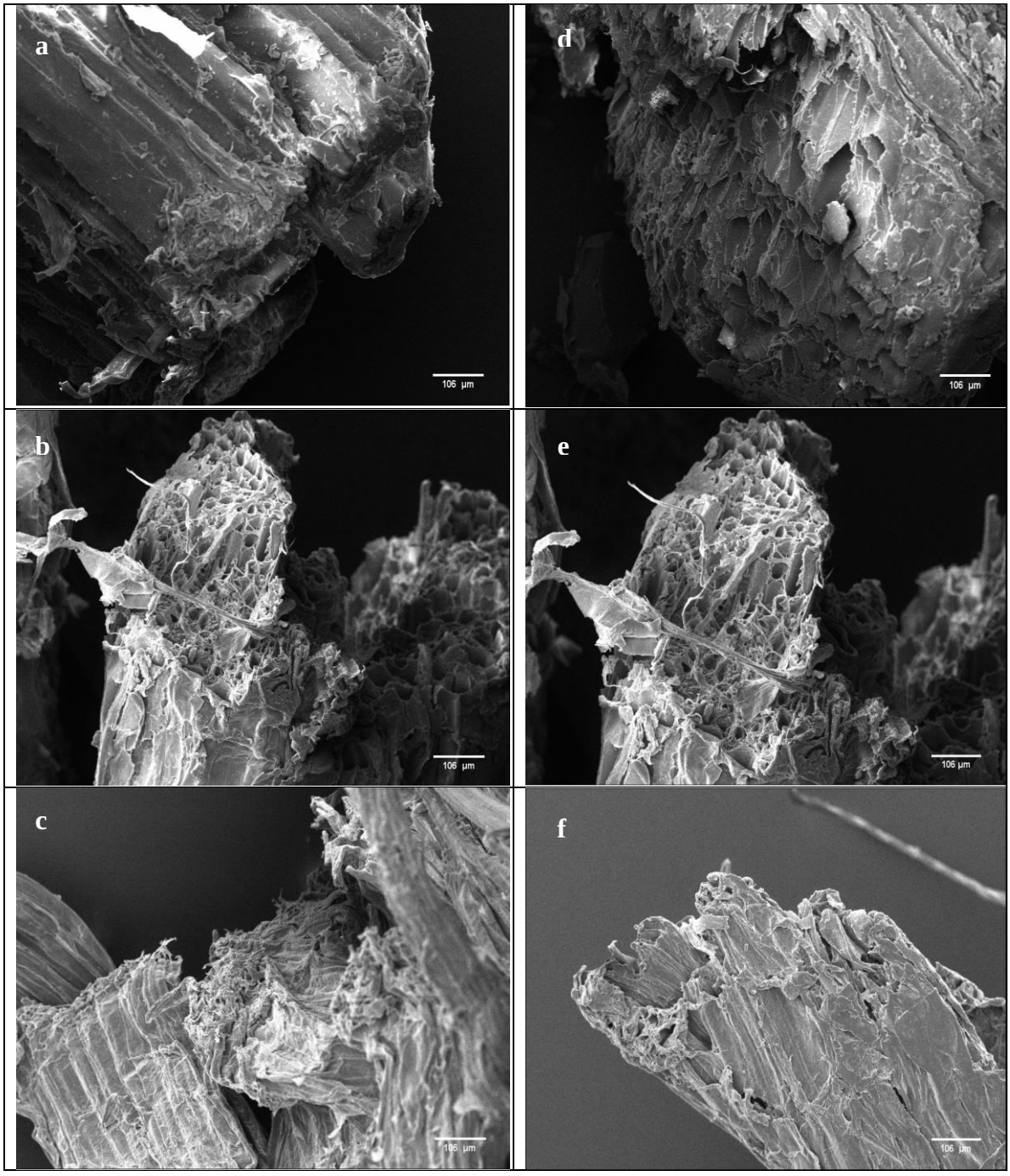


Fig. 9. SEM images of mill-run bagasse (a) raw, (b) acid-pre-treated, (c) acid/alkali-pre-treated; and depithed bagasse (d) raw, (e) acid-pre-treated, (f) acid/alkali-pre-treated

Scanning electron microscopy (SEM)

SEM was used to study the morphology of raw and regenerated sugarcane bagasse after acid/alkali pre-treatment. Figure 9 shows the SEM images at 800× magnification. The images show that the surfaces of the raw fibres (Fig. 9a, d) appear smooth and the surfaces of the pre-treated fibres appear rougher with more porosity (Fig. 9b, c, e, and f). The changes may be attributed to the disruption of the structure and reduction in crystallinity. One of the main objectives of pre-treatment is to increase the surface area and pore size for enzymatic hydrolysis (Alvira *et al.* 2010); therefore, this objective was met, as a remarkable increase in porosity was observed after pre-treatment for both mill-run and depithed bagasse.

Thermal analysis

Differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA) were used to study the thermal behaviour of the raw and pre-treated substrates from ambient temperature to 800 °C at a heating rate of 10 °C/min under a nitrogen flow. Figures 10 and 11 show the DSC and TGA curves for depithed (raw, acid-, and acid/alkali-pre-treated bagasse) and mill-run (raw, acid-, and acid/alkali-pre-treated bagasse). The DSC curves (Fig. 10) show an exothermic peak at approximately 100 °C, which was due to the removal of moisture when the sample was heated. The TGA curves (Fig. 11) exhibited three degradation steps.

The initial weight loss can be observed between 30 and 100 °C in all scans, which may be attributed to the evaporation of loosely bound moisture on the surfaces of the samples. The second step was observed between 250 and 350 °C, and the third step occurred between 350 and 500 °C. The exothermic peaks that appeared at 350 and 400 °C can be attributed to charring (Yang *et al*. 2007). Table 4 shows the weight loss and degradation temperatures of bagasse fibres.

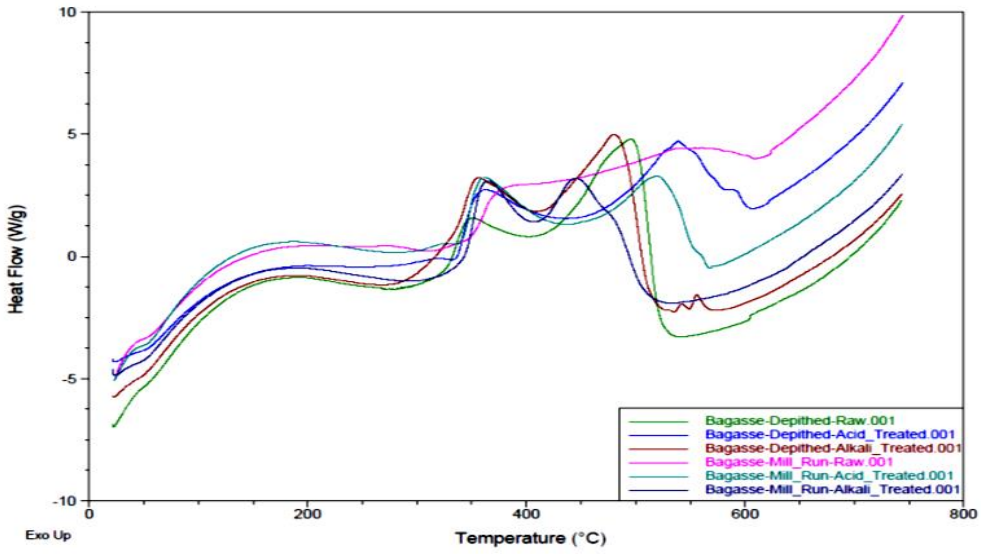


Fig. 10. DSC thermograms for raw and pre-treated mill-run and depithed bagasse

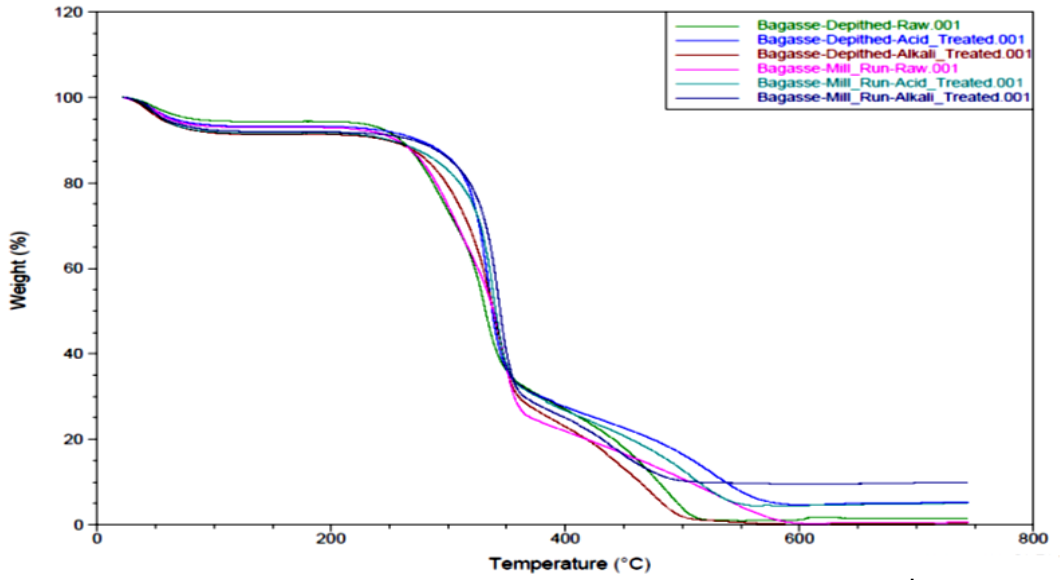


Fig. 11. TGA thermograms for raw and pre-treated mill-run and depithed bagasse

Table 3. Thermal Properties of Bagasse Fibres

Bagasse sample	Pre-treatment	Weight Loss (%)			Degradation temperature (°C)		
		100 °C	350 °C	400 °C	100 °C	350 °C	400 °C
Depith	Raw	5.7	60.7	31.9	192.1	351.0	495.0
	Acid	6.8	59.2	28.1	200.3	362.7	539.3
	Acid/alkali	8.7	62.8	28.1	190.9	356.2	480.2
MR	Raw	6.9	66.6	25.8	201.1	374.6	561.4
	Acid	8.4	59.6	27.4	186.8	362.1	519.6
	Acid/alkali	8.1	62.2	20.2	190.9	364.7	445.1

Depith - Depithed sugarcane bagasse
MR - Mill run sugarcane bagasse

CONCLUSIONS

1. The pre-treatment of the substrates with dilute sulfuric acid followed by sodium hydroxide proved to be successful, as the pore size of the regenerated pulps increased, which can be seen in the SEM images and the compositional analysis also showed the amount of hemicellulose and lignin that was removed from the sugarcane bagasse by the acid-alkali pre-treatment. This led to increased accessibility of the substrate to cellulase enzyme for hydrolysis. Other physical changes on the treated bagasse were determined by FTIR and TGA/DSC analysis.

2. The pre-treated substrate (10% w/v) when hydrolysed using an enzyme dosage of 30 FPU/gds (depithed bagasse) /40 FPU/gds (mill-run bagasse) with a surfactant of 0.4% Tween® 80 for 20 h resulted in 609 mg/gds (depithed bagasse) and 604 mg/gds (mill-run bagasse) total reducing sugars. These were the optimised conditions.

ACKNOWLEDGMENTS

The authors are grateful for the support of the HESA IBSA Grant, National Research Foundation (NRF) South Africa, Durban University of Technology and the Department of Microbiology, University of Delhi South Campus, New Delhi, India for providing the infrastructure facility. RG and RCK also acknowledge the Central University of Haryana for providing financial support.

