# Optimising the Pre-treatment Effects of Protic Ionic Liquids on Lignocellulosic Materials

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# Abstract

Ionic liquids as ‘green solvents’ are becoming ever more important in a wide variety of chemical applications. Certain ionic liquids, based on the imidazolium cation in particular, have been shown to be suitable for solubilising lignocellulosic materials, such as woods, rapid growing grasses and waste agricultural products, which do not interfere with food based crops. For these reasons, from waste or unneeded biomass materials, useful products can be produced, which have fewer ethical and economical issues.

The major difficulty with exploiting lignocellulosic materials is the pre-treatment step, to be able to access the chemical products within biomass. Current methods of pre-treatment to produce biofuels involve concentrated corrosive acids or high temperature and pressure reactors (steam explosion), both of which are environmentally unfriendly. Ionic liquids can be designed to be environmentally friendly, as well as potential biodegradability, depending on the choice of the ions used in synthesis.

This work has successfully shown the use of diethanolammonium chloride, (which is cheap and biodegradable), to pre-treat miscanthus with a 12 times improvement of sugar release compared to 4 times improvement with dilute acid. Similar improvements have been observed with poplar biomass. Lignin removal has been identified as the major benefit in this IL pre-treatment, while hemicellulose removal also played a role. Surface morphology is a factor but cellulose crystallinity was not determined to be a factor with this ionic liquid. A yield of 30% bio-ethanol was achieved with one step saccharification and fermentation but considerable improvements are required for the process to be more efficient.

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# Preface

This thesis was completed after many years researching the use of lignocellulosic materials as a future source for chemical production. I have always found renewable energy and materials a fascinating subject, even at GCSE level, and suppose it is somewhat fitting to spend my PhD discovering novel research in the topic.

Before my acknowledgements section there are a few personal thank you's that I felt were appropriate to mention. Appreciation has to be given to Jussi Sipilä and Paula Nousiainen from the University of Helsinki, without whom, I wouldn't have studied plant biomass research in the fourth year of my MChem, this paved the way into this PhD research.

Also thanks to John Slattery who originally approached me with this PhD topic, due to the abroad connection and the masters project, this started my PhD experience. It was a lucky moment and has led to 4 extremely enjoyable years at the University of York, exploring the importance in these plant biomass materials.

Doing a PhD for me was for two reasons. Firstly researching a topic I felt passionate about and being able to contribute further knowledge to society was a rewarding challenge. Secondly the PhD was also a chance for me to develop personally and professionally by being part of the chemistry department at the University of York, a graduate representative, a demonstrator/mentor, as well as a York city resident.

To sum up, in the great words of 'Hercule Poirot' we must realise two things as we go along our PhD journey:

Firstly, "it is the brain, the little grey cells on which one must rely." And this PhD has certainly tested that hypothesis and required many little grey cells.

Secondly, "one must seek the truth within - not without." Certainly something not to be forgotten during a PhD, otherwise you can easily become side tracked and forget the main reason behind your choices to complete a PhD, as well as the purpose.

***'This thesis is dedicated to the Gammons Family'***

# Acknowledgements

Over the past four years there are many people from the departments of chemistry and biology who I am grateful too for their help in completing my PhD. Firstly to my four supervisors who have given me ideas and support over the years. Seishi Shimizu for his creativity, inspiration and effective thesis writing sessions, Neil Bruce for the original start up ideas and equipment, Simon McQueen-Mason for his expertise with biomass and enzymes and John Slattery for the ionic liquid support, project development and generally putting up with me for the past 4 years.

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This work would not have been possible without help from the biology department, especially CNAP. The McQueen-Mason group have been fantastic over the years especially Luisa Elias, Katrin Besser, Susanne Bird and Poppy Marriot who have frequently helped with lab and experimental issues as well as in group meetings. For fermentation help, Alexandra Lanot and David Vaughan, without them the tests into SSF would not have been possible. For saccharification help over the years, Caragh Whitehead and Leonardo Gomez, they helped get the project started in the early days to which I am very grateful for. Finally the biggest thank you to CNAP has to go to Rachael Simister. Rachael has quite simply been a life-line over the past few years with help and support in all of the biology research. Without Rachael this project would have taken longer and been a lot more difficult to complete, so her help has been greatly appreciated.

Of course over the years I have made many friends in the chemistry department, all who have helped me both with work and just been great to be around. In the early days Tim James was great fun and helped start the ionic liquid research off. A massive thanks to double trouble, a.k.a Sharifa Hussein and Neetisha Mistry who have provided me with many a great memories over the past years and who's company and humour I still enjoy today. To past MChems including Rosanne Goodburn, Stephen McNicol, Jonathon Cottom and Christopher Sheerer for their

general good will, fun to be around and of course the banter! To Becci Bibby for initially providing the group with laughter and cakes, and then becoming a great friend. To Ollie Pickup for being a constant source of amusement for the group and David Johnson for great nights out. To Andrew Marriot and Luisa Ciano for being good friends, great neighbours and excellent spider catchers.

To my fellow fourth year PhD's, Lizzie Smith, Daniel Raines, Linda McAllister and Stephen Bromfield for all the fun over the years, pizza nights and general help to get through the PhD. And to more recent times in the group to Lucy Milner for her enthusiasm and interest in trying out fun new activities, Chris Cabry for a very memorable and enjoyable 4th year Christmas party. Lucia D'Andrea Rodríguez-Vida for injecting enjoyment into my last 6 months with fun conversations and creative English lessons. To Barby Procacci for our amazing dance moves and Jessica Milani for good office banter and chaining me to my desk for the last six months when needed.

To my home and old uni friends including Vicky Rimmer, Rebecca Goodfellow, Alison Orlandi, Jessica Hogg, Sara Southam, Vinny Evans, Julia Pritchard, Kelly Ready, Sean Perkins, Megan Harris, Katie Taylor, all who have been there for me over the years, from nights out to camping and city trips in London, Berlin and Lucerne. Such a good bunch of friends who are greatly appreciated.

Last, but certainly not least, the final thank you and the most important one of all, is to my parents. There direct help with the PhD project included dads help in printing all of the TAP reports, journals and the final thesis and mums help to proof read all the key work over the years, which was greatly appreciated. However more than that is many thanks for all the support in general over the past 4 years, well past 27 years actually, and without such help then making choices to stay in York, to live by myself, to be able to travel so much and enjoy so much would simply not be possible, for all of which I am grateful for!

To finish off the acknowledgements, a fun way to link all the great people above together in one simple way, and so to quote the words of a not so great man, 'Frank Gallagher' from the tv series Shameless, I have to say that "all of them to a man... know first and foremost the most vital necessity is this life.....they all know how to throw a PARTY!"

# Authors Declaration

I declare that the research detailed in this thesis is my own work and was conducted in the departments of Chemistry and Biology at the University of York, from October 2010 to September 2014 under the supervision of Dr. John Slattery, Dr. Seishi Shimizu, Prof. Neil Bruce and Prof. Simon McQueen-Mason. The research presented in this thesis is original work and has not been published elsewhere. The literature reviews and experimental research was conducted wholly by myself, unless stated otherwise below, where due credit has been given to my collaborators.

1. Saccharification High Throughput Robot- Caragh Whitehead and Rachael Simister - Figures 125 and 126.

2. Powder XRD - Natalie Pridmore - Figure 96.

3. SEM - Robert Mitchell - Figures 101 and 102.

4. Solid State NMR - Mattaus Bechman - Figures 133 and 134.

# Chapter 1
# Introduction and Literature Review

## 1.1 The Biorefinery Concept

Replacing petroleum as a carbon source for fuels and chemicals with a renewable source, is a goal for our future energy and chemical demands. Using lignocellulosic materials will play a key part in achieving those aims, and it will be biomass materials in biorefineries that will be converted into many chemical products. Lignocellulose can be classified into hardwood, grasses, agricultural residues and domestic solid waste, and is explained in full detail about plant biomass composition in section 1.2.[1] Figure 1 shows the renewable nature of plant biomass and the chemical cycle.

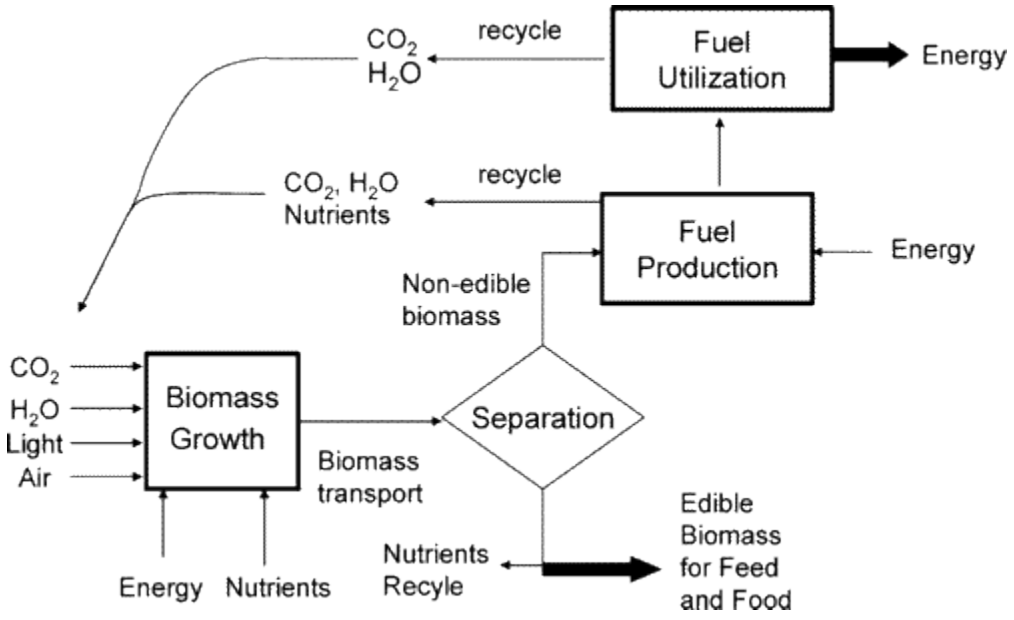


**Figure 1: Overall biomass cycle from growth to usage as a fuel product and recycled back to biomass growth. Figure adapted from reference 2.**

To access the useful chemicals in plant biomass, many methods have been employed in recent years, which include gasification, pyrolysis, thermo-catalytic processing, hydrolysis and fermentation.[3]

The principle aims of biorefineries are; (1) To reduce our reliance and use of oil and gas as fuels; (2) To produce chemicals from plant biomass as a renewable source.

Economic studies have shown that biorefineries with low risk and stable product markets are preferential to complex schemes, where a high diversity of co-products are being produced. The reason for this is the uncertainty of profit demands, which lies in the global market for the demand of each chemical product.[4] Hence it is indispensable for lignocellulose-derived products, such as biofuels, to fulfil the following criteria: efficiency in production, minimal raw material costs, potential for co-products, and economical competitiveness.[5] Figure 2 shows the overall view of a biorefinery and some of the available chemicals which are possibilities for production.

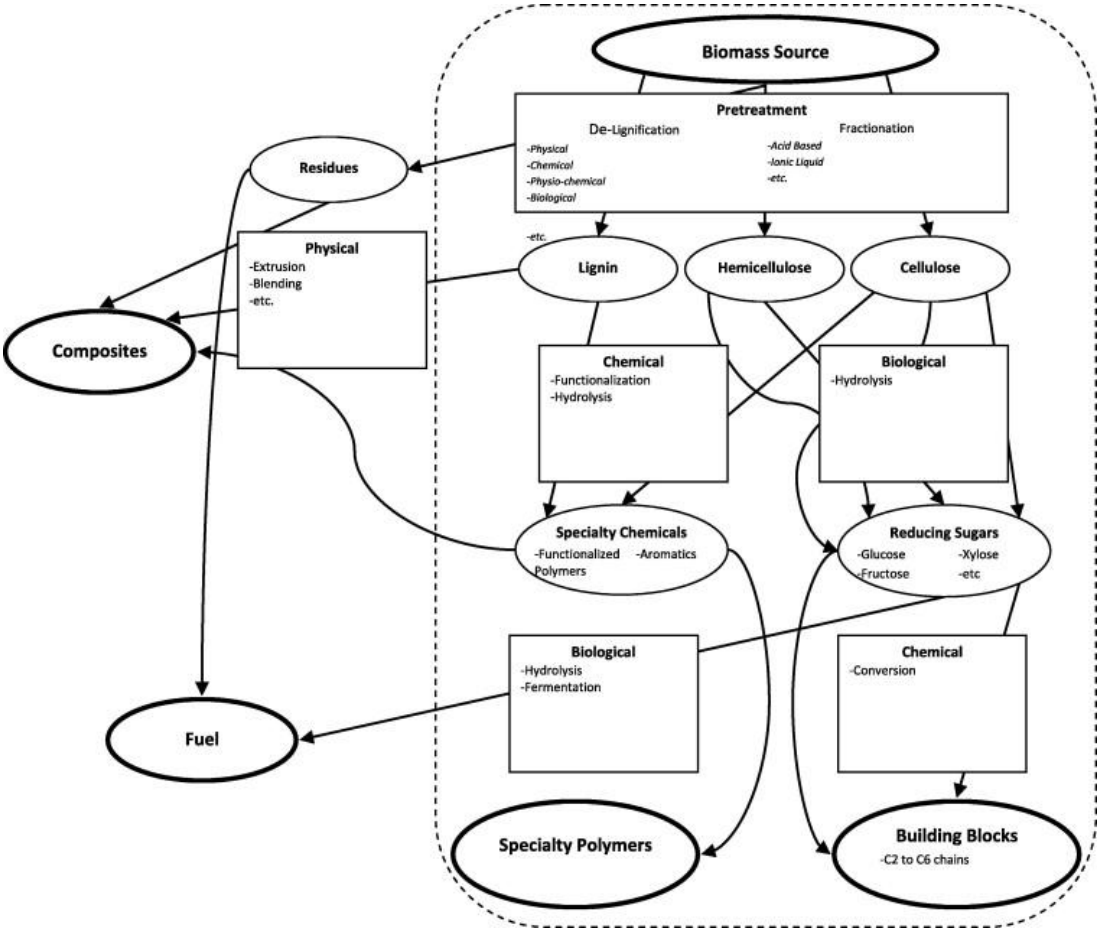


**Figure 2: The overall view of a biorefinery from biomass source to chemical building blocks modified from reference 6.**

This project will aim to look at the current flaws in certain concepts of the biorefinery and seek to discover solutions for future production.

## *1.1.1 The Chemical Processes of Biomass to Products*

There are three possible ways to convert lignocellulosic materials into platform chemicals, as summarised in Figure 3, which are: (1) Pre-treatment and hydrolysis; (2) Gasification; (3) Pyrolysis.[7] The goal of the present study, is to obtain bioenthanol from lignocellulose, namely the process (1). However, a brief overview of all three processes will be given below.

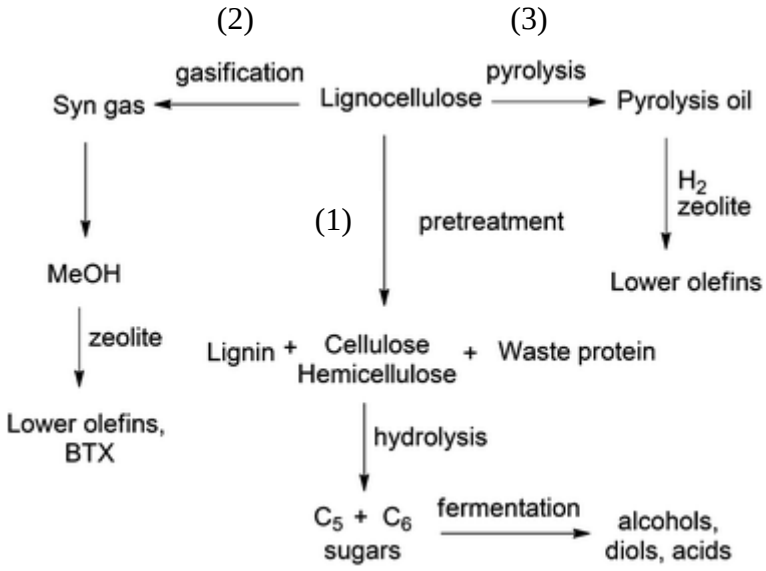


**Figure 3: Biomass processing pathways of the 3 key processes modified from reference 8.**

**1) Biomass pre-treatment and hydrolysis.**

This process requires two steps. The first step is some form of pre-treatment to remove hemicellulose or lignin, which can be achieved by steam explosion, dilute acid, treatment with ammonia gas and, as has been discovered recently, ionic liquids, to produce digestible polysaccharide polymers and monomeric sugars.[9]

The second step is to convert polysaccharides into sugars using acids (concentrated or dilute) or using enzymatic hydrolysis.[10] Although acids are inexpensive, > 65% sulfuric acid is used, which causes equipment corrosion and challenging acid recovery.[11]

In the case of biofuels, a third step of fermentation, with micro-organisms yeast, to form bioethanol is required.[9]

These processes, depending on the approach taken, currently can be inefficient and toxic. Hence, an optimal way of pre-treating plant biomass prior to hydrolysis will be explored in this thesis. To this end, all of the current pre-treatments currently being explored, will first be surveyed in detail (section 1.3), and hence compared to which advantageous properties of ionic liquids are beneficial for pre-treatment (section 1.4). It is for this reason, ionic liquids have already been employed in initial testing for pre-treatment of cellulose and lignocellulosic materials (sections 1.5 and 1.6). Yet the high cost of ionic liquids, as well as certain ionic liquids being toxic, have prevented their use in large-scale pre-treatments so far. Overcoming these issues, and using cheaper, environmentally friendly ionic liquids is the crux of this thesis (section 1.7).

**2) Gasification**

In this process a limited amount of oxygen is used to convert the biomass to syngas ($H_2$ + CO), with some $CO_2$, $CH_4$ and $N_2$.[7] The conversion is by solid, liquid or gas reactions involving partial oxidation, steam reforming, water-gas shift and methanation.[7] This method will not be discussed further in this thesis.

**3) Pyrolysis**

In this process biomass is treated thermally under anaerobic conditions at low pressures and high temperatures, often at several hundred degrees.[12] Bio-oil is the main product, which is rich in aromatic compounds, containing one to six carbons and can be reformed at biorefineries to biofuels and gas.[7-12]This method will not be discussed further in this thesis.

All of the three processes have problems during the biomass treatment including; (1) Lignin remaining in the biomass which is often just burnt; (2) 'Char' formation of intractable carbonaceous waste which cannot be used;[13] (3) A disadvantage with pyrolysis is the complexity of the chemical mixtures produced and separation issues; (4) In fermentation this requires extensive biomass pre-treatments which are costly, as well as the expensive separation and removal of chemicals from the broth.[14]
Figure 4 shows all of these biomass processing pathways and most of the chemical building blocks available to chemists.

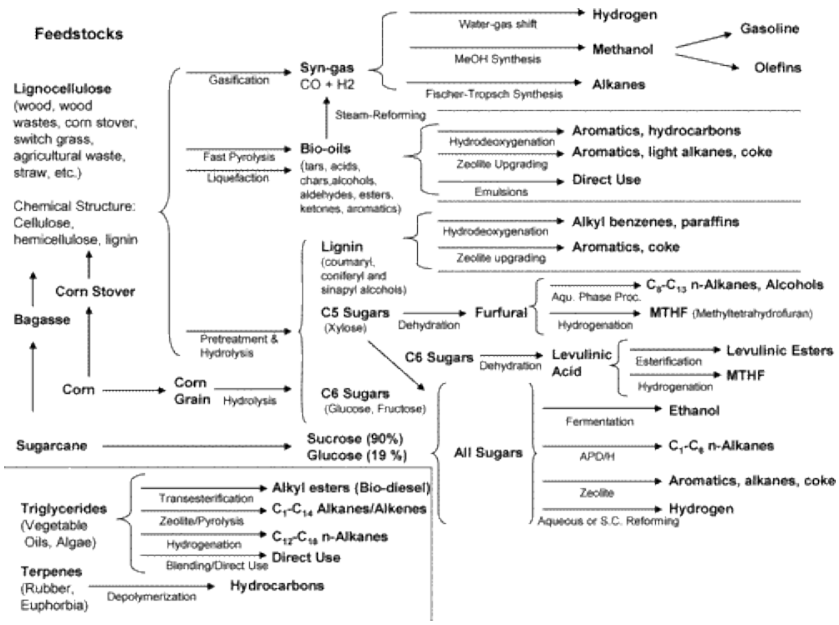


**Figure 4: Overall 3 biomass processing pathways, showing multiple useable chemical products modified from reference 2.**

## *1.1.2 Biofuels and Platform Chemicals*

The most important product from plant biomass for this project is biofuel production. The world produces approximately 87 gigaliters of liquid biofuels.[15] As time is progressing the importance of biofuels and their usage have become more apparent. Figure 5 shows the increase of bioethanol in Europe between 2004 and 2006.

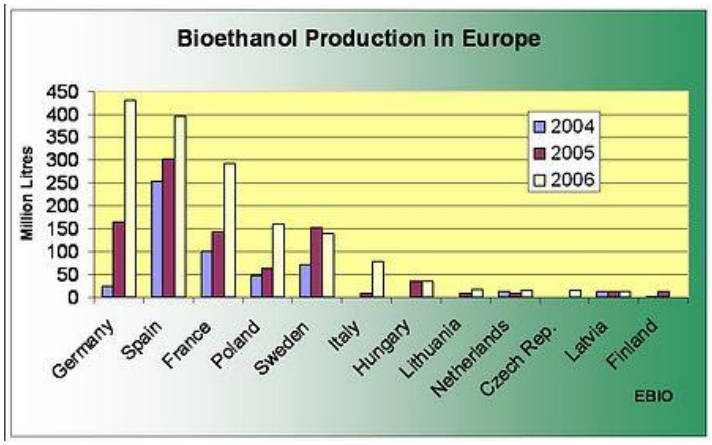


**Figure 5: Graph of bioethanol production in European countries modified from reference 15.**

Biofuels include, biomethanol, bioethanol, biodiesel, biogas, bio-char, vegetable oil and biohydrogen.[16] Bioethanol is the most widely used biofuel for transportation.

The main source of biofuels is from food crops and from vegetable oil, and in fermentation procedures, bioethanol and biodiesel can be produced. Biofuels currently have ethical implications, as the crops which should be sold for food consumption are in some cases being used for fuel, hence less food supply for an ever increasing population.[15] Other issues include cost of production and lack of sustainability.[17] However, the benefits of lignocellulosic materials is they are usually inexpensive feedstocks, they can reduce soil degradation, and they prevent indirect competition with food sources for biofuel production.[4]

The ability to use lignocellulosic materials would be of great advantage to the fuel industry, however, improved technologies are needed for the effective conversion of non-edible parts of plants to fuels and hence commence the development of 2$^{nd}$ generation biofuels.

Table 1 shows the benefits of using biofuels in society, and hence the need for further research of lignocellulosic materials as the source feedstock.

**Table 1: Advantages of using biofuels.[16]**

| **Economic impacts** | **Sustainability** |
|---|---|
| | Fuel diversity |
| | Increased number of rural manufacturing jobs |
| | Increased income taxes |
| | Increased investments in plant and equipment |
| | Agricultural development |
| | International competitiveness |
| | Reducing the dependency on imported petroleum |
| **Environmental impacts** | Greenhouse gas reductions |
| | Reducing of air pollution |
| | Biodegradability |
| | Higher combustion efficiency |
| | Improved land and water use |
| | Carbon sequestration |
| **Energy security** | Domestic targets |
| | Supply reliability |
| | Reducing use of fossil fuels |
| | Ready availability |
| | Domestic distribution |
| | Renewability |

For the biofuel production to be entirely renewable, all of the sugars should be retrieved from polysaccharides, and the energy necessary for the process should be obtained from renewable resources or burning other waste biomass components like lignin.[15] However, such goals are far from having been achieved. It is because of the following problems which run through the whole process from feedstock to product usage:

**1) Feedstock**

Wood is a major source that contributes to lignocellulosic feedstock for biofuels, but it has to be managed effectively. To maximise the amount of woody biomass available, the best way is to cut the plant near to the ground level after the end of the growing season.[15] This approach minimizes the loss of mineral nutrients, soil erosion and organic carbon emissions.[15] However, there are issues with alcohol generation from farm and forest waste, and so this does struggle to fulfil the desired role as next generation biofuels due to technological challenges.[15]

**2) Solvent processing**

Finding a suitable solvent media for plant biomass for cellulose and lignin proves to be a challenge itself. Presently recalcitrance is a problem which is limiting brewers as they are only converting just 40% of the energy content into ethanol.[18] This is compared to fermentation which converts 90% of simple sugars to ethanol. Therefore the maths involved indicates that to produce cellulosic ethanol plants you need far more raw material to make the ethanol than with fermentation.

**3) Mixing with conventional fuels**

The final issue with biofuels presently, is blend wall compatibility problems. Blend wall means the amount of biofuel that can be mixed with standard petroleum. At the minute it is a 10% share and so the demand for bio-source ethanol isn't high and so simple sugar fermentable process can fill this current gap.[18] However, in the future E85, which is 15% blend, will be implemented and potentially higher blends as times goes by will be needed, increasing the demand and the improved technologies for ethanol to be sourced from lignocellulosic materials.

Finally, in today's world, biofuels are about 2.7% of the global transportation energy usage, which is mostly due to ethanol from corn and sugarcane and diesel from soybean and rapeseed.**[17]** The demand for bioethanol will continue to increase over time. However, as stated, these are first generation biofuels and large scale usage of others are yet to come into fruition. Lignocellulosic ethanol should be commercialised during the next decade as an important source for transport fuel.[4]

## Chemicals from Biomass

As mentioned previously, there are many useful platform chemical molecules from biomass as shown in Figure 6. This thesis did not set out to produce any of these chemicals, as bioethanol was our target molecule, but the section below summarises the two main platform chemicals from biomass which are also targets for future IL pre-treatment.[19]

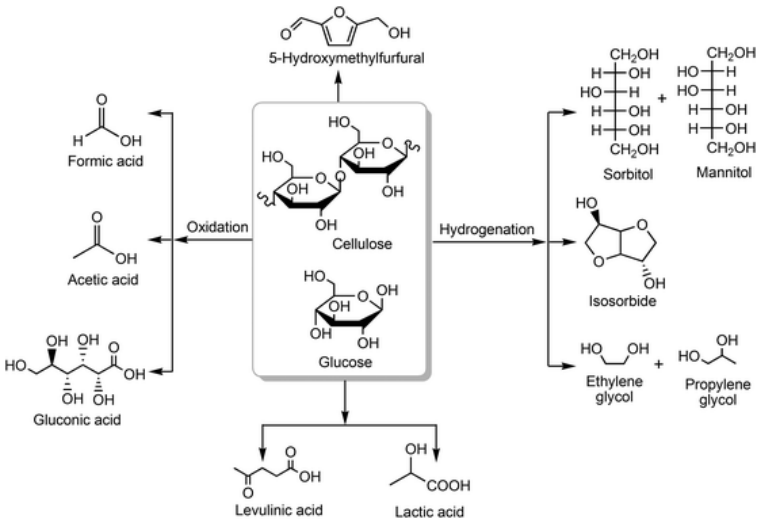


**Figure 6: Conversion of cellulose and glucose in ionic liquids to usable chemical products, modified from reference 20.**

### 1) 5-hydroxymethylfurfural

5-hydroxymethylfurfural (HMF) is one of the top chemical building blocks from plant biomass, and is important due to its great application as an intermediate in fine chemicals, plastic resins, pharmaceuticals and liquid transportation fuels.[21]

HMF is a scaffold for further chemicals or fuels and it is carbon neutral. In research literature the use of $CrCl_2$ and $RuCl_3$ as catalysts to convert cellulose into 5-hydroxymethyl-2-furfural has been successful.[22] The different steps require the different catalysts and in this work the ionic liquid [EMIM] Cl was used. Cr metal can assist the isomerisation from $C_1$-aldose to $C_2$ ketose by coordination to the hemiacetal portion of aldose.

The main use of furfural derivatives, is as a raw material in the synthesis of non-petroleum derived chemicals like furfuryl alcohol, furan and

methyltetrahydrofuran.[23] They can also be used to make bioplastics, biofuels, gasoline blendstock, and in agrochemicals.[23]

## 2) Levulinic acid

Levulinic acid is very versitile, and can be used as a building block for polycarbonates, fuel additives, polyacrylates and herbicides. Levulinic acid is one of the top 12 building block candidates reported by NREL in the USA.[24]

Figure 7 shows all of the potential chemical products from glucose and reinforces the importance of all of the available chemicals from plant biomass.

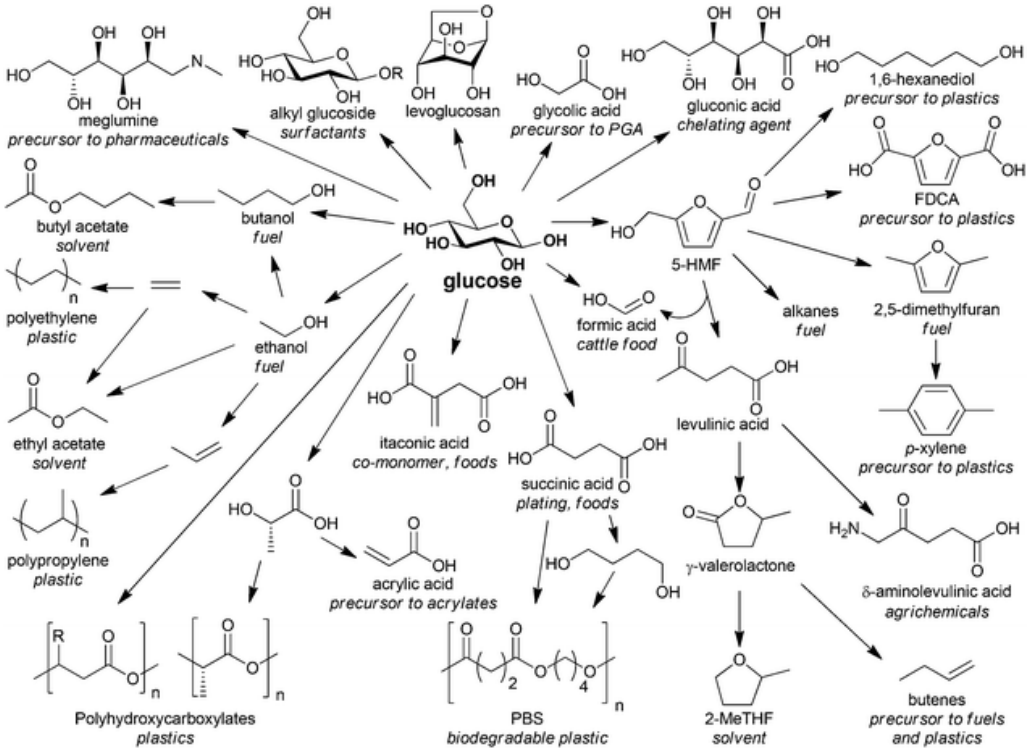


**Figure 7: Glucose and all of its potential chemical products and pathways modified from reference 25.**

### *1.1.3 The Research Project*

Previous approaches to pre-treatment, as has been summarised above with details in section 1.3, suffer from environmental issues and from impurities formed during the pre-treatment.[26] To overcome these problems, the present research aims are to use ionic liquids as a solvent medium to pre-treat the components of plant biomass in preparation for either chemical, or in our case, bio-catalysis. This is because ILs are non-volatile solvents, which can be made from benign chemical sources and selectivity of ILs on biomass pre-treatment has already been shown to be promising by previous studies.[128-134] Hence the goal is to replace the processes currently used at biorefineries and in industry, where methods such as acid and steam explosion, followed by hydrolysis with cellulase enzymes are employed.[27]

In using an anti-solvent like water, ethanol or acetone, it is possible to recover lignocellulosic components from ILs, as they will precipitate out due to their insolubility.[27] These products can be analysed using a variety of techniques including powder X-ray diffraction, FT-IR, NMR, SEM, GC, HPLC, TGA where appropriate, and all of these techniques are employed during the project. Figure 8 shows the key project aim, by using ILs to access the plant polymers and hence available chemical products in plant biomass, and the details of our project goals are in section 1.7.

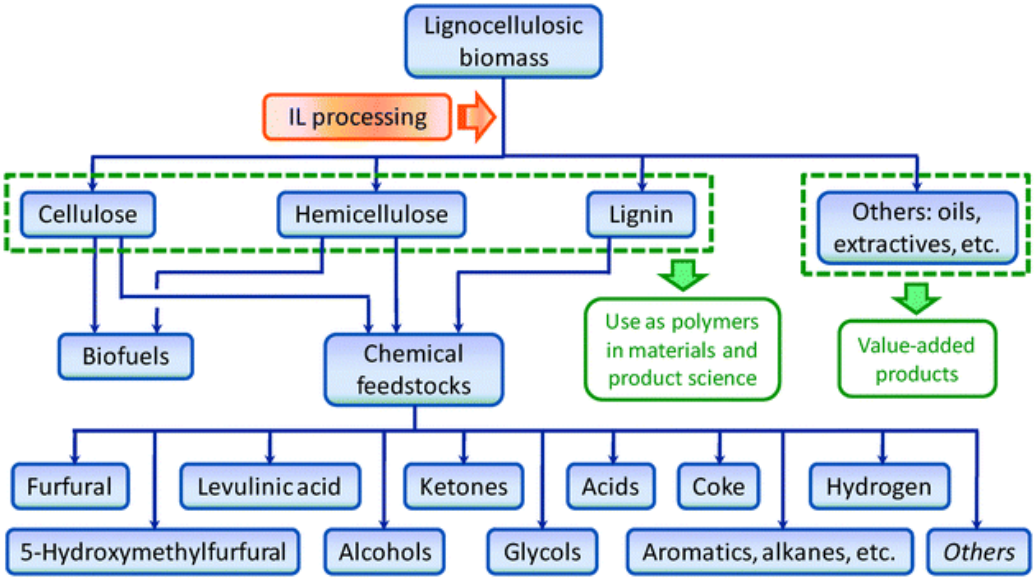


**Figure 8: Flow diagram showing potential uses and routes for lignocellulosic processing to chemical products modified from reference 28.**

# 1.2 Plant Biomass

## *1.2.1 Definition and Classification*

Biomass, in general, is derived from biological material from living or recently living organisms and hence is a renewable source of energy. In the past, most biomass has been incinerated to generate heat or electricity. The term biomass excludes organic materials such as fossil fuels which develop via geological processes and have been converted into oil and coal.[29]

Biomass can be regarded as, the total mass of living organisms, in a given area or of a given species, usually expressed as a dry weight.[29] Processed biomass is referred to as feedstocks available for energy use and has been classified into 4 sub-categories by the European committee for standardisation in 2003-2006.[29] These categories are;

Woody biomass

Herbaceous biomass

Fruit biomass

Blends and mixtures

Table 2 shows examples of biomass materials in each category except for number 4, blends and mixtures, which is dependent on the previous 3 categories.

**Table 2: Biomass classification system and corresponding materials.[29]**

| 1. Woody Biomass | 2. Herbaceous Biomass | 3. Fruit Biomass |
|---|---|---|
| **Whole tress with or without roots** | Cereal Crops | Berries |
| **Stemwood** | Grasses | Stone/kernel fruit |
| **Logging residues** | Oil seed crops | Nuts and acorns |
| **Stums/roots** | Root crops | - |
| **Bark** | Legume crops | - |
| - | Flowers | - |

Common industrial biomass materials include miscanthus, hemp, poplar, corn, switchgrass, sorghum, willow, sugarcane, eucalyptus and palm oil.[29]

## *1.2.2 Lignocellulosic Materials*

The study of lignocellulosic materials is a relatively new area of research which has advanced greatly over the last few decades as further interest into the structure and uses of these renewable sources has become apparent. Improving our fundamental knowledge in this subject helps drive industrial developments, as understanding the structure and reactions to form this material makes us utilize it as a renewable chemical source.

Lignocelluosic materials can refer to the main tissue of the stems, roots and branches of grasses and so called 'woody' plants, after the bark has been removed.[30] Plants are an organic material designed by nature to withstand harsh external thermal, chemical, mechanical and biological factors, and hence are very resistant to destruction of the cell wall, this is known as recalcitrance.[30-31]

Plant cell walls are a multifaceted micro-structural system of a lignin and hemicellulose matrix, supporting cellulosic fibrils packed in bundles.[32] The complexity in chemistry and morphology of cell walls have occurred over millions of years of evolution to protect the cell walls from deconstruction.[32]

These 3 main plant components of cellulose, hemicellulose and lignin are shown in the schematic in Figure 9 and explained in detail in sections 1.2.3-1.2.9. These three polymers are the most important parts of plants, but cellulose is considered the real structural component of the cell because lignin and hemicellulose can be removed without destroying the shape of the cell,[32] whereas, if cellulose is removed the cell is completely destroyed.

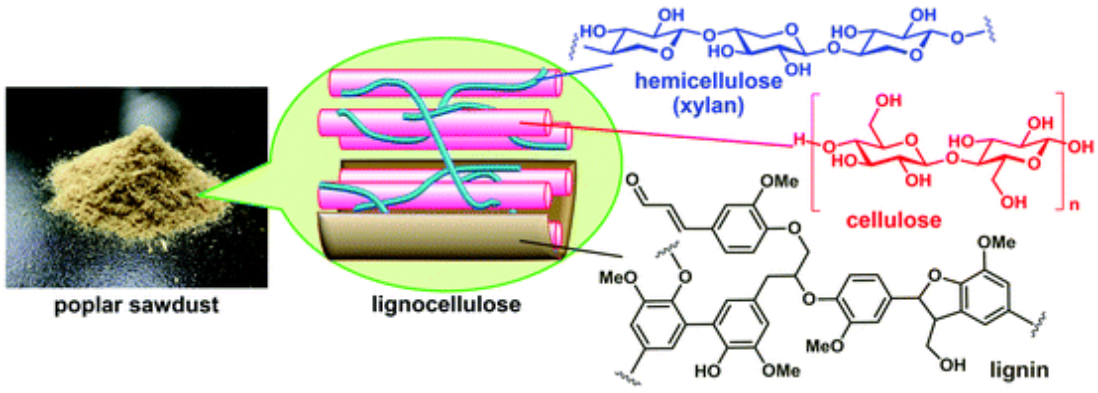


**Figure 9: Sub-structure of plant cell walls modified from reference [25].**

Plants, as a product of biological development, are complex substances both anatomically and chemically. They are non-uniform even within a single species and vary somewhat between individuals of the same species and often greatly between different species.[32] The rough composition of the natural polymers per material on average is between 38%-50% for cellulose, 23%-32% of hemicellulose and 15%-25% of lignin as shown schematically in Figure 10. However this does depend on the classification, as woody biomass often contains more lignin than herbaceous biomass. Also in grass based materials often less polysaccharide linkages to lignin occur, whereas in hardwoods these bonds dominate.[33]

From the beginning of time, lignocellulosic materials have been one of man's most important and valuable resources and today are still one of the most widely used raw materials, especially wood. Wood is majorly used in applications such as the pulp and paper industry, the fuel industry, the construction industry for houses, for boats and furniture and even for leisure activities, including art and sculptures. The great advantage that wood has over lots of other raw materials is the fact that it is essentially a renewable source and hence is considered an environmentally friendly resource. In modern times the emphasis to search for a renewable chemical source is becoming ever more crucial. However, great care must be taken to look after the heritage and habitat of this natural resource as complete ignorance could cause great problems for future generations, hence the need for sustainability within the wood industry, good management is essential.

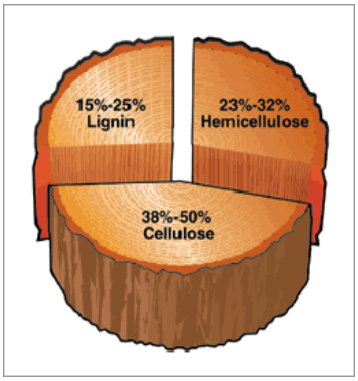


**Figure 10: Average composition of the 3 natural polymers in wood.[32]**

Table 3 shows the individual cellulose, hemicellulose and lignin fractions for a variety of plant biomass materials and demonstrates how they differ. Hence, when choosing biomass materials for chemical processes, knowing these relative amounts is important for improving the results.

**Table 3: Cellulose, Hemicellulose, and Lignin Contents in Common Agricultural Residues and Wastes.[34]**

| Lignocellulosic material | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| Hardwood stems | 40−55 | 24−40 | 18−25 |
| Softwood stems | 45−50 | 25−35 | 25−35 |
| Nut shells | 25−30 | 25−30 | 30−40 |
| Corn cobs | 45 | 35 | 15 |
| Grasses | 25−40 | 35−50 | 10−30 |
| Paper | 85−99 | 0 | 0−15 |
| Wheat straw | 30 | 50 | 15 |
| Sorted refuse | 60 | 20 | 20 |
| Leaves | 15−20 | 80−85 | 0 |
| Cotton seed hairs | 80−95 | 5−20 | 0 |
| Newspaper | 40−55 | 25−40 | 18−30 |
| Waste papers from chemical pulps | 60−70 | 10−20 | 5−10 |
| Primary wastewater solids | 8−15 | - | - |
| Solid cattle manure | 1.6−4.7 | 1.4−3.3 | 2.7−5.7 |
| Coastal bermudagrass | 25 | 35.7 | 6.4 |
| Switchgrass | 45 | 31.4 | 12 |
| Swine waste | 6.0 | 28 | Na |

## *1.2.3 Cellulose Structure and Composition*

Cellulose is the most abundant and non edible biomass resource on earth.[24] Cellulose has the formula $(C_6H_{10}O_5)_n$ and is known as a natural high polymer. Cellulose is structurally very strong, with a specific tensile strength of ca. 5200 kN m $kg^{-1}$, which is 18 times that of titanium.[35] In lignocellulosic biomass cellulose is part of the bio-composite in a hierarchical structure.[35] The glycosidic bond in cellulose is very resistant to hydrolysis and it is believed that the uncatalysed half-life of cellulose is ca. 5 million years.[36]

Cellulose is a polysaccharide consisting of a linear chain of hundreds to thousands of β(1→4) linked D-glucose units[36] and the monomer repeating structure is shown in Figure 11. Cellulose chains are un-branched and can contain up to 5000 glucose units, as the glucose units alternate up and down (in reference to the $C_6$ OH position), hence cellulose can be regarded as a series of cellobiose units joined together.[37] Cellulose is formed by a condensation reaction with removal of water. The hydroxyl groups at the C2, C3 and C6 positions of cellulose are susceptible to chemical reactions including etherification and esterification.[37]

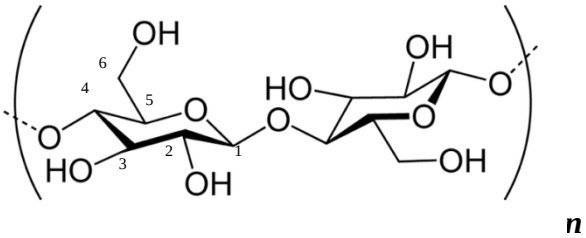


**Figure 11: Cellulose structure modified from reference 36.**

The glucose monomer units are highly soluble in water whereas cellulose is not. This is a kinetic phenomenon[36] as the hydroxyl groups on cellulose would indicate it could hydrogen bond to water and hence be solubilised, but the extensive hydrogen bond network between neighbouring hydroxyl groups in cellulose creates an impregnable barrier preventing the water molecules from penetrating or solvating cellulose, as shown in Figure 12. This means that cellulose is not thermoplastic[36] which is again due this extensive hydrogen bond network and therefore does not melt.