REFERENCES CITED

Alam, M. Z., Moniruzzaman, M., Sujan, S. M. A., Hossain, M., and Jamal, M. S. (2013). "Enzymatic saccharification of bagasse: Effects of different pretreatment methods," *Int. J. Renew. Energy Res.* 3(2), 230-234.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., and Negro, M. J. (2010). "Pre-treatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Bioresour. Technol.* 101(13), 4851-4861. DOI: 10.1016/j. biortech. 2009.11.093

Aswathy, U.S., Sukumaran, R.K., Devi, G.L., Rajasree, K.P., Singhania, R.R., and Pandey, A. (2010). "Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy," *Bioresour. Technol.* 101 (3), 925-930. DOI: 10.1016/j. biortech.2009.08.019

Balat, M., Balat, H., and Öz, C. (2008). "Progress in bioethanol processing," *Progr. Energy Combust. Sci*. 34(5), 551-573. DOI: 10.1016/j.pecs.2007.11.001

Borjesson, S., Peterson, R. and Tjemeld F. (2007). "Enhanced enzymatic conversion of softwood lignocellulose by poly (ethylene glycol) addition," *Enzyme Microb. Technol*. 40(4), 754-762. DOI: 10.1016/j.enzmictec.2006.06.006

Canilha, L., Chandel, A. K., dos Santos Milessi, T. S., Fernandes Antunes, F. A., da Costa Freitas, W. L., Almeida Felipe M. G., and da Silva S. S. (2012). "Bioconversion of sugarcane biomass into ethanol: An overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification and ethanol fermentation," *J. Biomed. Biotechnol.* 2012, 1-15. DOI: 10.1155/2012/989572

Cao, S. (2012). "Effect of surfactants on the pretreatment of sugarcane bagasse with dilute ammonia," Master's thesis, Louisiana State University, pp. 6.

Chandel, A. K., Antunes, F. A., Anjos, V., Bell, M. J. V., Rodrigues, L. N., Polikarpov, I., de Azevedo, E. R., Bernardinelli, O. D., Rosa, C. A., Pagnocca, F. C., and da Silva S.S. (2014). "Multi-scale structural and chemical analysis of sugarcane bagasse in the process of sequential acid-base pretreatment and ethanol production by *Scheffersomyces shehatae* and *Saccharomyces cerevisiae*," *Biotechnol. Biofuels.* 7(1), 63. DOI:10.1186/1754-6834-7-63

Colom, X., Carrillo, F., Nogués, F., and Garriga, P. (2003). "Structural analysis of photodegraded wood by means of FTIR spectroscopy," *Polym. Deg. Stab.* 80(3), 543-549. DOI: 10.1013/S0141-3910(03)00051-X

da Silva, A. S., Inoue, H., Endo, T., Yano, S., and Bon, E. P. S. (2010). "Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation," *Bioresour. Technol.* 101(19), 7042-7409. DOI: 10.1016/j.biortech.2010.05.008

Das, S., Bhattacharya, A., Haldar, S., Ganguly, A., Gu, S., Ting, Y. P., and Chatterjee, P. K. (2015). "Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: Comparison between artificial neural network and response surface methodology," *Sustainable Mater. Technol.* 3, 17-28. DOI: 10.1016/j.susmat.2015.01.001

Demibraş, A. (2000). "Mechanisms of liquefaction and pyrolysis reactions of biomass," *Energy Convers. Manage*. 41(6), 633-646. DOI: 10.1016/S0196-8904(99)00130-2

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure & Appl. Chem.* 59(2), 257-268.

Girio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., and Bogel-Łukasik, R. (2010). "Hemicellulose for fuel ethanol: A review," *Bioresour. Technol.* 101(13), 4775-4800. DOI: 10.1016/j.biortech.2010.01.088

Gupta, R., Khasa, Y. P., and Kuhad, R. C. (2011). "Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials," *Carbohydr. Polym.* 84(3), 1103-1109. DOI: 10.1016/j.carbpol.2010.12.074

Gupta, R., Sharma, K. K., and Kuhad, R. C. (2009). "Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498," *Bioresour. Technol.* 100(3), 1214-1220. DOI: 10.1016/j.biortech.2008.08.033

Han, L., Feng, J., Zhang, S., Ma, Z., Wang, Y., and Zhang, X. (2012). "Alkali pretreated of wheat straw and its enzymatic hydrolysis," *Braz. J. Microb.* 43(1), 53-61.DOI: 10.1590/S1517-83822012000100006

Hinterstoisser, B., and Salmén, L. (1999). "Two-dimensional step-scan FTIR: A tool to unravel the OH-valency-range of the spectrum of Cellulose I," *Cellulose* 6(3), 251-263. DOI: 10.1023/A: 1009225815913

Ivanova, N. V., and Korolok, E. V. (1989). "IR spectrum of cellulose," *J. Appl. Spec.* 51(2), 847-851. DOI: 10.1007/BF 00659967

Kuhad, R. C., Gupta, R., and Khasa, Y. P. (2011a). "Bioethanol production from lignocellulosics: An overview," in: *Waste from Wealth*, B. Lal and P. M. Sharma (eds.), Teri Press, Delhi, India, pp. 53-106.

Kuhad, R. C., Gupta, R., and Singh, A. (2011b). "Microbial cellulases and their industrial applications," *Enzyme Res.* 2011, 1-10. DOI: 10.4061/2011/280696

Kuhad, R. C., Gupta, R., Khasa, Y. P., and Singh, A. (2010). "Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation," *Bioresour. Technol.* 101(21), 8348-8354. DOI: 10.1016/j.biortech.2010.06.043

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P. (2009). "Methods for pre-treatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Ind. Eng. Chem. Res.* 48(8), 3713-3729. DOI: 10.1021/ie801542g

Kurakake, M., Ooshima, H., Kato, J., and Harano, Y. (1994). "Pretreatment of bagasse by nonionic surfactant for the enzymatic hydrolysis," *Bioresour. Technol.* 49(3), 247-251. DOI: 10.1016/0960-8524(94)90048-5

Li, K., Wan, J., Xiao, W., Wang, J., and Zhang, J. (2016). "Comparison of dilute acid and alkali pretreatments in production of fermentable sugars from bamboo: Effect of Tween 80," *Ind. Crops. Prod.* 83, 414-422. DOI: 10.1016/j.indcrop.2016.01.003

Manzoor, A., Khokhar, Z., Hussain, A., Uzma, A., Syed, Q., and Baig, S. (2012). "Dilute sulphuric acid: A cheap acid for optimization of bagasse pre-treatment," *Sci. Int. (Lahore)* 24(1), 41-45.

Mosier, N., Wyman, C., and Dale, B. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresour. Technol.* 96(6), 673-686.

Nasirpour, N., Mousavi, S. M., and Shojaosadati, S. A. (2014). "A novel surfactant-assisted ionic liquid pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis," *Bioresour. Technol.* 169, 33-37. DOI: 10.1016/j.biortech.2014.06.023

Nystrom, J. M., and Allen, A. L. (1976). "Pilot scale investigations and economics of cellulose production," *Biotechnol. Bioeng. Symp.* 6(6), 55-74.

Oh, S. Y., Yoo, D., Shin, Y., Kim, H. C., Kim, H. Y., Chung, Y. S., Park, W. H., and Youk, J. H. (2005). "Crystalline structure analysis of cellulose treated with sodium hydroxide and carbon dioxide by means of X-ray diffraction and FTIR spectroscopy," *Carbohydr. Res.* 340 (15), 2376-2391. DOI: 10.1016/j.carres.2005.08.007

Pandey, A. K. and Negi, S. (2015). "Impact of surfactant assisted acid and alkali pretreatment on lignocellulosic structure of pine foliage and optimization of its saccharification parameters using response surface methodology," *Bioresour. Technol.* 192, 115-125. DOI: 10.1016/j.biortech.2015.04.054

Pandey, K. K. (1999). "A study of chemical structure of soft and hardwood and wood polymers by FTIR spectroscopy," *J. Appl. Polym. Sci.* 71(12), 1969-1975. DOI: 10.1002/(SICI)1097-4628(19990321)71:12<1969::AID-APP6>3.0.CO;2-D

Pandey, K. K. (2005). "Study of the effect of photo-irradiation on the surface chemistry of wood," *Polym. Deg. Stab.* 90(1), 9-20. DOI: 10.1016/j. polymdegradstab.2005.02.009

Pandey, K. K. and Pitman, A. J. (2003). "FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi," *Int. Biodeterior. Biodegradation* 52(3), 151-160. DOI: 10.1016/S0964-8305(03)00052-0

Qing, Q., Yang, B., and Wyman, C. E. (2010). "Impact of surfactants on pretreatment of corn stover," *Bioresour. Technol.* 101(15), 5941-5951. DOI: 10.1016/j.biortech.2010.03.003

Rabelo, S. C., Carrere, H., Filho, R. M., and Costa, A. C. (2011). "Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept," *Bioresour. Technol.* 102(17), 7887-7895. DOI:10.1016/j.biortech.2011.05.081

Rainey, T. J. (2009). *A Study of the Permeability and Compressibility Properties of Bagasse Pulp*, Ph.D. dissertation, Queensland University of Technology, Brisbane, Australia.

Ramachandriyaa, K. D., Wilkinsa, M., Atiyeha, H. K., Dunforda, N. T., and Hiziroglub, S. (2013). "Effect of high dry solids loading on enzymatic hydrolysis of acid bisulfite pretreated Eastern redcedar," *Bioresour. Technol*. 147, 168-176. DOI: 10. 1016/j. biortech.2013.08.048

Rocha, G. J.M., Martin, C., Souto-Maior, A. M., Baudel, H. M., and de Abreu C. A. M. (2011). "Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production," *Biomass and Bioenergy* 35(1), 663-670. DOI: 10.1016/j.biombioe.2010.10.018

Sarkar, N., Ghosh, S. K., Bannerjee, S., and Aikat, K. (2012). "Bioethanol production from agricultural wastes: An overview," *Renewable Energy* 37(1), 19-27. DOI: 10.1016/j.renene.2011.06.045

TAPPI 211 om-93. (1996). "Ash in wood, pulp, paper and paperboard: Combustion at 525 °C," TAPPI Press, Atlanta, GA.

TAPPI T203 om-83 (1983). "Alpha-, beta-, and gamma- cellulose of pulp," TAPPI Press, Atlanta, GA.

TAPPI T208 om-84 (1984). "Moisture in wood, pulp, paper and paperboard by toluene distillation," TAPPI Press, Atlanta, GA.

TAPPI T222 om-83 (1983). "Acid-insoluble lignin in wood and pulp," TAPPI Press, Atlanta, GA.

TAPPI T223 hm-84 (1984). "Pentosan in wood and pulp," TAPPI Press, Atlanta, GA.

Tu, M., and Saddler, J. N. (2010). "Potential enzyme cost reduction with the addition of surfactant during the hydrolysis of pretreated softwood," *Appl. Biochem. Biotechnol.* 161(1-8), 274-287. DOI: 10.1007/s12010-009-8869-4

Wahid, Z., and Nadir, N. (2013). "Improvement of one factor at a time through design of experiments," *World Appl. Sci. J.* 21, 56-61. DOI: 10.5829/idosi.wasj.2013.mae.99919

Wen, W., Xinshu Z., Zhenhong, Y., Wei, Q., Qiong, W. and Xuesong, T. (2013). "Effects of ions and surfactant on enzymatic hydrolysis of sorghum bagasse," *Ciesc. J.* 64(10), 3767-3774. DOI: 10.3969/j.issn.0438-1157.2013.10.038

Wilke, C. R. (1978). "Process development studies on the bioconversion of cellulose and production of ethanol," Lawrence Berkeley Laboratory Report LBL-6881.

Yang, H. P., Yan, R., Chen, H. P., Lee, D. H., and Zheng, C. G. (2007). "Characteristics of hemicellulose, cellulose and lignin pyrolysis," *Fuel* 86(12-13), 1781-1788. DOI: 10.1016/j.fuel.2006.12.013

Yang, M., Zhang, J., Kuittinen, S., Vepsäläinen, J., Soininen, P., Keinänen, M., and Pappinen, A. (2015). "Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone–butanol ethanol fermentation," *Bioresour. Technol.*189, 131-137. DOI: 10.1016/j.biortech.2015.04.008

Yao, R., Qi, B., Deng, S., Liu, N., Peng, S. and Cui Q. (2007). "Use of surfactants in enzymatic hydrolysis of rice straw and lactic acid production from rice straw by simultaneous saccharification and fermentation," *BioResources* 2(3), 389-398.

Zhou, Y., Chen, H., Qi, F., Zhao, X. and Liu, D. (2015). "Non-ionic surfactants do not consistently improve the enzymatic hydrolysis of pure cellulose," *Bioresour. Technol.* 182, 136-143. DOI: 10.1016/j.biortech.2015.01.137

Article submitted: October 6, 2015; Peer review completed: January 22, 2016; Revised version received and accepted: May 28, 2016; Published: June 2, 2016.
DOI: 10.15376/biores.11.3.6267-6285

Pretreatment of South African sugarcane bagasse using a low-cost ionic liquid: Comparison of whole bagasse and depithed, fibrous and pith fractions

Clementine L. Chambon[a], Thandeka Y. Mkhize[b], Prashant Reddy[b], Agnieszka Brandt[a], Nirmala Deenadayalu[b], Paul S. Fennell[a] and Jason P. Hallett*[a]

[a] Department of Chemical Engineering, Imperial College London, Exhibition Road, SW7 2AZ, UK; [b] Department of Chemistry, Durban University of Technology, P.O. Box 1334, Durban 4000, South Africa

Abstract

This study describes the pretreatment *via* fractionation of a range of preparations of South African sugarcane bagasse into a cellulose-rich pulp and a low-sulfur lignin, using an inexpensive ionic liquid-water mixture. We optimized pretreatment conditions for whole and industrially depithed bagasse, assessing fractionation efficiency by residual lignin analysis and enzymatic saccharification. Pretreatment under the optimized condition of 120°C for 4 h using laboratory-depithed long fiber bagasse

*** Corresponding author:** Dr. Jason P. Hallett

Full postal address: Department of Chemical Engineering, Imperial College London, Exhibition Road, SW7 2AZ, UK. Email: j.hallett@imperial.ac.uk

produced a pretreated solid with 75% of the lignin removed and enabled the release of 68% glucose contained in the bagasse. Lignin was isolated after adding water as an antisolvent. Significant improvements in lignin recovery were obtained for laboratory-depithed bagasse compared with whole- and industrially-depithed bagasse, although glucose yields were comparable. This study shows that sugarcane bagasse can be successfully pretreated using low-cost ionic liquids and indicates that a depithing step may be unnecessary when applying ionic liquid pretreatment prior to cellulose saccharification.

Keywords
Ionic liquid; Depithing; Sugarcane bagasse; Pretreatment; Biofuel

1. Introduction

Rising global demands for energy, volatility of petroleum prices and an urgent need to decrease greenhouse gas (GHG) emissions associated with the use of fossil fuels have driven the push for replacing petroleum with biomass. First generation biofuels, mainly bioethanol produced from corn and wheat grains as well as sugarcane juice, are already in use as alternative transportation fuels, but they have garnered criticism due to competition with food sources (Havlík et al., 2011) and suffer from limited availability. In addition, the majority of first generation biofuels only offer moderate CO_2 savings compared to petroleum (Tindade et al., 2010). Lignocellulosic biomass comprising forestry, agricultural and agro-industrial residues is an abundant, renewable and potentially carbon-neutral alternative to petroleum. For biomass to make a meaningful contribution to CO_2 emission reductions and the conversion to be economically viable,

all parts of the biomass must be transformed into fuels, materials, and chemicals, with high energy efficiency and minimized cost. The selection of a low-cost, abundant substrate and its efficient utilization are hence critical bottlenecks in the development of large-scale biofuels and renewable chemicals industry.

Agricultural residues such as sugarcane bagasse represent a huge, underutilized source of carbon, with over 360 million metric tons (180 million dry tons) available for the production of renewable fuels and chemicals such as ethanol. This conversion would also diversify the product portfolio of the sugarcane industry, making it more resilient and competitive (Werther et al., 2000). In addition, the successful utilization of sugarcane bagasse as a chemical feedstock relies on the development of cost-competitive conversion technologies, including for the first step, the biomass deconstruction and fractionation, *a.k.a.* pretreatment.

Sugarcane (*Saccharum officinarum*) is a major crop in South America, Asia and Africa, consisting of sucrose sugar stored in the stalk. The fibrous lignocellulosic residue remaining after sugar extraction is known as bagasse. The sugar and ethanol industries currently produce 1,800 million tons of sugarcane per year (based on 2013 data) in over 80 countries, particularly in tropical regions and including parts of South Africa (FAO, 2014). Under current practice, each ton of sugarcane delivered to sugar mills yields around 300 kg of moist bagasse. The bagasse is burned to provide process energy and sometimes electricity. However, it is well documented that with currently available boiler technology, the energetic requirements of sugar and ethanol mills can be met by incinerating half of the produced bagasse (Dixit et al., 2010). Valorisation of both the sugarcane juice and the surplus bagasse has the potential to double bioethanol production per hectare without expanding cane fields or jeopardizing food and feed

production (UNICA, 2013). Fractionation of bagasse into sugars and lignin at the pretreatment stage would also produce a useful lignin stream that can be utilized in a variety of ways (Ragauskas et al., 2014), further boosting the economic and environmental viability of sugarcane-based biorefineries. Non-energy recovery applications of bagasse lignin are already a priority in South Africa, where LignoTech South Africa have commercialized the production of lignin-based products such as dust suppressants (Dustex South Africa, 2015).

Sugarcane bagasse is a heterogeneous, low density lignocellulose with a wide range of particle sizes and a high moisture content, typically 40-50% by weight (Pandey et al., 2000). Bagasse consists of three components, namely pith, fiber and rind. Whole bagasse contains up to 65% fiber, which is the most useful component. The non-fibrous 'pith' or parenchymatous tissue found predominantly in the center of the sugarcane stalk represents about 30%, and the dense non-fibrous 'rind' or epidermis surrounding the stalk accounts for the remaining 5%. The pith is made of up non-fibrous, spherical particles with a low aspect ratio, has a low density and is hygroscopic, resulting in various problems during processing (Pandey et al., 2000). Pith also has a higher ash content and poorer fiber morphology than the remainder of the bagasse, which is problematic for paper manufacture and biofuels production processes (Anzaldo et al., 2001). Removal of the smaller pith particles from bagasse prior to stockpiling is also used to reduce hazards through dust formation and spontaneous combustion (Rainey et al., 2013). Efficient depithing to separate the rind, pith and other fine extraneous material from the exploitable fibrous portion of bagasse has long been considered key to utilization of bagasse (Lois-Correa, 2012). As a result, commercial depithing has been in use for many years to increase the fiber content of bagasse from 60 to 80% before

paper pulping(Anzaldo et al., 2001). The depithing is performed in two stages (moist and wet depithing) to remove the pith and undesirable short fibers that are unsuitable for pulping as well as reducing the ash content of the material (Lois-Correa, 2012).

Pretreatment of lignocellulosic biomass, including bagasse, has been widely demonstrated to improve its enzymatic digestibility and subsequent fermentation (Zhang et al., 2012), accomplished by partial solubilization of hemicellulose and occasionally by removal of lignin, and by increasing the accessible surface area of cellulose. Various methods (*e.g.* alkali pretreatment, hydrogen peroxide, steam explosion, organosolv and other solvents) have been studied. Ionic liquids (ILs) are a large and varied group of low-melting organic salts, some of which have potential to be utilized as industrial solvents for lignocellulose pretreatment. In comparison to conventional (organic) solvents, they exhibit advantageous properties including non-flammability and low pressure processes (Clough et al., 2013). A range of ILs have been identified as effective pretreatment solvents that result in isolating a cellulose-rich pulp with greatly enhanced saccharification yields (Brandt et al., 2013). However, the most intensely studied candidate ILs, namely the cellulose-dissolving 1-ethyl-3-methylimidazolium acetate [C_2C_1im][OAc] and other imidazolium cation-based ILs, are prohibitively expensive and thermally unstable at typical pretreatment temperatures, which will undoubtedly limit their large-scale applications (Clough et al., 2013; George et al., 2015). In addition, the presence of water in the cellulose-dissolving ILs has been reported to be detrimental to their ability to swell and dissolve cellulose and hence their pretreatment efficiency. Therefore, extensive drying of the raw materials and ILs is usually necessary prior to pretreatment, resulting in a significant energy penalty, especially for wet feedstocks such as bagasse.

These drawbacks have motivated an intensive research focus in the development of moisture-tolerant and/or inexpensive (aqueous) ILs (Verdia et al., 2014). Hou *et al.* (2013) have demonstrated the use of amino acid ionic liquid cholinium lysine [(Ch][Lys]), the first report of the use of moisture-tolerant ILs for bagasse pretreatment (Hou et al., 2013). Sugar yields of 80% for glucose and 84% for xylose were obtained by enzymatic hydrolysis using bagasse without size reduction, though biomass loadings were limited to 5 wt%; moreover, this ionic liquid is thermally unstable and likely to be expensive to synthesize, limiting its applicability in commercial-scale processes (Earle et al., 2006). Deep eutectic solvents, for instance urea-choline chloride mixtures, are also being explored as less costly alternatives to neat ILs for biomass pretreatment (Francisco et al., 2012) However, these mixtures also suffer from low thermal stability at elevated temperatures (Parnham et al., 2006).

Our recent work has reported the development of a novel lignocellulose pretreatment process using inexpensive protic ionic liquids that could be manufactured at bulk scale at a cost as low as $1.24 per kg, *i.e.* at price parity with common organic solvents (Chen et al., 2014; George et al., 2015). These $[HSO_4]^-$-based ionic solvents were shown to be capable of selectively extracting lignin from lignocellulose such as *Miscanthus giganteus* and switchgrass, yielding a cellulose-rich pulp whilst significantly reducing the generation of sugar degradation products that are inhibitory to enzymes or fermenting microorganisms (George et al., 2015; Gschwend et al., 2015). The recovered lignin has been shown to be depolymerized and unadulterated, thereby contributing an additional stream with potential value as a precursor for the production of renewable aromatic chemicals (Brandt et al., 2015). The $[HSO_4]$- based ionic liquids have also been shown to be significantly more stable than $[C_2C_1im][OAc]$ (George et al., 2015).

Previous studies on bagasse pretreatment with ionic liquids have all faced at least one of several drawbacks that limit their large-scale application, namely low biomass loadings, the use of expensive ILs, thermally unstable ILs, moisture-intolerant ILs, excessive size reduction (Diedericks et al., 2011; Hou et al., 2013; Wang et al., 2015). In contrast, the Ionosolv process uses IL-water mixtures and is therefore highly moisture-tolerant.

The aim of the present study was to examine the delignification and production of sugars from whole and depithed South African sugarcane bagasse and to compare industrially depithed bagasse with bagasse separated into three fractions in the laboratory: long fibers, short fibers and pith bagasse. We have used the low-cost and proven ionic liquid $[HNEt_3][HSO_4]$ (Gschwend et al., 2015) to pretreat these five bagasse fractions, with a view to selectively extracting lignin and achieving high recoverable sugar yields for a subsequent fermentation. This study used washed and air dried bagasse to be as close to an industrially relevant scenario as possible, as storage of dried residues for processing later in the season is common in the sugarcane industry. The influence of a significant amount of pith present in the feedstock is discussed and a comparison of the impact of industrial and laboratory depithing processes on bagasse pretreatment is assessed *via* Klason analysis of the lignin content and enzymatic saccharification of the pretreated pulp.