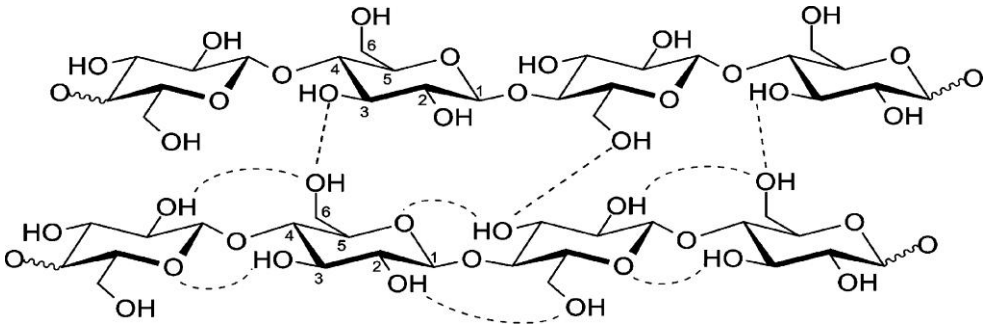

**Figure 12: Extensive H-bond network inter- and intra-molecular, modified from reference 38.**

Cellulose cannot be solubilised in any typical organic solvent because of this extensive hydrogen bond network, but it can be solubilised by treatment with sodium hydroxide and carbon disulphide.[36] It can then be regenerated by acidification of the solution,[39] however, this will not work with just strong sodium hydroxide. Cuprammonium hydroxide (copper oxide and ammonium hydroxide) will solubilise cellulose because it forms a complex with the copper and ammonium ions.[38]

The Kraft process[40] is another industrial procedure using an alkaline solution of sodium sulphide to process cellulose.[40] All of the procedures involving cellulose require alkaline conditions and modify the polymer afterwards. In industry, purification of cellulose by removing lignin and waxes can cause some degradation of the cellulose.

A system to classify the types of cellulose from where they have originally come from is measured by solubility. The most common type of cellulose is α-cellulose and this is virtually intact and does not dissolve in 17.5% aqueous sodium hydroxide.[10] If the cellulose can dissolve in this solution but not dilute acid then it is β-cellulose and if it dissolves in both solutions it is γ-cellulose.[10]

Celluloses' role in plant material is the major structural component of plant cell walls and it aggregates forming the fibres in the natural composite which is lignocellulose. The fact that cellulose is water insoluble, it creates this strong self supporting structure which is essential for the plant cell wall.[11] Cellulose forms microfibrils which are approximately 100 polymer chains in length[3]. It is believed that these microfibrils which form the structure in the cells are not all homogeneous and are ordered differently throughout the cell wall. In some parts they are ordered perfectly in three dimensional order to form crystalline micelles and in other regions

appear to be random and hence are amorphous[3]. It is in these amorphous regions where covalent bonds between cellulose and lignin occur.

Cellulose accessibility should be affected by crystallinity, but also by several other parameters like the lignin and hemicellulose contents and distribution, porosity and particle size.[41] There are four different crystalline allomorphs, which have been identified by characteristic X-ray diffraction patterns or using solid-state $^{13}C$ NMR spectra. These allomorphs are labelled as cellulose I, II, III and IV.[42] Cellulose I is the most abundant form and is the type present in nature. Cellulose II can be prepared by two distinct routes: mercerization (alkali treatment) and regeneration (solubilisation and subsequent recrystallisation).[42] Celluloses $III_I$ and $III_{II}$ can be formed from Cellulose I and II, respectively, by treatment with liquid ammonia, and the reaction is reversible.[42] Celluloses $IV_I$ and $IV_{II}$ can be obtained by heating celluloses $III_I$ and $III_{II}$ respectively.[42]

The complete understanding of cellulose has not been concluded, due to it being a complex mixture of different forms. However it is known that cellulose I (abundant in nature) is a mixture of two distinct crystalline forms, namely cellulose $I_\alpha$ (triclinic) and $I_\beta$ (monoclinic), which have both been verified using solid-state $^{13}C$ NMR.[42]

Cellulose forms about 30% of the carbon in the biosphere, about $10^9$ tonnes is being formed and hydrolysed every year.[14] Therefore the extraction and use of cellulose is an important feedstock for chemistry and an important target for energy conversion.[43] Improving this process is of great use in the future as explained further in section 1.3. Its benign properties including biodegradability, biocompatibility, and regenerative properties make cellulose an environmentally friendly raw material.[43] The current uses of cellulose include cellulose fibres for textiles, the paper industry, filters, food additives and in thin films like cellophane.[14,44]

## *1.2.4 Hemicellulose Structure and Composition*

Hemicellulose is the next most abundant renewable polymer on the planet after cellulose and represents about 20-30% of lignocellulosic biomass.[15] The hemicellulose polymer is very similar to cellulose but the chain length is much smaller (shorter in length) and they are dominated by a hydrogen bond from the C3-OH of one sugar to the ring oxygen of the preceding sugar.[14] A common feature is that the main chain is decorated with side groups, some of which can contain a carboxylic group. Hemicellulose monomers are a broad range of different polysaccharides containing 5 and 6 membered ring sugars like xylose, mannose and glucose for example, Figure 13 shows a sample of typical hemicellulose and Figure 14 shows the common sugars in hemicellulose.

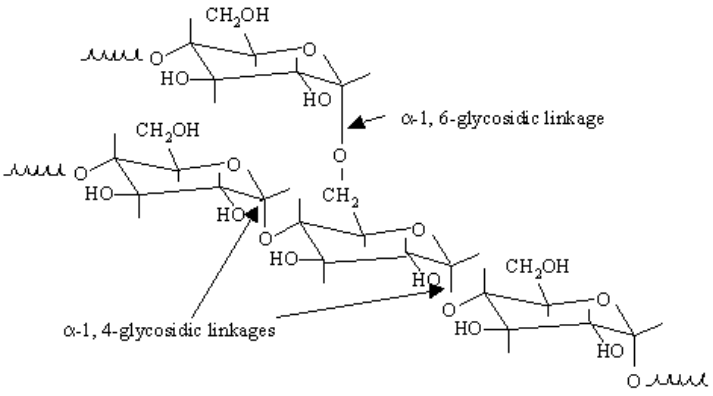


**Figure 13: Hemicellulose structure.[15]**

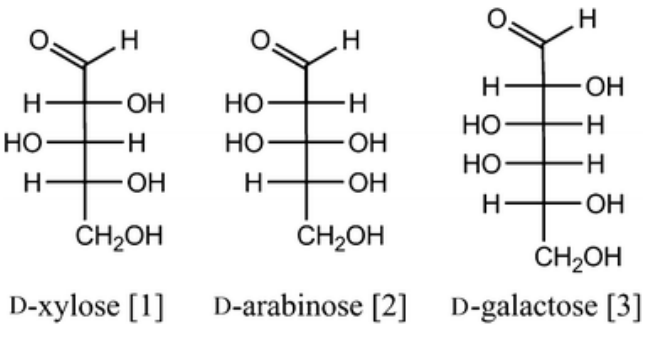


**Figure 14: Common sugars found in hemicellulose.[39]**

If certain sugars of the same form are linked together, like xylose, then this forms the polymer xylan. Xylan is the most abundant and most important hemicellulose polymer. The composition and structure of xylan is more complicated than that of cellulose and can vary quantitatively and qualitatively in various woody plant species. Xylan is composed of $\beta$-1,4-linked xylose units forming a xylan backbone and contains side chains connecting to the backbone, they may be esterified by phenolic acids, which crosslink xylan and lignin in the cell wall matrix.[16]

Hemicelluloses are not studied as much as cellulose because there is a smaller amount of them present and they are more complicated in their structure, but they can be separated from cellulose by extraction with dilute alkali and this is often required in pre-treatment processes as discussed in section 1.3.

## *1.2.5 Lignin Structure and Composition*

Lignin is a multifunctional natural polymer that has the potential to be developed into a major industrial raw material for a multitude of applications. Lignin is an extremely complicated molecule and its complete structure is still being assembled.[45] Unlike most natural polymers, i.e. cellulose, which consist of a single monomer and intermonomeric linkage, lignin is a network polymer made up of oxidative coupling of three major $C_6$-$C_3$ units with many carbon-carbon and ether linkages.[21]

Lignin is covalently linked to polysaccharides, forming a lignin-hemicellulose network made up of benzyl-ether, benzyl-ester, and phenyl-glycoside bonds.[46] Although there is no individual monomer for lignin there are 3 major sub-units in the bio-synthetic pathway to the lignin polymer, shown in Figure 15. This shows a sample of the lignin polymer and the common phenolic acids, which are the starting materials in the lignin polymer structure.[18] A three carbon chain attached to a 6 membered ring which are called phenyl-propanes is the main constituent. This then may have no, one or two methoxy groups attached to the ring.[6] The amount of methoxy groups varies depending on the biosynthetic pathway the polymers were produced by. Proof of these common structures can be found by degrading lignin with hydrogen on a metal catalyst.[47]

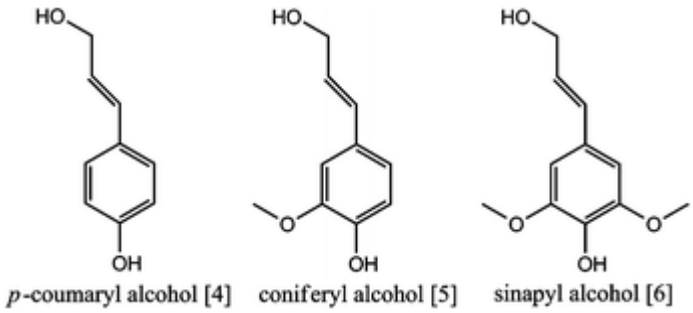


**Figure 15: Lignin structure of the phenolic acid monomer starting materials.[21-39]**

The phenyl-propane groups are joined together by carbon-carbon or carbon-oxygen-carbon bonds of which there are many varieties and hence very few of them are the same linkage. Also upon degradation many different products are formed depending of the types of bond cleavage and the bio-synthetic pathway of the original lignin. Molecular weights of up to 17 million have been reported for spruce lignin.[6]

Because of lignin's complex structure it is difficult to remove lignin from the plant cell wall due to it being very inert and insoluble; hence it is not usually isolated in its pure form to study. However, portions of lignin can be extracted with cold alcohol. Common lignin pre-treatment methods include, physical (Ball milling), solvent fractionation (organosolv process/phosphoric acid), chemical (acidic/alkaline) or biological (fungi), all explained more in section 1.3.[48]

In the plant cell wall lignin is the essential inert structural material and as previously mentioned it fills the spaces between cellulosic fibres. Lignification cements the cellulose fibres together and consequently stiffens the plant and protects it from physical and chemical damage.[6] Lignin is a strong supporting structure hence it plays no active role in the life of the plant. Usually plants contain 12-25% lignin[6] and the polymer can be regarded as a group of amorphous, high molecular-weight, chemically related compounds.

*In vitro*, lignin and corresponding extracts have shown antifungal and antimicrobial activity, as well as antioxidant and flame-retardant properties.[49] Uses of lignin currently include adhesives, synthetic rubber and oxidation to vanillin which is a flavouring agent.[6] However large amounts of lignin is burnt as fuel.

After cellulose and hemicelluloses, lignin is considered to be the most abundant natural polymer present on Earth. It is estimated that currently planet Earth contains 300 billion metric tons of lignin, with an annual biosynthetic rate of production of 20 billion metric tons. [23] Thus, with a perceived shortage of petroleum-based materials as well as a desire to utilise 'green materials', the chemistry and technology of lignin is seeing renewed awareness. This encourages great interest into this area of chemistry, however, the utilization efficiency of this lignocellulosic bioresource is highly dependent on its structural properties, such as the relative content, composition, accessibility, and reactivity of three cell wall components, namely, cellulose, hemicelluloses, and lignin.[21]

Therefore, the ability to fully characterise the structure of these components is essential in understanding the function and the most efficient applications for these molecules. Some chemists believe that lignin has little use other than to be burnt as fuel but there is a large amount of value chemicals present in the structure that if they could be extracted would provide a great source of new renewable chemicals.

## *1.2.6 Pectin and Wax's*

Pectin is a structural heteropolysaccharide which is found in the cell walls of plants as shown by Figure 16. Its purpose is to cross link and bind the cell wall together and is hence found primarily in the leafy part of plants.

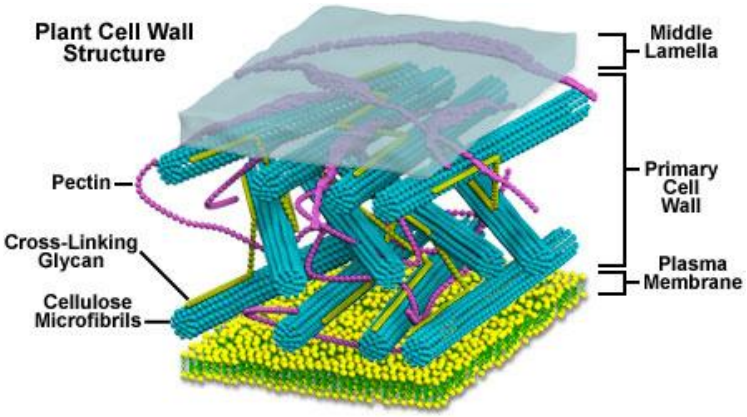


**Figure 16: Plant cell structure from reference 23.**

As with all the polymers in biomass the amount varies greatly between plants and within the plant overtime, as with age pectin is broken down by enzymes causing a weakness in the structure. Chemically pectin is bonded typically by 1,4-linked α-D-galactouronic acid units. A typically section of pectin is shown below in Figure 17.

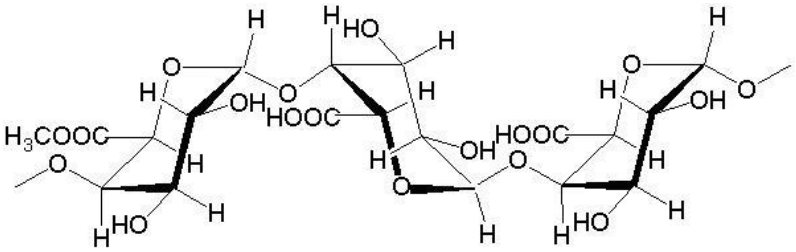


**Figure 17: Pectin structure (D-galacturonic acid).[23]**

Plants secrete waxes which are needed for processes like evaporation and hydration. Waxes are usually long chained apolar molecules consisting of a variety of esters and acids. Figure 18 shows a sample wax molecule. Waxes only comprise of about 1% of the plant structure and once again this can vary massively between species and in the same species but is usually always quite a low amount. During ethanol washing of plant materials the waxes are easily removed, or supercritical $CO_2$ can be used for this process.

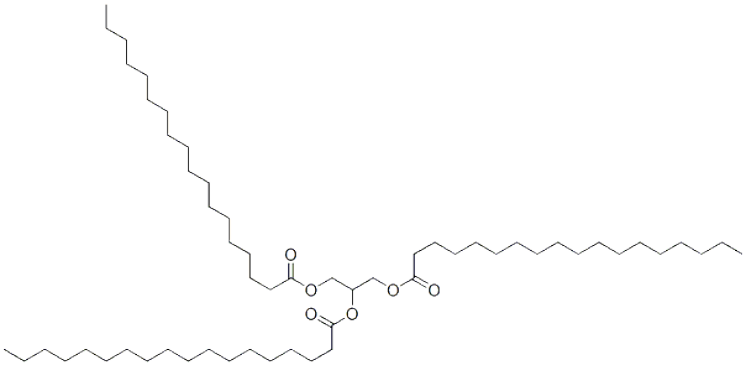

**Figure 18: Chemical structure of wax.[23]**

## *1.2.7 Starch*

Starch is a polysaccharide containing glucose, like cellulose, however the linkage is not a $\beta$-glycosidic bond. Due to this, part of starch is soluble in hot water (called amylose) whilst some (called amylopectin) is not. The starch is found in the seeds, roots and stems of plants and is valued as the food source.[50] For these reasons it is not considered ethical to use starch as a fuel source or for renewable chemicals.

## *1.2.8 Proteins and Trace Metal Elements*

Amino acids polymerised into macromolecular compounds are present in the plant material as well. However, these are insignificant compared to the three main polymers and serve no function for chemical platform molecules.

Trace elements in plants can include metals such as K, Ca, Mg, Al, Si, P, Na and Fe.[50] The amounts of these metals in the biomass is insignificant and the composition depends on the region where the plant is grown.

## *1.2.9 Glycosides and the Anomeric Effect*

A glycoside, is a sugar molecule that is attached to a non-carbohydrate moiety. The bond is usually referred to as a glycosidic bond, it is attached most often through the anomeric carbon and this bond is often broken during metabolic processes within nature. The anomeric carbon is defined as, the carbon in position C1 adjacent to the oxygen heteroatom present in the ring. The anomeric effect is a stereo-electronic effect which explains the preferred conformation of substituents on pyranose rings to lie axial instead of the usually sterically less hindered equatorial position, this is shown in Figure 19. An overwhelming number of glycosides occur in nature, mainly in plants. The designation glycoside is used for the acetal derivatives of the cyclic forms of sugars in which the hydrogen atom of the hemiacetal hydroxyl group has been replaced by an alkyl, aralkyl or aryl group.[24] If the sugar molecule is glucose then the term glucoside can be used, otherwise all other sugars are referred to as glycosides. The glycosidic bond attaches lignin units to cellulose and therefore investigations into the catalytic breakdown of this bond are extremely important in separating cellulose from lignin and for potential biofuel production.

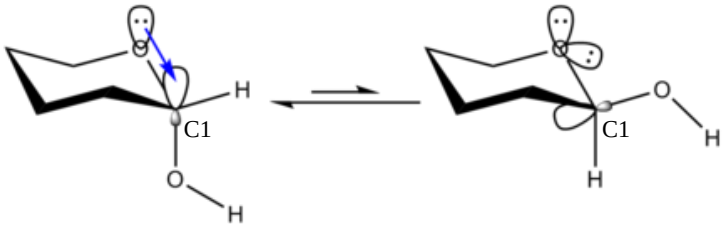


**Figure 19: Anomeric effect modified from reference 24.**

## 1.3 Pre-treatment Methods

Pre-treatment is essential in modifying plant biomass structure to enable access to the recalcitrant polymers in the cell walls. It is a necessary procedure in the conversion of lignocellulosic materials to biofuels and platform chemical molecules. The pre-treatment cost, is labelled as the second most expensive step of cellulosic ethanol production, after the cost of the feedstock,[51] and hence careful consideration of the available options is important.

The hydrolysis rate of amorphous cellulose is 30 times faster than crystalline cellulose, making pre-treatment essential for any increase in enzyme hydrolysis, as this decrystallises cellulose in plant biomass.[35]

Lignin has been identified as the major factor in recalcitrance of plant cell walls for saccharification during enzyme hydrolysis.[52] The problem with lignin is the presence of phenylpropanoid in the polymer, which is in the vascular tissues and fibres, and this inhibits enzymes and yeast in the process.[52]

There are numerous requirements of a pre-treatment method as follows;[2]

1. Low cost of the chemicals for pre-treatment
2. Minimal waste production
3. Fast reactions
4. Non-corrosive chemicals
5. Promote high yields in enzyme hydrolysis
6. Low enzyme loading of digestible cellulose in 5 hours to 3 days
7. Recovery of lignin for valuable conversion to chemical products

Based on these requirements there are multiple current methods employed for plant biomass pre-treatment including; (1) Mechanical; (2) Thermal; (3) Acid; (4) Alkaline; (5) Oxidative; (6) Ammonia expansion; (7) $CO_2$; (8) Biological; and (9) Ionic liquids.[26,53,54] The benefits and challenges to all the above methods will be discussed and the advantages and disadvantages summarised at the end.

### *1.3.1 Mechanical Pre-treatment*

The most common type of mechanical pre-treatment is milling. This often occurs for most biomass samples as reducing the size of the lignocellulosic material is important for the whole process. The reduction in size can often reduce the crystallinity, reduce the degree of polymerisation and increases the available surface area for hydrolysis. This process can increase the yield by up to 25 % and reduce the hydrolysis time by 23-59 %.[26] Milling also has the advantage there is no additional solvent required, so no chemical work up is required which often lead to inhibitors like furfural being created.[55]

However, the issue with milling is it is a very high energy process and for this reason it is not economically feasible on large scales. Also, below a 40 mesh particle size, any further improvement on the hydrolysis of the biomass is not achievable.[26]

Other studies for milling have included 'mix-milling', where biomass is milled in the presence of a solid weak-acidic catalyst. This solid-solid reaction increases the rate constant 13 times in comparison to just milling the biomass.[56] The use of ultrasound has also been used, as sonicated cellulose has been reported as being more accessible.[57] This technique could be used in unison with other methods as it creates stable colloidal suspensions of cellulose with a significant reduction in particle size.[57]

### *1.3.2 Thermal Pre-treatment*

Thermal pre-treatments include the use of hot water and steam explosion on breaking down the plant biomass. If the temperature starts to rise above 150-180 °C hemicellulose, shortly followed by lignin, will start to dissolve in water. The exact temperature depends on the exact composition of the polymers based on the biosynthetic pathways.

The hemicellulose can forms acids, which can hydrolyse the rest of the biomass polymers. The lignin compounds formed are phenolic, like vanillin, which in most cases have the inhibitory effect on enzymes and yeast. If the temperature is raised to 220 °C ethanol production can be completed inhibited due to furfurals and other soluble lignin compounds present.[26]

Steam pre-treatment/explosion is where the biomass samples are usually heated to 240 °C under high pressures for a few seconds, to minutes, in flow reactors. The steam can be released and the biomass cooled down quickly after the process, this is known as steam explosion. Both processes solubilise hemicellulose and give access to cellulose without the formation of inhibitors, however, the benefits of the explosion method are disputed in the literature and high temperatures and energy input are required.[26] At high pressures there is also a considerable safety risk.

Liquid hot water is also used as a method to remove hemicellulose and the pH must be controlled between 4-7 due to acids released. However, although the risk is lower than steam explosion, inhibitors can still be produced in the process.

### *1.3.3 Acid Pre-treatment*

This pre-treatment is performed with both dilute and concentrated acids to solubilise the hemicellulose component. Usually sulfuric, nitric and hydrochloric acids are used for the pre-treatment. Acid pre-treatment is performed over a temperature range of 120-210 °C, with 4 wt % acid loading and pre-treated for minutes, to hours, depending on a batch or flow-through reactors.[47] Sometimes the acid insoluble lignin can increase during the pre-treatment due to modification of the carbohydrates present.[47]

With concentrated acids, this is highly toxic, corrosive and difficult to recover after pre-treatment, as well as producing inhibitors including furfurals and humins.[58]

In cellulose the acid can alter the crystalline structure, swelling the material, as the acid facilitates water in penetrating the cellulose crystals, expanding the surface area.[59] This expansion promotes single polymer chains to be formed, followed by the breakup of these molecules. However, hydrolysis of the cellulose at the reducing ends occurs, forming glucose which is washed away after pre-treatment and reduces the effective yield.[59] It is known with dilute acid pre-treatments a recovery of only 50 % of the sugars is possible, this is due to conversion of sugars to other chemical products.[60]

In hemicellulose, mostly xylan is hydrolysed by the acid and hence can be removed from the biomass. Monomers, furfural and HMF are produced in acidic environments, which as previously discussed, inhibit enzymes and yeast and over

time these inhibitors increase.[61] Lignin can precipitate in acid pre-treatments as all effects are more profound in concentrated acids compared to dilute acids.

A selective oxalic acid-catalyzed hydrolysis has shown promise at removing hemicellulose at mild temperatures.[62] This is a single process to separate the wood polymers, which leaves a xylose rich hemicellulose aqueous solution, a lignin fraction in an organic phase and the cellulose pulp.[62]

In acid treatments, some new methods using superacids are also in development.[63] Comparisons to current acid pre-treatments including HCl, $H_2SO_4$ and HF, with HF-$SbF_5$ are being used to depolymerise cellulose to glucose at just 0 °C.[63]

### *1.3.4 Alkaline Pre-treatment*

The reactions in alkaline pre-treatments involve solvation. In essence, the biomass material is swollen, allowing enzymes to access the cellulose content. With strong alkali pre-treatments the polysaccharides are 'peeled' as the end groups are hydrolysed and removed.[26] However, this 'peeling' can result in loss of carbon from the polymers and $CO_2$ is produced.

If lower temperatures and aqueous potassium hydroxide is used, then xylan can be removed without this degradation as explained above. Lime is also reported to work more effectively than sodium hydroxide.[26]

A negative effect of the pre-treatment with alkaline, as well as the usual inhibitors present, is some of the alkali is consumed by the biomass. This effect causes modification to the cellulose, but the cellulose form produced can be more dense and more stable then the native form.

### *1.3.5 Oxidative Pre-treatment*

This pre-treatment takes advantage of oxidising agents like hydrogen peroxide or peracetic acid in water. These compounds can remove hemicellulose and lignin due to multiple reactions, including cleavage of alkyl or ether linkages, electrophilic substitution or oxidative cleavage of aromatic nuclei.

However, as the oxidant is not selective, then losses of cellulose often occur and some compounds formed from lignin are inhibitors to yeast and enzymes. Peracetic acid, however, is more selective and only oxidises the lignin content as tested on

sugarcane bagasse at ambient temperatures. The yields are improved from 6.8 % to 98 % with this pre-treatment.[26]

Other negatives also include sugars being lost, due to non-selective oxidation and the pH control of the materials is not compatible with enzyme hydrolysis.

### *1.3.6 Ammonia and Carbon Dioxide Pre-treatment*

Ammonia pre-treatment, (referred to as ammonia fiber expansion, AFEX), is conducted with 1:1 biomass loadings at ambient temperatures, lasting for 10-60 days or if completed at 120 °C, then for several minutes to an hour in pressured vessels. The pressure is released causing the ammonia to expand quickly, which causes disruption to the biomass network, aiding hydrolysis. This can cause mechanical disruption but also modification of the hemicellulose and lignin polymers but not cellulose.[64] A six-fold increase in enzyme activity was reported due to swelling of cellulose and delignification, however, this ammonia work is in very early days and any consequences and problems have not been fully studied or reported yet.[26]

$CO_2$ pre-treatment is used at high pressures and temperatures up to 200 °C for several minutes. The effect causes an acidic liquid which can hydrolyse the hemicellulose. Supercritical $CO_2$ can also be used which has been reported to increase yields of glucose in bagasse from 50 to 70 %.[26]

### *1.3.7 Biological Pre-treatments*

The most useful biological source of pre-treatment is white rot fungi, as they can completely degrade parts of the plant cell wall, as the fungi can degrade lignin components to small molecule products.[64] The major benefit of using this approach is it is environmentally friendly. *Phanerochaete chrysosporium* is most commonly used as it contains extracellular oxidative ligninolytic secreted enzymes.[64]

### *1.3.8 Combination Pre-treatments*

It has been reported that a way to improve the steam explosion techniques or hot water extraction is to add acid, as this catalyses the solubilisation of hemicellulose. This improves the solubility as the soaked biomass contains $SO_2$ which is converted to $H_2SO_2$, however, after a while all this is removed, which the acid replenishes.[26]

Another combination method is steam pre-treatment plus alkaline mixtures, often lime is added. This improves the digestibility on low lignin content biomass but not high lignin content. Additionally a benefit to this combination is lime is relatively cheap and safe and that the calcium can be regained afterwards.

Finally, steam pre-treatment has been combined with oxidative pre-treatment. The production of furfural content was low, a benefit for enzyme and yeast treatments down the line. However, parts of the hemicellulose were converted to water and $CO_2$ which can easily be removed, but decreases sugar production.[26]

### *1.3.9 Dehydration products*

In section 1.3 during many of the different pre-treatments, dehydration products have been produced. Figure 20 shows the dehydration pathway of cellulose and hemicellulose to the 4 key dehydration products during pre-treatment; (1) HMF; (2) Furfural; (3) Levulinic acid; (4) Formic acid.

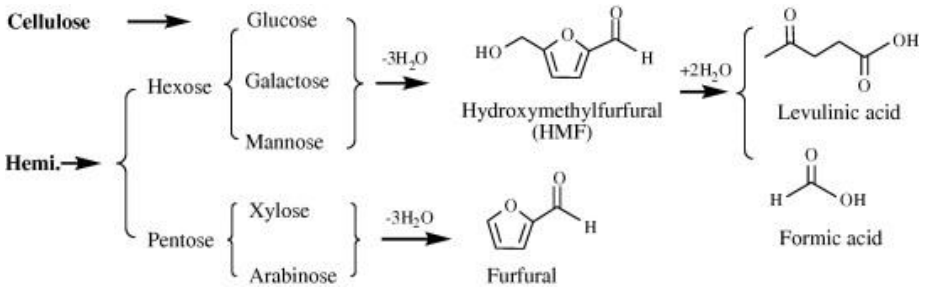


**Figure 20: Dehydration pathway from Cellulose/hemicellulose to levulinic, formic acid, furfural and HMF. Modified from reference 65.**

Section 1.1.3 explained how these chemical products themselves are very useful platform chemicals, however, during pre-treatment they are unwanted due to their negative effect on enzymes and yeast further down the processing pathway.

Figure 21 and Figure 22 show the mechanisms of how sugars can be converted in HMF.

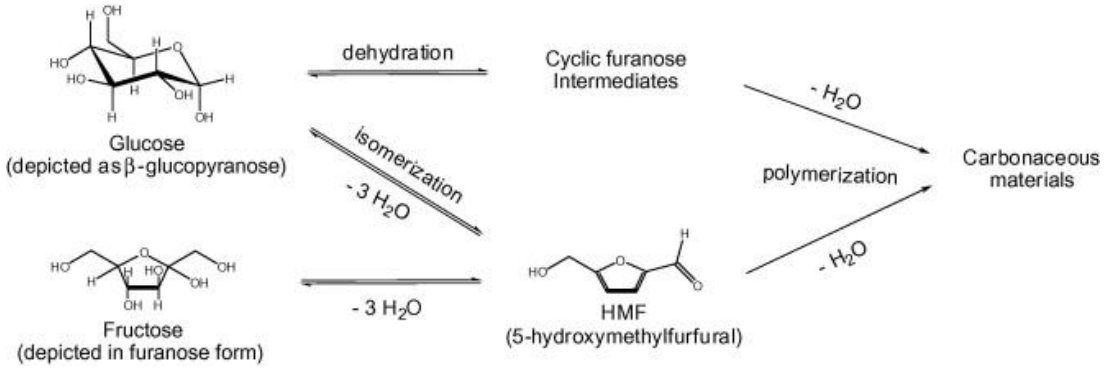


**Figure 21: Sugar units and how they are connected to HMF via chemical pathways.[67]**

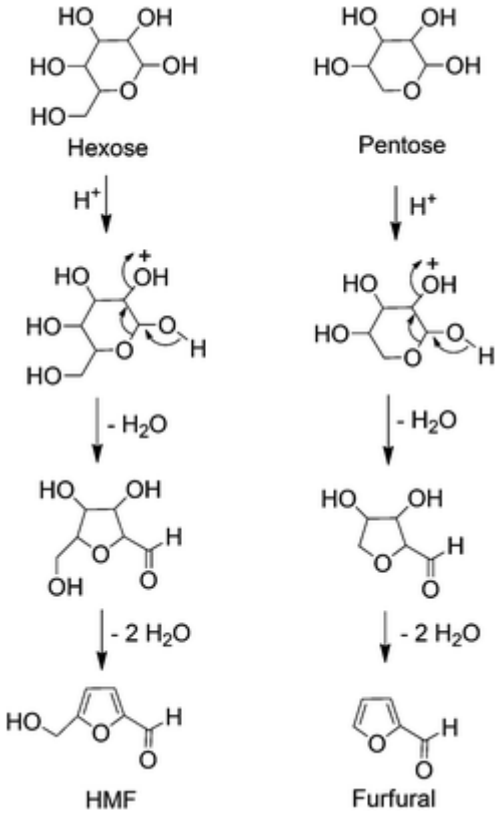


**Figure 22: Dehydration pathway for 6 and 5 membered rings.[8]**

For example, the dehydration of carbohydrates readily leads to substituted furans, levulinic acid derivatives and other functional small molecules.[68]

Figure 23 shows additional dehydration products from sugars, all of which can be produced during plant biomass pre-treatment.

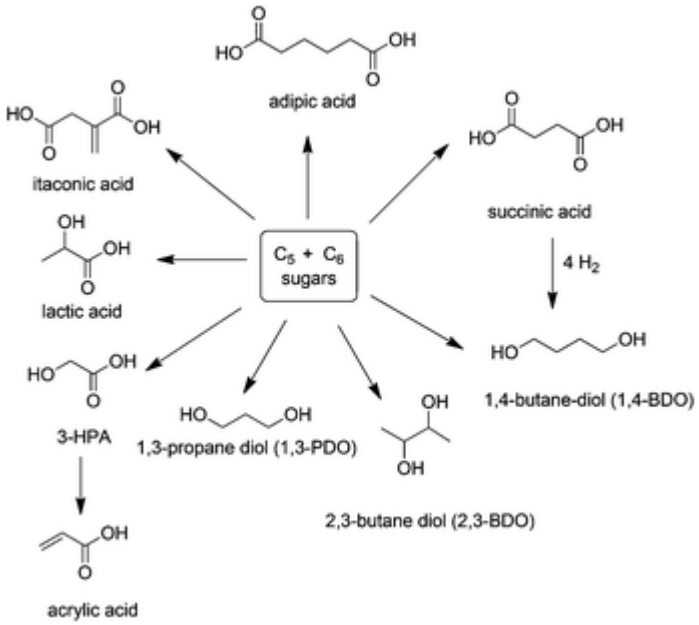


**Figure 23: Additional dehydration products from sugars[8]**

### *1.3.10 Current Industry Pre-treatment Examples*

Table 4 shows current industrial examples of pre-treatments and which ones are used for biomass processing. The main observation is all of these methods are in the pilot stage, as using lignocellulosic materials as renewable chemical sources is in the early stage of development.

**Table 4: A partial listing of companies developing ethanol-from-cellulose technologies from reference 60.**

| Company & headquarters location | Technology | Primary feedstock | Ethanol capacity | Comments |
|---|---|---|---|---|
| **BCI, Dedham, MA** | Dilute acid | Bagasse | 7560 million L/yr | Plant up and running in 2002 |
| **Bioengineering Resources, Fayetteville, AR** | Thermochemical gasification with fermentation | Wood | | Pilot plant operating |
| **Ethxx International, Aurora, ON** | Thermochemical gasification with catalytic conversion | Wood | | Pilot plant operating |
| **Fuel Cell Energy, Lakewood, CO** | Thermochemical gasification with catalytic conversion | Wood | | Pilot plant operating |
| **Iogen, Ottawa, ON** | Enzymatic | Oat hulls, switchgrass, wheat straw, and corn stover | 378 million L/yr (1 million gpy) | Experimental plant operating |
| **Masada, Birmingham, AL** | Concentrated acid | MSW | 3780 million L/yr (10 million gpy) | Plant up and running in 2002 |
| **Paszner Technologies, Inc, Surrey, BC** | Acidified aqueous acetone process | Wood | | Commercial plants under construction |
| **PureVision Technology, Ft. Lupton, CO** | Enzymatic | Wood | | Constructing pilot plant |

### *1.3.11 Pre-treatment Conclusion*

In conclusion, pre-treatment and plant biomass structure modifications, are most successful when lignin and hemicellulose are phase separated, as well as there being an increase in the porosity in the plant cell wall.[69]

Avoiding increasing the cellulose crystallinity, dehydration of sugar molecules and synthesis of HMF impurities, which inhibit enzyme and yeast activity, should all be avoided. Table 5 overleaf, summarises the key advantages of each of the pre-treatment methods discussed in section 1.3.

However the true effectiveness of all pre-treatments is extremely sensitive to the biomass choice and operating conditions. These should carefully be considered as different pre-treatments will be more successful on an individual biomass material.

**Table 5: Advantages and limitations of current conventional pre-treatments of lignocellulosic materials from reference 34.**

| Pre-treatment Process | Advantages | Limitations and Disadvantages |
|---|---|---|
| **Mechanical** | Reduces cellulose crystallinity | Power consumption usually higher than inherent biomass energy |
| **Steam Explosion** | Results in hemicellulose degradation and lignin transformation; cost-effective | Destruction of a portion of the xylan fraction; incomplete disruption of the lignin−carbohydrate matrix; generation of compounds inhibitory to microorganisms |
| **AFEX** | Increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes | Not efficient for biomass with high lignin content |
| **$CO_2$ Explosion** | Increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds | Does not modify lignin or hemicelluloses |
| **Acid Hydrolysis** | Hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure | High cost; equipment corrosion; formation of toxic substances |
| **Alkaline Hydrolysis** | Removes hemicelluloses and lignin; increases accessible surface area | Long residence times required; irrecoverable salts formed and incorporated into biomass |
| **Organosolv** | Hydrolyzes lignin and hemicelluloses | Solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost |
| **Pyrolysis** | Produces gas and liquid products | High temperature; ash production |
| **Biological** | Degrades lignin and hemicelluloses; low energy requirements | Rate of hydrolysis is very low |

### *1.3.12 Using Ionic Liquids for Pre-treatment*

The aim of this project is to replace current methods of pre-treatment with IL pre-treatments. Using ILs as pre-treatment solvents offers advantages over conventional methods mentioned previously, namely; (1) They modify the physicochemical properties of the biomass components, by either reducing cellulose crystallinity or removing lignin content; (2) Isolation of lignin from remaining polymers; (3) Fractionation of carbohydrate polymers from biomass.[1] (4) Ionic liquids have the ability to dissolve numerous biopolymers without derivatising the material. This makes them very flexible and useful to study for a variety of processes.[70]

Figure 24 shows the glucose yield comparison of ILs versus other pre-treatments and how it is comparable depending on the cation and anion choice.

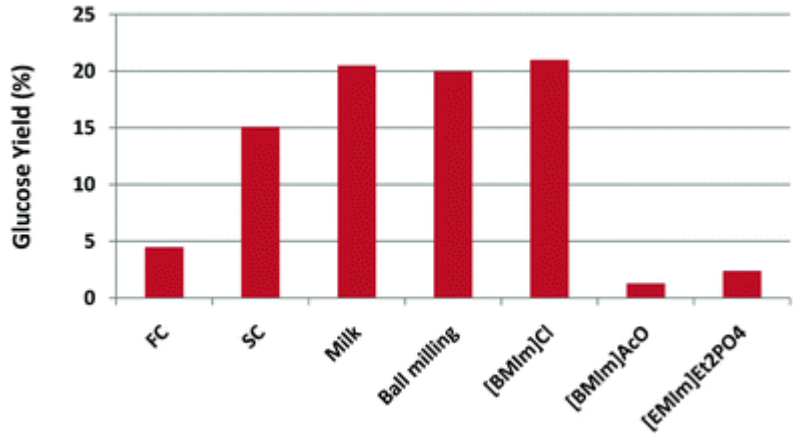


**Figure 24: Effects of pre-treatments on hydrolysis of cellulose using a $SO_3H$ catalyst. Conditions include 0.5 g cellulose, 0.5 g catalyst, 5 mL $H_2O$, 350 W, 150 °C, 1 hour. Modified from reference 57.**

There are many examples of ILs tested with biomass pre-treatment as sections 1.5 and 1.6 explain. However, the current disadvantages with ILs as pre-treatments are; (1) The price of IL precursors are high; (2) Toxicity of ILs is often unknown; (3) Environmental problems with scale up due to acids and bases required in synthesis.[55] Hence the goal of this thesis is to reduce these disadvantages of ILs whilst keeping the advantages.

# 1.4 Ionic Liquids

## *1.4.1 Definition and Classification*

Ionic liquids (ILs) are defined as molten salts that have a melting temperature below 100 °C[71] and at these temperatures are liquids, comprising exclusively of ions.[72] In essence, the only theoretical difference between ILs and molten salts is the temperature they melt at, however, the practical difference of using these materials is significant. Especially with room temperature ionic liquids (RTILs), as these are liquid below 25 °C and hence can be handled in the lab, along with standard organic solvents.[71] Ionic liquids can be liquid in a temperature range from 100 °C to as low as -96 °C[73] and some ILs have a wide liquidus range in excess of 400 °C.[73] ILs are considered as advanced solvents which can be tailored to the requirement or application required, they are classed as designer solvents.[74]

Prominent chemists including Welton, Holbrey and Seddon have reviewed the applications for ILs, which fall mainly into green chemistry and physical chemistry discussions.[72] For the purpose of this thesis, green chemistry is the main objective with regards to biofuel production from lignocellulosic materials, using ILs. Ionic liquids mostly comprise of organic cations and inorganic ions.[71] The different types of the most common cationic salt structures of ionic liquids are *N*-alkylimidazolium, *N*-alkylpyridinium, alkylammonium and alkylphosphonium, shown in Figure 25, as well as some common anions.