2. Materials and methods

2.1 Materials

All chemicals used were reagent grade and purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Whole sugarcane bagasse (*Saccharum officinarum*) (WB) was sourced from a local pulp and paper mill in the Kwazulu-Natal province of South Africa. Depithed bagasse (DB) was obtained from the same mill, where biomass was subjected to an industrial depithing process, a two-stage mechanical process that removes pith, short fibers and undesirable particulates (e.g. sand) to leave behind

depithed bagasse. All feedstocks were washed to remove adhering inorganic debris and air-dried at room temperature in order to prevent microbial degradation during shipping. They were ground and sieved (0.18-0.85 mm, US mesh scale -20/+80) and stored air dry in sealed plastic bags at ambient temperature. In a second set of experiments, WB was ground and subjected to a laboratory fractionation process by sieving (Chinsamy et al., 2004) (Fig. 1) to obtain pith bagasse (PB), short fiber (SFB) and long fiber bagasse (LFB). An overview of all lignocellulosic feedstocks used in this study is shown in Table 1.

2.2 Ionic liquid synthesis

Triethylammonium hydrogen sulfate $[HNEt_3][HSO_4]$ was synthesized as described previously (George et al., 2015). Briefly, triethylamine (126.49 g, 1.25 mol) was added dropwise to a solution of 5 M H_2SO_4 (250 mL, 1.25 mol of H_2SO_4) stirred in an ice bath. A rotary evaporator at 40ºC was used to reduce water content to 20 wt%. A mixture of 80 : 20 wt/wt $[HNEt_3][HSO_4]$: H_2O was prepared and used for all pretreatment experiments. The water content was verified by Karl-Fischer titration (METTLER TOLEDO V20) in triplicate. The acid-base ratio (1:1) was verified by determining the density of 80 : 20 wt/wt $[HNEt_3][HSO_4]$: H_2O (Anton Paar DMA 38 density meter; accuracy ±0.0001 g cm^{-3}) was 1.1910±0.0004 g cm^{-3}.

2.3 Ionic liquid pretreatment

The protocol for Ionosolv pretreatment developed by Brandt et al. (2011) was adapted. 1.00±0.05 g air-dry biomass were placed in a wide-mouthed 15 mL pressure-resistant

glass tube with screw-on Teflon cap and O-ring seal (Ace Glass #8648-04 tube with #15 front-seal plug, Vineland, NJ, USA). 10.00±0.05 g of 80 : 20 wt/wt $[HNEt_3][HSO_4]$: H_2O solution was added, the tubes sealed and the contents mixed with a vortex shaker. The samples were then placed into a preheated convection oven (HeraTherm OMH60, ThermoScientific) pre-heated to 120ºC for between 1 – 24 hours. After the pretreatment period, the tubes were removed from the oven and allowed to cool to room temperature. Experiments were carried out in triplicate.

After the pretreatment, the pulp/ionic liquid mixture were washed four times with ethanol (40 mL each). The ionic liquid/ethanol mixture and pulp were separated by centrifugation at 4,000 rpm for 50 min. The solids were re-suspended in fresh ethanol after each washing step and the supernatant was consecutively collected. The carbohydrate-rich material (solids or 'pulp') was transferred into a cellulose thimble and Soxhlet extracted with refluxing ethanol (150 mL, 24 h). The thimbles were then left to dry on the bench overnight. The Soxhlet extraction washings were combined with the ethanol washings from the previous steps and evaporated *in vacuo* at 40°C, leaving a dried ionic liquid/lignin mixture. Lignin was precipitated by adding 40 mL of deionized water to this mixture. The precipitated lignin was isolated by centrifugation as above and the supernatant decanted and collected. Lignin washing was repeated three more times. The lignin precipitate was dried *in vacuo* at 45ºC for 24 h. The air-dried pulp yield was determined by weighing the recovered biomass from the cellulose thimble and the oven-dried weight was determined. The dried lignin yield was recorded.

2.4 Enzymatic saccharification assay

Saccharification assays were carried out in triplicates with blanks (also in triplicates). The assay was adapted from NREL/TP-510-42629 (Selig et al., 2008). 100±10 mg (calculated on an oven dried weight basis) of air-dried biomass or pulp was placed in a 30 mL Sterilin container. Three blanks were run where biomass was replaced with 100 μL of purified water. 9.9 mL solution consisting of 5 mL 0.1 M sodium citrate buffer at pH 4.8, 40 μL Tetracycline T7660 solution (10 mg/ml in 70% ethanol), 30 μL of Cycloheximide C7698 (10 mg/mL in purified water), 4.71 μL purified water and 20 μL enzyme solution (NS22201, Novozymes) were added to each sample, the tubes sealed and placed in a rotary shaker-incubator (New Brunswick Scientific Innova®42, USA) at 50°C and 250 rpm for 7 days. The enzymes mixture NS-22201 is an experimental enzyme formulation that was kindly provided by Novozymes and is not currently commercially available. This mixture contains endoglucanases, exoglucanases and cellobiases and has a specific activity of 70 ± 12 μmol min^{-1} ml^{-1}. After the saccharification period had elapsed, 1 mL of solution was filtered through a 0.2 μm PTFE syringe filter. Samples were run on a Shimadzu Prominence HPLC equipped with a refractive index detector, deashing cartridges and an Aminex HPX-87P column (Biorad, 300 x 7.8 mm, prepacked HPLC carbohydrate analysis column) with purified water as mobile phase (0.6 ml min^{-1}). The column temperature was 85°C and the acquisition was run for 40 min. Calibration standards with concentrations of 0.1, 1, 2 and 4 mg mL^{-1} of glucose, xylose, mannose, arabinose and galactose and 8 mg mL^{-1} of glucose were used. Glucose and xylose yields were calculated based on the glucan and xylan content of untreated biomass (see ESI).

2.5 Analytical procedures

2.5.1 Compositional analysis of untreated substrate

Prior to undertaking compositional analysis, the extractives were removed from untreated biomass using NREL-TP-510-42619 (Sluiter et al., 2005). Moisture content of untreated material was determined following NREL/TP-510-42618 (Sluiter et al., 2008). The moisture content of untreated bagasse was 6.8% (WB), 5.2% (DB), 8.2% (PB), 6.6% (SFB) and 6.7% (LFB). Results of all biomass compositional analyses are reported on a dry weight basis.

Compositional analysis of untreated extractives-free biomass was carried out following NREL/TP-510-42618 (Sluiter et al., 2008).Analysis was carried out in triplicate. To summarize, solids remaining after two-stage acid hydrolysis were held at 105°C overnight. The mass of the ash-only fraction was then determined by heating the solids to 575°C for 4 h in a programmable muffle furnace (Nabertherm Controller P330 LT 5/13, Nabertherm GmbH, Germany). The acid-soluble lignin (ASL) content of the sample was determined by measuring the UV absorption of the acid hydrolysis supernatant at 240 nm wavelength and an absorptivity of 25 L $g^{-1}cm^{-1}$. Total lignin was calculated as the sum of acid soluble and acid insoluble lignin. HPLC analysis of glucose, xylose, mannose, arabinose and galactose was performed on a Shimadzu HPLC with an AMINEX HPX-87P Column (Biorad, 300 x 7.8 mm, pre-packed HPLC column) with refractive index detection. The content of carbohydrates, lignin and ash were expressed as a fraction of the sum of all components (normalized to 100%).

2.5.4 Size exclusion chromatography (SEC)

1-methyl-2-pyrrolidinone (NMP) eluent was obtained from Rathburn Chemicals Ltd (peptide synthesis-grade, UV absorbance cutoff 265 nm). SEC experiments were performed using lignins isolated from depithed bagasse (DB) and long fiber bagasse (LFB) using a Mixed-D column (5 µm particle size, 300 mm x 7.5 mm I.D.; Polymer Laboratories, UK) packed with polystyrene/polydivinylbenzene beads. The Mixed-D column was operated with 0.5 mL min^{-1} NMP as eluent at 80°C with a Knauer M100 isocratic HPLC pump. A Perkin-Elmer LC 290 variable wavelength UV-absorbance detector was used and routinely set to 270 and 300 nm. The system was calibrated using linear polystyrene standards with M_P values of 580, 970, 2,960, 19,880, 185,400, 523,000 and 5,000,000. Three linear PS standards across with Mp values of 970, 2960 and 19,880 were eluted to verify the calibration prior to each set of SEC experiments. The calibration curve used was $y = 0.4038x+10.632$, $R^2 = 0.9746$, where y = log[molecular weight (Da)] and t = elution time (min), valid between t = 11-19 min. SEC experiments were performed in triplicate. Polystyrene does not represent the geometry of the highly branched lignin macromolecule, so the molecular weights reported bear the risk of a systemic error; however, the relative trends in molecular weight distribution observed are valid. The ratio between the excluded (t = 0 – 15 min) and retained (t = 15 – 30) peak areas, i.e. $A_{E/R}$, was calculated.

2.5.5 Wavelength-dispersive X-ray fluorescence (WD-XRF)

Whole bagasse and pith bagasse ash were obtained by ashing in a programmable muffle furnace (Nabertherm Controller P330 LT 5/13) according to the following heating program: 25 → 105 ºC with a heating rate of 20ºC min^{-1}, plateau of 30 min at 105 ºC, 105 → 350 ºC with a heating rate of 3ºC min^{-1}, plateau of 30 min at 350ºC, 350 → 575

ºC with a heating rate of 3ºC min^{-1}, plateau of 1 h at 575 ºC, and annealing to room temperature. WD-XRF spectrometry was used to determine the chemical composition of whole bagasse and pith bagasse ash. Quantitative analysis was carried out in duplicate by ITRI Ltd (UK) using a Panalytical Axios mAX WD-XRF spectrometer.

2.5.6 Scanning electron microscopy (SEM)

The morphologies of untreated and pretreated bagasse solids were visually observed using scanning electron microscopy (JEOL JSM-5610 LV, JEOL Ltd, Japan). The pulp samples were mounted on a 1 cm^2 metal sample holder using a carbon black sticker. The sample was then gold-coated in an argon atmosphere using a sputter coating inside an EmiTech K550 (Emitech Ltd, Adhfort, UK). Representative images of pretreated sugarcane bagasse were acquired with a 20 kV accelerating voltage at magnifications ranging from 50x to 500x.

3. Results and discussion

3.1 Untreated bagasse composition

The results of the compositional analysis of the five bagasse fractions prior to pretreatment are shown in Fig. 2. These fractions comprise whole bagasse (WB), depithed bagasse (DB) obtained after industrial depithing, and a further three fractions obtained by a laboratory depithing process to produce long fiber bagasse (LFB), short fiber bagasse (SFB) and pith bagasse (PB). The pith fraction (1.4%) had the highest ash content, though not significantly higher than whole bagasse (1.2%). This may be because pith particles and other inorganic material adhering to the pulp were removed

during the washing process, effectively reducing the 'ash' (i.e. inorganics) content of WB compared with unwashed WB, which typically has ash contents of up to 5-6 wt% (Anzaldo et al., 2001). Though the fractions exhibited total lignin contents between 22.6 and 24.1 %, the holocellulose (cellulose and hemicellulose) to lignin ratio of the bagasse fractions was around 3:1 for all fractions within experimental error (Table S1, see ESI).