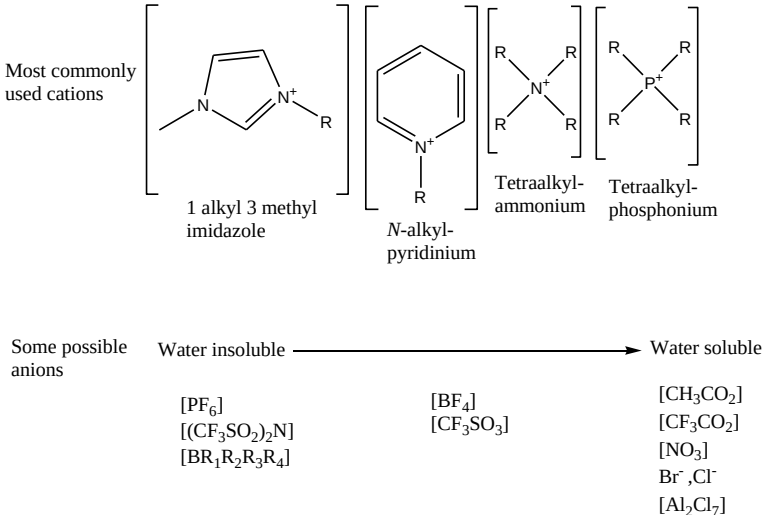


**Figure 25: Examples of cations and anions commonly used in ionic liquid synthesis modified from reference.[73]**

### *1.4.2 History and Discovery*

The history of ionic liquids dates back to the 19th century when the term molten salts was more commonly used to inclusively refer to these molecules[71] and the first report of a room-temperature molten salt, ethylammonium nitrate (m.p. 12 °C), was in 1914.[74] The first generation of ionic liquids was first studied in detail in the 1970's and were the organic chloroaluminates, comprising of aluminium(III) chloride, *N*-alkylpyridinium or 1,3-dialkylimidazolium chloride.[75-72] However, these materials are extremely moisture sensitive and undergo rapid hydrolysis so require an inert atmosphere, hence the next step was to form air and moisture stable ionic liquids.[74] 1-ethyl-3-methylimidazolium soon followed with varying anions as these ILs are air and water stable.[75]

A question has been asked, are they a new class of materials or are they the re-invention of molten salts? Inorganic molten salts are usually cheap due to being sourced from natural minerals, but ionic liquids including imidazole based, can vary expensive, usually ca. £175/kg, from Aldrich. The use of these materials though is a new class and papers in ionic liquid publications have risen significantly over the past few decades.

### *1.4.3 Protic Versus Aprotic ILs*

Aprotic ILs currently are the majority, and were researched more predominately first. The cations are organic with anions either organic or inorganic. They contain no protons around the positively charged centres. The most common examples are imidalolium ILs and there are fewer examples of ammonium salts.[76]

Protic ILs are synthesised *via* simple transfers of a proton from a Brønsted acid to a Brønsted base.[77] There is the potential of proton tunability, due to reversibility of the reaction. Protic ILs can be more conductive then the aprotic cases. A classic example is ethylammonium nitrate (EAN) formed from nitric acid and ethylammonium.[78] Protics are popular because of the easier synthesis, the low cost, the biodegradability but the aprotics are good model systems for the study of physical properties.

### *1.4.4 Physical Properties*

Ionic liquids have a multitude of important and useful properties due to their ionic nature, listed below.

**1. Melting Point**

As by the definition of ILs, the melting point has to be less than 100 °C. Variations, including RTILs, are more useful practically. In the case of the imidazoliums, the longer the R group on the cation the lower the melting point, giving rise to a tuneable melting point. [79] Low melting points are associated with an IL structure that is low in symmetry and has weak intermolecular interactions, such as a lack of H-bonding, and with a good distribution of charge over the cation. For the anion, increasing the size will lower the melting point.[79]

**2. Vapour Pressure**

ILs have no measurable vapour pressure, thus making them environmentally friendly due to no vapour being released into the atmosphere. Problems including azeotrope formation between solvents and products, do not arise in ILs.[79] Currently it is not possible to measure vapour pressure/enthalpies of vaporization of ILs with current technologies.[80] This is because extreme experimental conditions of high temperatures are required, which is close to the IL decomposition temperature. Also, extremely low pressures are required, which are difficult to calibrate.[80]

Due to this negligible vapour pressure, ILs are also non-flammable, another safety benefit. However, a caveat to a non-flammable material is that it is not necessarily non-combustible, hence careful consideration of ILs around heat sources is still important. The current definition of flammability, is linked to the flash point of a liquid, with different regulatory bodies stating different temperatures. However, with ILs forming a new class of solvents where, liquid/vapour/air mix is unlikely, potentially a new definition of flammable solvents is required to truly judge the fire safety of ILs.[81]

**3. Thermal Stability/Heat Capacity**

ILs have high heat capacity, which is limited to the strength of the heteroatom-carbon and heteroatom-hydrogen bonds in the structure. Some quaternary ammonium chloride salts decompose at 150 °C, where as others like [EMIM] Cl decompose much higher at 300-400 °C. [79]

**4. Density**

The general trend with density of ILs, is that it decreases as the size of the organic cation increases. This again, allows IL property tuning with function depending on the cation design. [79]

**5. Viscosity**

Viscosity is based on the ILs ability to form hydrogen bonds and van der Waals forces. An example is the C2-H on the imidazolium cation and a chloride anion. These observations are supported by IR and X-ray spectroscopy. [79] When the IL charge is more distributed, then less H-bonding occurs and lower viscosity is observed. The cation structure is important, as mobile side chains with lower molar mass reduces the viscosity. Long chains or fluorinated alkyl chains, increase the viscosity due to van der Waals forces. Viscosity can also be lowered by either an increase in the temperature or organic co-solvents. [79]

**6. Solubility and Solvation Capabilities**

ILs are great solvents for many applications of chemistry including organic, inorganic and materials chemistry. For example with 1-octene in tosylate melts, the solution becomes more non-polar and can significantly increase solubility. Varying alkyl groups and chains will all affect reactant solubility. [79] Anion tuning for water solubility is possible due to H-bonding interaction differences. IL structures can also be used to tune solvent polarity and the use of [BMIM] [$PF_6$] as a non-polar stationary phase in HPLC has been reported.[79]

## 7. Environment, Clean or Green Process

ILs can reduce side reactions, reduce solvent or catalyst consumption and due to their solubility can form biphasic systems, fill in miscibility gaps, and distillation of volatile compounds is possible. The caveat with ILs in their toxicity and disposal procedures are mostly currently unknown. [79]

All of the benefits above are of course cation and anion dependent, but as this section shows there are many tuneable properties that ILs contain which can be made use of in chemical procedures.

### *1.4.5 Benefits and Challenges*

In chemistry the reliance on solvents is incredible and often forgotten as a major part of all reactions; hence in modern times where the environmental concerns are becoming ever more apparent, the need for newer technologies is present. Solvents can become an issue for the main reason that they are used on such a large scale in industry and because of their volatility they are very difficult to recover.[82]

Ionic liquids are useful because they act as solvents for both organic and inorganic reactions, as well as combinations of a wide range of different reagents. Usually ionic liquids are polar but it is known that increasing the chain length of the alkyl substituent's on either the cation or anion leads to greater lipophilicity of the ionic liquid.[82] Ionic liquids are considered to be 'green' solvents, because they are environmentally friendly solvents and this is primarily due to them being non-volatile. However, they are not to be treated as always environmentally friendly as some can be quite toxic depending on their properties, which cations/anions are used and their synthetic route.

No molecular solvent (other than molten polymers) comes even close to the low volatility of ionic liquids.[71] Having an incredibly low and somewhat negligible vapour pressure makes them more efficient as solvents than your common organic solvents as they are generally safer, simpler to use and can be recovered easily. However, as a word of caution protic ionic liquids can be volatile as they have an acidic proton and as previously mentioned this can equilibrate between the cation and anion of the ionic liquid and the neutral molecular species which consequently with increased temperature readily evaporates.[83] Further advantages of ionic liquids are they have great stability thermally, chemically and electrochemically.[71]

Economically speaking ionic liquids are usually quite cheap, the protics, and consequently are available on industrial scale quantities. However, imidazolium based ionic liquids can be quite expensive and poor recovery of ionic liquids also makes them expensive so these recycling issues need to be improved for wide scale use. Their negligible volatility is also useful with high vacuum line systems where usually contaminants can cause problems with oil pumps, however, equally in this case they can cause problems as they don't evaporate and so can't be removed from the system.[82] Ionic liquids have good solubility with gases like $H_2$ and CO so can be

used in catalytic reactions including hydrogenations and carbonylations.[84] Ionic liquids containing chloroaluminate ions are good Lewis and Brønsted acids.[84]

With all these advantages you would expect to see ionic liquids in wide spread use but one of the main reasons why ionic liquids are not more commonly used by chemists, is due to chemists being unfamiliar with their properties and how they act as solvents. Also some of the ionic liquids have practical issues, like viscosity, as they are usually quite viscous so mixing your reagents with them could increase the time for some reactions to take place.

Based on all of the factors described in section 1.4.4 this leaves ILs with many benefits over organic solvents. Table 6 shows these comparisons of organic and ionic solvents.

**Table 6: Properties of organic and ionic solvents and the potential advantages available, modified from reference.[72]**

| **Property** | **Organic Solvents** | **Ionic Liquids** |
|---|---|---|
| **Number of solvents** | > 1, 000 | > 1, 000, 000 |
| **Applicability** | Single function | Multi-function |
| **Vapour pressure** | Obeys Clausius-Clapeyron Equation | Negligible under normal atmospheric conditions |
| **Flammability** | Usually flammable | Usually nonflammable |
| **Chirality** | Rare | Common and tunable |
| **Catalytic ability** | Rare | Common and tunable |
| **Tunability** | Limited solvents available | Unlimited range "designer solvents" |
| **Polarity** | Conventional concepts apply | Questionable |
| **Solvation** | Weakly solvating | Strongly solvating |
| **Cost** | Normally Inexpensive | 2 to 10 times the cost of organic solvents |
| **Recyclability** | Green imperative | Economic imperative |
| **Density ($g/cm^3$)** | 0.6-1.7 | 0.8-3.3 |
| **Viscosity (cP)** | 0.2-100 | 22-40, 000 |
| **Refractive index** | 1.3-1.6 | 1.5-2.2 |

VOC's like DCM and ethers have significant environmental impacts and their current worldwide usage is around £4 000 000 000 worth p.a. The Montreal Protocol requires re-evaluation of many industrial and commercial processes. Examples include the DuPont Hypalon® plant in Northern Ireland which closed down due to chlorinated hydrocarbon solvents being used.[76]

High solubilities in ionic liquids, means only small reactor volumes are needed. ILs can dissolve organic, inorganic, organometallic and polymeric materials, but not glass.[76] They can be useful for solubilising gases or in catalytic hydrogenations or carbonylations.

They exhibit Brønsted, Lewis and Franklin acidity, as well as superacidity.[76] [EMIM] $AlCl_4$ is a replacement for HF due to its comparative acidity but it is much easier to handle.[84] The comparison of no effective vapour pressure, means ILs are much easier to contain compared to standard organic solvents.[84] Also, water sensitivity in modern ILs does not affect some of the applications of these solvents.[76]

Weakly coordinating anions like $[BF_4]^-$ allow ILs to be polar yet non-coordinating solvents, making them very efficient in stabilising cationic intermediates without reducing the rate of reaction. [84]

**Challenges with ILs**

These can include issues with mathematical modeling, plus temperature and pressure ranges and paramangnetism of some ILs. Current methods can produce errors in the measurment of thermophysical data-acquired in commercial instruments as different structures, hence properties and errors of 3% in density, 5% in thermal conductivity and 20% in viscosity and heat capacities can be noted.[85]

Water content of ILs is extremely important, as the addition of this solvent effects key properties like viscosity and solvation. Challenges are that all ILs are hygroscopic, even the hydrophobic ones, so anhydrous ILs over time hydrate and gain water. Key points to consider with ILs are adsorption of water onto the IL surface, diffusion of water from the surface of ILs to bulk solution and any water-ion complexes formed.[86]

Volatile products can be removed from ILs *via* distillation. If the products are non-volatile, then the use of a solvent is required. Supercritical $CO_2$ can also be used, which would make the process environmentally applicable, if it is possible. [84]

### *1.4.6 Structure and Interactions*

The degree of order in molten salts and ionic liquids is similar to solids, determined by neutron and X-ray diffraction.[87] There is a 10-15% volume expansion during transition from crystalline to liquid state, ion-ion and atom-atom distances are similar. The long range order is not lost due to Coulombic forces between cations and anions of the salts.[88] Coulombic interactions give rise to ion pairs forming and higher ion clusters, shortening interionic distances and lowering the coordination number of ions, resulting in an increased volume upon melting.[88]

In ILs, the asymmetry opposes strong charge ordering due to ionic interactions, causing the system to not crystallise. The second difference of ILs compared to organic solvents is the cooperative network of hydrogen bonds between cations and anions, causing entropic structural directionality.[87] Imidazolium based ILs also have 3D interactions of π-stacking or alternating cation/anion chains.

ILs are not homogeneous solvents due to hydrogen bond networks with polar and non-polar nanodomains, hence indicating 'supramolecular' fluids. They are classed as entropic drivers referred to as the 'IL effect' - spontaneous, well-defined and extended ordering[87-89] Preferential solvation of reactive species in nano-structured domains during any reaction is the outcome of using ILs as solvents.[90]

## *1.4.7 Synthesis and Methodology*

Two basic ways to make ionic liquids are by metathesis, involving a metal or a salt, and acid-base neutralisation, as shown in Figure 26.[73]

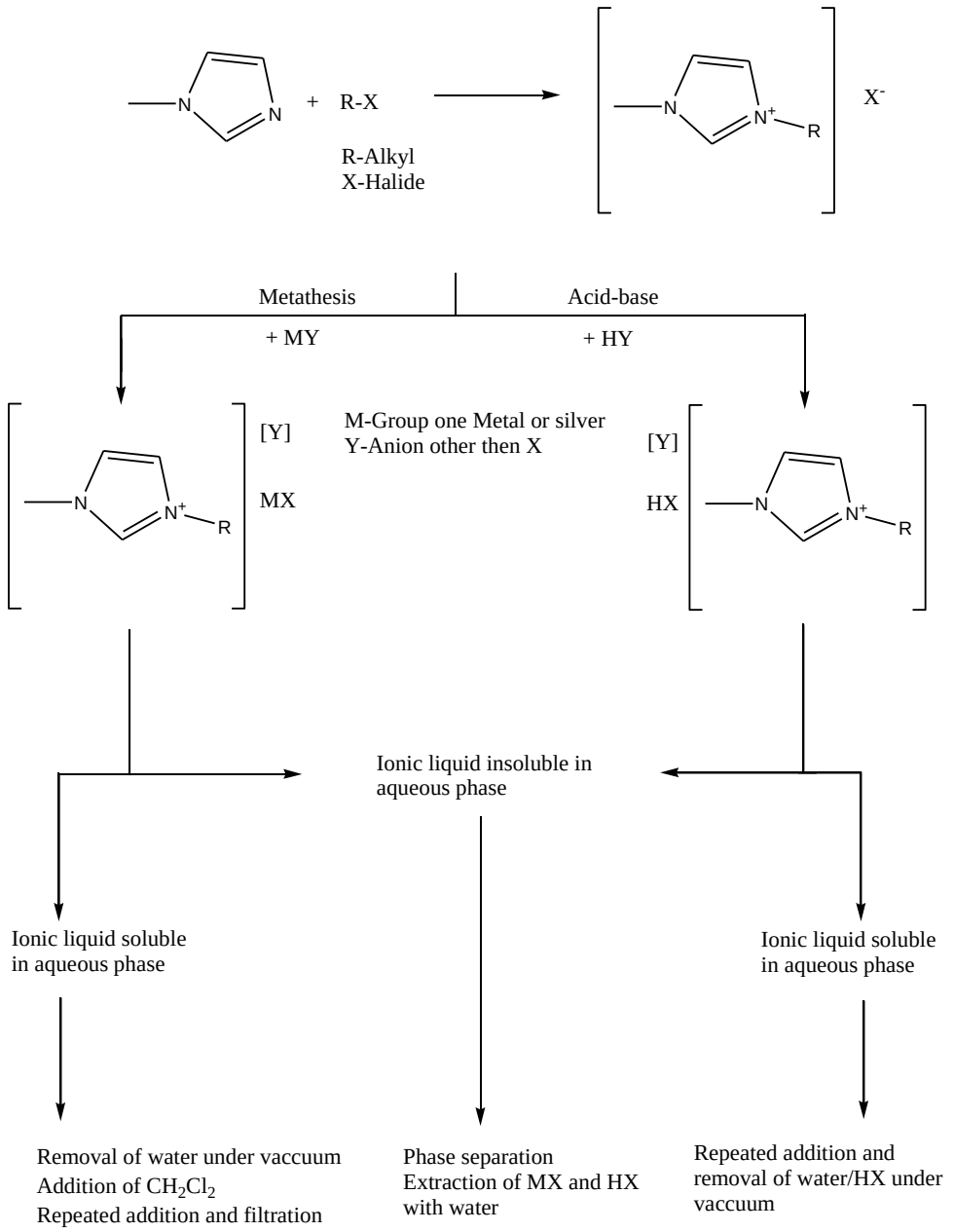


**Figure 26: Ionic liquid synthetic routes modified from reference 73.**

Water and chlorides impurities can effect transition metal catalysts in reactions like hydrogenation. Purity can often be neglected by researchers when synthesising ILs especially for trace level contamination made clear by melting points variations in some cases varying from 15 °C - 5.8 °C,[73] hence results of chemical reactions will be dependent on purity which is often not reported. Physical properties will depend

on purity including viscosity and density. It can be difficult to determine where the impurities come from due to multiple steps in the synthesis but further purification is often quite complicated. Chloride content can be determined by chloride-selective electrode from calibration curves obtained from aqueous solutions. This can be complicated though due to hydrogen bonding in the cation.[73] Issues also begin with water as this can hydrate metals causing reduction in activity, however, as ILs are so hydroscopic storage becomes an important concern.[73]

Metathesis with silver salts produces the cleanest ionic liquids, but requires molar equivalents of AgCl and hence is expensive. Acid-base method will be chloride free but other impurities can be present. $[C_4MIM][PF_6]$ can absorb up to 0.16 mole fraction of water from the atmosphere.[73] Chloride increases viscosity where as water decreases viscosity. Consideration must be considered when synthesising the material of choice.

There are difficulties that occur with ionic synthesis for example the preparation of the [EMIM] cation is much more difficult due to the volatility of the halogenalkane starting material[82] whereas [BMIM] cation starting material is perfectly fine. These problems in synthesis can be easily overcome by using a simple sealed tube system for example. The synthesis of ionic liquids usually isn't that difficult but it's the purity that can be difficult. Purity of some ionic liquids can be achieved by dissolving the liquid in acetonitrile and mixing with activated charcoal for 24 hours. It can then be filtered over neutral alumina. This usually removes any impurities causing colouration.

Ionic liquids are mostly air and moisture stable making them generally fine for use in open air chemistry. However ammonium and imidazolium salts are hygroscopic[82] and therefore in open vessels will pick up water from the atmosphere, so if the solutes being used are air or moisture sensitive then obviously the chemistry needs to be done in an inert atmosphere. Applications of ionic liquids include use in synthesis, catalysis, biocatalysis, separation technology, electrochemistry, analytical chemistry and nanotechnology.[93] Ionic liquids have a wide range of properties making them designer solvents, as they can be changed and modified for certain applications, for example being hydrophobic or hydrophilic.[74] On a side topic some issues with fluorine ionic liquids are that it diminishes the ionic liquids ability at

hydrogen bonding. Also with $[BF_4]^-$ and $[PF_6]^-$ anions care should be taken as they can hydrolyse to form hydrofluoric acid.[79] Hydrogen bonding between cations and anions is important for solvation effects for dissolved particles and transition states in chemical interactions, due to the interaction of a solvating ion and solute competing with the interaction of the counter ions, hence understanding the system will aid design of ionic liquids. In summary, as the previous sections have explained there are many advantages of ionic liquids and if designed efficiently could provide us with some great industrial improvements over the future years to come.

### *1.4.8 Purity*

Having impurities of ions used in the IL synthesis or water can significantly affect the type of research taking place. It has been shown that $Cl^-$ ions can coordinate to metal transition states[73] and water can also do the same, significantly affecting any rates of reaction. Trace level contamination is very difficult to analyse and this in the past has been ignored by many chemists. This can be seen as some ILs have reported literature melting points that can span 10 °C, in the case of [EMIM] $[BF_4]$.[73]

Hence, synthesising ILs pure is not a trivial task and every care must be taken. If the physical properties like melting point are dependent on purity then so will any catalytic effects desired from the IL.

### *1.4.9 Are Ionic Liquids Green?*

There is a strong connection between green chemistry and solvent properties. Due to the negligible vapour pressure there is a wide temperature range where the ILs are a liquid, making them the 'ultimate non-volatile organic solvent'. From this point of view ionic liquids are green.[71]

However, many ionic liquids are synthesised from toxic precursors or are toxic themselves. Further need is being placed on synthesising ionic liquids from renewable sources. Recent examples of this include using levulinic acid and phenol, both capable of being produced from lignin, in order to synthesise Brønsted acidic ILs for ester production.[95]

If an IL in synthesised from renewable chemicals and is inherently non-toxic and biodegradable then it is indeed green, but only when all of those criteria are met.

### *1.4.10 Industrial Applications of Ionic Liquids*

There are many examples of ILs being used in industry and below is just an example of a few current methods;

They can be used in synthetic reactions, separations, extractions, electrochemical, nanotechnology, biotechnology and engineering.[96] In biocatalysis, in 1984, the enzyme alkaline phosphatase is relatively stable in a 4:1 (v/v) mixture of the IL triethylammonium nitrate mixed with water.[84]

Reversible ionic liquids are also possible, a recent example for the acylation of cellulose has been reported[97] involving a heterocyclic ring system with $CO_2$ and methanol as reactants in the solvent DMSO, which can all be converted back to molecular materials and distilled to be separated. The IL acts as a catalyst and can be fully recycled.[97]

Ionic liquids are helping to solve many environmental challenges too, including cellulose and biomass pre-treatment as explained further in sections 1.5/1.6. Research is currently being undertaken in depolymerisation of non-natural polymers by recycling waste plastics to get access to nylon for example. Another important area is recovering metals including silver and palladium from complex slags and alloys using ILs. Finally $CO_2$ capture due to the rise in emissions from sources including fossil fuels is a worldwide problem and initial research has shown promise at using ILs to solve this issue dissolving 1 mol of $CO_2$ per mol of IL.[98]

ILs are useful solvents for biomolecules because they can increase shelf life of proteins, increase thermal stability, enhance folding of proteins and can act as additives in protein crystallisation.[99]

# 1.5 Cellulose and Ionic Liquids

## *1.5.1 The Start of a New Process*

The history into cellulose solubility in ionic liquids actually began as early as 1934, when Graenacher discovered that you could use molten N-ethylpyridium chloride, in the presence of nitrogen-containing bases, to dissolve cellulose.[100] The reason behind this solubility was most likely due to the high chloride concentration which is able to break the strong hydrogen bond network within the cellulose polymer units and hence forth this aids dissolution.

This hydrogen bond breaking effect is a driving force when considering the design of the ionic liquids for cellulose dissolution, as the interaction of the basic anion to hydrogen bond to cellulose is crucial at disrupting the highly ordered intra- and intermolecular hydrogen bond network.[101] Therefore hydrophilic ionic liquids like [BMIM]Cl and [AMIM]Cl have previously been used and show high activity at dissolving cellulose, achieving up to 25% saturation under currently optimum conditions.[100,102]

The role of the cation is less understood in cellulose dissolution but it is believed that electrostatic interactions do occur and the cation does play an active role in solubility. This role could be the strength of the interaction between cation and anion and hence, how does this affect the ability of the anion to interact with cellulose? It is most likely if any interaction between the cation and the polysaccharide takes place, then it is a Van der Waals interaction and is a fraction of the overall energy compared to anion-carbohydrate interaction.[103]

## *1.5.2 What Makes a Good Cation and Anion for Cellulose Dissolution?*

Figure 27 and Figure 28 show the structures of effective and ineffective IL's for cellulose solubility recently reported.[104] The effective cellulose solvents, comprises of imidazolium and pyridinium cations.[104] These are planar structures with a delocalized positive charge within the ring. The anions are all basic, fairly small, with good ability to form hydrogen bonds, some with multiple acceptor sites.

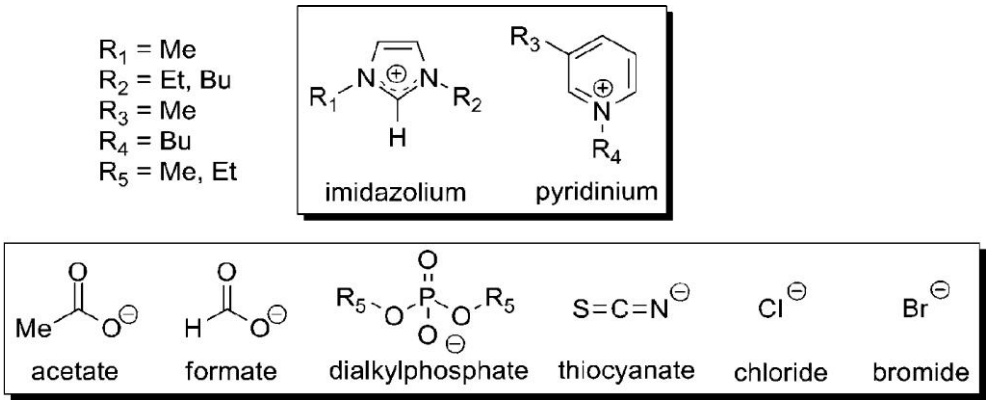


**Figure 27: Effective ILs for cellulose solubility modified from reference [104]**

Figure 28, the ineffective cellulose solvents, have piperidinium and pyrrolidinium as cations.[104] Pyrrolidinium and piperidinium do not delocalise the positive charge and have tetrahedral bulky structures. However, it's the anion which is more important in cellulose solubility and these anions are very bulky with their ability to hydrogen bond being poor; hence these are ineffective IL's for cellulose dissolution.

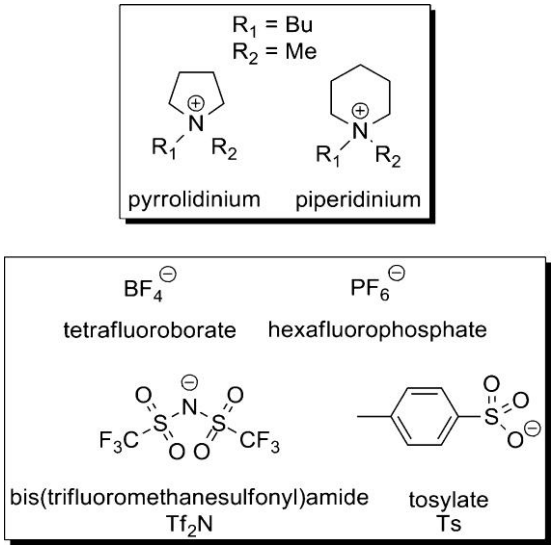


**Figure 28**: **Ineffective IL structures for cellulose solubility modified from reference [104].**

Cellulose pre-treated in [BMIM] containing Cl, Br, [SCN], [OAc] and other moderately basic anions have previously been explored and dissolve the cellulose successfully forming viscous solutions.[105] [BMIM]Cl is recorded to work the best, due to its short alkyl chain length and basic anion. Acetate anions are a close second to chloride and as they are less corrosive than chloride anions, and hence acetates are preferred to be used in reactions.[64] Longer cation chains are less efficient at dissolving cellulose as they reduce the effective chloride concentration within the liquids and hence reduce the effect of breaking down the hydrogen-bond network. Also with increased chain length the molecule becomes more hydrophobic, making the interaction with cellulose less likely. [105]

Table 7 shows most of the currently tested imidazolium ionic liquids and their percentage solubility with various IL anions.[106] It further illustrates that short chain imidazolium ions and hard, basic, nucleophilic anions are the best at dissolving cellulose.

**Table 7: Overview of the results from the dissolution studies for imidazolium based ionic liquids.**[106]

| IL/anion | $C_2$MIM | $C_3$MIM | $C_4$MIM | $C_5$MIM | $C_6$MIM | $C_7$MIM | $C_8$MIM | $C_9$MIM | $C_{10}$MIM |
|---|---|---|---|---|---|---|---|---|---|
| $F^-$ | 2% | | | | | | | | |
| $Cl^-$ | 10–14% | no sol. | 20%[a] | 1% | 6% | 5% | 4% | 2% | no sol. |
| $Br^-$ | 1–2% | 1–2% | 2–3% | 1–2% | 1–2% | 1% | 1% | 1% | no sol. |
| $I^-$ | | | 1–2% | | | | | | |
| $SCN^-$ | | | 5–7% | | | | | | |
| $BF_4^-$ | | | no sol. | | | | | | |
| $PF_6^-$ | | | no sol. | | | | | | |
| $NO_3^-$ | | | no sol. | | | | | | |
| $NTf_2^-$ | | | no sol. | | | | | | |
| $F_3CSO_3^-$ | | | no sol. | | | | | | |
| $EtSO_4^-$ | no sol. | | | | | | | | |
| $(CN)_2N^-$ | no sol. | | | | | | | | |
| $TsO^-$ | 1% | | | | | | | | |
| $AcO^-$ | 8% | | 12% | | | | | | |
| $R_2PO_4^-$ | 12–14% | | no sol. | | | | | | |

[a] 25% under microwave irradiation according to Rogers *et al*[BILD82]

Plotting the results from Table 7, of solubility versus the chain length of the imidazolium Cl IL's, leads to the odd-even effect as shown in Figure 29.

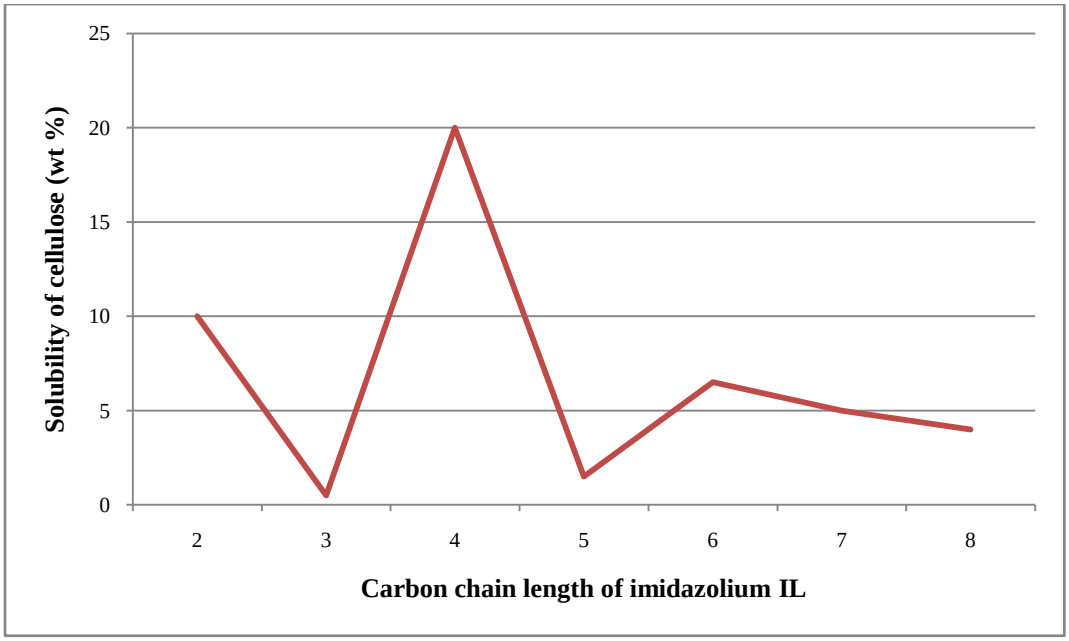


**Figure 29: Imidazolium Cl chain length versus cellulose solubility from reference 106.**

As can be seen in Figure 29, a very large odd-even effect is observed, before cellulose solubility drops off with the longer chain lengths of imidazole.[107] The reduction in solubility with increased chain length has already been explained but the initial odd-even effect is quite strange. Within nature, odd-even effects are present so this is not unusual, but the extreme differences in this case between 2, 3 and 4 carbons is quite unusual. To go from 0.5 % solubility with 3 carbons to 20 % with 4 carbons is very drastic and as of yet this trend has not been explained in the literature.**[108]**

Table 8 overleaf, shows the ILs tested to date for cellulose dissolution and the conditions used. Methods currently explored in cellulose dissolution involve conventional and microwave heating. Conventionally ca. 100 °C is used to dissolve cellulose as less than this seems to be inefficient at solubility. A potentially more energy efficient way is to use microwave heating as this can significantly accelerate the dissolution process and requires less energy input, however, care must be taken as over use can cause ionic liquid decomposition and this will become even more of an issue if using protic ionic liquids.[109]

As well as Table 8, appendix 1 shows an extensive comparison of ILs used so far on solubility studies on cellulose, polysaccharides, oligomers and monomer sugars.

**Table 8: Selected results for cellulose dissolution in ILs[28] For the cellulose column, the numbers in brackets correspond to the DP values where known.**

| IL | Cellulose | Method | Solubility (wt %) |
|---|---|---|---|
| **[$C_2$mim]Cl** | Avicel | Heat, 100 °C | 10 |
| **[$C_3$mim]Cl** | Avicel | Heat, 100 °C | 0.5 |
| **[$C_4$mim]Cl** | Avicel | Heat, 100 °C | 20 |
| **[$C_4$mim]Cl** | Pulp (1000) | Heat | 10 |
| **[$C_4$mim]Cl** | Pulp (1000) | Microwave | 25 |
| **[$C_5$mim]Cl** | Avicel | Heat, 100 °C | 1.5 |
| **[$C_6$mim]Cl** | Pulp (1000) | Microwave | 5 |
| **[$C_6$mim]Cl** | Avicel | Heat, 100 °C | 6.5 |
| **[$C_7$mim]Cl** | Avicel | Heat, 100 °C | 5 |
| **[$C_8$mim]Cl** | Avicel | Heat, 100 °C | 4 |
| **[Amim]Cl** | Pulp (650) | Heat, 80 °C | 14.5 |
| **[Amim]Cl** | MCC | Ultrasound | 27 |
| **[$C_4$mmim]Cl** | Pulp (569) | Heat, 90–130 °C | 12.8 |
| **[$C_4$mmim]Cl** | Pulp (286) | Heat, 90 °C | 9 |
| **[$C_4$mmim]Cl** | Pulp (593) | Heat, 90 °C | 6 |
| **[$C_4$mmim]Cl** | Pulp (1198) | Heat, 90 °C | 4 |
| **[$C_4$mPy]Cl** | Pulp (593) | Heat, 105 °C | 37 |
| **[$C_4$mim]Br** | Pulp (1000) | Microwave | 5–7 |
| **[Ammim]Br** | Pulp (286) | Heat, 80 °C | 12 |
| **[Ammim]Br** | Pulp (593) | Heat, 80 °C | 4 |
| **[Ammim]Br** | Pulp (1198) | Heat, 80 °C | 4 |
| **[$C_4$mim][SCN]** | Pulp (1000) | Microwave | 5–7 |
| **[$C_2$mim][OAc]** | Avicel | Heat, 100 °C | 8 |
| **[$C_2$mim][OAc]** | Avicel (225) | Heat, 110 °C | 28 |
| **[$C_4$mim][OAc]** | MCC | Heat, 70 °C | 28.5 |
| **[$C_4$mim][OAc]** | Avicel | Heat, 100 °C | 12 |
| **[Amim][HCOO]** | MCC | Heat, 85 °C | 22 |
| **[$C_4$mim][HCOO]** | MCC | Heat, 70 °C | 12.5 |

Table 8 continued.

| **IL** | **Cellulose** | **Method** | **Solubility (wt %)** |
|---|---|---|---|
| **$[C_4mim][HCOO]$** | Avicel (225) | Heat, 110 °C | 8 |
| **$[C_4mim][(C_6H_5)COO]$** | MCC | Heat, 70 °C | 12.0 |
| **$[C_4mim][(NH_2)CH_2COO]$** | MCC | Heat, 70 °C | 12.0 |
| **$[C_4mim][OHCH_2COO]$** | MCC | Heat, 70 °C | 12.0 |
| **$[Bu_4P][HCOO]$** | Avicel (225) | Heat, 110 °C | 6 |
| **$[C_4mim][HSCH_2COO]$** | MCC | Heat, 70 °C | 13.5 |
| **$[C_2mim][(CH_3CH_2O)_2PO_2]$** | Avicel | Heat, 100 °C | 12–14 |
| **$[C_1mim][(CH_3O)_2PO_2]$** | Avicel | Heat, 100 °C | 10 |
| **$[C_2mim][(CH_3O)(H)PO_2]$** | MCC | Heat, 45 °C | 10 |
| **Key for abbreviations of IL cations: $[C_nmim]^+$, 1-alkyl-3-methylimidazolium (*n* = number of carbons in the alkyl chain); $[C_nmmim]^+$, 1-alkyl-2,3-dimethylimidazolium (*n* = number of carbons in the alkyl chain); $[Amim]^+$, 1-allyl-3-methylimidazolium; $[Ammim]^+$, 1-allyl-2,3-dimethylimidazolium; $[C_4mPy]^+$, 1-butyl-3-methylpyridinium; $[Bu_4P]^+$, tetrabutylphosphonium.** | | | |

## *1.5.3 How do the IL Interactions Aid Cellulose Dissolution?*

Figure 30 shows the interaction of [BMIM]Cl with cellulose and a schematic of the hydrogen bonds which are formed.

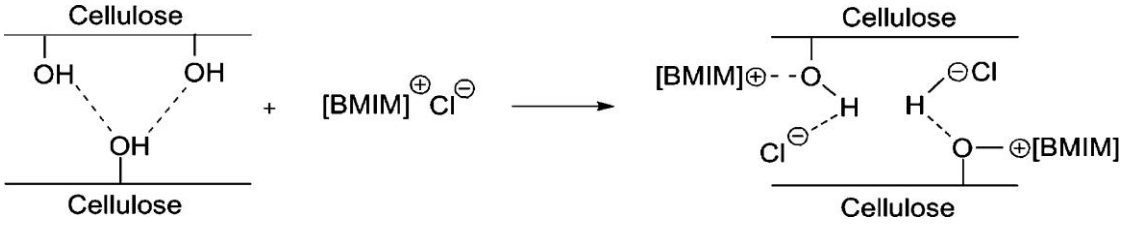


**Figure 30: Schematic representation of interactions of [BMIM] Cl on surface of cellulose[110]**

Mechanisms of Cl anions have been proved by $^{35/37}$Cl NMR relaxation studies.[110] [BMIM] Cl displays the highest hydrogen bonding basicity among the most common ionic liquids. [BMIM] salts which bear weaker hydrogen bond acceptors or non-coordinating groups as anions, are poor cellulose solvents.[110] Cellulose dissolution has been confirmed on the atomic level by high-resolution $^{13}$C NMR studies. [100] $^{13}$C NMR studies of cellulose in [BMIM] Cl solution show that the polymer is disordered in this medium, indicating that its hydrogen bonding network is indeed disrupted.

Cellulose can be regenerated from the ionic liquid by using polar protic organic solvents like ethanol, acetic acid or water and is reportedly done so with the same degree of polymerisation and polydispersity as previously, but the morphology is changed and the cellulose microfibrils are fused into a homogeneous macrostructure.[100]

It has been determined for [EMIM] [OAc] the hydrogen bond formation is the major driving force for cellulose solubility. Hydrogen atoms of cellulose hydroxyl groups H-bond with [OAc] and oxygen atoms of cellulose hydroxyl groups H-bond with H2 position on [EMIM].[111]

One additional thing which is important in understanding IL dissolution is knowledge of the water concentration because water will decrease cellulose solubility, as water competitively hydrogen bonds to cellulose microfibrils.[100]

However, having a completely water free sample is unnecessary (as well as unlikely) as some water being present is essential at preventing the $Cl^-$ ions from destroying

the carbohydrate rings and forming dehydration products. This is discussed further in section 1.5.4.

There are various methods which can be used to recover IL after the cellulose has been precipitated out and separated which include evaporation, ionic exchange,[112] ion-exclusion chromatography,[100] pervaporation, reverse osmosis and salting out.[110] However, in general it has been reported that complete reuse and recycling is not always possible depending on the IL's used. The ability to find an IL which is bio-degradable is also a goal within this research. Figure 31 shows all the potential issues with IL's for cellulose solubility which must be overcome where possible for this thesis.