3.2 Comparison of whole and industrially depithed bagasse

Whole, industrially depithed and laboratory depithed fractions of bagasse were pretreated with 4:1 wt/wt $[HNEt_3][HSO_4]$: H_2O solution and fractionated into (1) a cellulose rich material, (2) a lignin and (3) the recovered ionic liquid solution. The schematic of a typical mass balance is shown in the ESI† (Fig. S2).

3.2.1 Pulp, lignin and mass recoveries

Whole bagasse and industrially depithed bagasse were pretreated at 120ºC for between 1 and 24 h to find the optimal time for the fractionation at 120°C and assess the effect of industrial depithing. The cellulose-rich pulp and lignin recoveries are tabulated in Table S2. WB and DB were seen to give very similar mass recoveries within experimental error. Pulp yields were minimized after 16 h, suggesting maximal lignin and/or hemicellulose removal; this coincided with a maximum in lignin recovery at 76% within 16 h. After 16 h, pulp yields increased whilst lignin recoveries decreased, which may indicate the formation of less soluble, highly condensed lignins or insoluble carbohydrate degradation products called "pseudo-lignin". These undesirable products (formed as a consequence of severe pretreatment conditions, long time, high

temperature, high acidity) are known to adhere to the pulp surface and may contribute to reducing pretreatment efficacy at longer pretreatment times (Gschwend et al., 2015).

3.2.2 Delignification

Delignification of the pulp was calculated using the Klason lignin analysis protocol and expressed relative to the percentage of lignin present in untreated dry biomass. The delignification, lignin recovery and glucose yields obtained were tracked with pretreatment time (Fig. 3a,b). Delignification progressed with increasing treatment time, and was slightly higher in depithed material compared with whole bagasse. Maximal delignification values of 57% and 63% were obtained at 4 and 8 h for whole and depithed bagasse, respectively, before falling sharply towards negative values at 24 h. The maximal delignification values obtained for bagasse using $[HNEt_3][HSO_4]$ solution were ~70% of those obtained for Miscanthus under similar conditions (120°C, 4h), where 85% delignification was obtained. Lignin recovery was found to exceed delignification (i.e. lignin removed from the pulp) beyond 8 h, indicating the formation of additional lignin-like material at longer treatment times. A previous study with varying IL acid:base ratios has shown that the lignin yield can exceed delignification under acidic conditions, likely due to the formation of hemicellulose degradation products that precipitate with the lignin fragments (Verdia et al., 2014). It should be noted that water-insoluble sugar degradation products (pseudo-lignin) or condensed lignins adhering to the pulp can be detected as acid-insoluble lignin by the gravimetric Klason test (Gschwend et al., 2015). Large errors at 24 h may be attributed to substrate heterogeneity due to local differences in lignin redeposition onto cellulose microfibrils,

discussed in Section 3.5. Quantitative removal of lignin during IL pretreatment is rarely observed (Gschwend et al., 2015).

3.2.3 Enzymatic hydrolysis

The saccharification yield after subjecting the recovered pulp to enzyme hydrolysis is an important measure of the effectiveness of the pretreatment. Sugarcane bagasse samples were treated with a non-commercial Novozymes enzyme preparation both before and after pretreatment; the glucose and xylose yields obtained are shown in Fig. 4. Under optimal pretreatment conditions (120^{o}C, 4 – 8 h), glucose yields reached 69% and 65% of the theoretical maximum relative to the glucan content in native biomass found in whole and depithed bagasse, respectively This corresponds to a respective 5.3 and 8.1-fold increase in enzymatic saccharification yields compared with untreated biomass yields. Depithing seems to improve sugar accessibility of bagasse compared to untreated biomass, possibly due to (thermo)chemical changes undergone during industrial depithing that contribute to loosening the lignocellulose matrix. However, the absolute glucose yields obtained were very similar for the five different bagasse types. These results agree with observations by Hernandez-Salas *et al.* (2009) who used Novozymes enzyme preparations on bagasse following acid hydrolysis, where the amount of glucose liberated following enzymatic hydrolysis was similar for depithed and whole bagasse (Hernandez-Salas et al., 2009). However, the authors report a higher alcohol yield using depithed bagasse following fermentation, despite similar sugar yields, which may be due to the presence of different carbohydrate degradation products or other impurities. Decreasing glucose release beyond 8 h may confirm the presence of

pseudo-lignin or other degradation products that are inhibiting access of the enzymes to the cellulose fibrils.

Xylose yields (Fig. 4) were optimized within 1 – 2 h and reached maximal yields of 29% and 33% for whole and depithed material. The maximal amount of glucose released from enzymatic hydrolysis of whole and depithed bagasse was consequently double the amount of xylose liberated. The enzymatic hydrolysis yields fall short of the 87% and 64% glucose and xylose yields achieved using $[C_2C_1im][OAc]$ under similar pretreatment conditions (120ºC, 0.5 h) (Qiu et al., 2012). However, these studies use very expensive imidazolium-based ILs which have additionally been shown to be structurally unstable under biomass pretreatment conditions due to thermal degradation, making their use unfeasible in a commercial process employing temperatures above 100°C (Clough et al., 2013) Moreover, the use of ILs based on the $[HSO_4]^-$ anion enables the pretreatment of wet feedstocks containing up to 50% moisture, including raw sugarcane bagasse; $[OAc]^-$-based ILs would not be capable of handling such feedstocks due to the process requirement of dry conditions, potentially elevating energy requirements of the pretreatment to unsustainable levels. The saccharification yields obtained using $[HNEt_3][HSO_4]$: H_2O mixtures were 75% as high as $[C_2C_1im][OAc]$; this result is highly promising given that this ionic liquid is 40 times less expensive.

3.3 Comparison of long fiber, short fiber and pith bagasse

Pretreatment of three bagasse fractions obtained from a laboratory depithing process, described previously (Chinsamy et al., 2004), was compared at the optimum condition

identified in Section 3.2 (120°C and 4 h), under otherwise identical conditions. The three fractions were obtained by sieving to decreasing size fractions, yielding long fiber bagasse (LFB), short fiber bagasse (SFB) and pith bagasse (PB). To avoid interfering particle size effects, the particle size distributions of PB, SFB and LFB were verified, shown in Fig. S2 (ESI†). Though the distribution of particle sizes was fairly similar within the size fraction chosen for pretreatment (0.18-0.85 mm), the fractions have different aspect ratios (length of fibre particles vs. thickness). The surface area to volume ratio is hence likely to differ for LFB (low), SFB (moderate) and PB (high), which may impact the pretreatment, though it was not accounted for in this study.

The key indicators for pretreatment of all five sugarcane bagasse fractions analyzed following pretreatment in $[HNEt_3][HSO_4]$ solution for 4 h at 120°C are shown in Fig. 5.

3.3.1 Pulp, lignin and mass recoveries

Pulp yields for laboratory depithed feedstocks, shown in Fig. 5, were between 39-45% and significantly lower than those for whole and depithed bagasse (both 59%) under the same pretreatment conditions. Lignin recoveries were 71-89% for all three feedstocks which is markedly higher than those for whole and depithed bagasse (10-11%) indicating superior lignin removal. Given the similar compositions of all untreated biomass types and that the holocellulose : lignin ratios are ca. 3 : 1 for all feedstocks (Fig. 2, Fig. S1), the difference in pulp yields is likely to be attributable to differences in lignin extraction as well as hemicellulose solubilisation.

3.3.2 Delignification and lignin recovery

The residual acid-insoluble lignin content in the pulp was 7%, 10%, 10% for LFB, SFB and PB, respectively, matching the Klason lignin content of the cellulose pulp obtained from WB and DB (Fig. 5). Long fiber bagasse had the lowest residual lignin content, which may be explained by its low surface area to volume ratio. Reduced lignin re-deposition onto the pulp surface would result in LFB having the lowest pseudo-lignin content. However, no significant difference was obtained for SFB and PB, despite SFB having a lower surface area to volume ratio. Similarly, a stark improvement in delignification was seen for LFB, where the delignification (70%) and lignin recovery (87%) were particularly high. This indicates that a large proportion of the lignin extracted into the liquor is recovered as a precipitate. Our data show that a high lignin recovery is accompanied by a decrease in residual lignin in the pulp, suggesting that the high lignin precipitate recovery for LFB is due to particularly low lignin solubility in the antisolvent water rather than the formation of insoluble compounds under harsh conditions that contribute to the precipitate mass. Fig. S1 shows a typical mass balance for pretreatment of LFB. Over 89% of lignin present in untreated LFB was recovered by precipitation and 68% of the total glucan found in untreated LFB was released as glucose during saccharification. It was hence demonstrated that the Ionosolv process can be successfully applied for deconstruction of sugarcane bagasse.

3.3.3 Enzymatic hydrolysis

The results of the enzymatic saccharification assay are shown in Fig. 5. Glucose yields of 65-68% were obtained for the fractions obtained by laboratory sieving, with no

improvement in glucose yield obtained for LFB which had the highest delignification and a lower residual lignin in the pulp. This result reflects the similar glucan contents of the starting materials (Fig. 2). Xylose yields were limited to 5% for all three materials, and were similar or lower than those obtained for untreated material. This decrease in xylose yields following pretreatment was not observed in whole and depithed bagasse (Fig. 4) where xylose yields reached 21 – 25% after 4 h pretreatment. As no significant removal of xylan from fibrous and pith fractions was observed in compositional analysis following laboratory depithing and sieving (Fig. 2), it is possible that there are less hemicellulose sugars available for enzymatic hydrolysis following pretreatment. It is likely that hemicellulose extraction during pretreatment was more effective, accelerating the dissolution of xylan into the ionic liquid liquor, resulting in lower xylose yields after enzymatic saccharification (Fig. 5). This is supported by lower cellulose pulp yields observed for LFB, SFB and PB. Alternatively, differences in the surface to volume ratios (i.e. different particle size distributions) of each fraction may be responsible for differing xylan solubilities. Interestingly, the removal of xylan did not result in a greater glucose yield for these fractions. However, better removal of hemicelluloses might have facilitated lignin extractability; the effect of particle size distribution and its possible role in enhancing hemicellulose removal following laboratory depithing remains to be further investigated.

3.4 Impact of depithing on bagasse pretreatment

Depithing is usually considered an essential first step prior to biomass pretreatment to separate undesirable pith, sand and other inorganic components from the exploitable fibrous portion of bagasse. This study compared both commercially depithed and

laboratory depithed material to investigate whether a depithing step is necessary prior to bioethanol production. Sieving bagasse in the laboratory is likely to remove a higher proportion of larger pith than commercial depithing (ca. 40% of total material removed compared with ca. 30% in a commercial depither) (Rainey et al., 2013).

Pretreatment trials were performed under identical conditions using industrially depithed bagasse (obtained from a two-stage commercial mechanical process) and laboratory depithed bagasse (obtained from an automated pith-fiber separator at ambient temperature). In spite of the high energy intensity of depithing, the ash content of whole and depithed material was not significantly reduced after depithing (1.2% to 0.7% for industrial depithing). This could be due to the removal of adhering sand, pith and residual sugars from the whole bagasse prior to shipping from South Africa to the UK, which may have artificially reduced the pith content of whole bagasse. Moreover, the ash content of the pith fraction was not markedly higher than that of the fibrous fractions (1.5% in PB vs. 1.2% and 0.4% for SFB and LFB, respectively; see Fig. 2). The starkest differences in the pretreatment outcomes of industrially and laboratory depithed material lie in the pulp yield, xylose yields and lignin recoveries. Pulp yields for laboratory sieved fractions were significantly lower (40-45%) than for whole and depithed bagasse (59%). We believe this reflects an increased removal of hemicellulose sugars from laboratory depithed material. This agrees with the observation that xylose yields for laboratory sieved fractions are poorer following pretreatment, as hemicelluloses are dissolved in the liquor and hence unavailable for enzymatic hydrolysis. Lignin recoveries were markedly higher for industrially depithed material (71-87% vs. 44-50%), which may be due to enhanced lignin extraction due to rapid hemicellulose removal, or to lower lignin solubility in the antisolvent water. In the best

case, IL pretreatment was able to extract and isolate 87% of the native lignin present in long fiber bagasse, which was quantitatively recovered as a precipitate.