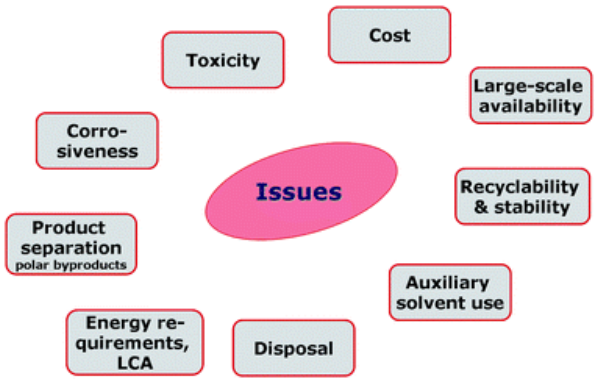


**Figure 31: General issues to be addressed, relating to ionic liquids in biomass processing modified from reference 111.**

Protic ionic liquids, including [DEA] Cl, are extremely rare in the cellulose literature. Virtually no research work for lignocellulose degradation has been complete and this is most likely due to the complexity of the protic IL's and their potential equilibrium from IL back to acid/base mixtures. However, one paper does report a study of diethanolamine and triethanolamine with formic acid and acetic acid and concludes that their ability to dissolve cellulose is inefficient.[113] The study is purely a qualitative one where mixtures of cellulose and IL formed a turbid dispersion. Their use in research on plant biomass has yet to be complete. These ILs offer reduced cost and increased bio-degradability over aprotic imidazolium ILs.

In conclusion the required factors for cellulose dissolution are;[104]

(1) A cation which is aromatic and can delocalize the positive charge.

(2) A second heteroatom in the ring, which can be favourable and generate dipoles between IL and polymer.

(3) The anions ability to hydrogen bond is crucial.

(4) Anions which are small in size or have several hydrogen bonding positions is beneficial.

### *1.5.4 Cellulose and Water Interactions*

Performing high yielding hydrolysis of cellulose into monosaccharides should, when using water in a chloride based ionic liquid which also contains an acid catalyst, lead to 90% yields of glucose from cellulose.[56] Pre-treating cellulose in $H_2SO_4$ and [EMIM] Cl has been performed but this produced 5-hydroxymethylfurfural (HMF) with moderate yields of glucose.[56]

Water plays an important role in preventing the dehydration reactions of glucose by the $Cl^-$ ions, as the water solvates $Cl^-$. A simple trade off occurs between using water to stabilise glucose as a product and stopping dehydration of the glucose, as well as ensuring complete solvation of cellulose in the first place to obtain a maximum possible yield. Hence gradual addition of water over a time period of ca. 2 hours has been required to achieve this goal.[56]

An experiment in hydrolysing crude biomass with the same $Cl^-$ based ionic liquid, enabled the intermolecular polymeric interactions to be disrupted and produced a 70% yield of glucose and 79% yield of xylose using a two stage process.[56] The benefits of this included an inexpensive chemical catalyst rather than enzymes, production of high sugar yields in only a few hours at only 105 °C and it avoided hazardous concentrated acids by using catalytic amounts of dilute acid. The potential drawbacks of this process could be highly viscous biomass which might require special handling and on a large scale could cause issues, plus water evaporating costs could be a big issue. A final note is that as imidazolium ionic liquids are expensive and so the complete recovery of them will be required to make the process economical viable.[56]

This section demonstrates how water can be useful and problematic in IL cellulose pre-treatment but is a factor that should be considered when designing the pre-treatment. It also implies that the need for ILs to be water free is not necessary.

The use of hydrophobic ionic liquids has shown the ease of separating glucose after hydrolysis by simple water extraction and it being IL free, in a 51% yield. Microwaves efficiently accelerated the reaction time to only 15 minutes, however, for these hydrophobic ILs to work effectively LiCl was added in 12 M HCl, which is not sustainable. [114]

## *1.5.5 Cellulose Hydrolysis and Degradation*

As previously mentioned in section 1.3, pre-treatment methods are required in converting cellulose into a more accessible form so it can be hydrolysed and converted into fuels.

The critical concentration for cellulose dissolution changes depending on which acid is used. Sulfuric acid is ca. 60-65% by weight; hydrochloric acid is ca. 40% by weight and phosphoric acid is ca. 83% by weight.[58] The acids break down cellulose into oligomers, then monomers and finally dehydration products. Rates of acid hydrolysis of glycosidic bonds in polysaccharides can vary greatly between the positions of glycosidic bond, for example 1 - 6 linkages in cellobiose are much harder then 1 – 2 linkages to hydrolyse.[59]

Alkaline degradation is also possible but the mechanisms are much less understood, although it is known that alkaline degradation occurs at the reducing end of the polysaccharide.[59] As well as breaking the polysaccharides down into monomers, again the monomers themselves can be broken down. The standard potential small molecule degradation products of cellulose in ionic liquid with acid present, are shown in Figure 32.

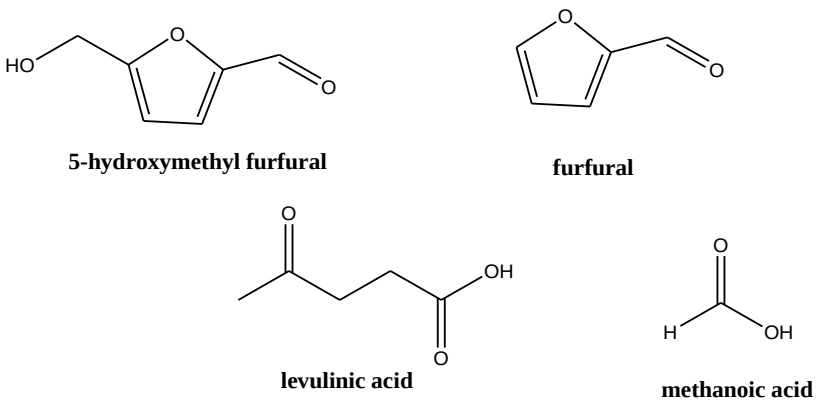


**Figure 32: Potential degradation products of cellulose in ionic liquid[115]**

5-hydroxymethyl furfural (HMF), furfural and levulinic acid are some of the main products possible to form upon breakup of the monomer sugar ring as previously discussed.[61] The results of cellulose hydrolysis can be proven using HPLC-MS, NMR and FT-IR. The change of structure and functional groups enables these

techniques to monitor the change, as the C=O bonds in particular are distinctive at proving production of the furfurals for example.

Acidic ionic liquids, like the one shown in Figure 33 (A), are an example of mutually compatible solvents. The imidazolium based ionic liquid component can solubilise cellulose, enabling the glycosidic linkages to become easily available for the acid component to hydrolyse them. This system can produce up to 24 % HMF and 17 % furfural with a 84 % conversion rate of microcrystalline cellulose at 150 °C.[116] The potential of developing this multi-functional IL could prove to be very important for the future, however, ionic liquid recycling issues could limit the reproducibility of such catalysts, because further runs showed that the catalyst conversion rates reduced significantly.[116]

Another ionic liquid in Figure 33 (B), is N-methylmorpholinium methyl sulfonate $[NMM]^+ [CH_3SO_3]^-$ and it is a protic ionic liquid which was found to show high catalytic activity on sucrose degradation to HMF.[61] Sucrose is a disaccharide comprised of glucose and fructose (a five membered sugar ring). A 47.5 % yield of HMF was achieved using this ionic liquid.[61] Fructose is dehydrated into HMF using [BMIM] Br with 100 % conversion.[117] An example of how ILs are used on cellulose/oligomer pre-treatments to produce chemicals.

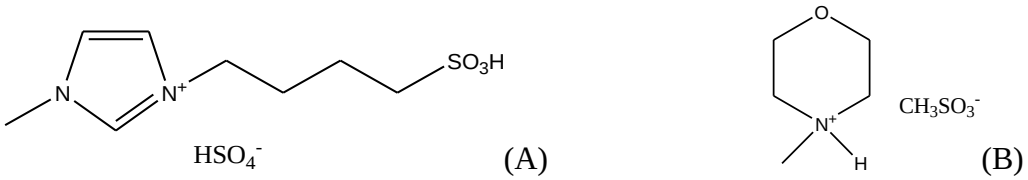


**Figure 33: 1-(4-sulfonic acid) butyl-3-methylimidazolium hydrogen sulfate (A) and N-methylmorpholinium methyl sulfonate (B).[61]**

Finally, it has been reported [BMIM] [OAc] does not behave as an inert solvent during cellulose pre-treatment. Using a $^{13}C$-isotopical label and a fluorescence label on 1-alkyl-3-methylimidazolium acetate, the ionic liquid has been shown to react at the C-2 reducing position of cellulose to form a carbon-carbon bond.[118] This reaction occurs more so with catalytic amounts of base present, like imidazole impurities.

Figure 34 shows the IL-carbohydrate unit and the * shows the position of the $^{13}C$ label.[118]

This result is relatively minor compared to the amount of units in the cellulose chain, however, it is unknown if this reaction occurs with some of the other keto/aldehyde groups and for derivatives of cellulose required for medicinal purposes these minor impurities can cause adverse effects.[118]

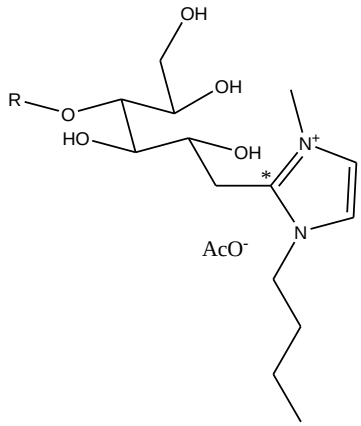


**Figure 34: *N*-butyl-*N*-methylimidizolium acetate to "sugar" structure.[118]**

### *1.5.6 Cellulose and Enzymes*

Enzymes are very important when it comes down to breaking apart the lignocellulosic structure in plants and the degradation of the linkage between lignin-carbohydrate compounds and their mechanisms, is important in understanding their chemistry, structure, uses and hence efficiency. Enzymes that rupture the ester linkage between carbohydrate molecules and phenolic acids such as ferulic acid (feruloyl esterase) and *p*-coumaric acid (*p*-coumaroyl esterase)[66] are considered as a new and important area of research because the esterases are suspected to play an important role in plant biodegradation and their detailed examination could provide further insight into cell wall structure.[66]

Cellulases are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (beta-glucosidase, EC 3.2.1.21) shown in Figure 35.[119, 120] The first two enzymes act directly on cellulose, yielding mainly cellobiose and glucose as the reaction products. The cellobiose is then hydrolysed to glucose by cellobiase.**[119]** Cellobiohydrolase degrades crystalline cellulose most efficiently. Both endoglucanase and cellobiohydrolase degrade amorphous cellulose.

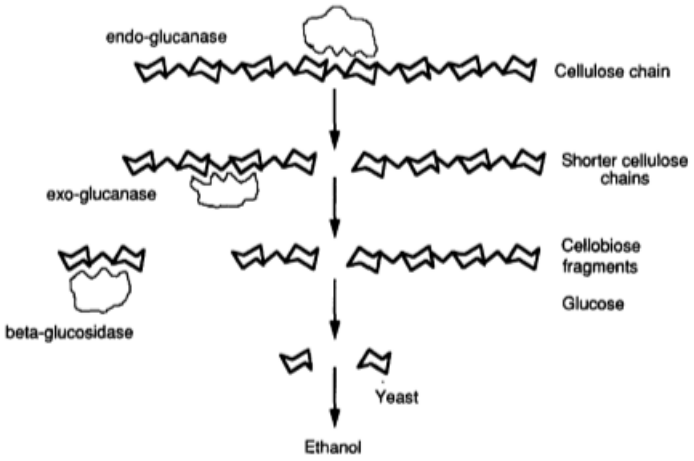


**Figure 35: Plant biomass to ethanol with enzyme functionality shown, modified from reference 53.**

Pre-treatment of cellulose with ionic liquid can become crucial for enzyme hydrolysis as this unravels the crystalline structure into an amorphous structure so

enzymes can reach the glycosidic bonds. This residual crystallinity will enhance saccharification, producing the glucose units.[9]

Saccharification, is the enzymatic breakdown of complex carbohydrates, like cellulose, into monosaccharides and this is explained further in chapter 2. The properties which make IL's good at lignocellulose solubility; high polarity, water solubility and negligible vapour pressure, will also obstruct the separation and complete recovery of the carbohydrates produced.[121] In order for this system to work on a large scale, good recovery is very important. Also, as it has been shown previously, $Cl^-$ and $[OAc]^-$ ions are needed for cellulose solubility, however, they also hold on to cellulose strongly preventing the enzymes from breaking down the structure and equally it can then denature them.[122] The enzymes performance in aqueous solutions can be greatly affected by inorganic species following the trend of the Hofmeister series.[123, 124]

The ability to reduce the effective concentration of the anion would be an advantage to the enzymes and so increasing the chain length on the cation would do this, however, by default this reduces the solubility of cellulose, as previously explained, in the ionic liquid and increase the viscosity.[73] Hence, one improved suggestion was to use glycols as they have low viscosities and melting points and still dissolve cellulose. Poly(ethylene oxide)s have therefore been included into the cationic or anionic designs.[122]

Another improvement is using hydrophobic IL's which can be used as a protective coating on cellulase enzymes, enabling this complex to be stable in cellulose dissolving IL's like [BMIM] Cl.[125] Figure 36 shows this system and how it works.

As previously stated [BMIM] Cl is one of the current successful ionic liquids for cellulose dissolution, however, it is poor at maintaining cellulase activity for the saccharification of cellulose. Hydrophobic IL's, for example [BMIM] $Tf_2N$, are good for retaining water molecules in the protein folded structure, which is essential for keeping the water shell protein microenvironment. This basically immobilises the enzyme with the hydrophobic IL and provides a source to act on the cellulose, however, a lot of IL can cause mass-transfer limitations from the bulk media to the enzyme, as well as mechanical stability depending on the thickness of the IL layer.[125]

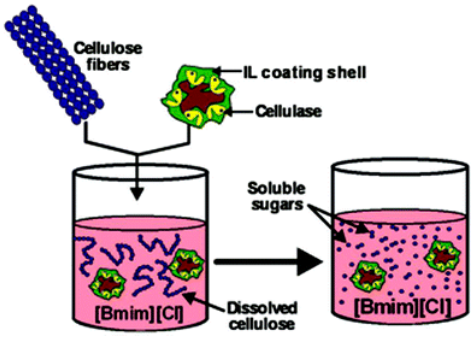


**Figure 36: Saccharification of cellulose dissolved in BMIM Cl catalyzed by immobilised cellulase which is coated with a hydrophobic IL modified from reference [125].**

## *1.5.7 Lignin in Ionic Liquids*

The most efficient ionic liquid for selective lignin dissolution has been reported as *N*, *N*-dimethylethanolammonium formate ([DMEA] formate), as shown in Figure 37.[75] The important reason for this is because lignin is a strong hydrogen bond acceptor due to its phenolic ether oxygen's and hence this forms a good interaction between solvent and solute. [DMEA] formate is also good as the chosen ionic liquid because of its strong electrostatic interactions which occur between the cation and anion in each pair but not between the ion pair and its neighbours in the bulk solvent.[75]

Successful isolation of lignin from artificial wood has been achievable but only due to the fact that no covalent bonds were present between lignin and cellulose. Other ionic liquids including [BMIM] Cl, [AMIM] Cl and [EMIM] [OAc] were also successful at dissolving lignin.[126] Although the chloride anions do have strong and successful abilities to dissolve lignin, as previously stated, they also dissolve cellulose and hence are not very selective resulting on poor separation when biomass is used, whereas the formates and acetates show high lignin solubility and selectivity.

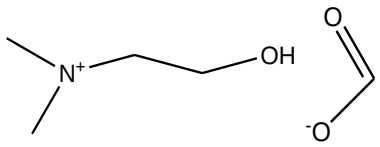


**Figure 37: Dimethylethanolammonium formate.**

[EMIM] [OAc] was used on biomass to remove ca. 40% of the lignin material and after this cellulase enzymes efficiently degraded cellulose into monomers. [EMIM] [OAc] has been shown to extract lignin from wood flour as well.[126] Complete lignin removal was not essential, as after 40% removal maximum cellulose digestibility was recorded.[126]

It is difficult to study lignin directly due to its non-uniformity, however, the use of model compounds such as alkene-substituted aromatics and simple ethers, which are more reactive then lignin, are hence deceptive model compounds.[127] Less reactive 4-ethylguaiacol is better for reactivity of lignin alkyl linkages when studied.

Challenges to address with lignin pre-treatment study as well as the source, include lignin self-condensation reactions.[127] However, as the goal in this thesis is to access the sugar part of biomass, further consideration of IL lignin pre-treatment is not considered.

## 1.6 Plant Biomass and Ionic Liquids

As well as studies on cellulose, ionic liquids have been used to study solubilisation and pre-treatment of plant biomass materials. The most common ionic liquid studied with lignocellulosic materials is the imidazolium based ILs, especially $[BMIM]^+$ and $[EMIM]^+$. The first success at dissolving wood powder in [BMIM] Cl was achieved by Rogers and co-authors in 2007.[128] This study showed how [BMIM] Cl was capable of partially dissolving un-treated wood and that the cellulose regenerated maintained the same purity and properties of before.[128] This IL dissolved both cellulose and lignin during the pre-treatment, breaking the non-covalent bonds, allowing for polysaccharide rich material to be hydrolysed. Although successful, [BMIM] Cl shows moderate toxicity and expense, so other ILs were sought after for this type of work.

Shortly after [BMIM] Cl, further studies with [AMIM] Cl also showed lignocellulosic solubility.[27] All of the ILs tested though, could only dissolve a small amount of biomass, and even then, the dissolution was never complete. Light scattering techniques were used to test ILs for biomass solubility and Kamlet-Taft parameters showed how the hydrogen-bond basicity of the IL, determined by the anion, was crucial for either solubility or biomass swelling.[27]

Table 9 summarises some of the initial key findings with ILs.

**Table 9: Solubility of lignocellulosic powders in ionic liquids during thermal heating; 'Loading' is the amount of biomass for which dissolution was attempted relative to the amount of ionic liquid. 'Solubilisation' is the percentage of the introduced biomass that was brought into solution (the remainder being undissolved residue). n.d.: not determined.[27]**

| Ionic liquid | Loading wt% (solubilisation %) | Feedstock | Particle size (mm) | Temperature and time |
|---|---|---|---|---|
| **[EMIM][OAc]** | 5 (92.2) | Pine | <0.125 | 175 °C, 30 min |
| | 5 (42.8) | Pine | <0.125 | 185 °C, 7 min |
| | 5 (93.5) | Pine | 0.250–0.500 | 110 °C, 16 h |
| | 5 (98.5) | Oak | 0.250–0.500 | 110 °C, 16 h |
| | 5 (75) | Beech | 0.100–0.500 | 115 °C, 24 h |
| | 5 (95) | Beech | 0.100–0.500 | 115 °C, 72 h |
| | 5 (40) | Spruce | 0.100–0.500 | 115 °C, 24 h |
| | 5 (75) | Spruce | 0.100–0.500 | 115 °C, 72 h |
| **[BMIM]Cl** | 5 (26.0) | Pine | 0.250–0.500 | 110 °C, 16 h |
| **[EMIM]Cl** | 3 (95.0) | Beech | n.d. (wood flour) | 120 °C, 24 h |
| **[C=$C_2C_1$im]Cl** | 8 (n.d.) | Spruce | 0.100–2.000 | 110 °C, 8 h |
| | 5 (26) | Pine | 0.450–0.650 | 100 °C, 15 h |
| | 3 (50) | Pine | 0.450–0.650 | 110 °C, 2 h, Microwave irradiation |
| | 10 (100) | Spruce, Eucalyptus | Ball milled for 48 h | 75 °C, 48 h |
| **[EMIM][$C_2C_2PO_4$]** | 4 (n.d.) | Wheat straw | <5 | 100 °C, 1 h |

There are a variety of factors that influence plant biomass pre-treatment including; (1) Biomass feedstock; (2) IL cation; (3) IL anion; (4) Pre-treatment time; (5) Temperature.[27,129] These factors need to be considered independently when designing new IL pre-treatment methods.

In general for (1) biomass feedstock, grass type biomass has been chosen as it is more easy to solubilise compared to woods. However, the effect of the biomass particle size plays a significant role in the ease of pre-treatment. Research has shown how pine and oak in [EMIM] [OAc] were significantly not solubilised for larger

particle sizes, sometimes incomplete within efficient time frames and temperatures.[129]

For (2) and (3), interactions between the IL and plant biomass include;

1. Ionic interactions
2. π-π interactions
3. Hydrogen bonding

It is believed that the π-π interactions of imidazolium ILs and lignins aromatic molecules, plays a vital role in removing and solubilising lignin from the biomass material.[130] The interactions disrupt the inter- and intramolecular hydrogen bonds in crystalline cellulose, as well as those between cellulose and lignin.[130]

[EMIM] [OAc] was recorded as being better than [EMIM] Cl as an alternative for plant biomass pre-treatment due to its lower melting point, lower viscosity, lower corrosive character and non-toxicity.[131] Chloride anions form non-directional hydrogen bonds which can act to bind the cellulose chains together.[103] Acetate forms bridging anions between strands, which do not cross-link cellulose as Figure 38 shows.

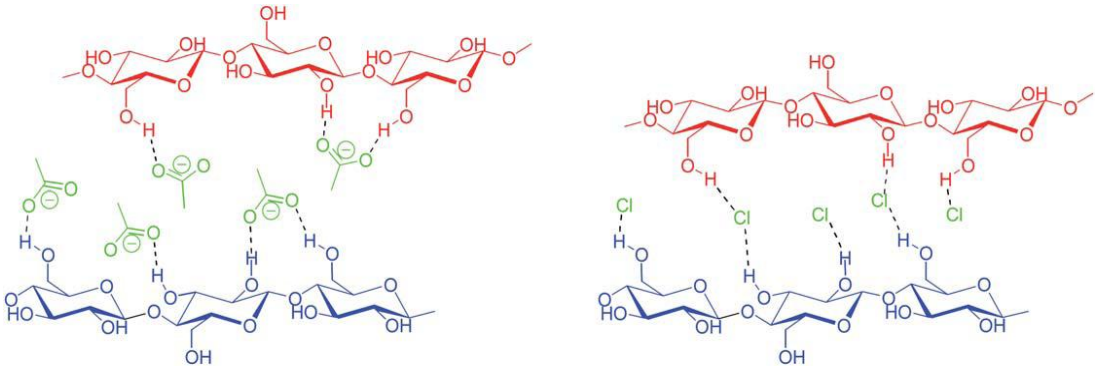

**Figure 38: IL anions hydrogen bonding with individual cellulose strands as modified from reference 38.**

Aprotic imidazolium ILs have been noted as being the most effective at pre-treating plant biomass. However, they are expensive for large scale production, even if recycled, re-used or aqueous solutions are possible.[132] From an environmental perspective imidazolium rings are poorly biodegradable and are significantly more toxic then non aromatic structures.[132]

For (4) and (5), between 60 minutes to 96 hours, and 80-200 °C has been used on various biomass materials as Table 10 shows.

**Table 10: Biomass processing with ionic liquids: conditions and products generated modified from reference 133.**

| Biomass | Entry | Conditions | Ionic liquids | Products |
|---|---|---|---|---|
| **Cellulose (dried)** | 1 | 10 wt%, 100 °C, or, 25 wt%, microwave heating | [$C_4$mim]Cl, [$C_2$mim][OAc] | Cellulose regenerated |
| | 2 | 5 wt%, 130–150 °C, 10–180 min | [$C_4$mim]Cl | Cellulose regenerated for successive cellulase hydrolysis |
| | 3 | 2.5–5.0 wt%, 100 °C, 16 h (addition of water) | [$C_2$mim][$HSO_4$] | Lower oligomers, DP depending on processing conditions |
| | | 2.5–5.0 wt%, 100–120 °C, 4–16 h (addition of various acids) | [$C_2$mim]Cl, [$C_4$mim]Cl, [$C_4$dmim]Cl | |
| | | 2.5–5.0 wt%, 80–120 °C, 24–96 h (addition of nucleophiles) | [$C_4$mim]Cl | |
| | | 2.5–5.0 wt%, ultrasound | [$C_4$mim]Cl | |
| | | 5 wt%, 100 °C, 5 h, Amberlyst 15DRY | [$C_4$mim]Cl | • Cellulose oligomers |
| | | | | • 30% total reducing sugars, of which 10% mono- and disaccharides |
| | | | | • Precipitation of oligomers by addition of water |

| | | | | |
|---|---|---|---|---|
| **Pine, poplar, eucalyptus, oak, straw, plywood, spruce, switchgrass** | 4 | 5–10 wt%, 100–130 °C, 8–16 h, 150 °C (micro-wave), 1 h | [$C_4$mim]Cl and other chloride-based ionic liquids, [$C_2$mim][OAc] | • Complete dissolution |
| | | | | • Cellulose separation from lignin and hemicellulose by |
| | | | | - Precipitation of cellulose |
| | | | | - Removal of lignin by extraction prior to precipitation of cellulose |
| | | | | • Some depolymerisation |
| | | | | • Direct hydrolysis with enzymes in ionic liquid not possible due to denaturation |
| | | | | • Selective precipitation of cellulosic and hemicellulosic oligomers suitable for fast enzymatic digestion in aqu. medium |
| | | | | • Direct acetylation |
| **Bagasse** | 5 | 6–10 wt%, 190 °C, 1 h | [$C_4$mim]Cl, +20 wt% aqu. NaOH/NaCl | • Complete dissolution |
| | | | | • Addition of aqu. NaOH-phase |
| | | | | • Precipitation of cellulose |
| | | | | • Lignin and hemicellulose dissolved in aqu. phase |

| | | | | |
|---|---|---|---|---|
| **Poplar** | 6 | 7 wt%, 100 °C, 24–72 h | [DBU-H]X, $X^-$ = $Cl^-$, $[CH_3SO_3]^-$, $[HCO_2]^-$, $[CH_3CO_2]^-$, $[(RO)_2PO_2]^-$ | • Complete dissolution |
| **Corn stalk, rice straw, pine, bagasse** | 7 | 5 wt%, 100–120 °C, 5–60 min | $[C_4mim]Cl$ + aqu. HCl, $[C_4mim]Cl$ + aqu. $CF_3CO_2H$, $[C_4mim][HSO_4]$ | • Complete dissolution of carbohydrates, not lignin<br>• Depolymerisation to lower oligomers<br>• Monosaccharide selectivity is function of time, water and acid concentration (*e.g.* formation of HMF and furfural) |
| **Poplar, bagasse, maple** | 8 | 5–10 wt%, 80–160 °C, 1.5–72 h (acetic acid) | $[C_1mim][CH_3SO_4]$, $[C_4mim][CF_3SO_3]$, $[C_2mim][ABS]$, [DBU-H] $X^-$= $[OTs]^-$, $[CF_3CO_2]^-$, $[HSO_4]^-$, lactate, $[SCN]^-$ | • Selective dissolution of lignin |

In conclusion, there are numerous factors which are beneficial for ILs to posses, if the pre-treatment of lignocellulosic materials is to be achieved;[27]

1. Short pre-treatment times
2. Can be used on multiple lignocellulosic materials
3. Relatively cheap to make and use
4. Can be re-used and recycled
5. Biodegradable or non-toxic
6. Specific to plant biomass fractionation.

It is the goal of ionic liquid researchers to discover designer ILs which are capable of the above.

## 1.7 Research Project Aims and Goals

The aim of this research project are to optimise the pre-treatment of lignocellulosic biomass by using cheap, readily available, environmentally benign protic ILs, in order to achieve high efficiencies of sugar release during saccharification from biomass, and in turn, high yields of bio-ethanol from fermentation. To this end, the benefits of using alkylammonium ILs include reduced cost and low viscosity for plant biomass pre-treatment will be compared to aprotic ILs and current industrial methods.[132]

The only current research using alkylammonium ILs[113] reported their inefficiency at solubilising cellulose, however; (1) The chloride anion was not tested in these studies; (2) They were tested on pure cellulose and other literature states cellulose solubility is not essential for effective plant biomass pre-treatment.[134]

Protic ionic liquids, including [DEA] Cl, are extremely rare in the cellulose literature. Virtually no research work for lignocellulose degradation has been complete and this is most likely due to the complexity of the protic IL's and their equilibrium from IL back to acid/base reactants. However, one paper does report a study of diethanolamine and triethanolamine with formic acid and acetic acid and concludes that their ability to dissolve cellulose is inefficient.[113] The study is purely a qualitative one where mixtures of cellulose and IL formed a turbid dispersion. No research into any pre-treatment properties of these protic ammonium IL's has been completed.

The aims of this project will be achieved by carefully modifying each variable systematically as stated in Figure 39.

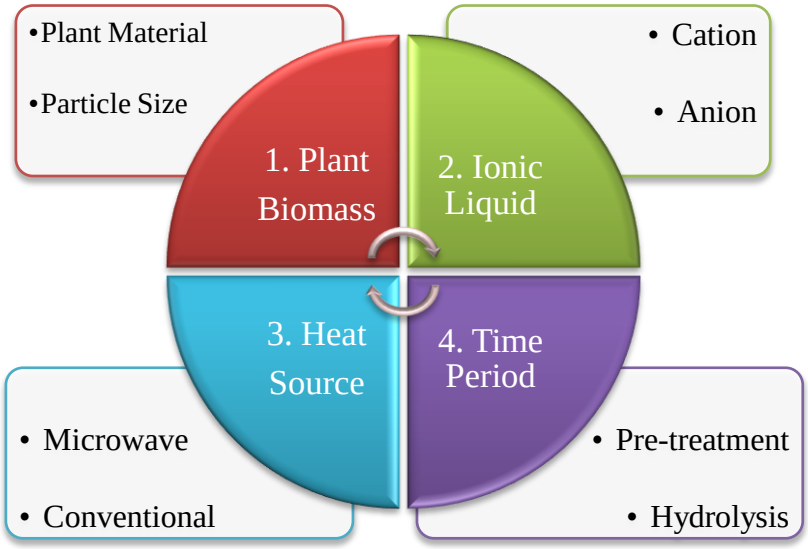


**Figure 39: Variability wheel, showing main methods of optimising IL pre-treatment.**

1) Plant biomass choice will be modified to discover which materials are most effectively pre-treated by ionic liquids.

2) Ionic liquid design to incorporate the best cation and anion choice by systematic modification of the structure of the IL and determination of its activity as a pre-treatment solvent to effectively release sugars from the biomass.

3) Comparison of the effectiveness of IL pre-treatments on the amount of sugars released using microwave and conventional heating.

4) Time periods of pre-treatment and hydrolysis to be optimised to increase benefit at lowest possible energy input.

In addition to the above variables, the comparison of all IL pre-treatments to conventional pre-treatment methods, dilute acid and base methods, as well as the use of co-solvents during IL pre-treatment will be compared.

The pre-treated biomass from all of the studies above will be analysed to understand structural and chemical composition. Methods including powder X-ray diffraction, thermogravimetric analysis, infrared, scanning electron microscopy and polymer compositional analysis will be used.

The use of cellulose as a model compound in order to better understand the origin of pre-treatment effects will be explored in addition to standard biomass tests above.

Finally, fermentation of hydrolysed sugars from pre-treated biomass will be attempted to investigate the final part of the pathway from biomass to biofuels. The yield from IL pre-treatment and how this method can be implemented for future research will be studied.

# Chapter 2

# Ionic Liquids and Biomass Saccharification Studies

## 2.1 Saccharification Introduction

### *2.1.1 Definition, Procedure and Aims*

In this chapter the effectiveness of IL pre-treatment will be analysed based on the variability wheel in the project aims, section 1.7, and measured using saccharification. Saccharification is the process of breaking down complex carbohydrates into simpler monosaccharides using enzyme mixtures. Cellulase enzymes are commonly used for biomass pre-treatment and there are a wide range of these enzymes commercially available for hydrolysis of lignocellulosic materials.[135] The major enzymes in biomass hydrolysis come from the fungus *Trichoderma reesei.*[136] The amount of glucose yielded from a chosen biomass sample will vary a lot depending on the source of the sample, the pre-treatment and the enzymes chosen, hence careful consideration is important. This hydrolysis outcome will be used as a measure for determining the effectiveness of the IL pre-treatment.

Some of the most important parameters when choosing enzymes for potential large scale applications are cost, enzymatic activity, stability and availability.[135] In research work, where a large amount of samples are required to be analysed, then using a high throughput assay for biomass digestibility is beneficial. Robotic systems, which work by taking aliquots from the hydrolysis mixture and analysing for the amount of sugars released, can be very useful for such projects.[137] These high throughput robots are tested against controls to ensure accurate results. The benefits of the robots include sensitivity, robustness and reliability and[137] this allows important results to be achieved for large numbers of plant tissues, as well as for the screening of large populations for studies into mutant identification and genetic association.[137]

Based upon this recent development, a high throughput approach has been used in this research work to analyse large samples of IL pre-treatments. One goal of lignocellulosic hydrolysis, as well as improving the current methods employed, is to simplify the amount of steps between plant material and biofuels. Currently, even if

IL's become better as a pre-treatment compared to steam explosion, they still have to be completely removed from the biomass before saccharification begins. Research has shown that a single vessel reaction involving IL pre-treatment followed by saccharification to sugars and then fermentation to biofuel, bio-ethanol, has been achieved all in one step, however, this work is in early days.[79]

Starting with cellulose, [EMIM] [DEP] was used as a pre-treatment with IL-resistant yeast and enzymes, with ca. 90% yields of ethanol and ca. 82% recovery of the IL.[79] However, these are initial results and are still a way off from being practical used.

Combining IL pre-treatment with saccharification, using halophilic enzymes, with minimal washing and high recovery rates of IL has also been shown in the literature. *Hu*-CBH1 is a halophilic cellulase derived from halophilic archaeon (*Halorhabdus utahensis)* and it can function in high salt concentrations, at high temperatures and ~ 20% [AMIM] Cl.[138] This is possible due a surface rich in acidic amino acids. The negative surface charge can interact with the IL forming a hydration sphere, hence making the protein heat resistant.[138] Hence, this can hydrolyse the cellulosic polymers from the biomass during IL pre-treatment to release sugars.[138]

However other cellulase enzymes are usually not capable of this. It has also been found that residual $[EMIM]^+$, in biomass recovered after pre-treatment, was the primary source of inhibition on downstream microbial growth and ethanol production using *S. cereviase*.[139] Concentrations of less than 0.1 wt % of [EMIM] [OAc] were required to not have an effect on the *S. cereviase* and it is believed the inhibitory effect is due to both the cation and anion combination.[139]

Water has been shown to be the best solvent at washing biomass free of IL, however the removal of water is more difficult compared to other organic solvents. Other research groups use a 1:1 acetone/water mix to wash and precipitate carbon rich material.[140] Ethanol is used to wash biomass in some other IL pre-treatments and was used in the pre-treatment studies in this project.[139]

Inactivation of the enzyme, *Trichoderma reesei* cellulase, due to concentrated chloride anions from [BMIM] Cl has also been reported as another example of researched IL effects on enzymes.[82] The future of enzymes being capable of

functioning in IL's still has a lot of work to be done but hopefully in the future enzymes and IL's will work synergistically with one and other, making industrial scale processes economically and practically viable.

The aims of this project are to use protic ILs, which are cheap, readily available and biodegradable, to successfully pre-treat plant biomass for improved sugar release. Figure 40 shows the wheel of variability of all of the different variables planned to be modified during the IL pre-treatment and saccharification studies.

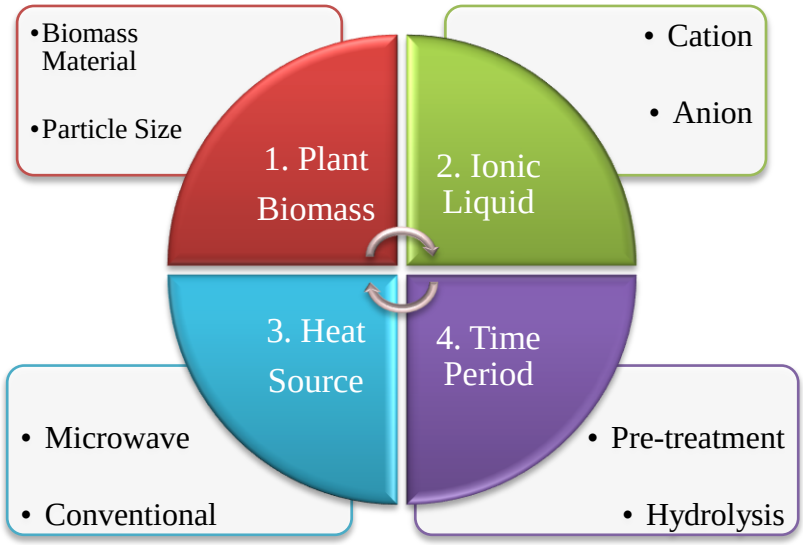


**Figure 40: Wheel of variables to modify during IL pre-treatment and saccharification experiments.**

In these experiments, as stated previously, it will be the sugar released after the biomass has been hydrolysed which will be used to assess how effective an IL pre-treatment has been. Table 11 shows the methods for hydrolysis of biomass and in this case enzymes have been chosen as the method of choice.

**Table 11: Comparison of hydrolysis methods for plant biomass.[16]**

| | Consumables | Temperature (K) | Time | Glucose yield (%) | Available |
|---|---|---|---|---|---|
| **Dilute acid** | < 1% $H_2SO_4$ | 488 | 3 min | 50-70 | Now |
| **Concentrated acid** | 30-70% $H_2SO_4$ | 313 | 2-6 h | 90 | Now |
| **Enzymatic** | Cellulase | 323 | 1.5 days | 75-95 | Now-2020 |

## *2.1.2 Biomass Loadings for Pre-treatment Experiments*

The importance of not having a high solid mixture to liquid mixture, is to ensure efficient enzyme hydrolysis. Not only will lignin content inhibit enzyme efficiency but reduced water content during hydrolysis prevents cellulase-substrate interaction and the increased sugar release during saccharification will also cause product inhibition.[141]

Water is crucial in hydrolysis for enzyme function and transport mechanism as well as mass transfer of end-products. Increased loading is preferred due to reduced costs, however, the negatives including increased inhibitors to enzymes and yeast preventing optimum function, are to be avoided. Also insufficient mixing with high biomass loadings is also common, as well as increased viscosity and lower ion mobility, which reduces ion effects.[142] Therefore, 12 to 15 percent loading is considered the maximum limit in which pre-treated biomass is effectively mixed and hydrolysed.[141] For these experiments a loading of 10 wt% biomass has been chosen.

## *2.1.2 Methodology for Saccharification*

In order to analyse the effectiveness of the ionic liquids for biomass pre-treatment, the plant biomass materials were analysed based on how much sugar was released after the pre-treatment, i.e the sugar conversion efficiency from the original biomass polymers. This sugar conversion was used as a quantitative method and in practical terms gave an indication of the efficiency of each individual pre-treatment variable. The methodology for the enzyme hydrolysis experiments is detailed in chapter 6, section 6.8 and is based on using MBTH to quantify the sugars released. Other methods could be used including the PAHBAH method, but MBTH was chosen due to it being a low cost method.[62]

As a consequence of using enzymes as a method of analysis, the experimental data is the absolute sugar yield from each set of experiments and this needs to be normalised in order to cross compare samples from different experiments, otherwise the data is non-comparable.

Hence, in chapter 2 the experimental results are shown in two different graphical formats. The first type which contains sugar conversion: 'nmols of sugars/ mg of material' on the y-axis, is the original experimental data. This is formatted for the

thesis and shows the exact amounts of sugars released in each experiment against the pre-treatment conditions used. This is important in order to quantify yields and as well as characterising the sugars produced. However, because the exact quantities of sugars released are dependent on the individual experiments due to the enzyme mixtures used in each experiment not being a 100% identical, an overall improvement ratio is taken.

Therefore the second set of graphs show the improvement factor and have 'improvement ratio' on the y-axis. The important part here is these graphs are based on the internal ethanol controls of each set of experiments, completed as a reference point to quantify how effective the IL or biomass variable being investigated is at sugar release. Assigning the ethanol control as a value of 1, all other internal results receive a ratio value, for example 4.5, and by doing this as all experiments contain internal ethanol controls, the ratios can be used as a cross comparison for other experiments.

Pre-treatment with ethanol was used as the control as it dissolves any sugars present in the biomass mixture originally but has little effect on the cell walls of the biomass during the pre-treatment.[143] An ethanol control can then be used to evaluate the relative improvement of the [DEA] Cl pre-treatment and to ensure any improved sugar release is due to the IL and not, either heating or mechanical stirring on the biomass.

## 2.2 Temperature Studies of Plant Biomass Pre-treated with Ionic Liquids

Biomass decomposition, as is the case for many chemical processes, is accelerated by increasing temperature.[27] However, increasing temperature also has disadvantages including: (1) 'Charing' of the sample; (2) Energy cost increase; (3) Decomposition of the ILs. For this reason, the choice of an optimum temperature is crucial for pre-treatment and maximising the benefits without the disadvantages.

Current research shows[61,102] that a range of temperatures have been used for ionic liquid pre-treatment on plant biomass. Table 12 shows examples of cellulose solubility in ILs over a range of temperatures. It is clear to see that at higher temperatures increased solubility is observed.

**Table 12: Solubilities of microcrystalline cellulose in the ILs at different temperatures[144]**

| **Entry** | **Solubility (g mol$^{-1}$) and temperature (°C)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **IL** | **40** | **50** | **60** | **70** | **80** | **90** | **100** | **110** | **120** |
| **1** | [$C_4$mim][$CH_3COO$] | 23 | 25 | 26 | 31 | 36 | 49 | 54 | 56 | 58 |
| **2** | [$C_1OC_2$mim][$CH_3COO$] | 8 | 17 | 21 | 23 | 29 | 39 | 44 | 49 | 56 |
| **3** | [$C_2$OHmim][$CH_3COO$] | | 1 | 18 | 22 | 26 | 30 | 33 | 34 | 34 |
| **4** | [$C_4$dmim][$CH_3COO$] | | 5 | 18 | 24 | 27 | 29 | 32 | 35 | 37 |
| **5** | [ph$C_1$mim][$CH_3COO$] | | 1 | 14 | 17 | 20 | 22 | 24 | 30 | 34 |
| **6** | [$C_2$mmor][$CH_3COO$] | | | 1 | 3 | 6 | 18 | 21 | 28 | 31 |
| **7** | [C=$C_2$mmor][$CH_3COO$] | | 1 | 2 | 4 | 12 | 17 | 21 | 24 | 28 |
| **8** | [C=$C_2$mpip][$CH_3COO$] | | | | | | 10 | 14 | 16 | 19 |
| **9** | [$C_4$mpip][$CH_3COO$] | | | | | 1 | 2 | 3 | 4 | 7 |
| **10** | [$C_4$mpyr][$CH_3COO$] | | | | | | | 1 | 2 | 3 |
| **11** | [$C_4$ebim][$CH_3COO$] | | | | | | | | | <1 |
| **12** | [$C_2$ebim][$CH_3COO$] | | | | | | | | | <1 |
| **13** | [$C_4$ebt][$CH_3COO$] | | | | | | | | | <1 |

In order to determine at which temperatures our biomass pre-treatments using ILs would work most efficiently, miscanthus was pre-treated with [DEA] Cl at 10 different temperatures. Starting from 50 °C and rising to 150 °C, increasing in 10 °C increments were the temperatures tested for effective pre-treatment.

Figure 41 shows the saccharification results per temperature for each pre-treatment. It is clear to see from the samples pre-treated at 50 °C to 100 °C there is a steady increase in the amount of sugars released per mg of sample. At 100 °C the amount plateaus off to a steady amount of ca 80 nmols of sugars per mg of material up to the 140 °C pre-treatment. The pre-treatment at 150 °C then starts to increase to 120 nmols of sugars per mg of material. This is expected and research shows that temperatures of 185 °C can greatly improve the dissolution of some of the plant polymers, with the added problem of IL degradation occurring.[140]

What is quite remarkable with this experiment is that the sugar conversion rate goes through to a plateau at ca 100 °C, which means that a rise of pre-treatment temperature up to ca 150 °C is unnecessary.

Therefore the standard temperature for all of pre-treatment experiments used was 100 °C unless stated otherwise.

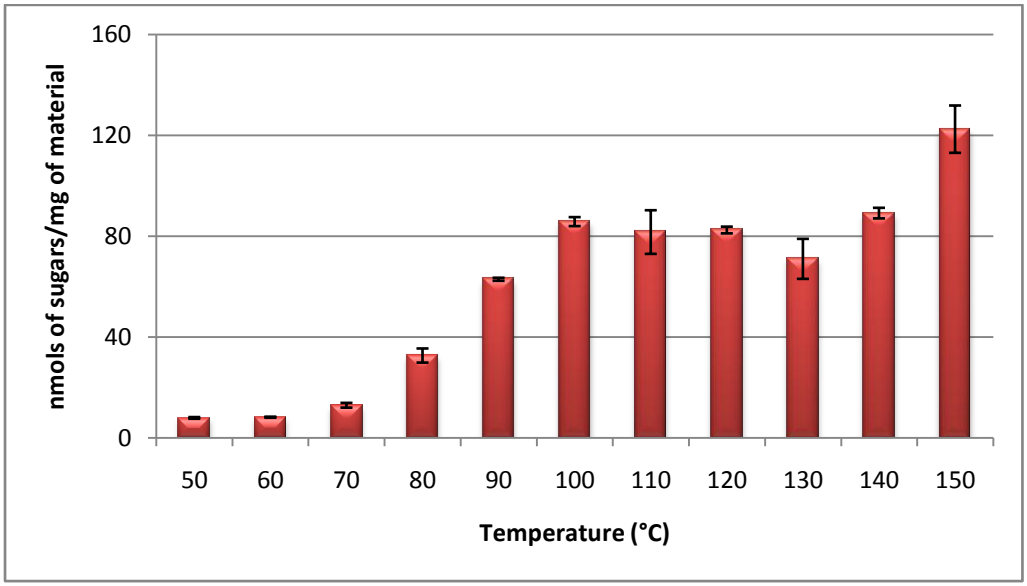


**Figure 41: Miscanthus pre-treated with [DEA] Cl over a range of 10 temperatures as shown, each for 24 hours.**

## 2.3 Plant Biomass Selection

To determine the most appropriate lignocellulosic material of choice for assessing the effectiveness of pre-treatment with ionic liquids, a selection of biomass materials were tested with [DEA] Cl. Maize stover, miscanthus, poplar, sugarcane bagasse and wheat are all potential sources of sugars for bio-fuel production.

In the following section, each biomass material will be discussed one by one as a potential for IL pre-treatment. The choice of the plant materials was designed to cover one from all of the biomass classifications as discussed in the introduction (1.2). Miscanthus is a grass-based material, poplar is a hard-wood, maize stover and sugarcane bagasse are waste agricultural products and wheat is a food based crop. Figure 42 shows the plant biomass samples chosen for these experiments.

**Figure 42: Plant biomass samples chosen. From left to right, miscanthus, poplar, wheat, maize stover, sugarcane bagasse.**

### *2.3.1 Maize Stover*

Maize stover is the waste material left over from corn after the harvest, including the stem, leaves and husk.[145] Hence, maize stover is an important feedstock to be tested for renewable chemical generation using ILs. Maize stover was pre-treated over a range of time periods with [DEA] Cl to see the effect of the IL over time on the biomass.

Figure 43 shows the saccharification results for maize stover pre-treated with [DEA] Cl at 100 °C. The best time period for maize stover pre-treated in [DEA] Cl is 72 hours, although within error, all except 60 minutes pre-treatment time are the same. After 5 hours of pre-treatment a significant amount of sugars had already been released indicating the added benefit of increased time period was not needed compared to the energy required. The total overall improvement outcome at 72 hours is two times the amount of sugars released after pre-treatment compared to the ethanol control. This improvement has shown that pre-treating maize stover with [DEA] Cl is beneficial as it releases more sugars.

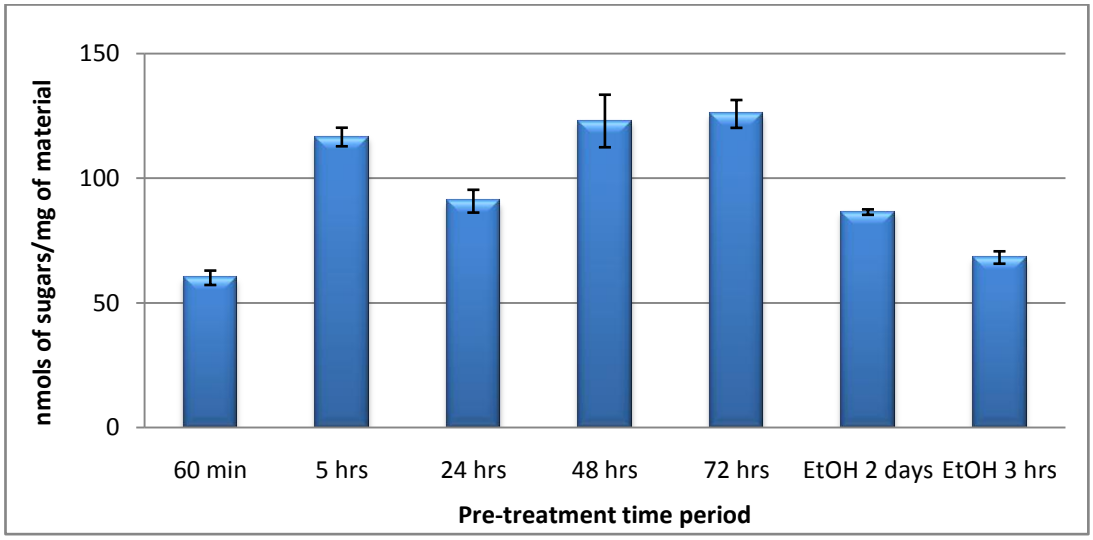


**Figure 43: Maize stover pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

## *2.3.2 Miscanthus*

Miscanthus is an energy crop with a genus comprising of 15 species of perennial grasses.[146] The grass is native to subtropical Africa and South Asia and is known as a non-wood rhizomatous C4 perennial grass with an estimated lifetime of 10-15 years.[146] Miscanthus can now also be found in Europe and is growingly being used as a candidate for energy crops due to its rapid growth, excellent productivity and high resistance to disease.[135,147] Its benefits outweighs other crops such as switchgrass and poplar, as it is more productive per acre than any other biomass.[148]

Figure 44 shows the saccharification results for miscanthus pre-treated with [DEA] Cl at 100 °C for a range of time periods. After 60 minutes of treatment with the IL ca. 70 nmols of sugars per mg of material had been released, which is already 7 times more than the ethanol control. This is an excellent improvement on the digestibility of miscanthus after just 60 minutes. Within error, 60 minutes, 5 hours and 24 hours pre-treatment had the same amount of sugars released and at 48 hours and 72 hours the peak release amount for the experiment was achieved. Overall, this total improvement shows an improvement factor of 12 times the amount of sugars released from the miscanthus after IL pre-treatment compared to the ethanol control. This excellent result shows how useful [DEA] Cl is as a pre-treatment solvent that allows easy access of cellulose to enzyme hydrolysis.

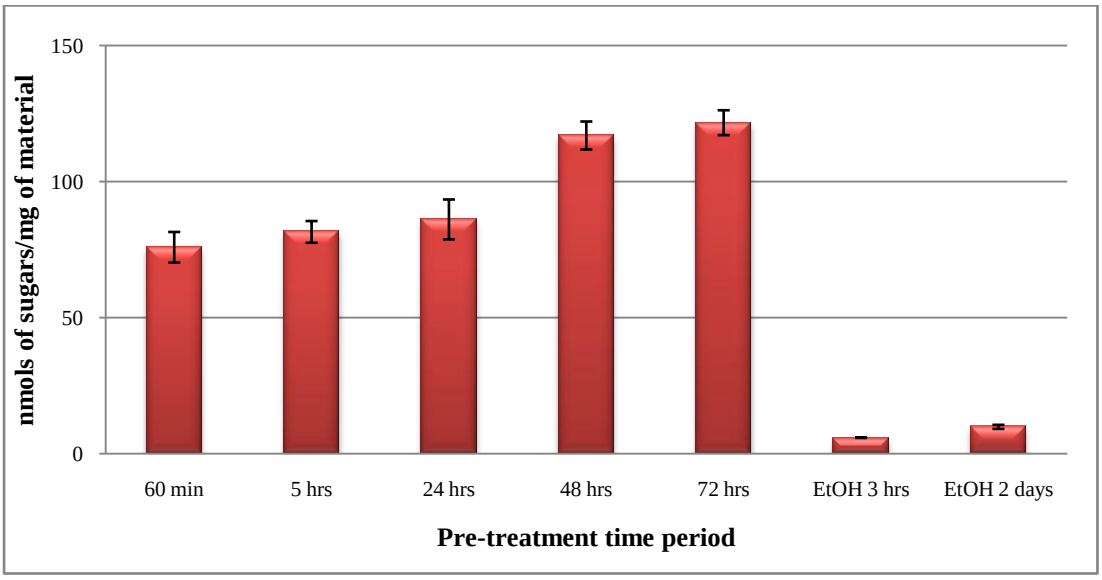


**Figure 44: Miscanthus pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

### *2.3.3 Poplar*

Poplar, along with willow, is a dedicated bioenergy wood, which are short rotation crops with high biomass yields and low agricultural inputs for long-term perennial cropping. Land quality required for the crop is also low, reducing competition with food based crops and allowing the use of unused land for energy generation.[149]

Figure 45 shows the saccharification results for poplar pre-treated with [DEA] Cl at 100 °C for a range of time periods. After just 60 minutes of IL pre-treatment ca. 30 nmols of sugars per mg of material had been released, which is 6 times greater than the ethanol control pre-treatment. A result which is lower than miscanthus but still a significant improvement after such short pre-treatment time. Within error, 60 minutes, 5 hours and 24 hours pre-treatment had the same amount of sugars released, with 48 hours and 72 hours being the peak amounts release in the experiment.

Overall, this improvement shows a maximum increase of 8 times the amount of sugars released from poplar compared to ethanol control. These results are very interesting as the [DEA] Cl pre-treatment clearly is very effective on both miscanthus and poplar samples, considering they are both very different species.

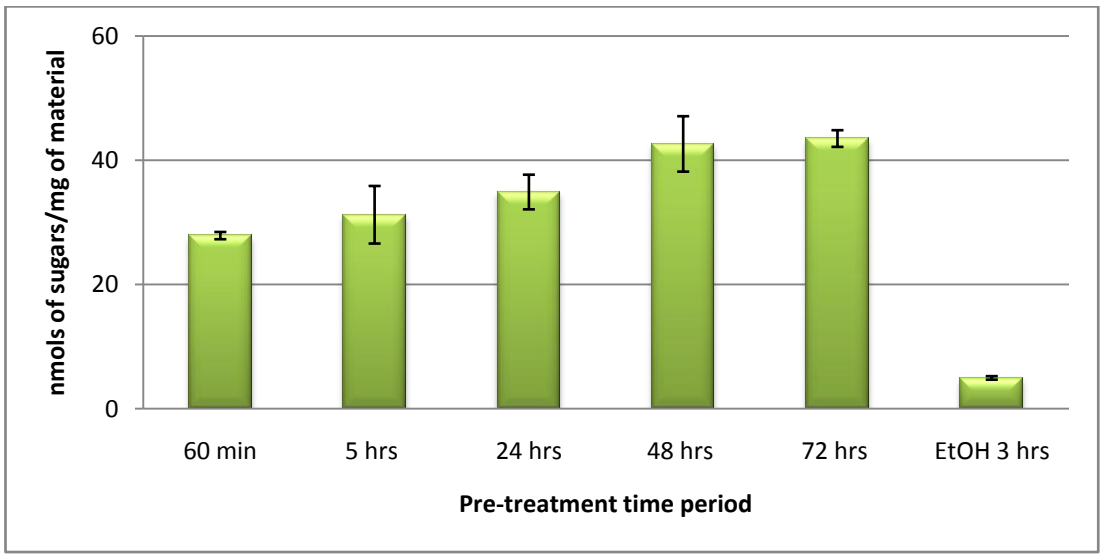


**Figure 45: Poplar pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

### *2.3.4 Sugarcane Bagasse*

Sugarcane bagasse is a byproduct from the sugarcane industry and hence is a useful waste product from industry. Most of the bagasse is usually burnt for generating power and is used as a fuel source for the sugarcane industry. It is the most abundant lignocellulosic material in India, second after Brazil, with 179 million tons of bagasse being produced annually,[150] hence maximising the usage of this material is important.

Figure 46 shows the saccharification results for sugarcane bagasse pre-treated with [DEA] Cl at 100 °C for a range of time periods. After 60 minutes of the IL pre-treatment ca. 45 nmols of sugars per mg of material had been released, which is 2 times more than the ethanol control. Within error, all the remaining time periods released a similar amount of sugar, indicating after 60 minutes of pre-treatment, no further improvement on digestibility occurred. Overall this improvement is considerably smaller than miscanthus and poplar IL pre-treatments.

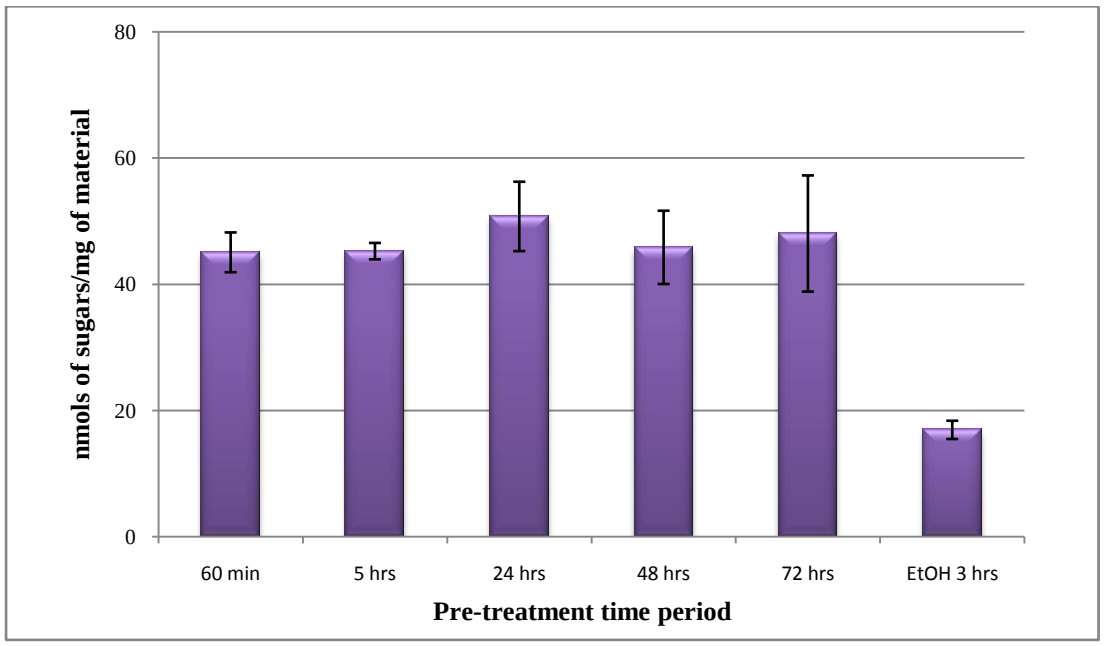


**Figure 46: Sugarcane bagasse pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

### *2.3.5 Wheat*

Wheat is the only food based biomass to be tested in this project, purely as a comparison to the lignocellulosic materials used. Figure 47 shows the saccharification results for wheat pre-treated with [DEA] Cl at 100 °C for a range of time periods. After 60 minutes of IL pre-treatment only a fractional increase in the sugars released compared to the ethanol control had been achieved. The 5 and 24 hour time periods of pre-treatment equally remained similar, with a slight improvement and increase at 48 and 72 hours, peaking at ca. 80 nmols of sugars per mg of material.

Overall, this improvement is much smaller again than the miscanthus and poplar effect, as there were only twice the amount of sugars released from the wheat compared to the ethanol control.

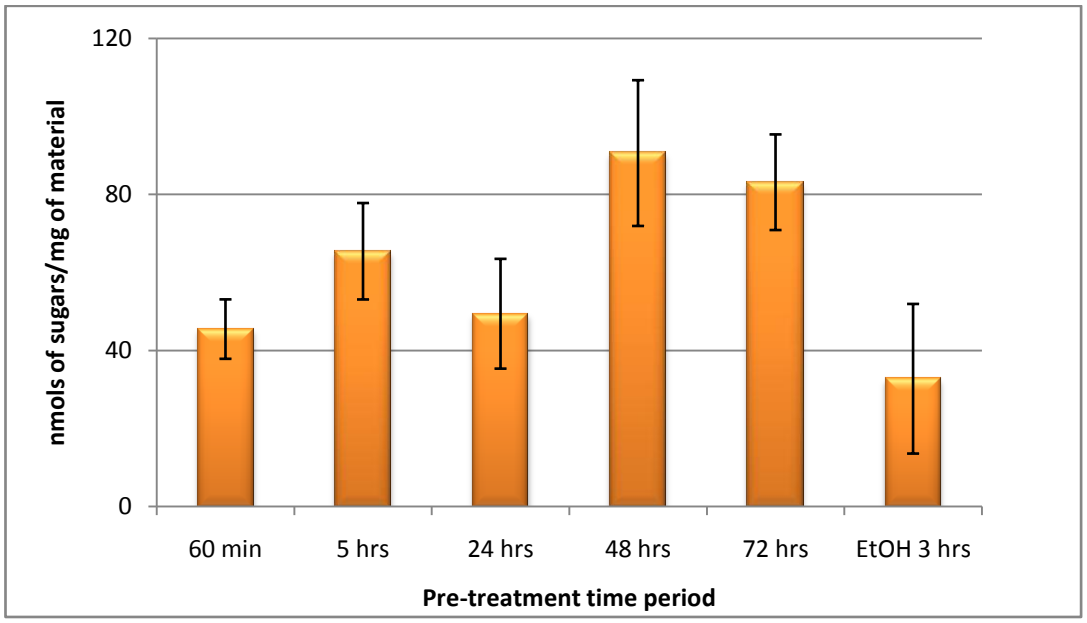


**Figure 47: Wheat pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

### *2.3.6 Summary of Lignocellulosic Materials Preferentially Pre-treated with [DEA] Cl*

[DEA] Cl was shown to successfully pre-treat biomass samples from all of the plant biomass classifications tested, which shows the versatility and effectiveness of the IL [DEA] Cl. The efficiency, however, varied quite a bit from each type of biomass with no clear trend between which biomass classification was best pre-treated by the IL. This was potentially expected due to the complex nature of plant biomass materials. Their non-uniformity and their unique biosynthetic pathways makes it difficult to predict their pre-treatment behaviour, without conducting experiments.[151] Chapter 3 will look into the structural and chemical components in the material after pre-treatment which could explain these differences.

In order to sum up all of the previous data in section 2.3 on how changing the lignocellulosic material of choice with the pre-treatment of [DEA] Cl improves the amount of sugar release, a comparison of the improvement factors based on each individual material is shown. To make comparisons, the normalisation which has been introduced in section 2.1.3 was indispensable, as comparing the exact value of the sugar release between samples does not give us any useful information. By considering the improvement factor between the time periods per biomass sample and the ethanol control, we get a good indication of which type of material works best with the IL pre-treatment. Figure 48 shows the graph based on the improvement factor for each material over time.

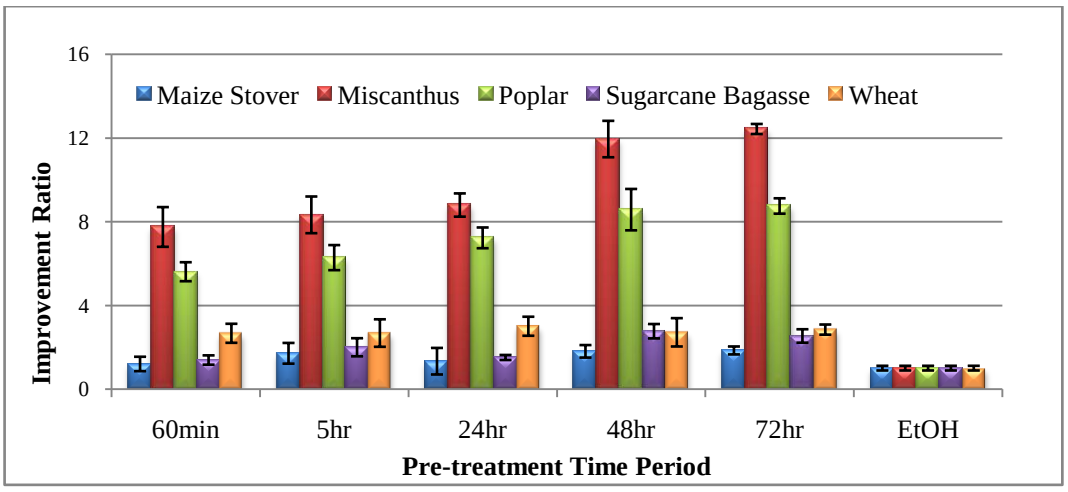


**Figure 48: Ratio of improvements from the various biomass materials pre-treated with [DEA] Cl compared to individual ethanol control pre-treatment.**

Figure 48 clearly demonstrates the significant improvement miscanthus and poplar (in red and green) have compared to maize stover, sugarcane bagasse and wheat. We can see that maize stover, sugarcane bagasse and wheat do have an improvement in sugar release but why for these materials it is considerably less will be investigated further in chapter 3. For the rest of the research miscanthus will be used as the main biomass source, with poplar as a comparison in some cases, as using these materials, ILs can pre-treat them most efficiently. The approximate yields under these conditions are calculated to be between 10-15%, however, until the enzyme mixture is used as manufactures recommendation, a true and reflective yield cannot be calculated.

## *2.3.7 HPLC Analysis of Miscanthus and Poplar Composition of Sugars Released After Pre-treatment with IL and Enzyme Hydrolysis*

IL pre-treatment has been most successful on saccharification efficiency for miscanthus and poplar. A question to be asked is has the IL pre-treatment modified certain sugar polymers more than the others? As this may, in turn, shed light to the particular efficiency on these samples. A way of testing this theory is to analyse the miscanthus and poplar to see the sugar composition of the pre-treated material and how this changes from before pre-treatment, HPLC was used to identify the sugars released from saccharification.[102,152]

The aim was to see if the IL had affected the ease of access for the enzymes to hydrolyse one biomass polymer over another. The nmols of sugars per mg of material released from the saccharification experiments are a mixture of the nine common sugars present in the plant polymers. Figure 49 shows the 5 common sugars present in plant biomass.

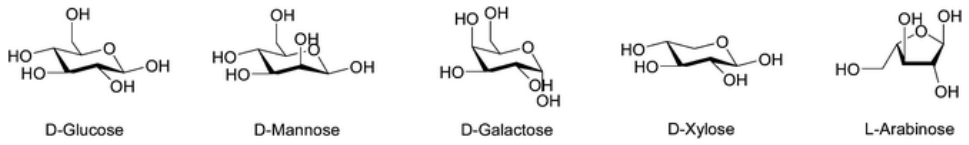


**Figure 49: Sugars commonly found in hemicellulose and cellulose in lignocellulosic materials.[27]**

In order to analyse these sugars and determine the relative composition of them present after hydrolysis, the saccharification residue was removed and analysed via HPLC.

Figure 50 shows the HPLC results from miscanthus pre-treated in [DEA] Cl over the range of time periods with an ethanol control. As the data shows, glucose is the main residue followed by xylose and mannose respectively. The other sugars are present in small quantities or not at all.

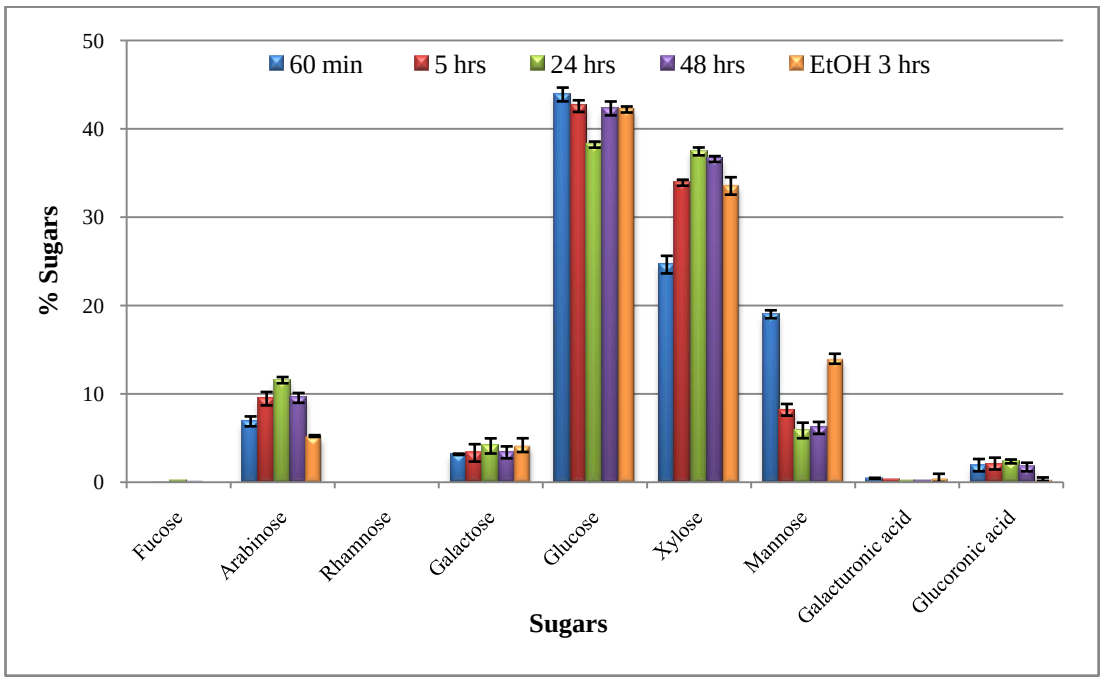


**Figure 50: Percentage sugars from miscanthus samples per each pre-treatment time period in [DEA] Cl.**

Studying Figure 50, the relative amount of xylose increases between the 60 minute pre-treatment time to 5 hours pre-treatment, potentially implying during the first 60 minutes of pre-treatment less of the hemicellulose polymer is solubilised or broken down. By 5 hours of IL pre-treatment all of the 3 key sugars are equal in distribution for the remainder of the time periods. Some sugars reduce after the 72 hour pre-treatment as possible degradation of the sugars could be occurring. Apart from the above, the ethanol control sugars remain similar to the IL, implying there is no alternative preference for either cellulose or hemicellulose in miscanthus.

Figure 51 shows the HPLC results from the poplar pre-treated in [DEA] Cl over the range of time periods. The graph shows that, glucose and mannose are the main residues with xylose slightly less in composition. Studying Figure 51, the relative amount of glucose increases from 60 minutes towards 48 hours pre-treatment, potentially implying during these time periods more of the cellulose is being pre-treated. However, at 72 hours this amount drops rapidly and seems to be replaced mostly by mannose, implying at this time, most of the hemicellulose is now being dissolved or broken down.

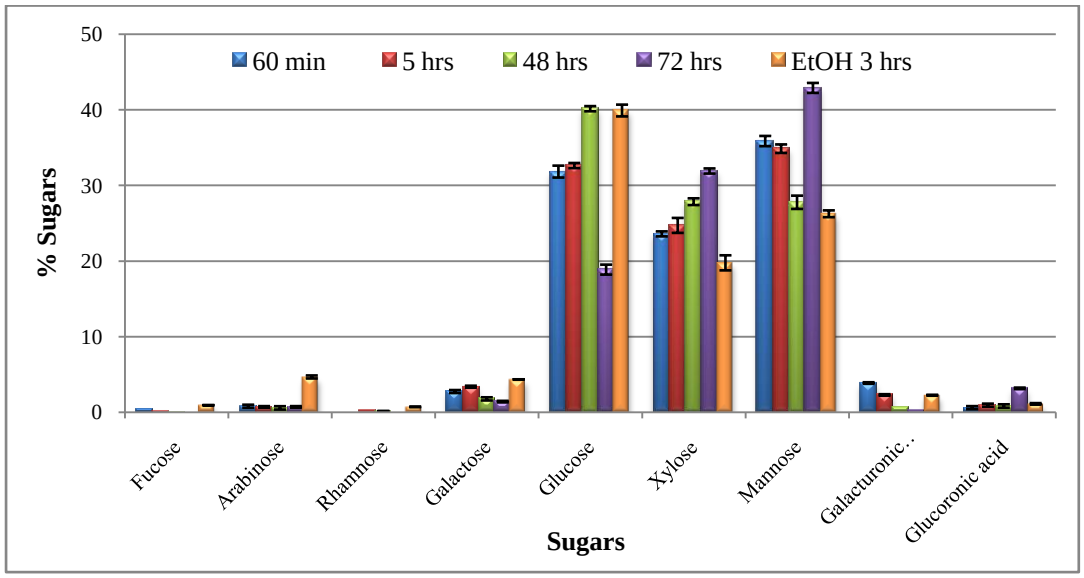


**Figure 51: Percentage sugars from Poplar samples per each pre-treatment and time period in [DEA] Cl.**

In conclusion [DEA] Cl pre-treatment did favour different sugars but only with small difference observed. Essentially, they were all hydrolysed with equal breakdown of the polymers over time, which makes one wonder why there has been an improvement for certain types of biomass over the others in the first place? It will later be shown in chapter 3 that it is lignin which plays a crucial role in determining the pre-treatment effectiveness, which as lignin contains no sugars, is not shown here using HPLC to analyse the sugar content.

## 2.4 Ionic Liquid Cation Alteration for Structure-Activity Relationship Study on Miscanthus Pre-treatment

In order to investigate how important the IL cation is on lignocellulosic pre-treatment, a range of structures identified by literature and based on aprotic and protic structures were synthesised, all made with chloride as the anion (section 6.4).

The strategy for modifying the cation structure was in order to understand the structural basis of this plant biomass decomposition efficiency. Therefore, systematic modification has been introduced to clarify the chemical structure responsible for effective saccharification of the plant biomass.

The role of the cation in plant biomass pre-treatment is not fully understood. Research shows that having a C-H$^{\cdots\cdots}$O hydrogen bond forming with cellulose allows increased rates of solubility.[144] However, this effect can be reversed with competing strong cation and anion interactions. A cation with acidic protons but without a highly electronegative atom or large substituents could be a rational choice.[144] Hence, for standard aprotic comparisons this method has been followed but does not fully apply to the cheaper, protic IL choices. This was because the ammonium protics for plant biomass pre-treatment have not been studied in full detail, hence a wider approach to cation choice was adopted.

### *2.4.1 Aprotic Cation Structures*

The aprotic ILs were synthesised based on the structure of the 1-methyl-3-butylimidazolium cation (BMIM), hence all cations have the *N*,*N*-butylmethyl chains where possible. This was chosen due to the previous research in the literature, which showed these types of structures to be effective at decrystallising cellulose for improved sugar release.[27] The other base structures for aprotic design were pyrrolidine, pyrrole, piperidine, pyridine and morpholine, as shown in Figure 52. The criteria for structure activity screening was;

1. Aromatic *vs* non-aromatic – size and steric issues, absence of phenolic groups or other large substituents, presence of π electrons and electrostatic interactions,[144] size issues to fit between polymer chains due to planar cations.[153]
2. 5 membered *vs* 6 membered rings – size effect as expected from previous studies, avoiding steric hinderence, shorter chain lengths, less hydrophobic and non polar.[27]

The structures in the red box in Figure 52 are to compare 5 membered rings which are aromatic and non-aromatic with the structures in the blue box being 6 membered rings both aromatic and non-aromatic. The morpholinium cation in the orange box is used to compare any potential benefits to having two heteroatoms in the ring, in this case oxygen and nitrogen.

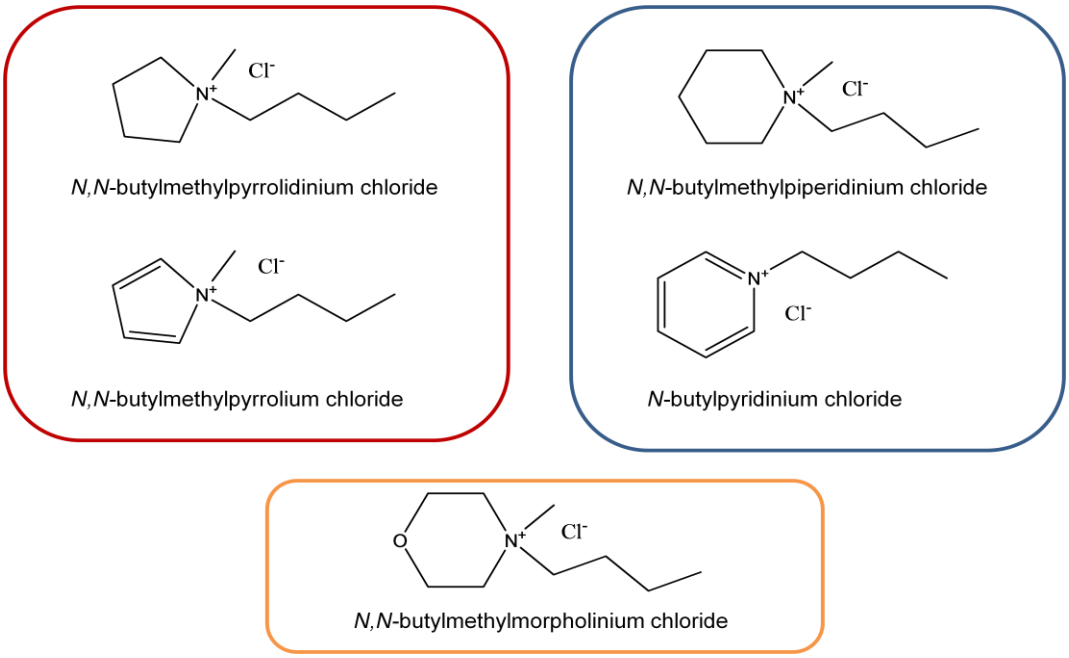


**Figure 52: Aprotic IL structures for structure-activity experiments on the pre-treatment of miscanthus for increased sugar release.**

The ionic liquids in figure 10 were abbreviated to the following; Butylmethylpyrrolidinium [BMPyr]

Butylmethylpyrrolium [BMP]

Butylmethylpiperidinium [BMPip]

Butylpyridnium [BPy]

Butlylmethylmorpholinium [BMMorph]

In all graph and tables these abbreviations are now used.

## *2.4.2 Protic Cation Structures*

The same process of structure comparison was applied to the protic ILs, these were all based around the diethanolammonium cation. This was chosen as alkanoates and alkanolammoniums are known to be biodegradable and to compare to [DEA] Cl already shown to be successful.[154] Removing the OH groups or exchanging the OH groups, were all examples of types of modification used to test the cation design and hence the structure versus activity relationship.

Figure 53 shows the diethanolammonium cation [DEA] with 3 other cations structurally related with two carbon chains on the nitrogen centre. Bis-2-methoxyethylammonium [B-2-MEA] compares the OH converted to $OCH_3$ groups, and dipropylammonium /diethylammonium [DPA]/[DethylA] show 3 and 2 carbon chains on the nitrogen in the absence of OH respectively.

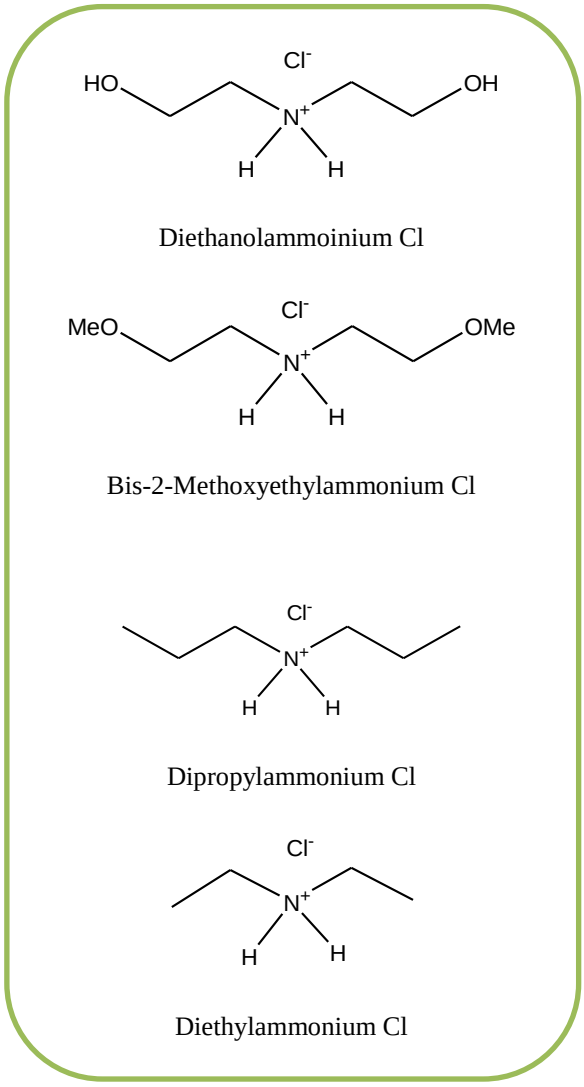


**Figure 53: Protic IL structures based on two carbon chains around the ammonium centre for pre-treatment with miscanthus for increased sugar release.**

Figure 12 shows the diethanolammonium cation with $Cl^-$ for comparison with single alkyl chains from the nitrogen centre. Ethanolammonium [EA] and propanolammonium [PA] both have one OH group, with either 2 or 3 carbons in the chain. Ethylammonium [EthylA] has a single 2 carbon chain without the OH group present. All of these 3 new structures have 3 protons around the nitrogen centre. The data should tell us how important the OH group is and whether the size of the chain length matters.

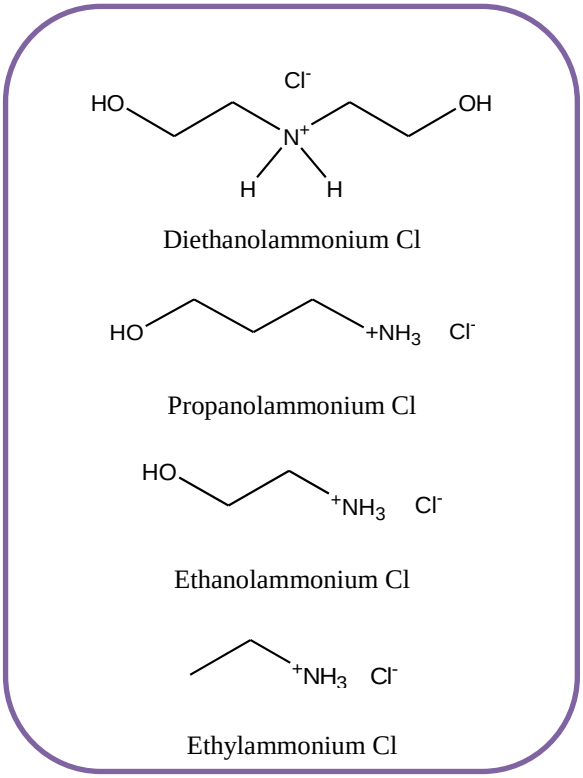


**Figure 54: Protic IL structures based on single carbon chains around the ammonium centre with/without OH groups for pre-treatment with miscanthus for increased sugar release.**

Finally choline $Cl^-$ was used as an aprotic IL with similar structure to the diethanolammonium cation. Choline chloride (vitamin $B_4$) is produced on the million metric ton scale per year as it's an additive for chicken feed. Hence this makes it very cheap, non-toxic and biodegradable IL.[155,156] In recent years, choline Cl has attracted significant attention as a safe alternative to imidazolium ILs.[143]

Cation modifications were made to compare dimethylethanolammonium [DMEA] and triethylammonium [TriethylA] cations with choline. Figure 55 shows the structures and as it shows all cations have 1, or in the case of choline no, protons around the nitrogen centre. The previous figures of protic ILs now also have examples of cations with 1, 2 or 3 protons around the nitrogen centre.

From Figure 53, Figure 54 and Figure 55 a detailed study into the structure-activity of the cations and the effects this has on the pre-treatment of miscanthus can be achieved for protic ammonium ILs.