Size exclusion chromatography (SEC) of the precipitated lignins from depithed bagasse and long fiber bagasse was performed, in order to compare their molecular mass distributions. The SEC chromatogram (see ESI Fig. S3) shows a bimodal distribution, with an excluded peak (short elution times) and retained peak (long elution times) corresponding to large and smaller molecular mass material, respectively. The ratios of the excluded peak area to the retained peak area, $A_{E/R}$, was compared, where a higher, $A_{E/R}$ indicates a greater proportion of large molecular mass material. Lignin isolated from long fiber bagasse is made up of a greater proportion of large molecules and is likely to have a higher average molecular weight than depithed bagasse lignin. Given the similar holocellulose : lignin ratios (Table S1) in both untreated bagasse fractions, the differences in lignin extraction are likely due to differences in lignin structure. The low solubility of high molecular weight lignin fragments extracted from long fiber bagasse is proposed as the major reason for its high lignin precipitate recovery. Long fiber bagasse pulp also had the lowest residual lignin content and showed the greatest improvement in glucose recovery (10.1-fold) compared with untreated biomass. This may be due to the lower surface area per unit volume for long fibers compared with short fibers, preventing lignin deposition onto the pulp surface. Despite these differences, glucose recoveries were similar for all five bagasse fractions (Fig. 5).

Depithing using a room-temperature laboratory separation of pith and fibrous fractions in bagasse seems to be useful where lignin extraction is a key objective. Given the higher lignin recoveries obtained, it is clear that depithed sugarcane bagasse fractionated with Ionosolv ILs is potentially particularly suitable source of lignins, with a low sulfur

and carbohydrate content (Brandt et al., 2015), providing an additional process stream suitable for further upgrading to value-added aromatic chemicals. However, where a high fermentable sugar recovery is considered paramount, as in most biofuels production processes, depithing seems to be an unnecessary step. Avoiding the high energy input and equipment costs of the depithing operation, estimated to be around US $5.50 per ton of bagasse, may also contribute to lowering operating and capital costs for sugarcane bagasse bioconversion, helping to accelerate the development of cost-effective biofuels production from bagasse. As discussed in detail by Rainey et al. (2013), depithing also has implications for the bulk density and compaction of stockpiled bagasse, its permeability and water holding capacity which must be considered prior to recommending using whole non-depithed bagasse as a feedstock for a commercial-scale biorefinery.

3.5. Scanning electron microscopy of cellulose pulps

The micro-structural features of untreated biomass and pretreated material produced at 120°C and 4 h of Ionosolv pretreatment were investigated using scanning electron microscopy (SEM). The morphologies of untreated pith and long fiber bagasse and the resulting cellulose pulps are shown in Fig. 6. Visual observance of substrate microstructure following pretreatment is crucial to supplement evidence for lignin re-deposition and disruption of the lignocellulose matrix obtained from chemical analysis.

Pith bagasse before treatment has an anisotrophic, amorphous, porous structure with a high aspect ratio and a rough surface (Fig. 6a,b). The pith consists of many hollow cavities (pore diameter ca. 100 μm), known as the lumen (Lee and Mariatti, 2008).

After pretreatment, disruption of the lignocellulosic structure occurred, resulting in a collapsed lumen and a lot of debris (results not shown). The fiber surface was seen to be smoother following pretreatment, which may be due to lignin deposition on the pulp surface, either during pretreatment or during washing. The redeposition of lignin onto the pith surface may play a role in limiting the glucose yields obtained from pretreated pith bagasse. The deposition of lignin droplets onto the surface of pretreated biomass has been reported previously (Selig et al., 2007) notably under acidic pretreatment conditions. These droplets may be detected using Klason analysis and are hypothesized to be composed of lignin and lignin-carbohydrate condensation products, which deposit on the pulp surface and inhibit enzymes. Untreated long fiber bagasse (Fig. 6c,d) has a complete and compact lignocellulosic structure, consisting of cellulose fibers bound by natural polymers including lignin and pectin. Fibrous bagasse has a much higher aspect ratio than pith bagasse and has a rough surface consisting of fibers of ca. 12 μm width. Mild swelling was seen to occur following pretreatment with a slightly increased fiber width of ca. 14 μm (Fig. 6d), an effect which may be attributed to disruption of hydrogen bonding between the fibers following pretreatment. This loosening of the lignocellulosic structure may explain the high delignification of 70% obtained for LFB after only 4 h of pretreatment. As with pith bagasse, the pulp surface appears to be smoother after pretreatment, which may be attributed to amorphous lignin deposition on the pulp surface during the ethanol washing step; however, this requires further verification.

4. Conclusions

80 wt% $[HNEt_3][HSO_4]$:H_2O solutions were shown to efficiently fractionate whole, depithed, fibrous and pith bagasse into a cellulose-rich pulp and a lignin precipitate. Laboratory depithing produced high purity cellulose pulps with 87% lignin recovery, and achieved glucose yields that were 75% of those obtained for dry $[C_2C_1im][OAc]$ by others. The results suggest moisture-tolerant ionic liquids are promising candidate solvent solutions for large scale processing of raw bagasse. Evidence is presented that suggests a costly depithing step prior to pretreatment and cellulose saccharification may be unnecessary in processes where a high yield of fermentable sugars is the priority of bagasse utilization.

Acknowledgments

The authors wish to acknowledge Imperial College London for a scholarship for CLC. Additional funding was provided by Climate-KIC and the European Institute of Innovation and Technology (EIT). Data about the specific activity of enzymes used was obtained by Dr. Alex P. S. Brogan and Wei-Ming Ng. The authors thank Chetna Vaghela for maintenance and technical assistance with HPLC instruments.

References

1 Anzaldo, J., Vargas, J., Turrado, J., Patt, R., Sanjua, R., 2001. Morphological and Chemical Composition of Pith and Fibers from Mexican Sugarcane Bagasse. Holz als Roh- und Werkst. 59, 447–450.

2 Brandt, A., Chen, L., Dongen, B.E. van, Welton, T., Hallett, J.P., 2015. Structural changes in lignins isolated using an acidic ionic liquid water mixture. Green Chem. Advance Ar. doi:10.1039/C5C01314C

3 Brandt, A., Grasvik, J., Hallett, J.P., Welton, T., 2013. Deconstruction of lignocellulosic biomass with ionic liquids. Green Chem. doi:10.1039/c2gc36364j

4 Brandt, A., Ray, M.J., To, T.Q., Leak, D.J., Murphy, R.J., Welton, T., 2011. Ionic liquid pretreatment of lignocellulosic biomass with ionic liquid–water mixtures. Green Chem. 13, 2489. doi:10.1039/c1gc15374a

5 Chen, L., Sharifzadeh, M., Dowell, N., Welton, T., Shah, N., Hallett, J.P., 2014. Inexpensive ionic liquids: [HSO4]−-based solvent production at bulk scale. Green Chem. 16, 3098–3106.

6 Chinsamy, S., Davis, S.B., Sahadeo, P., Kalidass, N., 2004. The development of an automated pith-fibre separator for the cane sugar industry. Proc. S. Afr. Sug Technol Ass 553–556.

7 Clough, M.T., Geyer, K., Hunt, P. a., Mertes, J., Welton, T., 2013. Thermal decomposition of carboxylate ionic liquids: trends and mechanisms. Phys. Chem. Chem. Phys. 15, 20480. doi:10.1039/c3cp53648c

8 Diedericks, D., Rensburg, E., García-Aparicio, M.D.P., Görgens, J.F., 2011. Enhancing the enzymatic digestibility of sugarcane bagasse through the application of an ionic liquid in combination with an acid catalyst. Biotechnol. Prog. 28, 76–84. doi:10.1002/btpr.711

9 Dixit, A.K., Thakur, V. V., Jain, R.K., Mathur, R.M., 2010. Efficient depithing of bagasse: A step towards sustained availability of raw material for paper industry to produce high quality paper. Cent. Pulp Pap. Res. Inst. 22, 163.

10 Dustex South Africa, 2015. http://www.dustex.co.za/ (accessed 18.11.15).

11 Earle, M.J., Esperança, J.M.S.S., Gilea, M. a, Lopes, J.N.C., Rebelo, L.P.N., Magee, J.W., Seddon, K.R., Widegren, J. a, 2006. The distillation and volatility of ionic liquids. Nature 439, 831–4. doi:10.1038/nature04451

12 Francisco, M., van den Bruinhorst, A., Kroon, M.C., 2012. New natural and renewable low transition temperature mixtures (LTTMs): screening as solvents for lignocellulosic biomass processing. Green Chem. 14, 2153. doi:10.1039/c2gc35660k

13 George, A., Brandt, A., Tran, K., Zahari, S.M.S.N.S., Klein-Marcuschamer, D., Sun, N., Sathitsuksanoh, N., Shi, J., Stavila, V., Parthasarathi, R., Singh, S., Holmes, B.M., Welton, T., Simmons, A., Hallett, J.P., 2015. Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. Green Chem. doi:10.1039/c4gc01208a

14 Gschwend, F.J. V., Brandt, A., Tan, B., Weale, J., Fennell, P.S., Hallett, J.P., 2015. A low-cost ionic liquid for lignocellulose pretreatment.

15 Havlík, P., Schneider, U. a., Schmid, E., Böttcher, H., Fritz, S., Skalský, R., Aoki, K., Cara, S. De, Kindermann, G., Kraxner, F., Leduc, S., McCallum, I., Mosnier, A., Sauer, T., Obersteiner, M., 2011. Global land-use implications of

first and second generation biofuel targets. Energy Policy 39, 5690–5702. doi:10.1016/j.enpol.2010.03.030
16 Hernandez-Salas, J.M., Villa-Ramirez, M.S., Veloz-Rendon, J.S., Rivera-Hernandez, K.N., Gonzalez-Cesar, R.A., Plascencia-Espinosa, M.A., Trejo-Estrada, S.R., 2009. Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresour. Technol. 100, 1238–1245.
17 Hou, X., Li, N., Zong, M., 2013. Facile and Simple Pretreatment of Sugar Cane Bagasse without Size Reduction Using Renewable Ionic Liquids − Water Mixtures. ACS Sustain. Chem. Eng. 1, 519–526.
18 Lee, S.C., Mariatti, M., 2008. The effect of bagasse fibers obtained (from rind and pith component) on the properties of unsaturated polyester composites. Mater. Lett. 62, 2253–2256. doi:10.1016/j.matlet.2007.11.097
19 Lois-Correa, J.A., 2012. Depithers for Efficient Preparation of Sugar Cane Bagasse Fibers in Pulp and Paper Industry. Ing. Investig. y Tecnol. 13, 417–424.
20 Nations, F. and A.O. of the U., 2014. FAOSTAT (Database). Latest update.
21 Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., 2000. Biotechnological potential of agro-industrial residues . I : sugarcane bagasse. Bioresour. Technol. 74, 69–80.
22 Parnham, E.R., Drylie, E. a., Wheatley, P.S., Slawin, A.M.Z., Morris, R.E., 2006. Ionothermal Materials Synthesis Using Unstable Deep-Eutectic Solvents as Template-Delivery Agents. Angew. Chemie 118, 5084–5088. doi:10.1002/ange.200600290
23 Qiu, Z., Aita, G.M., Walker, M.S., 2012. Effect of ionic liquid pretreatment on the chemical composition, structure and enzymatic hydrolysis of energy cane bagasse. Bioresour. Technol. 117, 251–6. doi:10.1016/j.biortech.2012.04.070
24 Ragauskas, A.J., Beckham, G.T., Biddy, M.J., Chandra, R., Chen, F., Davis, M.F., Davison, B.H., Dixon, R. a., Gilna, P., Keller, M., Langan, P., Naskar, a. K., Saddler, J.N., Tschaplinski, T.J., Tuskan, G. a., Wyman, C.E., 2014. Lignin Valorization: Improving Lignin Processing in the Biorefinery. Science (80-.). 344, 709–719. doi:10.1126/science.1246843
25 Rainey, T.J., O'Hara, I.M., Mann, a. P., Bakir, C.H., Plaza, F., 2013. Effect of depithing on the safety and environmental aspects of bagasse stockpiling. Process Saf. Environ. Prot. 91, 378–385. doi:10.1016/j.psep.2012.09.002
26 Selig, M., Weiss, N., Ji, Y., 2008. NREL/TP-510-42629, Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure LAP).
27 Selig, M.J., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2007. Deposition of Lignin Droplets Produced During Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. Biotechnol. Prog. 23, 1333–1339.
28 Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. NREL/TP-510-42618, Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP).
29 Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2005. NREL/TP-510-42619, Determination of Extractives in Biomass: Laboratory Analytical Procedure (LAP).
30 Tindade, L.M., Dolstra, O., Loo, E.N. (Robert) van, Visser, R.G.F., 2010. Section One - Towards Sustainability: Principles of Plant Production, in: The