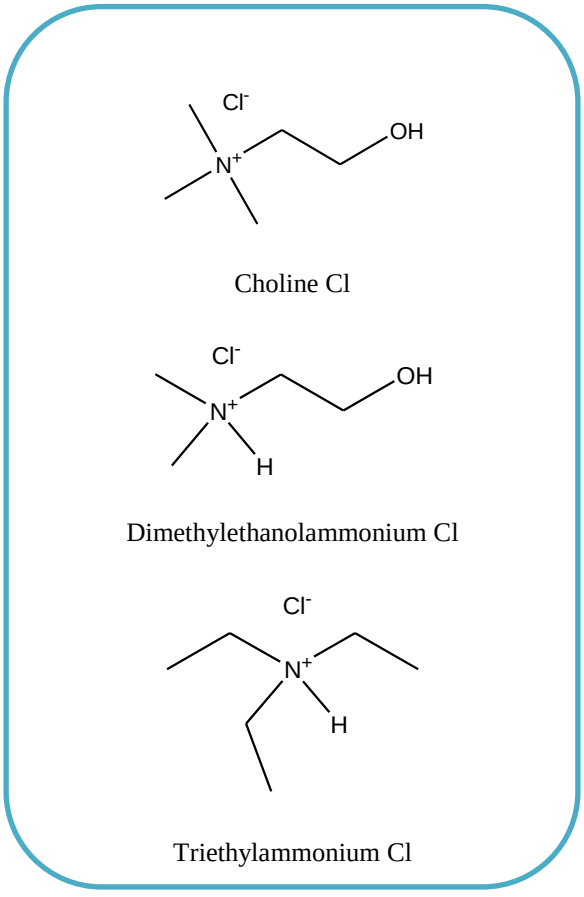


**Figure 55: Protic IL structures based on choline with only 1 proton around the nitrogen centre for pre-treatment with miscanthus for increased sugar release.**

### *2.4.3 Miscanthus Pre-treated by IL Cation Chlorides*

The question repeatedly asked is; what is the best anion structure for lignocellulosic IL pre-treatment, and what role does the cation play? To answer this question, saccharification efficiency measurements, spanning over a wider range of cation structures have been compared. Miscanthus has been chosen as a biomass sample for this study due to its successful pre-treatment with [DEA] Cl from the saccharification studies as shown is section 2.3.

Miscanthus was pre-treated by the ILs shown in figures 52, 53, 54 and 55 at 100 °C for 24 hours and the results from saccharification are shown in full in Figure 56. The results are ordered from most effective cation to least effective cation for miscanthus pre-treatment, based on the amount of sugars released. As Figure 56 shows [DEA] Cl is clearly the best cation of choice releasing ca. 85 nmols of sugars per mg of material compared to the ethanol control for the experiment releasing ca. 10 nmols of sugars per material.

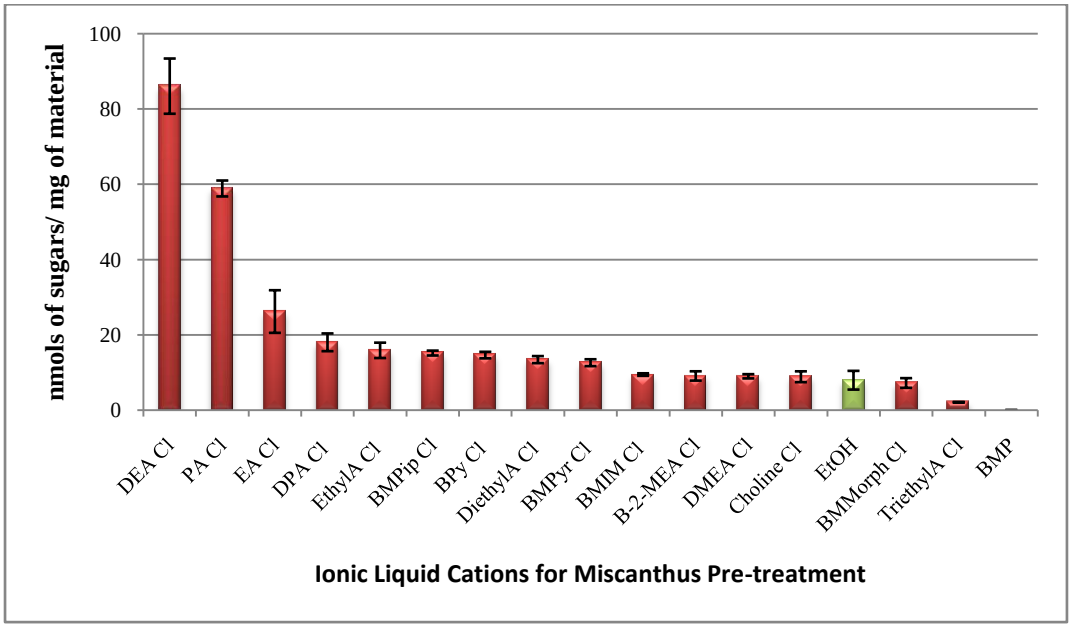


**Figure 56: Miscanthus pre-treated in chloride based ILs with different cations for 24 hrs. Sugar release is shown left to right from highest to lowest amount. The green bar shows the ethanol control.**

An initial structure-activity summary based on the data from Figure 56, is that all of the other ionic liquids performed better or equal as a pre-treatment on miscanthus then the ethanol control, except [TriethylA] and [BMP] cations. However, the improvement for most of the cations was only fractional compared with the control

and the significant difference and increase in improvement is only with the top 3 IL cations of [DEA], [PA] and [EA] respectively.

To understand in detail any structure-activity relationships, the following experimental data in Figure 52 will be discussed in full for each of the series of IL cations and then be converted to improvement ratios in order to see comparisons clearly across the series of ILs from figures 52-55.

### *2.4.4 Aprotic Cation Structure Improvement on Pre-treatment*

What is the structural basis of the cation's efficiency in saccharification of plant biomass? To answer this question, a close comparative examination into the structure of each cation is essential. Here, we focus specifically on the aprotic cations tested, in order to determine what makes the best IL cation for pre-treatment. The focus is on miscanthus to see the effect each IL structure had on the sugar release.

Figure 57 shows the improvement ratios for the 5 aprotic cation structures from Figure 52 as well as the [BMIM] cation as a reference. The graph shows that the [BMP] cation did not work, as the sample 'burned' during pre-treatment and what was left was a black carbon rich material that could not be hydrolysed.

The other samples all worked as pre-treatment solvents with [BMPip] and [BPy] being the most successful miscanthus pre-treatments. [BMPip] and [BPy] are based on piperidine and pyridine and hence both contain 6 carbons. The graph shows that six membered rings were therefore more successful than 5 membered rings. However, there is only a little difference in the improvement of sugar release from miscanthus regardless of whether the cation was aromatic or not, implying that these differences are not significant.

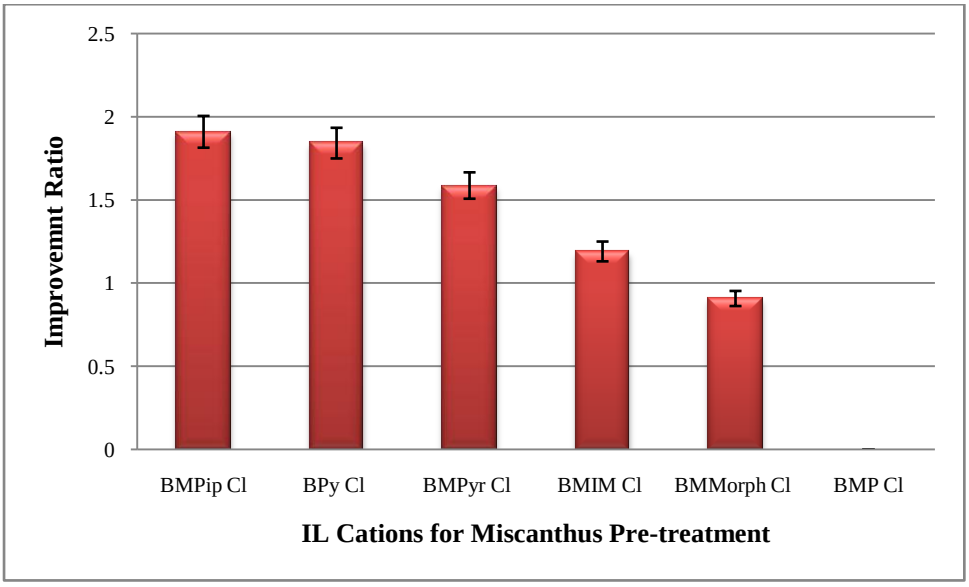


**Figure 57: Improvement ratio of IL aprotic cations for pre-treatment of miscanthus based on aprotic structure-activity design shown in Figure 52.**

However, overall the improvement in the aprotics with all the cations in general is very low and hence no significant conclusions can be made about the structure of the aprotics versus benefits of miscanthus pre-treatment. In this case all of the aprotic cations did not perform effectively as pre-treatment solvents, which is actually not a negative result. Our project aims are to use protic ILs, which are cheaper to synthesise, as the pre-treatment solvents and so having protic IL cation structures as the best pre-treatment solvents is more important and beneficial for the future of IL and plant biomass pre-treatment. This is because this project will require large scale pre-treatment of plant biomass, hence the pre-treatment method used needs to be economically feasible.

### *2.4.5 Protic Cation Structure Improvement on Pre-treatment*

In the previous subsection, aprotic cations have been shown not to improve miscanthus saccharification significantly. Will protic cations cause notable improvements on saccharification? The goal here is to continue to pave a way towards a structure-activity relationship. To this end, a systematic modification of the end group of the n-chain on the cation, as well as the chain length, have been introduced, to see how it affects saccharification. An addition benefit protic ILs have in the use in pre-treatment is having the feature of being tuneable due to the proton transfer equilibrium.[99] This allows for further observations, with some catalytic activity, to be made.

**1) The effect of the cation end group.**

Figure 58 shows the improvement ratio from protic IL structures from Figure 53 with 2 carbon chains off the ammonium centre. The data shows that although the size of the cation is important as literature states[27] over 6 carbons the results of biomass pre-treatment can decline.

In this experiment, removing those OH groups and replacing them with $OCH_3$ or $CH_3$ dramatically reduces the effectiveness of the cation with $Cl^-$ anions on miscanthus pre-treatment. The lowest improvement result was the bis-2-methoxy group but neither the diethyl or dipropyl units performed much better in the pre-treatment of miscanthus. These results indicate that the OH group is crucial in the successful pre-treatment of the biomass. This will be further investigated with structural and chemical studies of the pre-treated plant biomass in chapter 3.

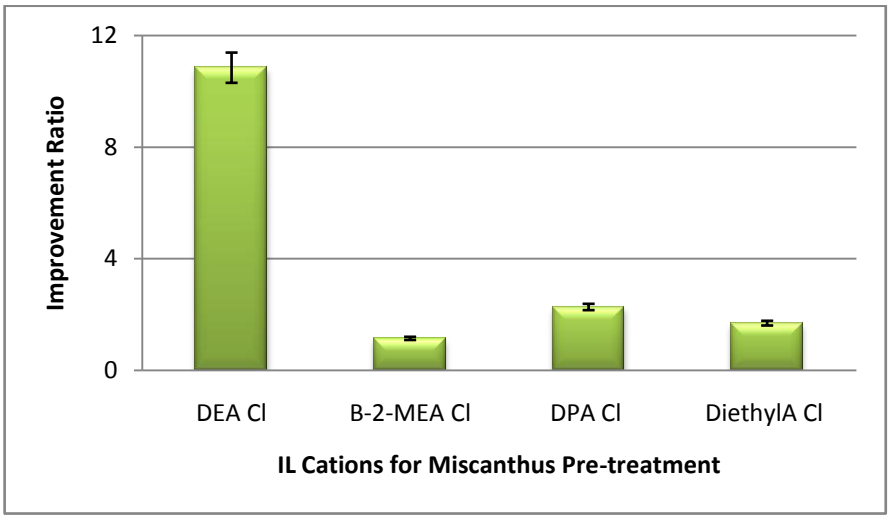


**Figure 58: Improvement ratio of IL cations for pre-treatment of miscanthus based on protic design shown in Figure 52.**

### 2) The effect of chain length

Figure 59 shows the improvement ratios for the cation structures from Figure 54 based how many OH groups are present, as well as chain length. The data shows that for ethylammonium there is little function in this IL as a pre-treatment solvent, so having more protons around the nitrogen centre also seems to have little effect on improving the pre-treatment compared to [DEA] Cl.

As can be seen from the graph, when you reduce the number of OH groups from 2 to 1 there is a dramatic reduction from 11 to 7 in the improvement ratio, from [DEA] to [PA] and [EA] Although as should be noted with [PA] there is also a chain length effect. However, this is still a significant improvement from the ILs for pre-treatment, just less effective then [DEA]. The data proves the presence of the OH on the protic IL cation is the most significant factor for effective pre-treatment on biomass.

Interestingly, between [PA] and [EA] there is a reduction from 7 to 3 within the improvement ratio. This result could potentially imply size of cation plays a role, or that the interaction between the IL cation and anion will affect the potential interactions with the biomass and IL. This is not what expected as usually increased chain length has a negative effect, but not in this case.

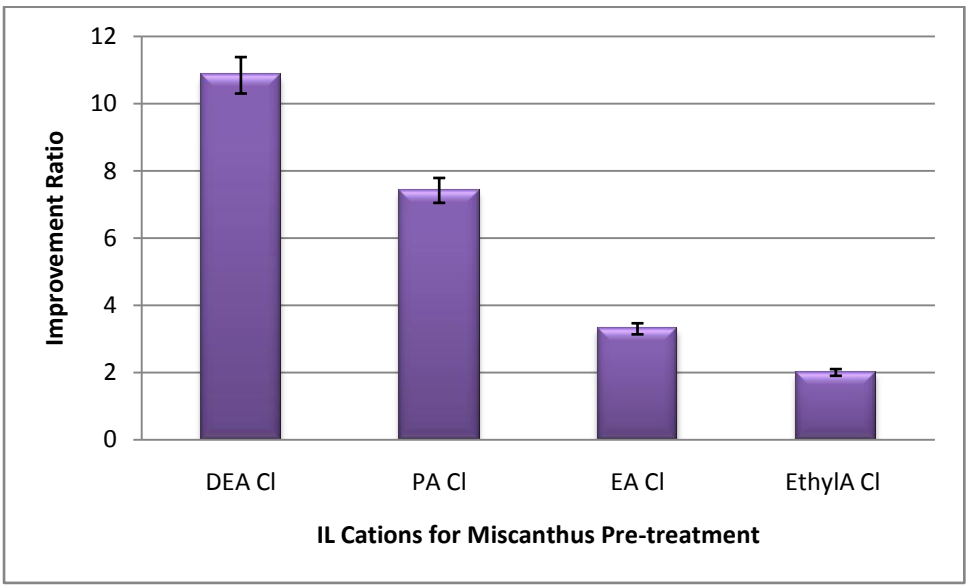


**Figure 59: Improvement ratio of IL cations for pre-treatment of miscanthus based on protic structure-activity designs as shown in Figure 54.**

### 3) Protons around the cation

In order to understand the origin of the difference between protic and aprotic ILs, a systematic modification has been introduced to control the number of protons around the IL cation to analyse the effect this had on miscanthus saccharification.

The last set of ILs are shown in Figure 60, which shows the improvement ratios for the protic/aprotic cation structures in Figure 55. The graph shows that with 1 OH group, as both choline and DMEA cations contain, the saccharification efficiency results are poor. This result was not expected based on the previous results from Figure 59, as the OH groups play an important role in successful miscanthus pre-treatment.

However, the reason for this reduction could be due to the choline Cl and DMEA Cl are solid ILs at 100 °C, and hence the pre-treatment effects between solid IL and solid biomass are significantly reduced compared to liquid IL and solid biomass. To test whether this pre-treatment would function with these ILs, higher temperatures would be needed but this could start to break down the biomass by thermal pathways and also goes against the green aims of this project. Therefore choosing ILs that are liquid below 100 °C is necessary for the environmental approach to be considered. In addition, the use of IL solvent mixtures may also play a role.

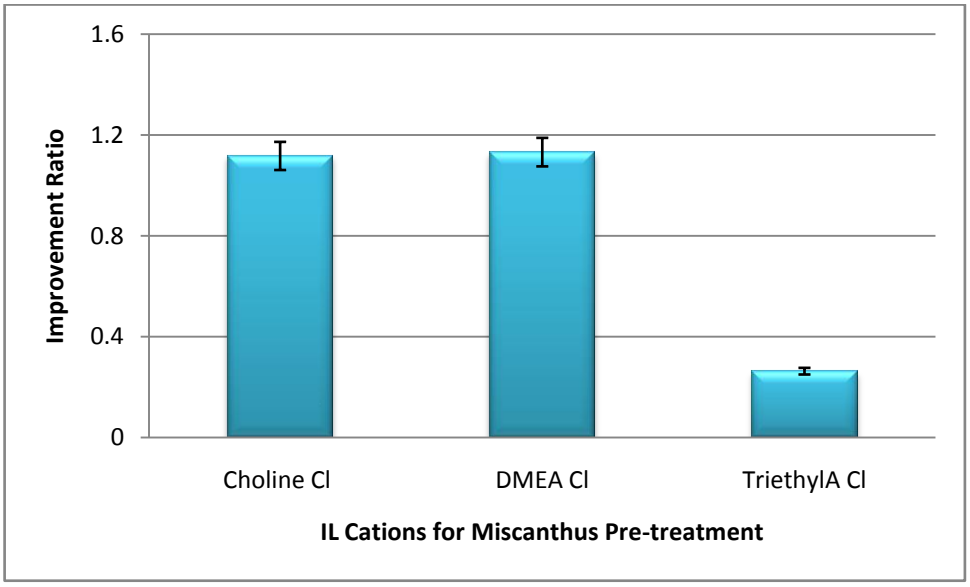


**Figure 60: Improvement ratio of IL cations for pre-treatment of miscanthus based on protic structure-activity designs as shown in Figure 55.**

### *2.4.7 Conclusions of IL Cation Design on Miscanthus Pre-treatment*

In summary all of the improvement ratio data from the aprotic and protic IL structures were plotted on the same graph for comparison. Figure 61 shows this comparison of all the improvement ratios from the previous 4 figures. As can be seen;

(1) There is only a little trend with the red bar aprotic cations, where 6 membered rings featured as being more effective then 5 membered rings, but no trend between aromatic *vs* none aromatic structures.

(2) No trend with the green bar protics, indicating that the OH functional group is important for effective miscanthus saccharification

(3) The blue bar protic ILs confirm that both protons around the nitrogen centre are important, but more so that the ILs need to be liquid at 100 °C

(4) The clearest trend is with the purple bar ILs, based on OH functionality and size of cation, it is clear to see that overall the presence of OH on the nitrogen chains is essential for effective miscanthus pre-treatment.

The reasons for these difference will be explored in detail in chapter 3.

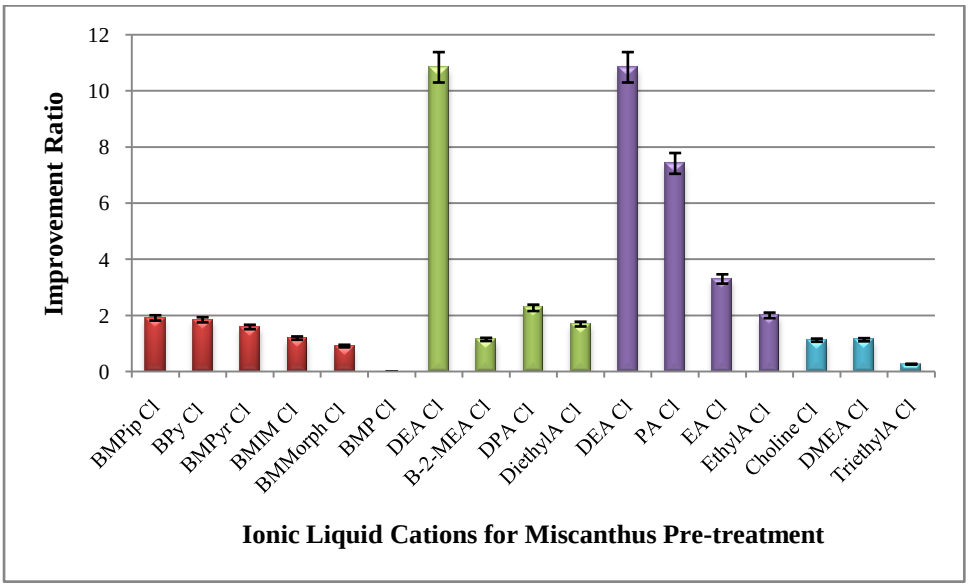


**Figure 61: Comparison of the cations used to pre-treat miscanthus in groups and in order from most effective to least effective. Figure 52 cations in red, Figure 53 cations in green, Figure 54 cations in purple and Figure 55 cations in blue.**

## *2.4.8 HPLC results of miscanthus pre-treated in aprotic ionic liquids*

To investigate whether the different cations had different pre-treatment effects on the sugar polymers, the amount of sugars released after hydrolysis were analysed using HPLC as in section 2.3. Figure 62 shows some of the aprotic cations used to pre-treat miscanthus. The data shows although there is some difference in the amount of xylose or glucose in the hydrolysis mixture after the incubation period, and this variation must be contributed to the IL cation structure, there is no structure-activity trend between the cation designs.

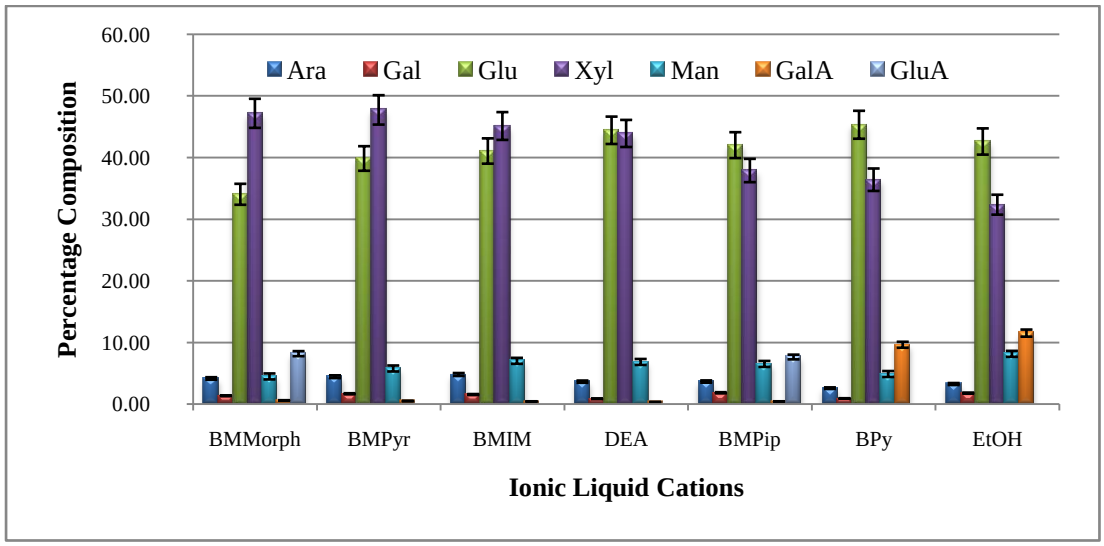


**Figure 62: HPLC of sugars released after pre-treatment of aprotic chloride ionic liquids.**

## *2.4.9 HPLC results of miscanthus pre-treated in protic ionic liquids*

To investigate the origin of this wide-variety in saccharification efficiency, the affinity of the ILs to the polymer chains may give us crucial information. This is because, upon saccharification, the ILs are expected to interact with the polymer fibres and disrupt the electrostatic and in some cases covalent bonding. HPLC is employed to address this because we can see how the sugar content changes and this can be linked back to the polymers. HPLC will not give us any information on lignin with it being a non-sugar based polymer.

In comparison to 2.4.8, Figure 63 shows some of the protic IL cations used to pre-treat miscanthus. The data shows greater differences in glucose and xylose released by the hydrolysis after IL pre-treatment, compared to the aprotic cations. However, again there is no trend between structure and activity of the cations of sugar release from the plant polymers. In the cases where the ILs function less effectively then [DEA] Cl, sometimes have mixtures containing more xylose, however, in other cases have mixtures with more glucose, hence from the HPLC sugar data no clear trends can be observed. Chapter 3 will probe these differences further.

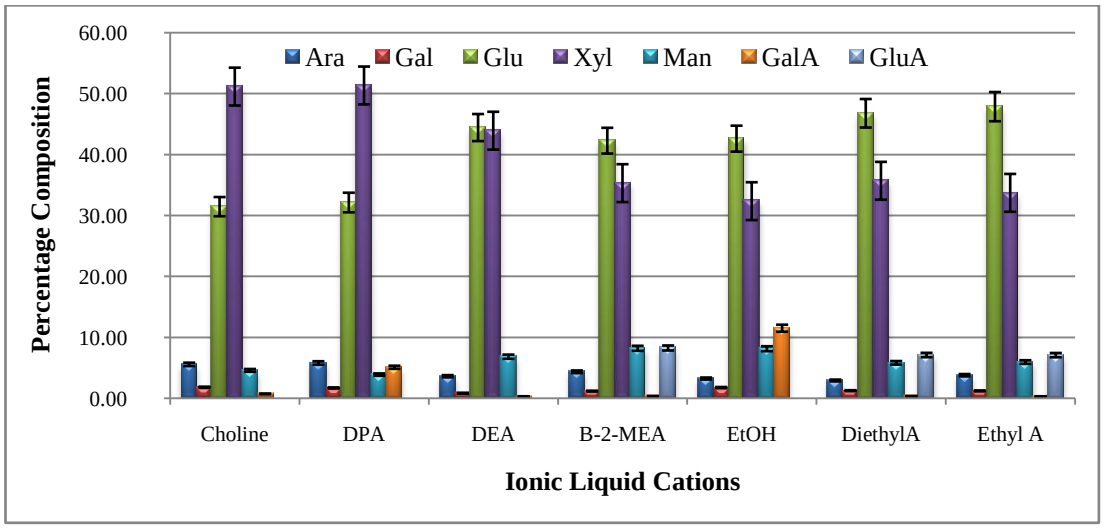


**Figure 63: HPLC of sugars released after pre-treatment of protic chloride ionic liquids.**

## 2.5 Ionic Liquid Anion Modification for Pre-treatment of Miscanthus

Having found that $[DEA]^+$ is the optimum cation tested for plant biomass pre-treatment in the previous subsection, now the choice of anions should also be considered and optimised in a similar manner. To do so, one has to bear in mind that a good IL anion in plant biomass pre-treatment should be two things; (1) Capable of making hydrogen bonds with the polymers (cellulose, hemicellulose or lignin), in order to open up the lignocellulosic materials; (2) Have a high hydrogen bond basicity.[157]

Research, based on Kamlet-Taft paramaters has shown, that an anion with a $\beta > 0.80$ exhibits sufficient swelling and dissolution of biomass.[157] Kamlet-Taft parameters quantify three polarity measurements of solvents. $\pi^*$ is general dipolarisability, $\alpha$ is the hydrogen bond acidity, $\beta$ is the hydrogen bond basicity. Initial research with [BMIM] as the cation has shown that Cl had a $\beta$ value of 0.83 and Ac had a $\beta$ value of 1.2, making both of these anions suitable choices with aprotic cations for biomass pre-treatment.[157]

The next set of pre-treatment experiments were designed to alter the anion and keep the cation the same. Diethanolammonium [DEA], was used, as this currently is the best choice for our pre-treatment as proven in section 2.4. Anions like $[BF_4]^-$, $[PF_6]^-$ and $[Tf_2N]^-$ were all automatically excluded from the study based on poor hydrogen bonding.

### *2.5.1 Anion Choice for Pre-treatment of Miscanthus*

According to research[153], as previously stated, effective anions for plant biomass pre-treatment should have the following properties:

- Highly basic anions
- Capable of hydrogen bonding

Therefore, the following are some anions which satisfy the above; Nitrate $[NO_3]^-$, acetate $[OAc]^-$, hydrogen sulphate $[HSO_4]^-$ and fluoride-doped [DEA] Cl. Figure 64 shows the chemical structures of the anions used in this section. For the fluorine-doped experiments, the source of the fluorine was TBAF with 5%, 10% and 15% weight doping used in [DEA] Cl. TBAF was chosen as this has been suggested as been able to dissolve cellulose effectively in combination with more toxic and non-recyclable solvents or aprotic ILs.[158]

[DEA] Cl was used as a control as well as the standard ethanol control. Saturated ammonium chloride solution ($NH_4$ Cl) was used as a none IL, to further prove the need for both the cation and anion to be present for the pre-treatment of plant biomass to function effectively in the presence of ILs. The pre-treatments were tested for both 5 hours and 24 hours, depending on the initial improvement, to determine if sufficient sugars were released from the miscanthus after 5 hours or if further time was needed.

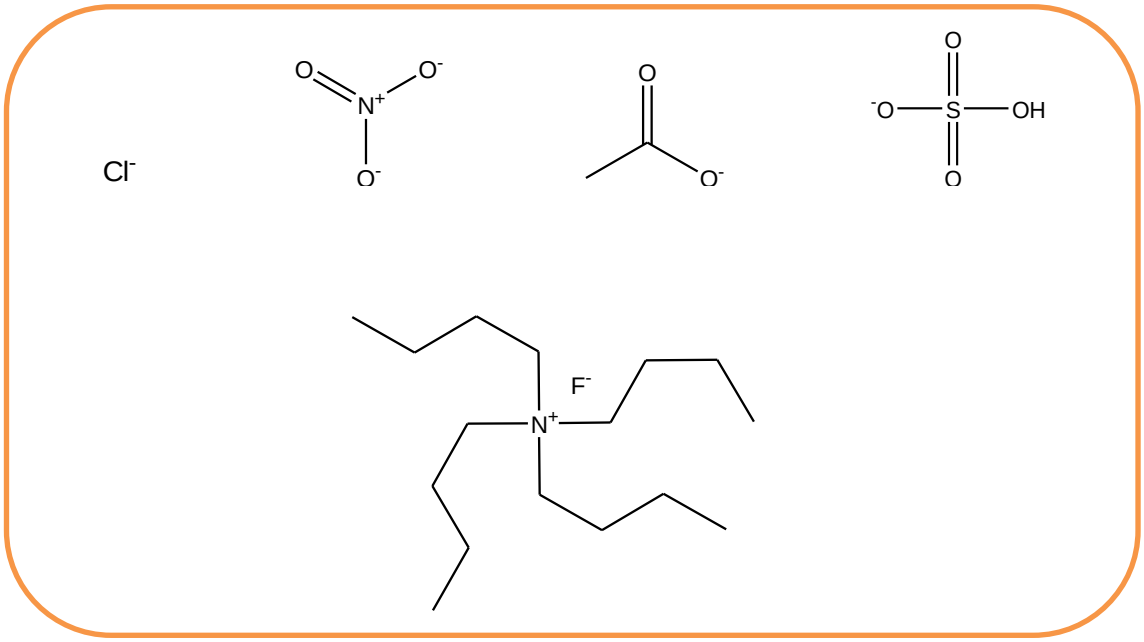


**Figure 64: Structures of anions used in miscanthus pre-treatment and TBAF.**

## *2.5.2 Anion Comparison Pre-treatments on Miscanthus*

All of the anions shown in Figure 64 were used with the [DEA] cation as a pre-treatment solvent on miscanthus. Figure 65 shows the saccharification results for the anion IL trend. The red bars are 5 hour pre-treatment times and the blue bars are 24 hour pre-treatment times, both at 100 °C. This is the initial sugar release data showing the nmols of sugars released per mg of material.

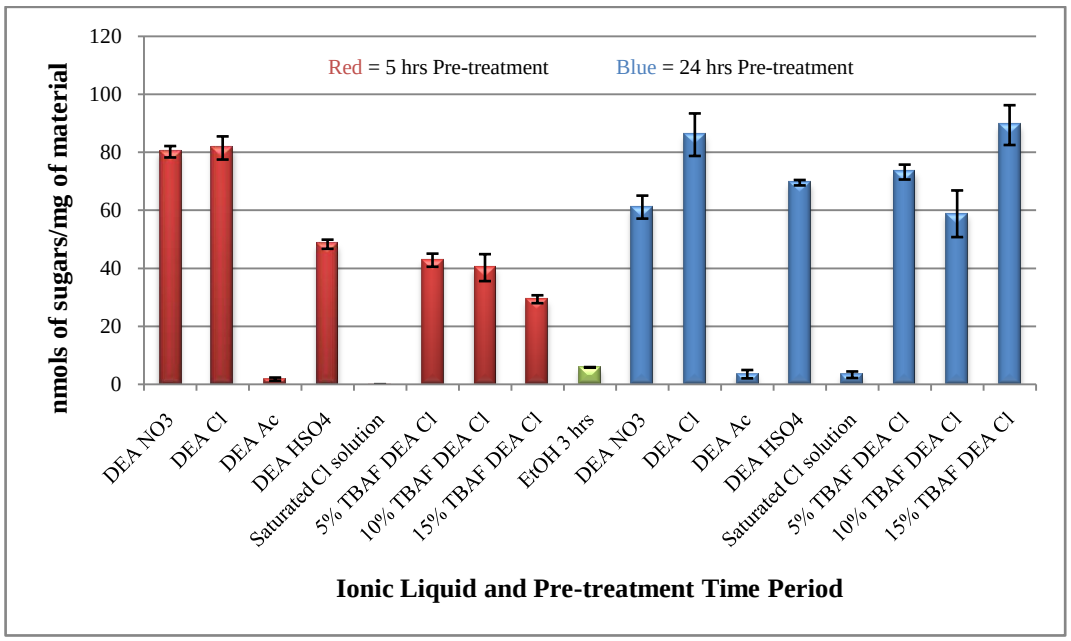


**Figure 65: Miscanthus pre-treated in diethanolammonium based ILs with different anions for 2 different time periods, 5 hrs and 24 hrs. The green bar shows the ethanol control.**

The order of anions in relation to improvement is Cl > $NO_3$ > $HSO_4$ > OAc. This is interesting as there is a link with the Hofmeister series[124, 159] in this order of improvement, however, as this series relates to proteins and is indicative of the water content of them, how well it truly relates to pre-treatment of plant biomass is unknown.

The results are also interesting as [DEA] Cl is clearly the most effective IL for pre-treatment of miscanthus, as at both 5 hours and 24 hours, the most sugars are released from this pre-treatment. The [DEA] $NO_3$ shows very promising initial results as after 5 hours ca. 80 nmols of sugars per mg of material are released, equal to that of [DEA] Cl. However, after 24 hours of pre-treatment the amount of sugars released after saccharification actually reduces with $[NO_3]^-$ to ca. 60 nmols of sugars

per mg of material. The reasons for this could be due to the $[NO_3]^-$ dehydrating sugars into reduced compounds like HMF or residual nitrate in the biomass samples, denaturing the enzymes during hydrolysis.

With the $HSO_4$ anion and the TBAF doped [DEA] Cl, the same trends occurred. As the time increased for pre-treatment with the miscanthus, the effect they had on the material did so also. Within error, all these pre-treatments produced the same result, regardless of the amount of TBAF in the [DEA] Cl, implying this had no added positive effect. However, overall the greatest improvement of these ILs was ca. 60 nmols of sugars per mg of material, which is significantly less than [DEA] Cl pre-treatment, so some apparent detrimental effect.

Current research suggests synthesising new fluorous ILs involving imidazolium cations have the potential to improve biomass pre-treatments due to fluorine-fluorine interactions, but extreme sensitivity to this is shown to the cation structure and smaller sized chemical units are preferred, hence no further work was completed with fluorine.[121]

The saturated ammonium chloride solution had virtually no effect on the miscanthus and the sugars released were less than the ethanol control. The [DEA] [OAc] also had little effect and is an unsuccessful pre-treatment solvent. This is interesting as when [OAc] is paired with [EMIM] it is a successful pre-treatment solvent, implying the reasons why [EMIM] and [DEA] are effective are notably different.

Figure 66, overleaf, shows the improvement ratios of the anions used in section 2.5.3, removing the saturated ammonium Cl solution and the doped TBAF [DEA] Cl samples, as these had no improvement effect on the pre-treatment.

As can be seen, the $Cl^-$ anion is the best overall with $[NO_3]^-$ being a close second. However, due to protic $[NO_3]^-$ ILs being reported as explosive,[78] with the potential strong oxidising properties of nitric acid under anhydrous conditions, a violent reaction can occur with amines as they are hypergolic. Therefore, $[NO_3]^-$ anions were not used for safety reasons in further experiments. A further example of how dangerous these anions can be lies in the fact that ethylammonium nitrate is used as monopropellant.[78]

The $[HSO_4]^-$ performed better at pre-treating miscanthus at longer times, but still significantly less effective than $Cl^-$. Acetate was ineffective at pre-treating the miscanthus with very poor sugar release after the pre-treatment as previously stated.

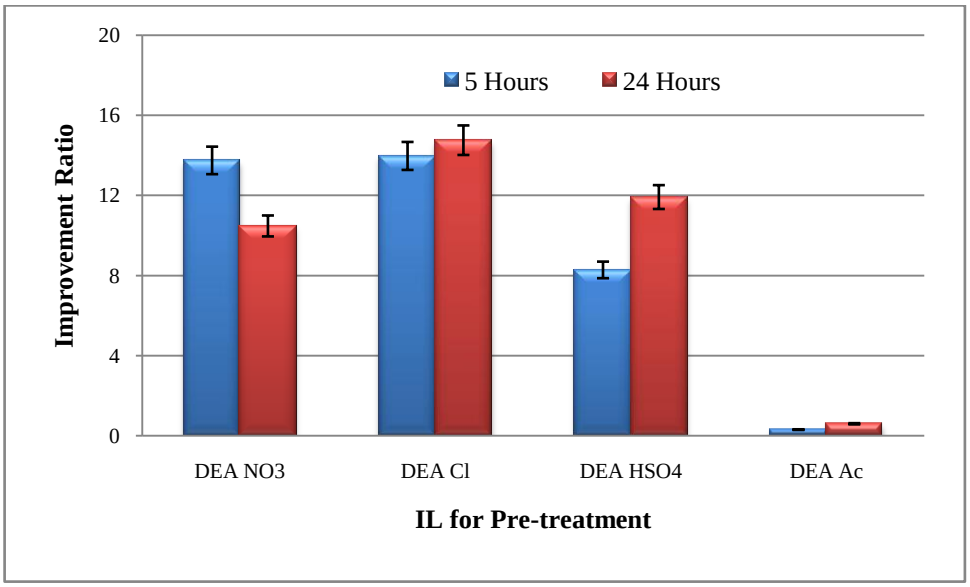


**Figure 66: Improvement ratio of anion variables for pre-treatment on miscanthus with [DEA] cation.**

Hence in summary, [DEA] with Cl is the most effective pre-treatment for miscanthus, but $NO_3$ can also be used to get the same high release of sugar results. However, if using $NO_3$ as a pre-treatment anion, great care should be taken when handling these ILs.

### *2.5.3 Aprotic IL Anion Changes with [BMIM]$^+$ Cation*

Having focused extensively on the protic cation with anion species dependence on miscanthus saccharification, here is a comparison with the standard aprotic cation [BMIM] and some anions.

Interestingly, although some reports[27] state that the acetate anion shows promise at dissolving cellulose, depending on the cation, in these studies the acetate proved to be ineffective at pre-treating miscanthus as shown in Figure 66. Also changing the cation to [BMIM] and testing with Cl and [OAc] also proved to be ineffective, as Figure 67 shows. The [OAc] did not improve the pre-treatment and both [BMIM] ILs are still far less effective than the [DEA] Cl IL for miscanthus pre-treatment.

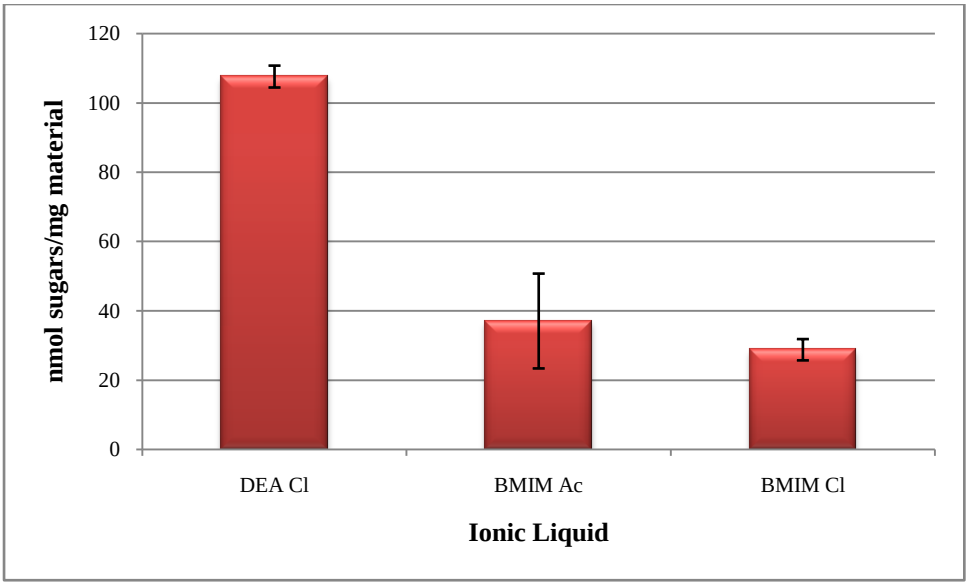


**Figure 67: Comparison of sugar release after pre-treatment with [BMIM] and either Cl or [OAc] as the anion and [DEA] Cl.**

# 2.6 Microwaves

## *2.6.1 Introduction*

In conventional heating, all the samples have been heated in an oil bath. Even though it is easy to set up, accessible and cheap, this method has various shortcomings, namely;

1. The time taken for heating the system
2. Uniformity of heating, especially as the IL system is viscous
3. Efficient heat transfer in viscous systems

To overcome these shortcomings, microwaves can be used as an alternative heat source. Microwave heating results in the volumetric heating of materials, compared to conventional heating which relates to heat conduction.[160] As microwave heating relates to polarisation of materials, polar solvents like ILs absorb microwave irradiation effectively and this gives ILs significant benefits over molecular solvents, due to their higher heat capacity.[160]

Microwave heating is core volumetric heating, where the whole volume is simultaneously heated by direct coupling of microwave energy between any molecules in the pre-treatment solution.[160]

Microwave technology is emerging as a useful tool for chemical processes including polymers and organic synthesis. Microwave irradiation, which has been proved to be a clean, fast and convenient energy source, has been used in enzyme-catalysed reactions[161] and to be an effective method at accelerating the rate of heterogeneous catalysis, but this is in debate.

Microwaves have been used to show the increased solubility of cellulose in [BMIM] Cl.[160]Microwave pre-treatment has been noted to destroy the surface structure of biomass particles, improving the effective interactions between the biomass and the catalyst.[162]

Table 13 shows examples of IL pre-treatment of cellulose and the comparison between conventional and microwave heating. As it shows effective solubilities of cellulose have been achieved in the microwave after short time periods, hence this improvement should also be possible with [DEA] Cl pre-treatment of biomass.

**Table 13: Solubility of Dissolving Pulp Cellulose in Ionic Liquids from [105].**

| Ionic Liquid | Method | Solubility (wt %) |
|---|---|---|
| **[$C_4$mim]Cl** | heat (100 °C) | 10% |
| | (70 °C) | 3% |
| **[$C_4$mim]Cl** | heat (80 °C) + sonication | 5% |
| **[$C_4$mim]Cl** | microwave heating | 25%, clear |
| | 3−5-s pulses | viscous solution |
| **[$C_4$mim]Br** | microwave | 5−7% |
| **[$C_4$mim]SCN** | microwave | 5−7% |
| **[$C_4$mim][$BF_4$]** | microwave | insoluble |
| **[$C_4$mim][$PF_6$]** | microwave | insoluble |
| **[$C_6$mim]Cl** | heat (100 °C) | 5% |
| **[$C_8$mim]Cl** | heat (100 °C) | slightly soluble |

For these experiments, all samples were pre-treated using an experimental setup where the temperature was kept constant at 100° C. The results show microwaves could be promising as a heating source for ionic liquid treatment of biomass. The power (W) required to keep the samples at the required temperature was minimal. After the initial input the ionic liquids maintain the temperature for 4-8 minutes, sample dependant. The pressure value was the same for all experiments and could be an indication of the water present in the sample if the pressure rose to excess and the pre-treatment automatically stopped.

The following section shows how time period, IL cation/anion and biomass sample choice, all can be pre-treated using the microwaves with effective results.

## *2.6.2 Time Period Investigation*

The aim here is to examine the effect of microwave time period on pre-treatment of plant biomass. To this end, miscanthus was pre-treated with [DEA] Cl in a microwave. The period for pre-treatment has been varied between 5 and 60 minutes.

Figure 68 shows this data and over time there is a slight increase from 5 minutes of pre-treatment in the microwave to 60 minutes pre-treatment, going from ca. 60 nmols of sugars per mg of material to ca. 80 nmols of sugars per mg of material.

However, this improvement is not significant over time and in fact it quickly saturates after the initial 10 minutes. Thus a microwave pre-treatment time of 10 minutes is deemed to be sufficient enough to successfully pre-treat the miscanthus for enzyme hydrolysis, with 60 minutes being the most effective time period.

Comparing this to the ethanol control, the 10 minute pre-treatment is 12 times more effective than no pre-treatment, which matches with conventional pre-treatment but after just 10 minutes compared to 24 hours. This is a significant improvement and reduction in energy required using the microwave over conventional methods.

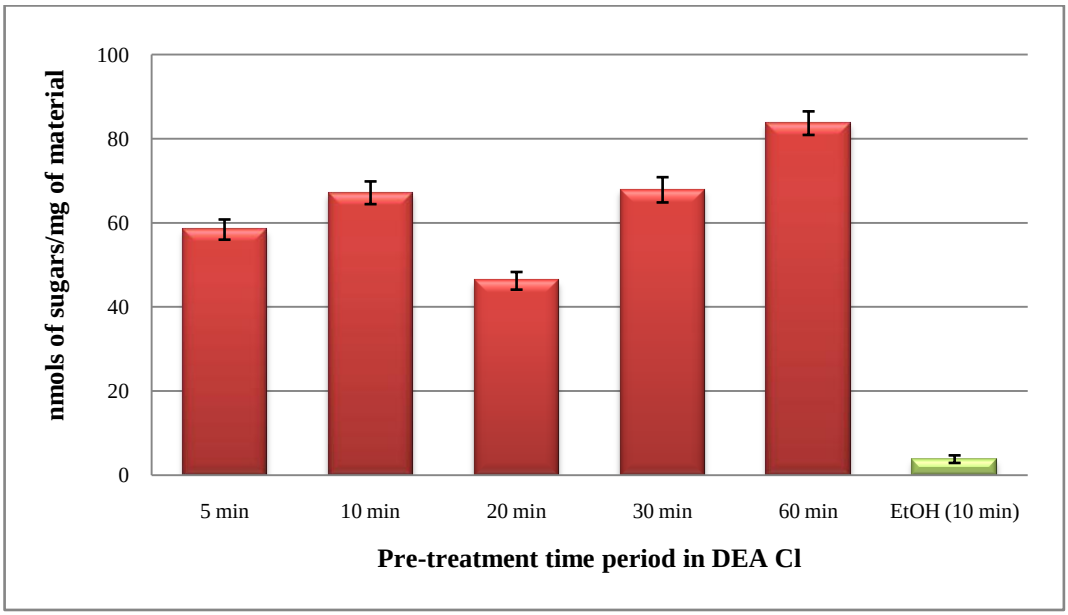


**Figure 68: Miscanthus pre-treated in [DEA] Cl over a range of time periods, using microwave heating. Ethanol control shown in green.**

### *2.6.3 Ionic Liquid Structure Modification for Miscanthus Pre-treatment*

In order to analyse if some of the IL structures from section 2.4 could pre-treat biomass more effectively with microwave pre-treatment over conventional pre-treatment, miscanthus and poplar were pre-treated in a range of ILs for 10 minutes in the microwave at 100 °C.

Miscanthus was chosen as a direct comparison to section 2.4. Poplar was chosen as a second biomass to compare with miscanthus and which showed promise from section 2.3. However, as poplar was the largest in particle size of all 5 biomass types, it would be interesting to see if poplar could be pre-treated successfully for very short time periods in the microwave, confirming the role of particle size with these protic IL pre-treatments.

The ionic liquids chosen were some of the cation choices in section 2.4, figures 52-55. [HMIM] Cl was used in both cases but due to potentially high viscosity of [HMIM] the samples did not work conventionally and were not shown in section 2.4, however, [HMIM] was re-tried with microwave pre-treatment. Also the anion $NO_3$ was used as a close comparison to Cl with the [DEA] cation.

Figure 69 shows the results for miscanthus pre-treatment with variations in the IL structure and Figure 70 shows the results for poplar pre-treatment with the same IL structure variations. The ionic liquids in both figures are presented in the same order with the ethanol control shown in green.

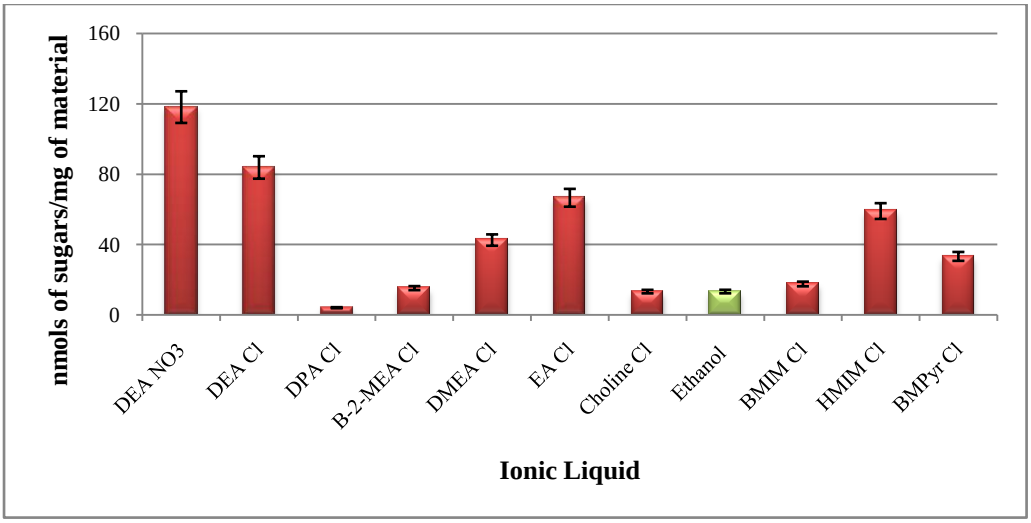


**Figure 69: Miscanthus pre-treated in ionic liquids for 10 minutes. Ethanol control in green.**

With miscanthus pre-treatment [DEA] Cl and [DEA] $NO_3$ continue to be the best IL choice, however, using microwave pre-treatment other ILs including [DMEA] Cl and [HMIM] Cl have improved performances in the microwave compared to the conventional pre-treatment.

With the poplar pre-treatments, [DEA] Cl and [DEA] $NO_3$ are once again the best IL choice, however, this time ILs like [DPA] Cl and [HMIM] Cl have improved performances on saccharification efficiency compared to conventional heating.

There are clearly some similarities in the ILs effective pre-treatments, which was expected, but interestingly some differences where some ILs are now functioning as potential effective pre-treatments on biomass, where as conventionally they didn't.

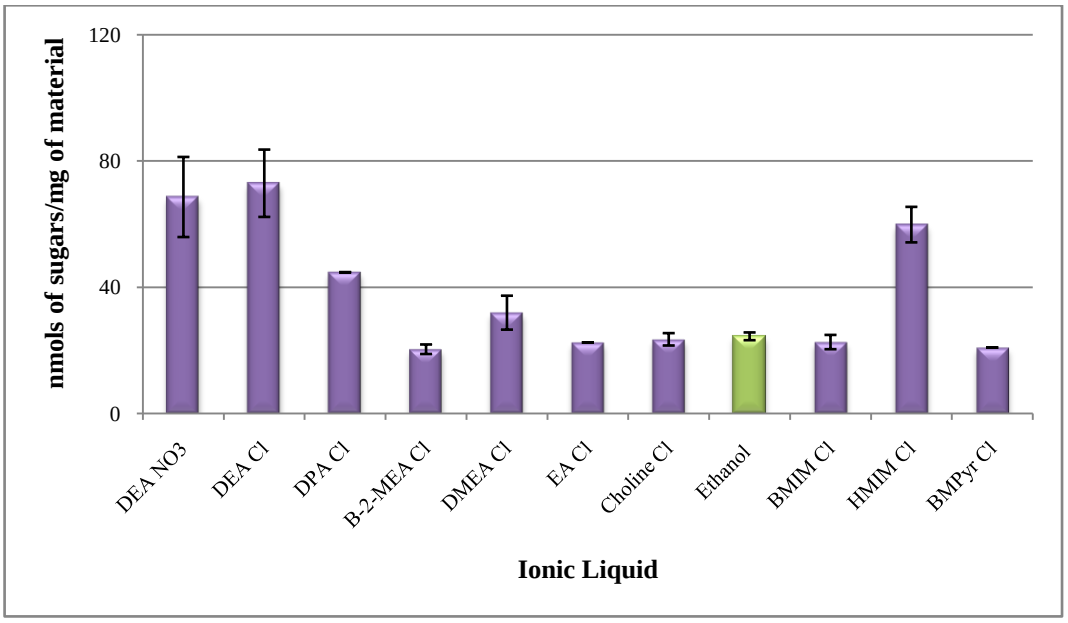


**Figure 70: Poplar pre-treated in ionic liquids for 60 minutes. Ethanol control shown in green.**

Figure 71 shows the improvement ratios of the pre-treatments of miscanthus and poplar with the variety of IL structures tested. From this comparison it is now clear to see which ILs have preferential pre-treatment with each biomass material, as well as how successful they are. [EA] Cl has little effect on poplar and so is not useful as a microwave pre-treatment, however, [HMIM] Cl shows promise with both materials, especially miscanthus in microwave pre-treatments.

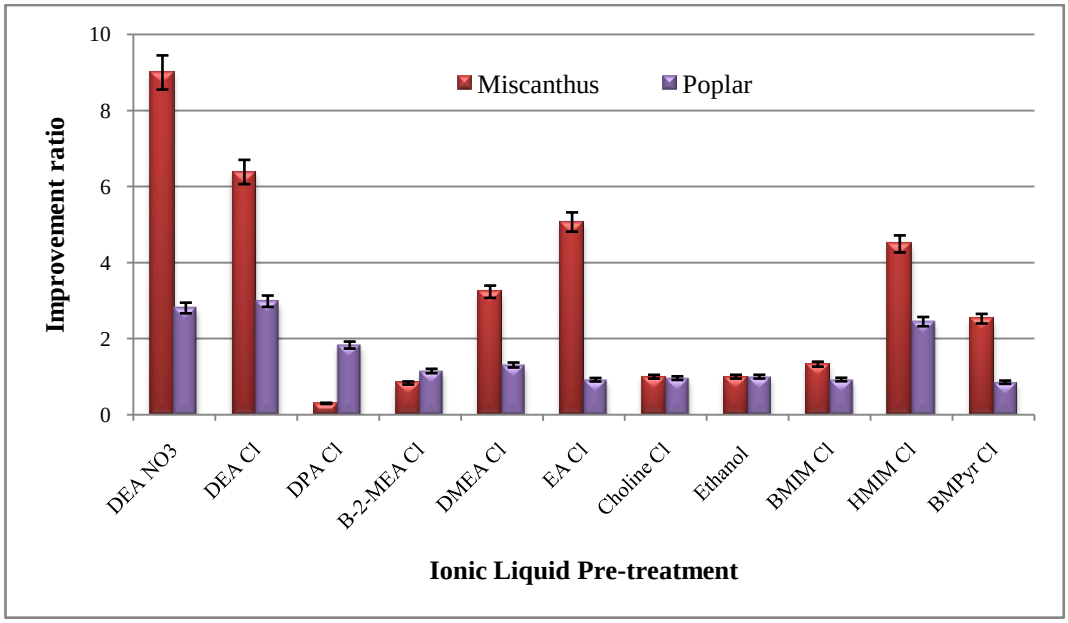


**Figure 71: Improvement ratios for miscanthus and poplar in ionic liquid.**

## Comparison of conventional versus microwave pre-treatment

Here the effectiveness of ILs have been compared between conventional heating and microwave heating pre-treatment. Figure 72 shows this comparison of the ILs used to pre-treat miscanthus under conventional conditions and using the microwave and there are a few interesting results.

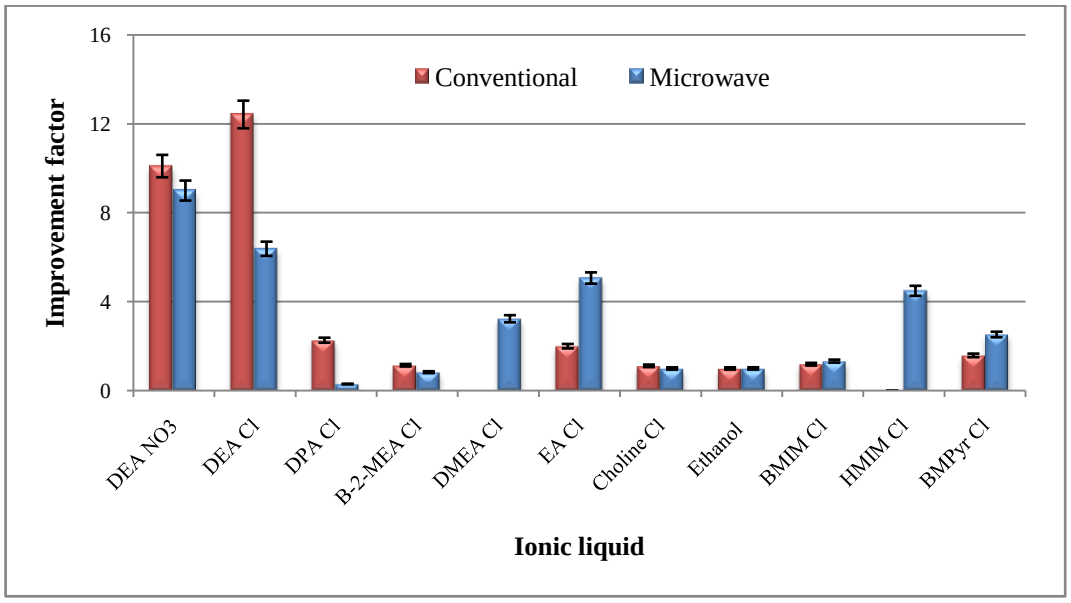


**Figure 72: Comparison of microwave pre-treatment to conventional pre-treatment conditions with a variety of aprotic and protic ILs tested**

Firstly, the best results obtained are with [DEA] Cl conventionally with a 12 times improvement compared to a 7 times improvement with the microwave. This is important as although the conventional results are higher, this is after 24 hours, compared to just 10 minutes in the microwave for a 7 times improvement on pre-treatment.

Secondly, the figure shows how some ILs which had a low effectiveness for the pre-treatment of biomass from conventional heating are now much more successful using the microwave. [HMIM] Cl for example has gone from having no effect to having a 4 times improvement after 10 minutes in the microwave. A potential reason for this is due to localised heating in the microwave. As the sample of miscanthus and IL is heterogeneous, there is a chance of localised heating to significantly higher temperature than 100 °C, which would account for reduced viscosity and hence more effective pre-treatment on a shorter time scale.

## *2.6.4 Biomass Variation pre-treated in [DEA] Cl*

As in section 2.3, the plant biomass choice was varied with pre-treatment in [DEA] Cl to compare and see if the microwave pre-treatment made further improvements on the sugar release for 10 and 60 minutes.

Figure 73 shows the 5 biomass samples pre-treated in the microwave with [DEA] Cl for 10 minutes at 100 °C. The order of efficiency for the different biomass material has more or less remained the same. However, a striking difference has been observed for poplar compared to the efficiencies observed in the conventional pre-treatment (section 2.3).

Firstly, wheat and sugarcane bagasse perform relatively poorly compared to miscanthus on sugar release, this is similar to conventional pre-treatment. The amounts of sugars released from maize stover matches the amount of sugars released from miscanthus which at first looks promising but maize stover is a much easier biomass to break down structurally as section 2.3 showed.

Finally the sugar release rate for poplar is the worst of all 5, the biggest change between using conventional pre-treatments on the biomass and microwave pre-treatments. Is this a particle size issue?

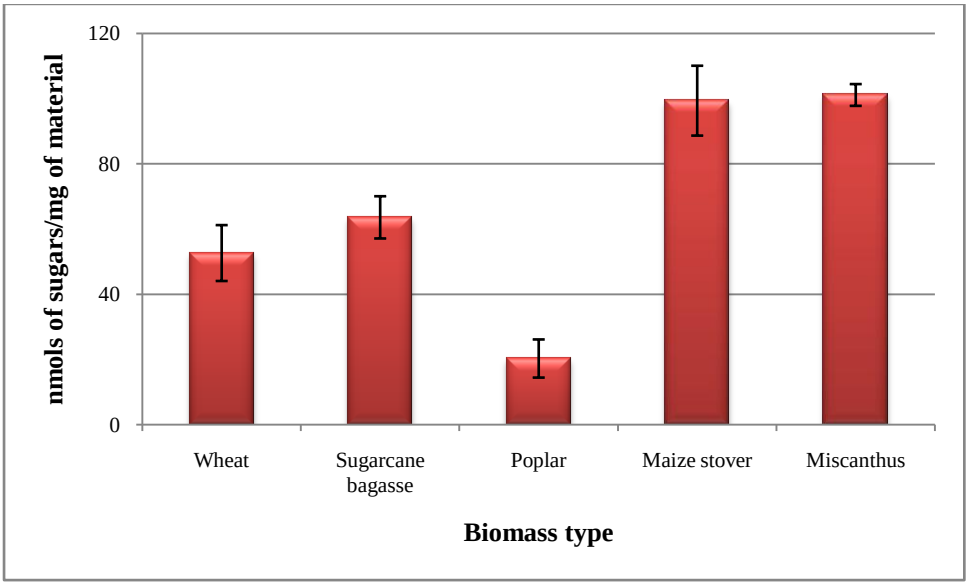


**Figure 73: Biomass types pre-treated in [DEA] Cl for 10 minutes.**

What is the cause of this discrepancy for poplar? To answer this question, we must compare the microwave pre-treatment results after 60 minutes to see if this is a time effect or microwave effect. Even though all of the other biomass materials remain virtually the same with the results after 10 minutes, poplar significantly improves.

Figure 74 shows the same 5 biomass samples pre-treated in [DEA] Cl but for 60 minutes in the microwave. In this figure we can see wheat, sugarcane bagasse, miscanthus and maize stover are all the same as the previous figure, however after 60 minutes the poplar sample has now improved greatly in line with the miscanthus and maize stover samples.

The reason why poplar started off as being pre-treated ineffectively by [DEA] Cl in the microwave after 10 minutes, could be due to the particle size of poplar being significantly bigger than the other 4 biomass samples. Hence for the effects of the IL to take place on the biomass a longer period of time is required.

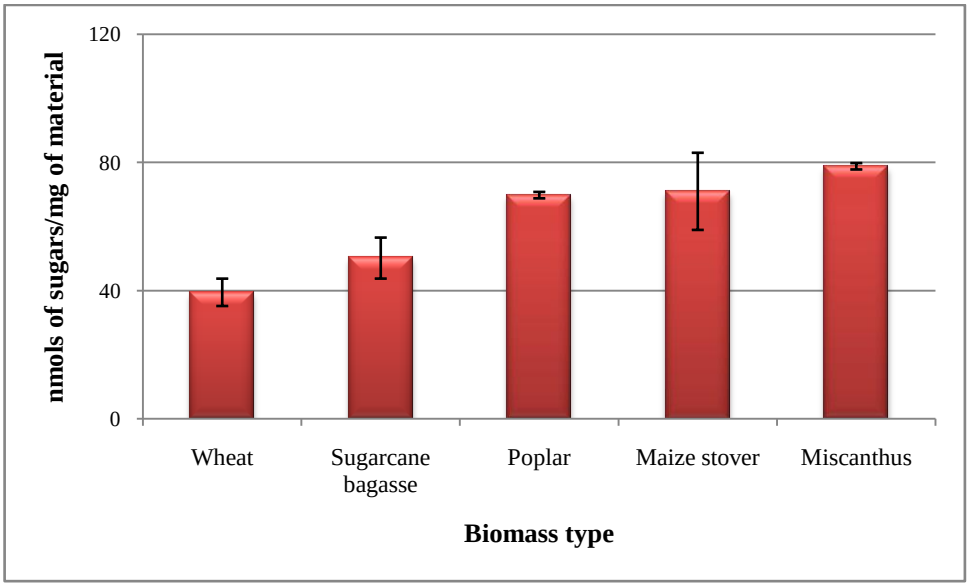


**Figure 74: Biomass types pre-treated in [DEA] Cl for 60 minutes.**

**Comparison of different biomass materials pre-treated with conventional heating and microwave pre-treatment**

In order to see the comparison between the microwave heating and conventional heating, Figure 75 shows 10 minutes in the microwave and 60 minutes conventionally. The data shows in most cases the two methods are comparable and no direct advantage is achieved in increasing the amounts of sugars released. However, using the microwave for pre-treatment does significantly reduce the time needed for the pre-treatment and hence the energy needed.

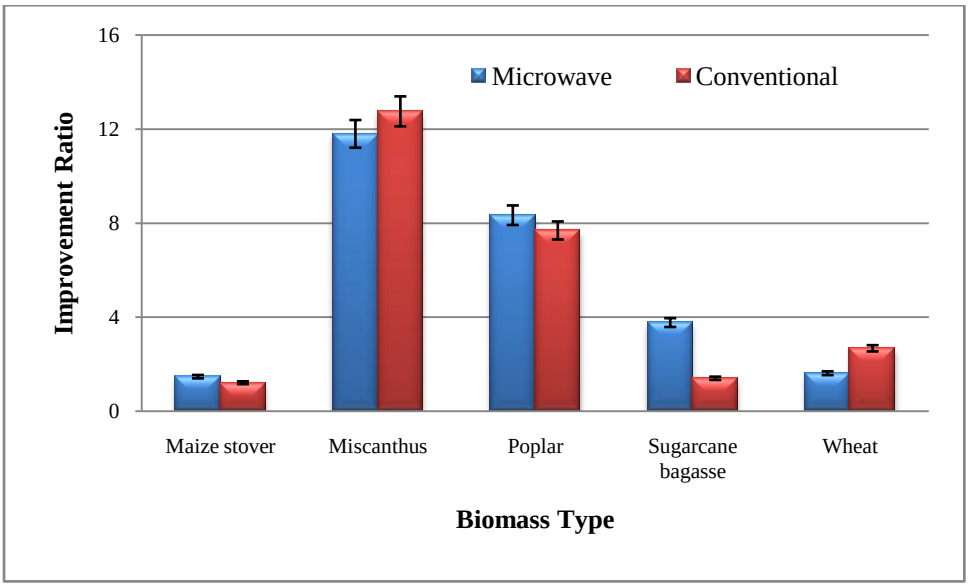


**Figure 75: Comparison of biomass types pre-treated in [DEA] Cl in the microwave for 10 minutes versus conventionally for 60 minutes.**

### *2.6.5 Summary of Using Microwaves with ILs as a Pre-treatment Method on Plant Biomass*

Section 2.6 has demonstrated how microwaves can be used to speed up lignocellulosic pre-treatment for sugar release.

**Time:** Currently, after 10 minutes of [DEA] Cl pre-treatment, miscanthus has a 7 times improvement ratio for sugar release. This is compared to a 12 times improvement on pre-treatment conventionally after 24 hours. This significant time improvement saves time and energy for the pre-treatment.

**Biomass:** For the different lignocellulosic pre-treatments the order of effectiveness has remained the same as conventional pre-treatment, with miscanthus > poplar > sugarcane bagasse > wheat > maize stover. However, for poplar the time required is significantly longer than the other 4 because of the increased particle size of poplar.

**Ionic Liquids:** [DEA] Cl is the most effective IL at pre-treating miscanthus in the microwave, the same as conventional conditions. However ionic liquids which are highly viscous, such as [HMIM] Cl, can now be used as effective IL pre-treatments. This opens up the possibility of using other IL designs, which conventionally would not work, using the microwave could be used.

# 2.7 Standard Industrial Comparisons of Plant Biomass Pre-treatments to Ionic Liquid Pre-treatments

## *2.7.1 Introduction*

In this section firstly, the [DEA] Cl was re-used to show that this IL is recyclable, as an industry process would require this. Also the miscanthus is pre-treated with fresh samples of [DEA] Cl in an attempt to see if a flow process would be beneficial for sugar release after the IL pre-treatments of biomass.

Chapter 1, section 1.3, all of the comparable pre-treatments were discussed and Table 14 shows the summary of all the methods overleaf. As the table shows there are many factors with effective pre-treatment and each different method has advantages with major effects and disadvantages with minor or no effects. For industrial comparisons, dilute acid and dilute base were used, as well as hydrothermal bombs to simulate water pressured reactions in the lab.

Also in this section, [DEA] Cl was used with acid and base pre-treatments, to see if multiple pre-treatment steps had any effect on the overall efficiency of the biomass pre-treatment. Supercritical $CO_2$ was used as a comparison to IL pre-treatment on miscanthus.

**Table 14: Comparisons of current biomass pre-treatments versus their effects on plant biomass.[2,26]**

| | Increase accessible surface area | Decrystallization cellulose | Solubilization hemicellulose | Solubilization lignin | Formation furfural/HMF | Alteration lignin structure |
|---|---|---|---|---|---|---|
| **Mechanical** | *** | *** | | | | |
| **ST/SE** | *** | | *** | * | *** | *** |
| **LHW (batch)** | *** | ND | *** | * | * | * |
| **LHW (flow through)** | *** | ND | *** | ** | * | * |
| **Acid** | *** | | *** | * | *** | *** |
| **Alkaline** | *** | | * | ** | * | *** |
| **Oxidative** | *** | ND | | ** | * | *** |
| **Thermal + acid** | *** | ND | *** | ** | *** | *** |
| **Thermal + alkaline (lime)** | *** | ND | * | ** | * | *** |
| **Thermal + oxidative** | *** | ND | * | ** | * | *** |
| **Thermal + alkaline + oxidative** | *** | ND | * | ** | * | *** |
| **Ammonia (AFEX)** | *** | *** | * | *** | * | *** |
| **CO2 explosion** | *** | | *** | | | |

* = Minor effect; *** = Major effect; N.D = Not determined

## *2.7.2 Re-using DEA Cl for pre-treatment of miscanthus*

A major disadvantage to ILs is there relatively high cost compared to common solvents.[1,126,140] A way to reduce these costs is recycling the IL. Recycling the IL after pre-treatment is important for both operational and solvent costs.[132,163]

In order to see how effective [DEA] Cl was at pre-treating multiple batches of miscanthus, the same batch of [DEA] Cl was re-used 8 times with 8 fresh samples of miscanthus. The [DEA] Cl was removed from miscanthus by standard ethanol washing and the ethanol was removed *in vacuo*. No further purification of the IL took place before it was re-used for 8 further pre-treatments.

Figure 76 shows the same amount of sugars were released, within error, after enzyme hydrolysis each time except for re-use 4. These results prove the activity of the IL on miscanthus is repeatable and the IL can be re-used without special purification. [DEA] Cl is therefore a promising biomass pre-treatment solvent that can be used for a series of pre-treatments.

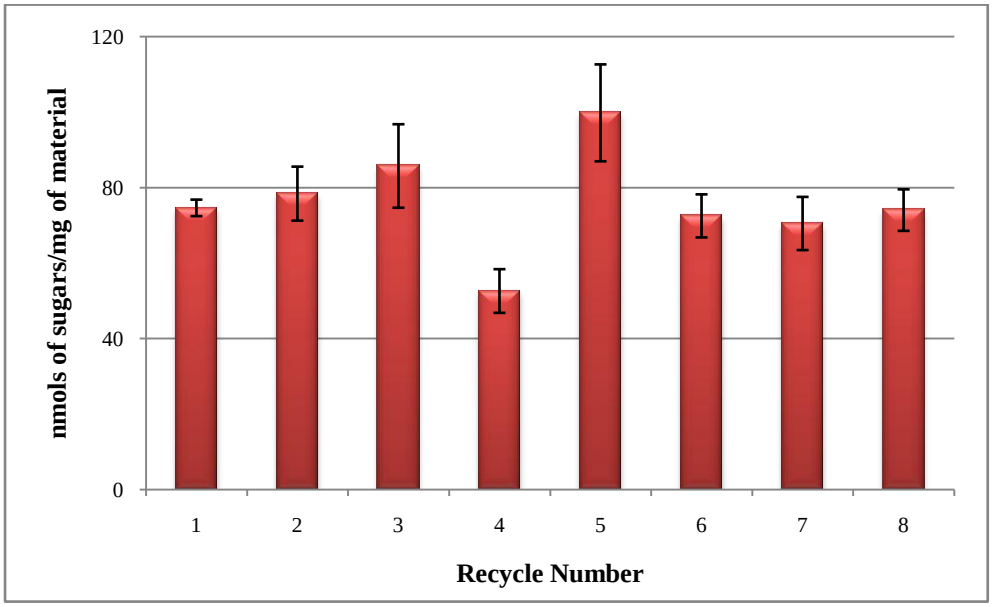


**Figure 76: [DEA] Cl Re-used 8 times with new samples of miscanthus.**

The reason why sample 4 has a lower sugar release amount is due to ethanol being present from the previous run. During the pre-treatment the sample was observed to bubble upon heating and after analysis ethanol was found to be present in this sample. This suggests that removing the ethanol completely from the IL prior to re-use is essential in optimising the pre-treatment, as if not the ethanol can act as an

anti-solvent and counteract the [DEA] Cl and pre-treatment. Otherwise [DEA] Cl can be re-used with the same effective and promising results on sugar release.

To further test this idea of re-use, miscanthus was repeatedly pre-treated with fresh samples of [DEA] Cl. This was to observe if any more sugars were released from the same sample of miscanthus after multiple pre-treatments with fresh [DEA] Cl samples.

Figure 77 shows miscanthus re-used 3 times for 3 pre-treatments with fresh [DEA] Cl each time. As can be seen from the sugar release amounts, within error, the same amount of sugars were released each time, further proving that the IL re-use in Figure 76 keeps its efficiency. Fresh [DEA] Cl does not aid any improvement on pre-treatment on the miscanthus then the re-used [DEA] Cl.

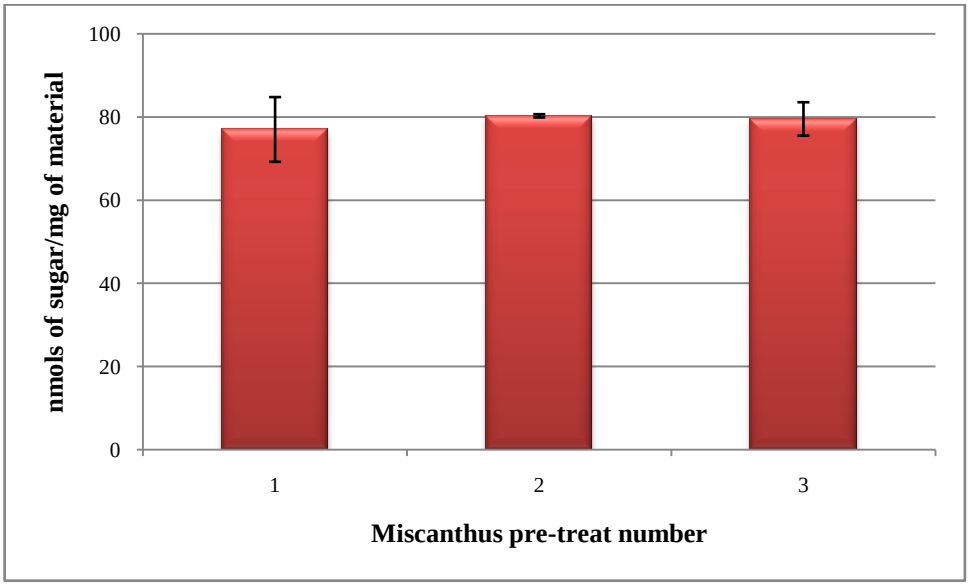


**Figure 77: Miscanthus re-used for pre-treatment with fresh samples of [DEA] Cl each for 24 hours at 100 °C.**

It is shown in chapter 3, that during 1 pre-treatment run with [DEA] Cl, between approximately 7-11 wt% of biomass can be lost in the IL. This loss currently does not affect the re-use of [DEA] Cl for pre-treatment, however, the recovery of this material from the IL would be a future goal for a higher product yield.

### *2.7.3 Hydrothermal Bombs: Steam Explosion Comparison*

How does IL pre-treatment stand up to the conventional industry method, such as dilute acid or pressurised water? In order to compare how effective this IL pre-treatment so far was in comparison to example industry methods, hydrothermal bombs were used in combination with dilute acid treatment. By heating hydrothermal bombs to 100 °C, a build up in pressure is achieved inside to mimic the concept of industrial steam explosion.

The IL, [DEA] Cl, is indeed comparable to acid pre-treatment and this is seen in Figure 36, where the results of the 4 pre-treatment types analysed, all with miscanthus as the plant biomass choice. [DEA] Cl pre-treatment is the current best IL to use, 0.1 M $H_2SO_4$ is used as the acid comparison and 2 controls of ethanol and water are shown. Figure 78 shows all pre-treatments which were carried out for 1 hour at room temperature as a control (in red) and at 100 °C (in blue) with 3 repeats of each pre-treatment (i.e 3 separate bombs per experiment).

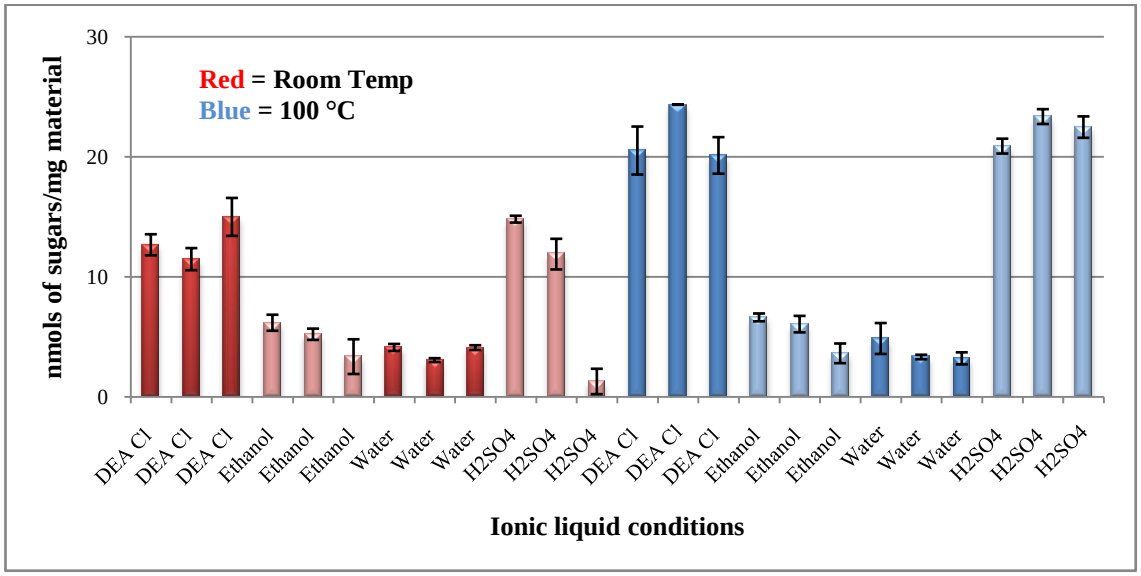


**Figure 78: Saccharification analysis of hydrothermal bombs of 4 pre-treatment types at room temperature and 100 °C.**

The results show that the 100 °C pre-treatment worked best, as after saccharification more sugars are released, this makes sense from previous work on temperature studies where heating the samples further was required to aid pre-treatment. Figure 78 shows that under these conditions, the controls were both much lower than the IL and acid pre-treatments at release sugars after saccharification, as expected, but also

that the IL and acid pre-treatments were comparable. This promising result, shows the capabilities of [DEA] Cl as a successful pre-treatment solvent for miscanthus under these conditions and how dilute acid pre-treatment with hydrothermal bombs is comparable.

## Conventional Stirring *Versus* Hydrothermal Bomb

The conventional industry method comparison involved using a hydrothermal bomb. To apply this to ILs is problematic, due to the high viscosity of ILs. This is because in the hydrothermal bombs no stirring takes place and this means less efficient mixing of the IL with the biomass. To check this hypothesis, the improvement ratio from the hydrothermal bomb experiments has been compared to that of conventional methods with stirring.

Figure 79 shows the improvement ratio from the hydrothermal bomb experiments, compared to the conventional pre-treatments of miscanthus with [DEA] Cl, [BMIM] Cl and ethanol control as previously seen. As the graph shows, the significant improvement in sugar release from the longer conventional IL pre-treatments is most likely due to the increased time period of pre-treatment with the IL, as the hydrothermal bomb experiments were only run for 1 hour. However, the mechanical stirring available during conventional studies, which was not available in the hydrothermal bomb experiments, will play a role, especially as these are viscous IL materials.

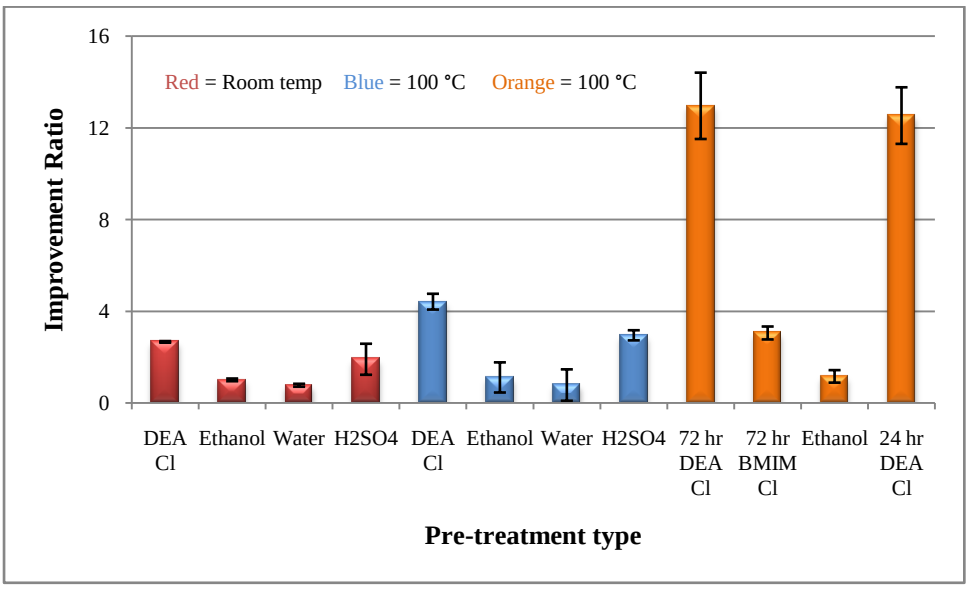


**Figure 79: Comparison of hydrothermal bomb pre-treatment versus conventional pre-treatment.**

### *2.7.4 Dilute Acid and Dilute Base Pre-treatment*

How do ILs compare to dilute acids and bases in the context of conventional pre-treatment? This question is important if ILs are to be used as a large scale pre-treatment method for lignocellulosic pre-treatment.