Biobased Economy: Biofuels, Materials and Chemicals in the Post-Oil Era. pp. 59–62.
31 UNICA, 2013. Session 5: Global approaches to support for advanced biofuels - how the need for a comprehensive policy framework is being addressed, in: Fifth Stakeholder Plenary Meeting of EBTP.
32 Verdia, P., Brandt, A., Hallett, J.P., Ray, M.J., Welton, T., 2014. Fractionation of lignocellulosic biomass with the ionic liquid 1-butylimidazolium hydrogen sulfate. doi:10.1039/c3gc41742e
33 Wang, G., Zhang, S., Xu, W., Qi, W., Yan, Y., Xu, Q., 2015. Efficient saccharification by pretreatment of bagasse pith with ionic liquid and acid solutions simultaneously. Energy Convers. Manag. 89, 120–126. doi:10.1016/j.enconman.2014.09.029
34 Werther, J., Saenger, M., Hartge, E.-U., Ogada, T., Siagi, Z., 2000. Combustion of agricultural residues. Prog. Energy Combust. Sci. 26, 1–27.
35 Zhang, Z., O'Hara, I.M., Doherty, W.O.S., 2012. Pretreatment of sugarcane bagasse by acid-catalysed process in aqueous ionic liquid solutions. Bioresour. Technol. 120, 149–56. doi:10.1016/j.biortech.2012.06.035

List of Figures

List of Tables

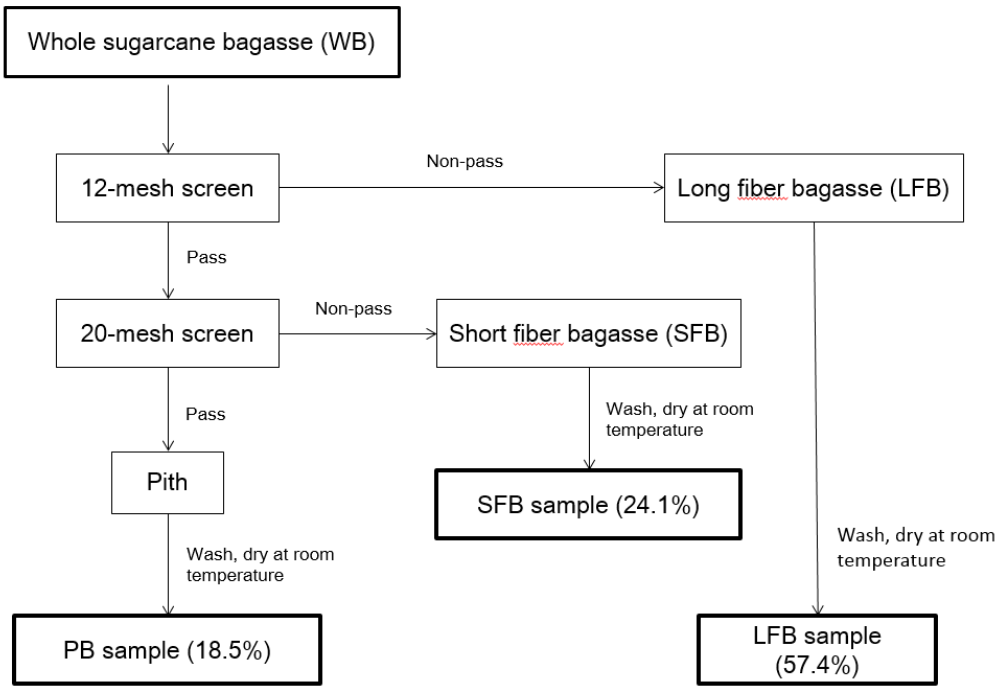

Whole sugarcane bagasse (WB)
12-mesh screen
Non-pass
Long fiber bagasse (LFB)
Pass
20-mesh screen
Non-pass
Short fiber bagasse (SFB)
Pass
Wash, dry at room temperature
Pith
SFB sample (24.1%)
Wash, dry at room temperature
Wash, dry at room temperature
PB sample (18.5%)
LFB sample (57.4%)

Figure 1.

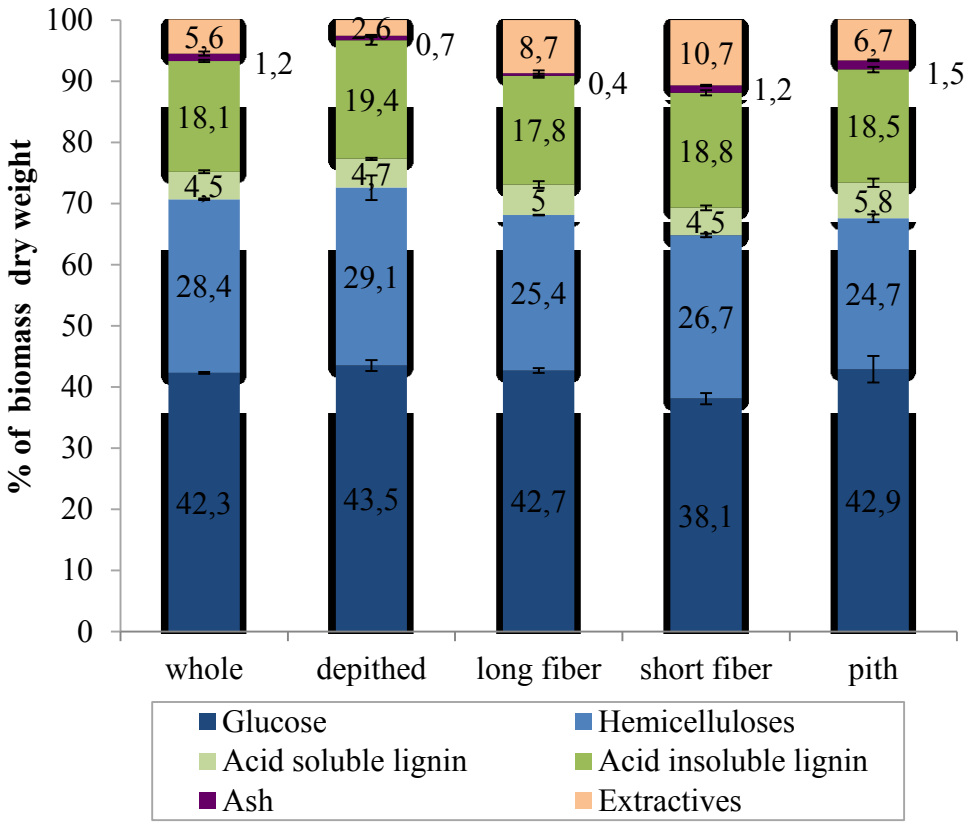

% of biomass dry weight
100
90
80
70
60
50
40
30
20
10
0
5,6
1,2
18,1
4,5
28,4
42,3
2,6
0,7
19,4
4,7
29,1
43,5
8,7
0,4
17,8
5
25,4
42,7
10,7
1,2
18,8
4,5
26,7
38,1
6,7
1,5
18,5
5,8
24,7
42,9
whole
depithed
long fiber
short fiber
pith
Glucose
Hemicelluloses
Acid soluble lignin
Acid insoluble lignin
Ash
Extractives

Figure 2.

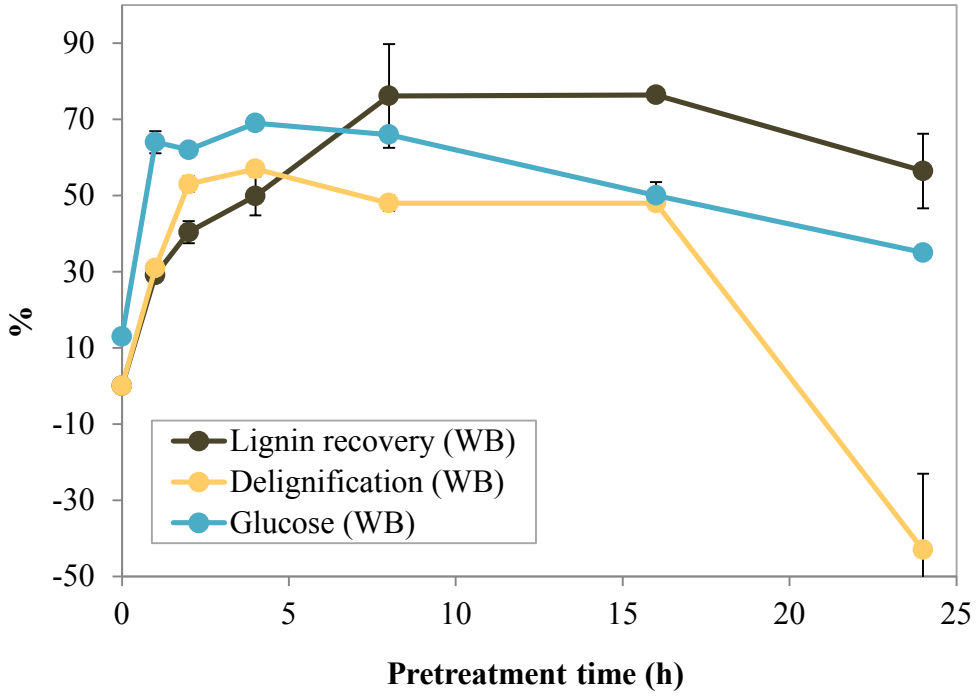


Figure 3a

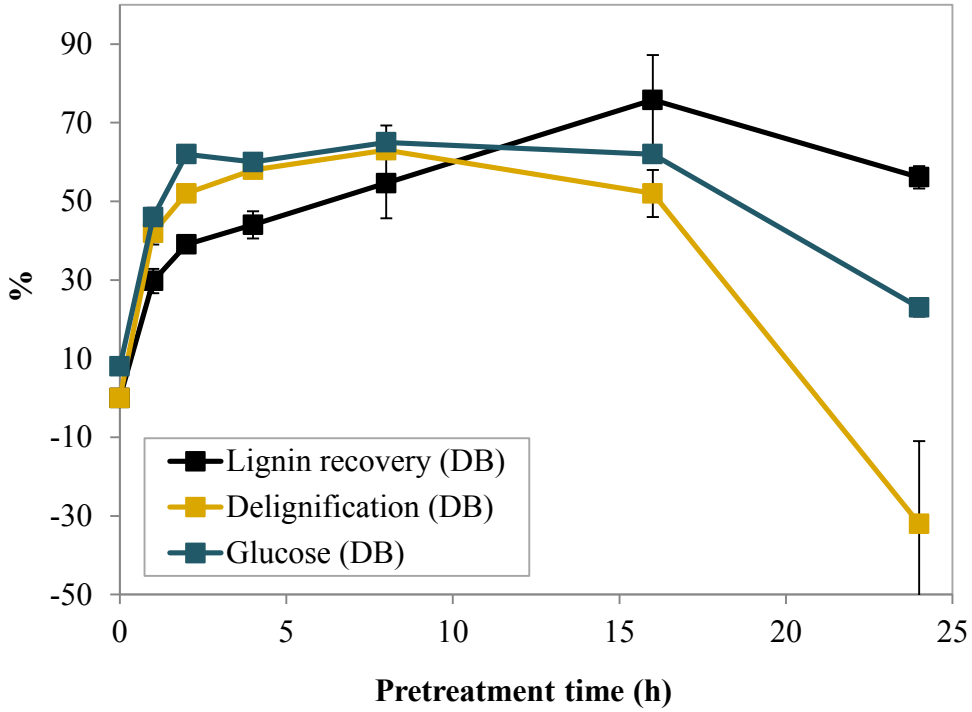


Figure 3b

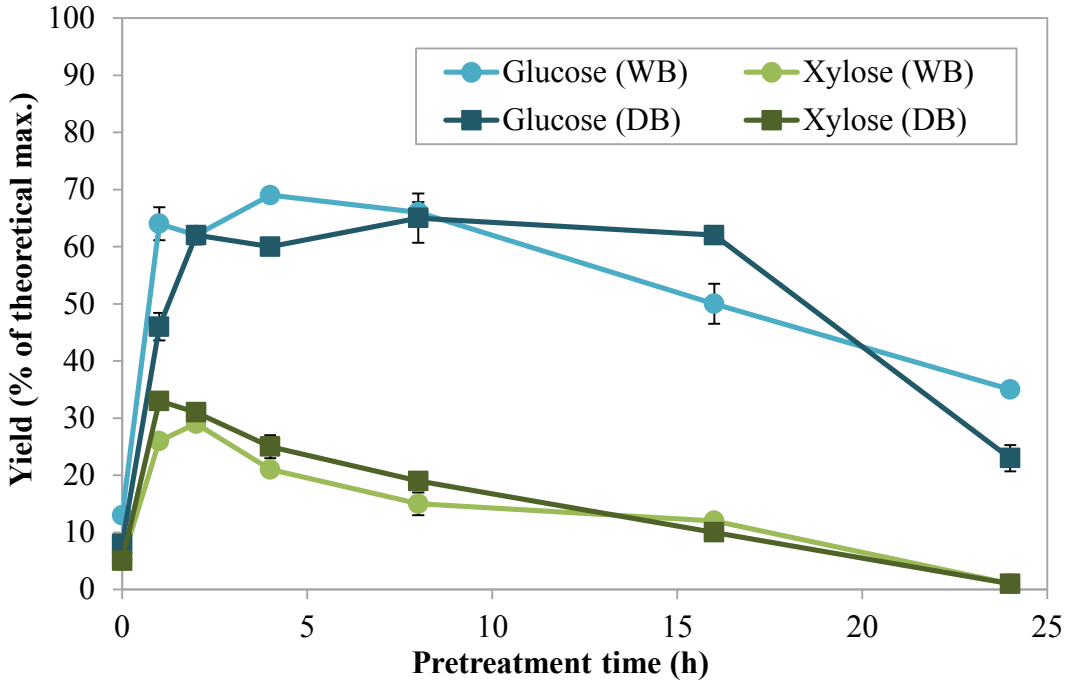

Glucose (WB)
Xylose (WB)
Glucose (DB)
Xylose (DB)
Yield (% of theoretical max.)
Pretreatment time (h)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25

Figure 4.

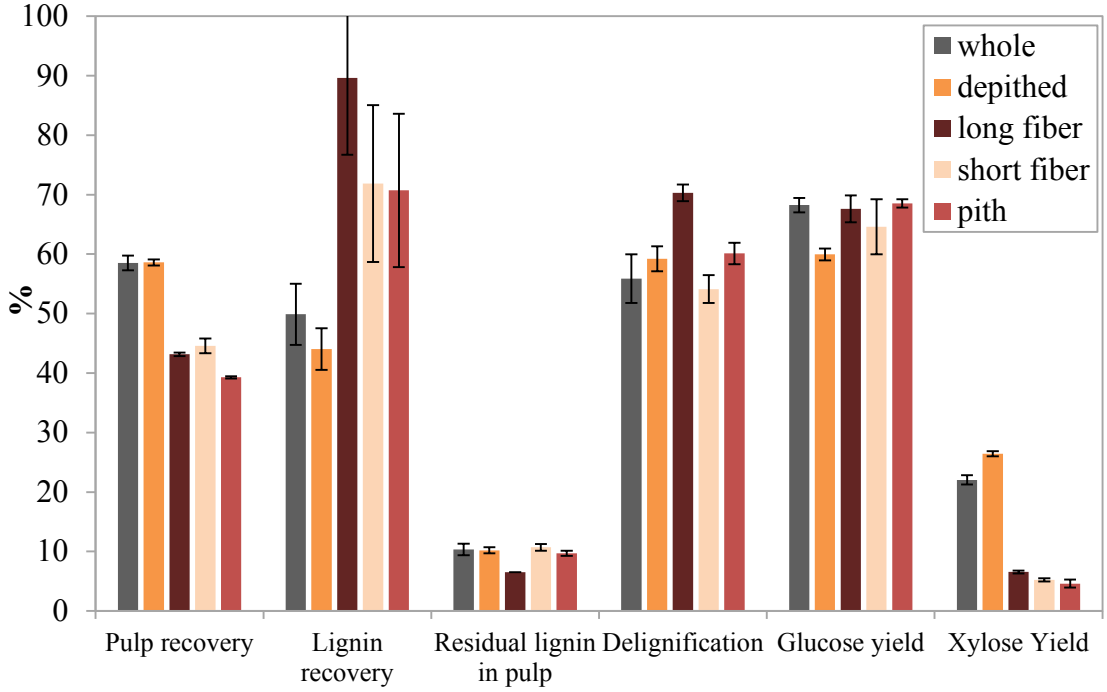

100
90
80
70
60
50
40
30
20
10
0
%
whole
depithed
long fiber
short fiber
pith
Pulp recovery
Lignin recovery
Residual lignin in pulp
Delignification
Glucose yield
Xylose Yield

Figure 5.

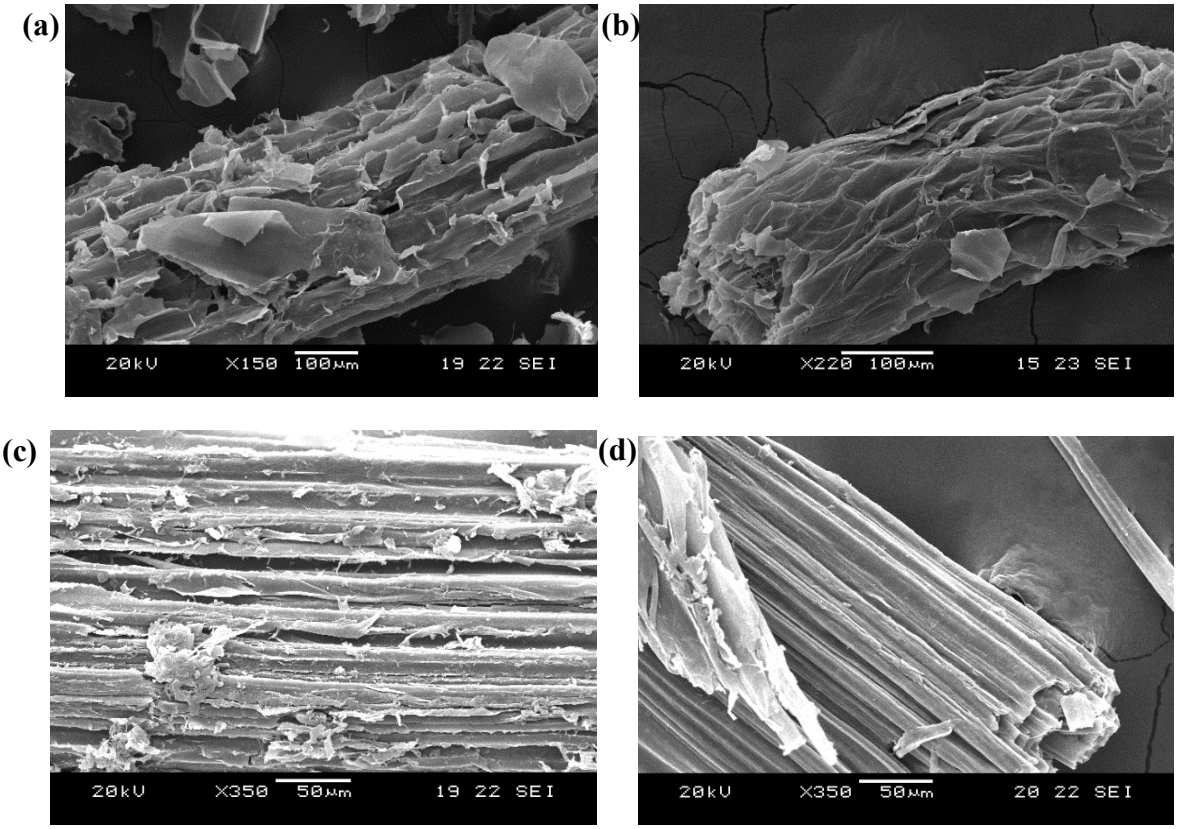

(a)
20kV X150 100µm 19 22 SEI
(b)
20kV X220 100µm 15 23 SEI
(c)
20kV X350 50µm 19 22 SEI
(d)
20kV X350 50µm 20 22 SEI

Figure 6.

Table 1.

Substrates
Whole bagasse (WB): Mixture of short fibers from the vascular bundle of sugarcane stalk (‘pith’) as well as longer fibers from the cortex of sugarcane stalk ‘rind’.
Depithed bagasse (DB): High density, fibrous material left over after removal of pit particles by industrial depithing.
Short fiber bagasse (SFB): Short fibers from the cortex of sugarcane stalk ‘rind’ with moderate length/width ratios; obtained from laboratory depithing process.
Long fiber bagasse (LFB): Longer fibers from the cortex of sugarcane stalk ‘rind’ with high length/width ratios; obtained from laboratory depithing process.
Pith bagasse (PB): Small, spongy spherical particles from the vascular bundle of sugarcane stalk. Low density, porous material with a higher ash content; obtained from laboratory depithing process.

Table 2.

Compound (wt%)	*Long fiber bagasse ash*	*Pith ash*	Trace elements (ppm)	*Long fiber bagasse ash*	*Pith ash*
SiO_2	54.57	70.06	Cu	136	<60
Al_2O_3	0.47	1.11	Cr	185	96
Fe_2O_3	0.74	1.35	Hf	<95	<95
Mn_3O_4	0.31	0.11	Ni	<60	<60
SO_3	2.17	1.76	Pb	<90	<90
CaO	11.15	13.07	V	<60	62
MgO	10.23	5.22	Zr	<90	<90
ZnO	0.08	0.06			
Na_2O	4.37	4.99			
K_2O	9.60	1.00			
P_2O_5	5.96	1.66			
TiO_2	0.09	0.17			
SrO	0.05	0.05			
BaO	0.08	0.09			
LOI[a]	N/A	N/A			

[a]Loss on ignition