One of the promising new techniques being trialled in comparison to ILs is dilute acid or base pre-treatment. In order to compare dilute acid and dilute base with the standard IL conditions for pre-treatment, 0.1 M $H_2SO_4$ and 0.1 M NaOH were used as pre-treatment solvents on miscanthus for 24 hours at reflux following the normal procedures. Figure 27 shows dilute acid compared to [DEA] Cl, [BMIM] Cl and ethanol pre-treatments. Dilute acid functions at ca. twice the improvement compared to [BMIM] Cl, however, [DEA] Cl is ca. three times more effective then the dilute acid. The dilute acid is hence an effective miscanthus pre-treatment solvent but not as promising as [DEA] Cl. This further proves from section 2.7.2 that the absence of mechanical stirring of the viscous IL effects the pre-treatment of miscanthus and its essential for it to occur if efficient sugar release for fermentation is to be obtained.

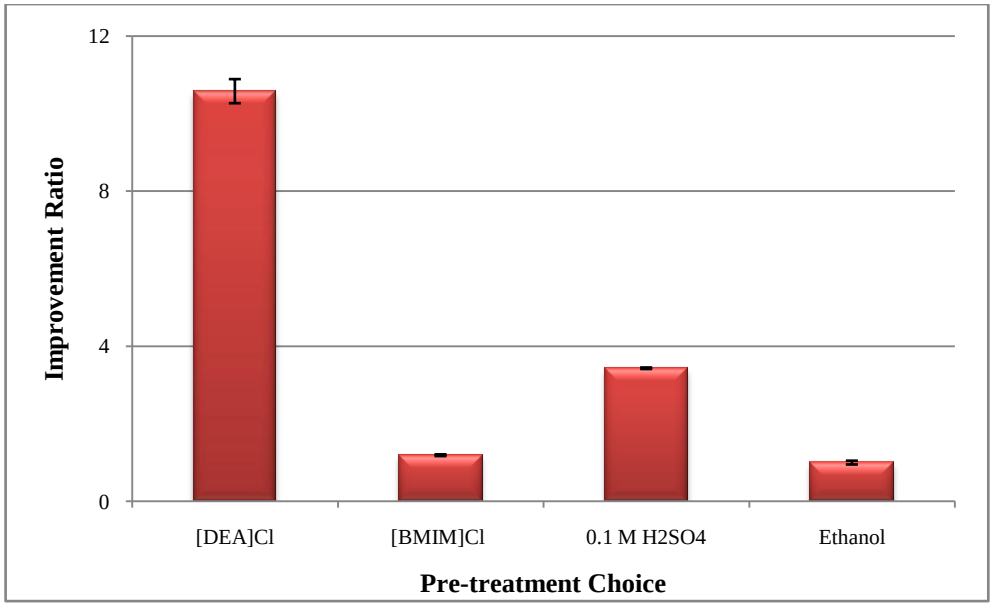


**Figure 80: $H_2SO_4$ as a pre-treatment for miscanthus versus [DEA] Cl, [BMIM] Cl and ethanol control saccharification results.**

Figure 81 shows the same ethanol, [DEA] Cl and 0.1 M $H_2SO_4$ pre-treatments on miscanthus used in Figure 80, with 0.1 M NaOH pre-treatment of miscanthus added. As the graph shows, the NaOH under these conditions is, within error, as effective as [DEA] Cl as a pre-treatment solvent. However, the error bars on this experiment are much more significant with the NaOH compared to any of the other pre-treatments, showing that the lowest point of error it is equal to the [DEA] Cl pre-treatment sugar release. This could be due to any residual base effecting the slightly acidic enzyme conditions in the enzyme buffer. Hence, depending on the amount of residual base in the hydrolysis mixture, the sugar release amount is affected.

Either way the 0.1 M NaOH is a comparable method to ILs in terms of effectiveness, but a fresh sample of base would be needed each time for use on biomass, whereas in theory the IL could be recycled, which would give the IL an advantage over the base.

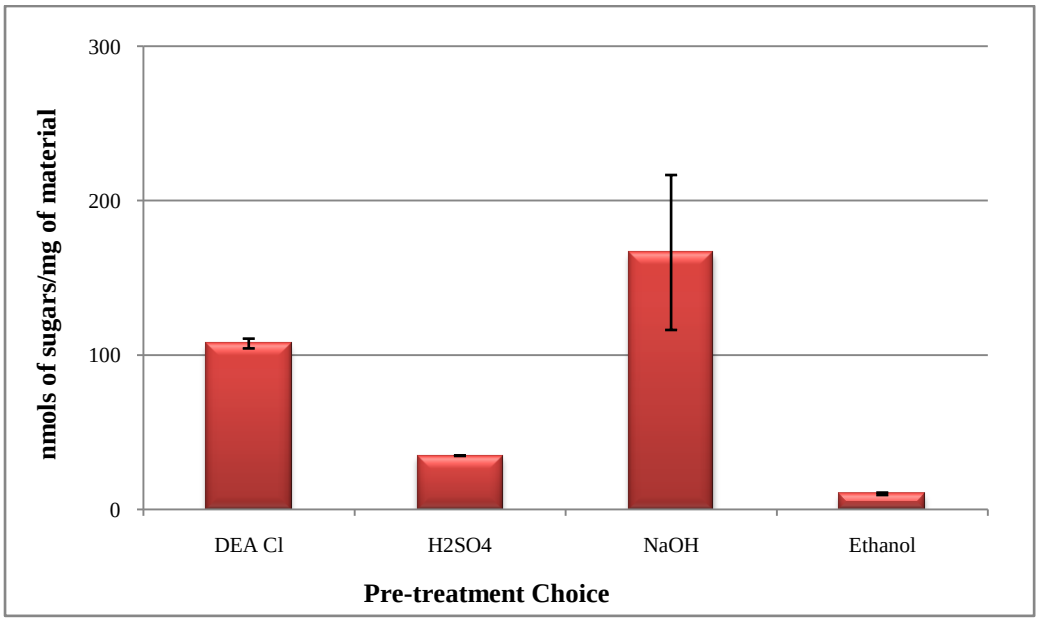


**Figure 81: NaOH as a pre-treatment for miscanthus versus [DEA] Cl, $H_2SO_4$ and ethanol control saccharification results.**

### *2.7.5 Pre-treatment Combinations with Dilute Acid, Dilute Base and Ionic Liquid*

To gain pre-treatment benefits that the NaOH and IL pre-treatments potentially have on miscanthus, a combination of pre-treatments were trialled to see if combining these steps had overall greater improvement on the digestibility of the plant material. Table 15 shows the order in which the pre-treatment of miscanthus took place. After each individual pre-treatment the miscanthus was washed with ethanol three times and then the second or third pre-treatment step took place. All pre-treatments were for 24 hours at 100 °C.

**Table 15: Pre-treatment combination experiments in order of each pre-treatment solvent.**

| Experiment | Pre-treatment 1 | Pre-treatment 2 | Pre-treatment 3 |
|---|---|---|---|
| **1** | NaOH | DEA Cl | - |
| **2** | $H_2SO_4$ | DEA Cl | - |
| **3** | DEA Cl | $H_2SO_4$ | - |
| **4** | NaOH | $H_2SO_4$ | - |
| **5** | NaOH | DEA Cl | $H_2SO_4$ |

Figure 82 shows the pre-treatment combinations and the sugar results of the enzyme hydrolysis.

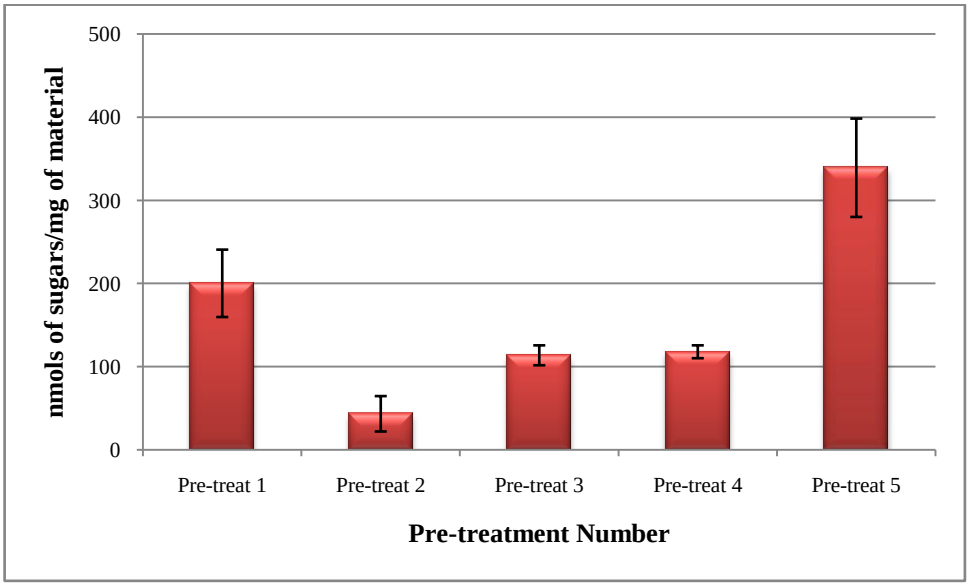


**Figure 82: Pre-treatment types of acid, base and IL combinations from Table 15 saccharification results.**

There are numerous observations to be noted from the figure which are interesting from combining these pre-treatments. Firstly experiment 2 was dilute acid followed by IL, compared to experiment 3 which was IL followed by dilute acid and there is a difference of ca. 50 nmols of sugars/ mg of material, which is double the improvement of experiment 3 compared to experiment 2.

This clearly shows the acid aids the digestibility of the plant material after IL pre-treatment, which does not occur the other way round. The reason for this could be due to polymers being removed, e.g lignin during the pre-treatment as explained in section 3.6. The better pre-treatments are obtained when dilute base is used, which increases more if it is the IL not acid used in combination with it, although when all 3 are used as pre-treatment solvents we obtain the best results of all.

This implies that whilst the three pre-treatment solvents in principal compete with each other to digest the plant material, their individual effects on the structural components of the miscanthus are all different. When used in unison their strengths combine and the overall effect is better than each individual solvent. This clearly leads the way to more experiments using pre-treatment combinations and in chapter 3 the need to look at the structural and chemical changes in the plant with each pre-treatment is required.

## *2.7.6 Supercritical $CO_2$ Pre-treatment*

Supercritical carbon dioxide ($sCO_2$) is a green solvent which has been applied to many biocatalytic systems and procedures.[164] Current research has shown the application of supercritical $CO_2$ in stripping useful waxes from plant biomass.[165] $sCO_2$ is a low environmental impact technology, that has been used previously to extract waxes from plant materials.

Usually in the pre-treatment experiments in this thesis, ethanol is used to strip out the removable simple sugars and waxes from biomass prior to pre-treatment, leaving the alcohol insoluble residue, in section 6.2. In order to determine whether $sCO_2$ was potentially more effective as a washing step compared to ethanol washing before IL pre-treatment, samples of $sCO_2$ treated miscanthus were processed with the IL pre-treatment versus standard conditions, before saccharification. Figure 83 shows 3 types of plant biomass washing; none (un-treated); $sCO_2$ washed and ethanol washed. After the wash stage each sample of biomass was pre-treated with 0.1 M NaOH, 0.1 M $H_2SO_4$ and [DEA] Cl before enzyme hydrolysis to show sugar release.

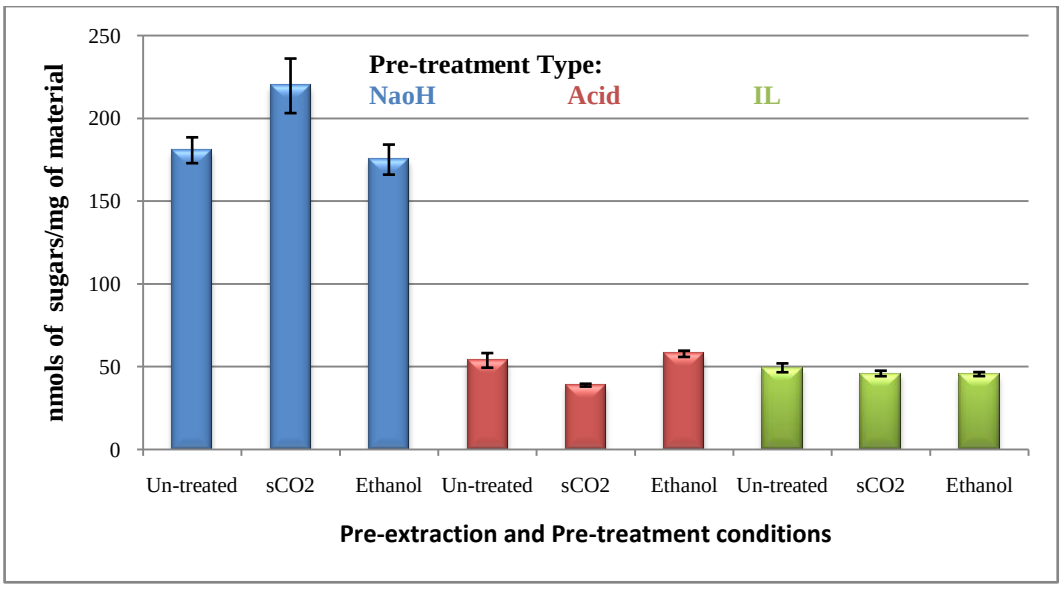


**Figure 83: Different pre-extractions compared with different pre-treatments to optimise sugar release for saccharification.**

Figure 83 shows that NaOH is in all cases the best pre-treatment with acid and IL being equal within error for all wash steps. The reason for this change compared to section 2.7.4 is because the miscanthus used in these experiments was from the same batch but of a larger particle size, hence in addition these experiments have

demonstrated the importance of controlling particle sizes for pre-treatment studies. In the case of acid and IL pre-treatments there is no variation in sugar release regardless of a wash step, or not. However, in the case of NaOH the $sCO_2$ wash step improved the digestibility of the plant material compared to none or ethanol wash.

### *2.7.7Summary of Pre-treatment Industry Comparisons*

Section 2.7 has shown how [DEA] Cl is comparable to some standard industrial pre-treatments. For steam explosion, the IL functioned better than both water and dilute acid under these conditions. In comparison to conventional dilute acid and dilute base pre-treatment, [DEA] Cl is better or equal to these pre-treatments, overall better if it can be recycled. There is evidence that pre-treatment combinations are very important at maximising the different effects each solvent has on the miscanthus, further optimisation on sugar release can be achieved this way. Finally the use of $sCO_2$ as a wash step can be used with ILs to increase valorisation of the material, but this is similar in its effectiveness to ethanol.

## 2.8 Investigating Ionic Liquid Mixtures for Pre-treating Miscanthus

Miscanthus performed the best amongst all the biomass materials in the IL pre-treatment studies. There were significant improvements over conventional pre-treatment methods, such as dilute acid and $sCO_2$. This motivates one to consider whether mixing ILs with other solvents could improve the efficiency further. An added benefit would be the reduction of IL amount needed, which would also reduce the cost.

Current literature has shown numerous IL mixtures or IL solutions to be a useful tool in chemistry research and for pre-treating plant biomass.[152] IL solvent mixtures offer control over the solutions density, vapour pressure, phase behaviour, viscosity, conductivity and diffusion co-efficient.[166]

Research has shown that ILs with increased water content can selectively dissolve the lignin component from the biomass and increase pre-treatment efficiency.[167] The added benefit with water/IL mixtures is they are cheaper to than pure ILs.

In this section we trial out some IL mixtures and the effects they have on the pre-treatment of miscanthus.

### *2.8.1 Water and [DEA] Cl Mixtures for Miscanthus Pre-treatments*

Initial studies began with water and [DEA] Cl mixtures, as this IL was shown to be the most effective pre-treatment solvent in earlier studies. It is known that water can act as an anti solvent on plant biomass with regards to pre-treatment, however, some new studies indicate it can act as a co-solvent.[168] IL water mixtures reduce viscosity issues, reduce energy inputs and costs for IL scale up and recycling. They also eliminate gel formation of the plant biomass polymers during pre-treatment.[168]

Results have shown with 50-80% aqueous mixtures of ILs in [EMIM] [OAc], successful glucose yields have been achieved. Correlation with Kamlet-Taft parameters have backed up this research.[168]

Some IL mixtures of [DEA] Cl with water were tested to see the effect on the sugar release from miscanthus after the pre-treatment. Figure 84 shows the hydrolysis results for the [DEA] Cl and water mixtures, which at first with small amounts of water present, the amount of sugars released from miscanthus drops by 25 %. This implies the water is acting as an anti-solvent and reducing the effects of the [DEA] Cl.

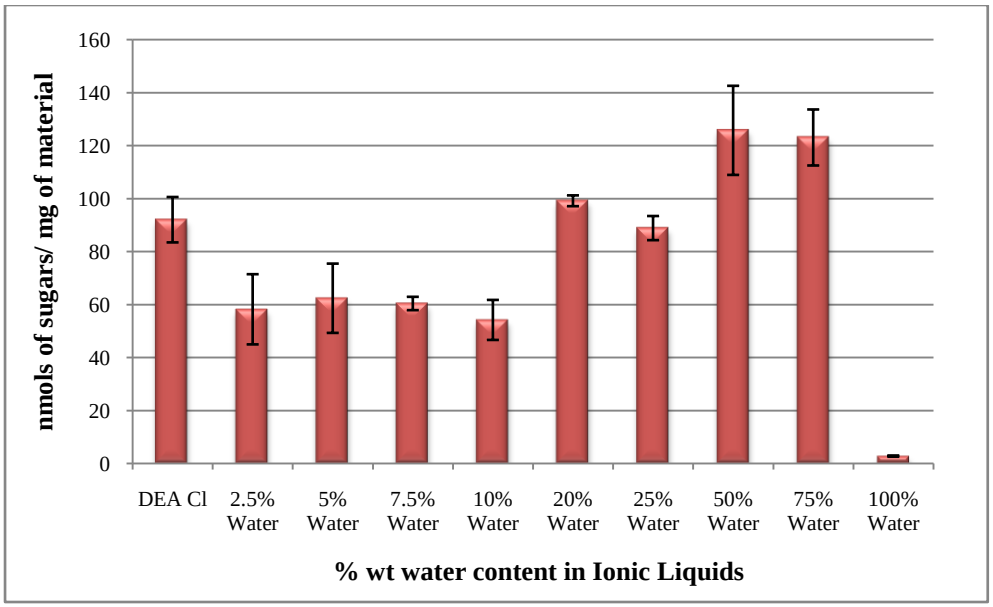


**Figure 84: Enzyme hydrolysis of [DEA] Cl and water mixtures used to pre-treat miscanthus for 24 hours at 100 °C.**

However, this reduction remains the same until a mixture of 20 wt % water and 80 wt % [DEA] Cl. At this point onwards, the amount of sugars released increase

towards a 50:50 mixture and then continues to increase until the mixture is 75 wt % water and 25 wt % IL. This observation implies, within error, the water and IL mixtures are now as effective as pure [DEA] Cl or slightly better. This data supports the idea of water acting as a co-solvent and provides the system with two advantages. Firstly, the use of less IL in the pre-treatment lowers the cost of pre-treatment. Secondly, as ILs have been noted to effect enzyme degradation[169], using less IL in the pre-treatment stage would prevent downstream issues with enzyme and yeast processes.

At 100 % water, as expected, there is virtually no pre-treatment of miscanthus and only a fraction of sugars are released. At which point in the mixture series, where the IL is saturated with water and the pre-treatment is reduced is currently unknown. This initial data shows the practical use in IL and water mixtures and much more work is required to fully optimise the system. Chapter 3 will discuss in more detail the reasons why this improvement in hydrolysis efficiency is observed.

In the absence of water completely, some research has shown saccharification yields drop dramatically, suggesting dry ionic liquid has a negative effect on the enzymes accessing the polysaccharides.[170]

### *2.8.2 DMSO and [DEA] Cl Mixtures for Miscanthus Pre-treatments*

A number of studies have shown that DMSO,[128] used individually and in combinations with ILs ([BMIM] Cl),[107] have improved the pre-treatment efficiency of plant biomass based on improving viscosity issues.[171] To see whether the same improvement could be observed for miscanthus, pre-treatment experiments were performed with pure DMSO and a 50 wt % mixture of DMSO and [DEA] Cl.

Figure 85 shows the enzyme hydrolysis results from [DEA] Cl and DMSO miscanthus pre-treatments and shows DMSO on its own has very little effect on the pre-treatment of miscanthus. A 50:50 mix of DMSO with [DEA] Cl has some improvement over pure DMSO on the pre-treatment, however, this is lower than pure [DEA] Cl on its own. Therefore the DMSO is hindering the ILs function and reducing its efficiency.

The results conclude that using a protic IL and DMSO mixture as a pre-treatment solvent for miscanthus is not effective and hence no more research into DMSO as a co-solvent were completed.

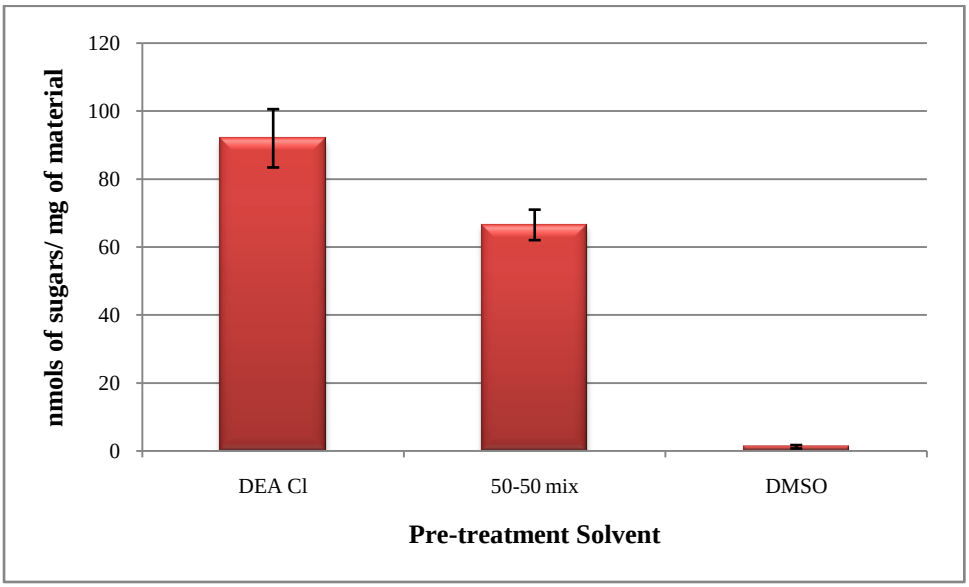


**Figure 85: Sugar release from miscanthus pre-treated with [DEA] Cl/DMSO mixtures for 24 hours at 100 °C.**

### *2.8.3 Dilute Acid and [DEA] Cl Mixtures for Miscanthus Pre-treatments using High-Through Robot the ChemSpeed*

Dilute acids and ILs have both contributed significantly to improve the efficiency of pre-treatment.[172-173] In previous examples [BMIM] Cl has been combined with acids over a range of mixtures.[173] Section 2.7 demonstrated using dilute acid and base pre-treatments followed by IL pre-treatment as a potential to improve pre-treatment efficiency, therefore is it possible to combine their strength to achieve a better efficiency through mixing the two?

To address this question and to be able to screen a greater number of mixtures, the use of the high-throughput robotic platform ChemSpeed synthesiser SLTII was employed as detailed in chapter 6, section 6.2. The use of the ChemSpeed allowed a more exact optimum mixture of dilute acid and IL ratio for pre-treatment to be discovered, in a much shorter period of time.

Table 16 shows the pre-treatment combinations of 0.1 M $H_2SO_4$ and [DEA] Cl by weight with miscanthus as the chosen biomass. $H_2SO_4$ was chosen as its been shown be more efficient at catalysing cellulosic breakdown.[173] HCl, $HNO_3$ and $H_3PO_4$ have also been used as added catalysts, $H_3PO_4$ being least effective.

**Table 16: Amounts of DEA Cl and 0.1 M $H_2SO_4$ for ChemSpeed experiment on pre-treating miscanthus.**

| **Solid Biomass 200mg** | **DEA Cl (g)** | **0.1 M $H_2SO_4$ (g)** | **Time (hr)** | **Temperature (°C)** |
|---|---|---|---|---|
| **Miscanthus** | 4 | 0 | 24 | 100 |
| **Miscanthus** | 3.8 | 0.2 | 24 | 100 |
| **Miscanthus** | 3.6 | 0.4 | 24 | 100 |
| **Miscanthus** | 3.4 | 0.6 | 24 | 100 |
| **Miscanthus** | 3.2 | 0.8 | 24 | 100 |
| **Miscanthus** | 3 | 1 | 24 | 100 |
| **Miscanthus** | 2.8 | 1.2 | 24 | 100 |
| **Miscanthus** | 2.6 | 1.4 | 24 | 100 |
| **Miscanthus** | 2.4 | 1.6 | 24 | 100 |
| **Miscanthus** | 2.2 | 1.8 | 24 | 100 |
| **Miscanthus** | 2 | 2 | 24 | 100 |
| **Miscanthus** | 1.8 | 2.2 | 24 | 100 |
| **Miscanthus** | 1.6 | 2.4 | 24 | 100 |
| **Miscanthus** | 1.4 | 2.6 | 24 | 100 |
| **Miscanthus** | 1.2 | 2.8 | 24 | 100 |
| **Miscanthus** | 1 | 3 | 24 | 100 |
| **Miscanthus** | 0.8 | 3.2 | 24 | 100 |
| **Miscanthus** | 0.6 | 3.4 | 24 | 100 |
| **Miscanthus** | 0.4 | 3.6 | 24 | 100 |
| **Miscanthus** | 0.2 | 3.8 | 24 | 100 |
| **Miscanthus** | 0 | 4 | 24 | 100 |

Figure 86 shows the saccharification results of [DEA] Cl and 0.1 M $H_2SO_4$ mixtures in ratios and the sugars released after hydrolysis. As the graph shows, the sugars released from miscanthus are, within error, equal from 100 % [DEA] Cl up until 25 wt % [DEA] Cl and 75 wt % 0.1 M $H_2SO_4$. This demonstrates the same results as shown in section 2.8.1 where the ILs pre-treatment 'effect' is still efficient with only 25 wt % content. Unlike section 2.8.1 there is no initial reduction in efficiency implying the acid is playing a separate role in the pre-treatment than just water. This is expected from section 1.3.

After 25 wt % [DEA] Cl and 75 wt % 0.1 M $H_2SO_4$ the function of the mixture as a pre-treatment solvent is greatly reduced. Using the ChemSpeed it is clear to see where this cut off in activity begins. The use of these acid and IL mixtures gives us an advantage of using combination pre-treatments to reduce cost and IL contamination. However, residual acid in the miscanthus can also cause downstream problems with yeast processing and the question of whether these mixtures can be recycled still remains?

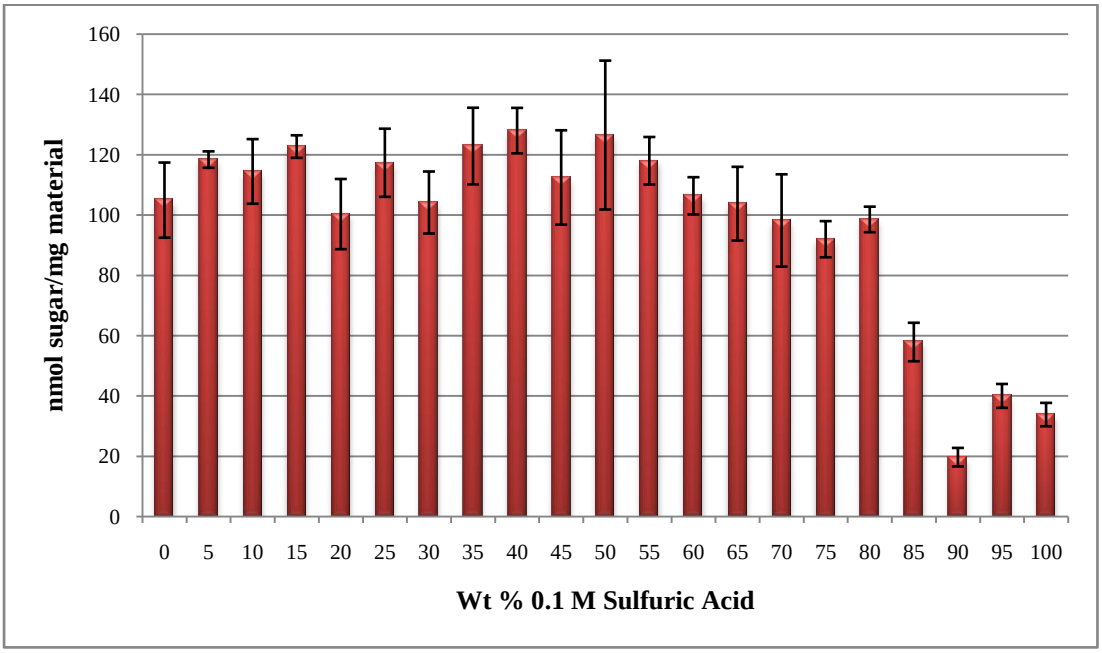


**Figure 86: ChemSpeed data for [DEA] Cl and 0.1 M $H_2SO_4$ mixture studies for the pre-treatment of miscanthus for 24 hours at 100 °C.**

## 2.9 Enzyme Hydrolysis Studies

The goal for the saccharification step, is to improve and optimise the amounts of sugars being released after enzyme hydrolysis. To this end, the following factors have been systematically varied:

- Time of enzyme hydrolysis
- Enzyme concentration (Based on manufacturers recommendation)

It is important to optimise enzymatic hydrolysis, and results reported for optimum conditions include 48 hours of incubation, a cellulase concentration of 7 U $ml^{-1}$ and a substrate concentration of 0.5%.[169] Although these optimisations will take place, the enzyme ratio will not be altered. This is because the detailed study of the enzyme hydrolysis step is outside the goals of this thesis. However, for future improvements, enzyme modification will play a role.

On top of the above, a one-pot reaction of pre-treatment and hydrolysis has been attempted to; (1) prevent the loss of sugars and; (2) reduce the time and cost of the processing pathway. Current research[174] has shown one-pot reactions to be possible depending on the conditions and the enzymes used, as shown in Figure 87.

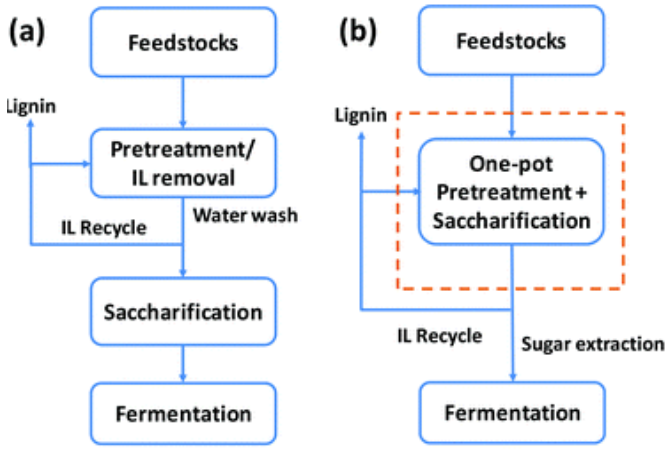


**Figure 87: Comparison of conventional biomass to biofuel pathway versus a one-pot pre-treatment and saccharification modified from reference 174.**

Research demonstrates using anaerobic, cellulolytic and thermophilic microbes to deconstruct plant biomass which could be industrial viable and require limited or no pre-treatment.[175] *N*-benzenesulfonyl types ILs have also been shown to be enzyme compatable,[176] and enzymes (*Streptomyces murinus)* has been reported of working in ammonium based ILs with slower rates of reaction compared to aqueous systems.[177]

### *2.9.1 Time Course of Hydrolysis Experiments*

To test the optimum incubation time period of enzyme hydrolysis in order to obtain maximum sugar release within the shortest time, systematic modification of the time period of incubation was controlled. Periods of 2 hours, 8 hours (standard time used), 24 hours, 72 hours, 96 hours and 120 hours were all tested.

Figure 88 shows the enzyme hydrolysis results for different incubation time periods for miscanthus pre-treated in [DEA] Cl and [BMIM] Cl respectively. From just 2 hours incubation there is a steady increase in sugar release with both IL pre-treatments towards the 72 hours incubation. At 72 hours the [DEA] Cl pre-treated sample plateaus off to a similar level for the remaining 2 time periods indicating maximum digestion of miscanthus has occurred. For [BMIM] Cl pre-treated miscanthus the sugar release continues to rise past 72 hours until the end of the incubation time period of 120 hours.

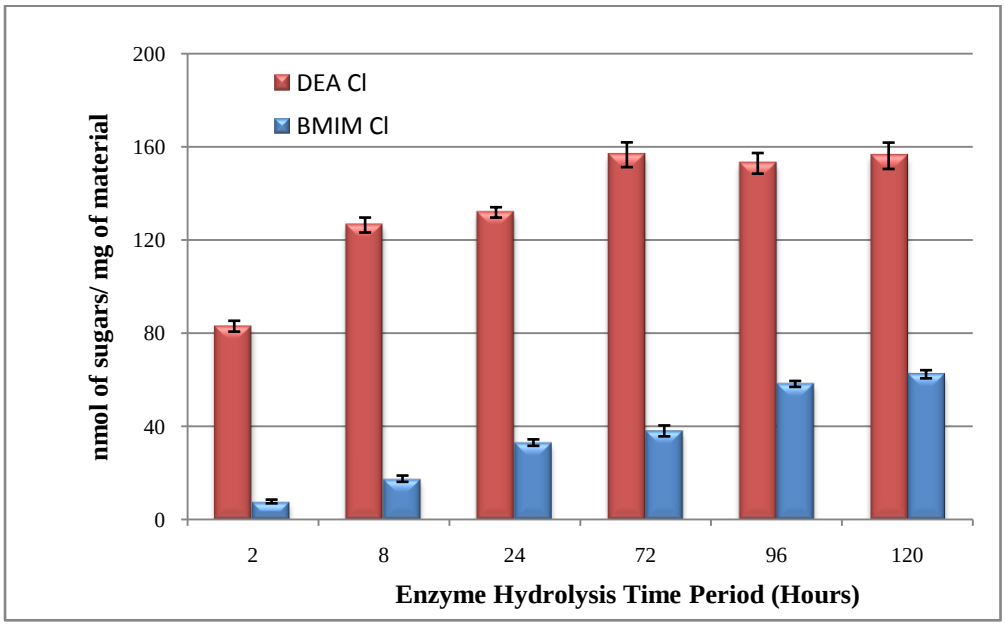


**Figure 88: Miscanthus pre-treated with [DEA] Cl and [BMIM] Cl over a range of enzyme hydrolysis time periods from 2 hours to 120 hours.**

These results for both ILs show that although 8 hours incubation for enzyme hydrolysis is enough time to gain sufficient sugar release, further benefits are achievable by allowing the incubation to be increased to 72 hours. After 72 hours, for [DEA] Cl pre-treated samples no further benefits are obtained from longer

incubation times, but for poorer performing pre-treatment ILs like [BMIM] Cl, further improvements are possible. This demonstrates how further improvements can be gained from poorer ILs at pre-treatment and leads the way to having individual incubation times depending on the IL chosen.

Hence in conclusion, the majority of improvement takes place by the standard 8 hour incubation time, so for the sake of energy efficiency, one may stop the hydrolysis after 8 hours.

### *2.9.2 Enzyme Concentration Based on Full Enzyme Loading Hydrolysis Experiments*

In an attempt to see the effect of enzyme concentration on the amounts of sugars released from hydrolysis, the enzyme loading of the saccharification experiment was altered to the manufactures recommended amounts, known as full loading (details in section 6.8). The purpose behind this reasoning is that research has shown the main factor affecting saccharification efficiency and the cost, is the amount of the enzyme used. Cellulase enzymes are commercially obtainable but reports show these can account for 30 % of bio-ethanol production cost, hence minimising the amount of enzyme used is crucial.[52] This experiment will aim to achieve 2 goals;

1. What further hydrolysis efficiencies can be achieved with full enzyme loading?
2. Based on full loading, what is the maximum yield of sugars our best IL pre-treatment [DEA] Cl can produce after hydrolysis?

This recommended enzyme concentration was used on hydrolysis of miscanthus pre-treated with [DEA] Cl and pre-treated with [BMIM] Cl, each for 72 hours at 100 °C. The dilute acid and [DEA] Cl pre-treated miscanthus samples in hydrothermal bombs from section 2.7, were also compared as a industry comparison. An ethanol control pre-treated sample was used to compare standard conditions. Aliquots of each hydrolysis solution were taken over a range of time periods, as in section 2.8.1, to monitor any change over the time course and hence to be able to calculate the maximum yield possible.

The results are shown in Figure 89 and show that for all pre-treatments the amount of sugars released increases until 8 hours of incubation with enzymes and then plateaus off. Therefore this shows how using full loading of enzymes has increased the rate at which [BMIM] Cl pre-treated miscanthus sugars are released, from section 2.9.1. The maximum amount of sugars from [DEA] Cl from this current data is ca. 330 nmol of sugars/ mg of miscanthus. For [BMIM] Cl this is considerably less at ca. 40 nmols of sugars/ mg of material as expected. The hydrothermal bombs, shown in green and purple, show the same trends as section 2.7, with [DEA] Cl in the hydrothermal bombs pre-treating the miscanthus better then dilute acid pre-treatment.

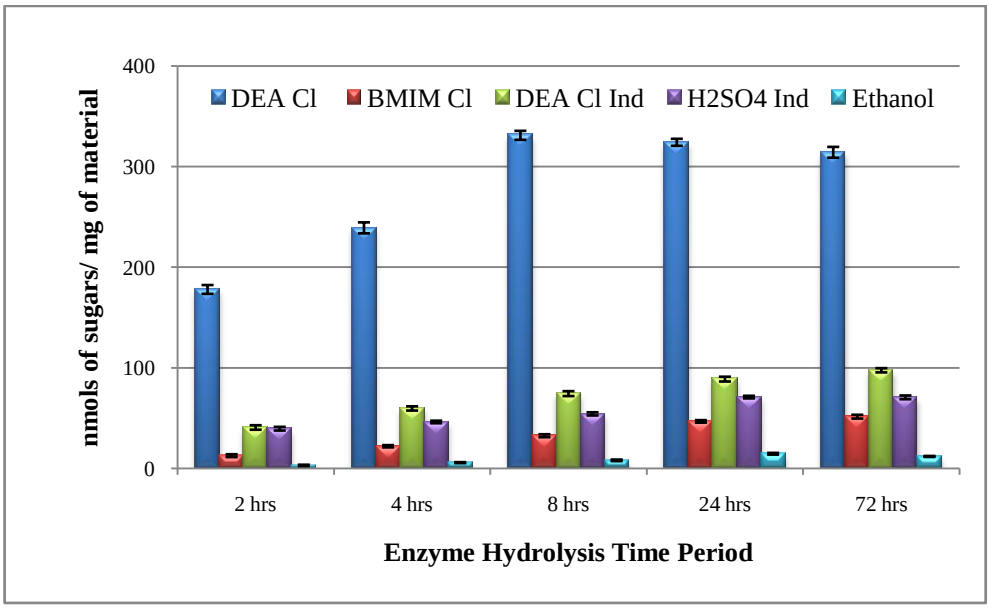


**Figure 89: Saccharification analysis of full enzyme loading of various pre-treatments of miscanthus in order to calculate maximum yield of sugars released.**

In order to calculate the maximum yield of miscanthus, the total sugar content in miscanthus needed to be calculated first. Miscanthus was analysed as in section 6.9 where this procedure will hydrolyse cellulose into glucose and via UV analysis the glucose content was calculated. Lignin is left behind after the reaction is complete. Using a standard glucose calibration graph the total sugars available for saccharification was calculated. The value for total amount of cellulose composition on the wild type miscanthus used in these experiments is 0.34 mg of cellulose per mg of material (error ± 0.04 mg). Using this figure as a maximum amount of sugar available and taking the maximum value achieved from the 72 hour incubation time

period with enzyme hydrolysis after pre-treatment with [DEA] Cl at 100 °C, the total achievable yield is 9.32%.

This 9.32 % figure assumes that all the sugar available from the pure miscanthus is available to be digested by the enzyme after IL pre-treatment. However, this is not the case as some of the sugars are hydrolysed and lost during the IL pre-treatment in the IL. Therefore performing compositional analysis on the 72 hour pre-treated miscanthus and comparing it to the enzyme hydrolysis composite is required. Therefore the actually available theoretical yield is now 0.17 mg after 72 hours IL pre-treatment, hence the percentage yield using full enzyme loading enzymes on [DEA] Cl pre-treated miscanthus is 40%. This is currently the highest yield using [DEA] Cl as the IL pre-treatment at 100 °C for 72 hours with full loading of enzymes.

### *2.9.3 Pre-treatment and enzyme mixtures*

Can pre-treatment and saccharification be done in a one-pot reaction? If they can, there are the following added benefits:

1. The overall process time can be shortened
2. Energy efficiency would be improved
3. Reduction in the loss of sugars due to the wash purification step

To this end, 6 types of pre-treatments were analysed. [DEA] Cl, [DEA] Cl:$H_2O$ 50:50 mixture, 0.1 M NaOH, 0.1 M $H_2SO_4$, water and ethanol were all used as pre-treatments on miscanthus for 24 hours at 100 °C, before the solutions were cooled and then directly added to the enzyme buffered solution. No washing or removing of the pre-treatment solvents took place.

Figure 90 shows that [DEA] Cl, [DEA] Cl/$H_2O$ and 0.1 M NaOH pre-treatment mixtures were completely inactive at digesting any miscanthus to release sugars. 0.1 M $H_2SO_4$, water and ethanol all had enzyme activity and sugar release, however, each samples hydrolysis activity was reduced compared to normal and the best outcome was 0.1 M $H_2SO_4$ combined pre-treatment/saccharification with ca. 17 nmols of sugars/mg of material.

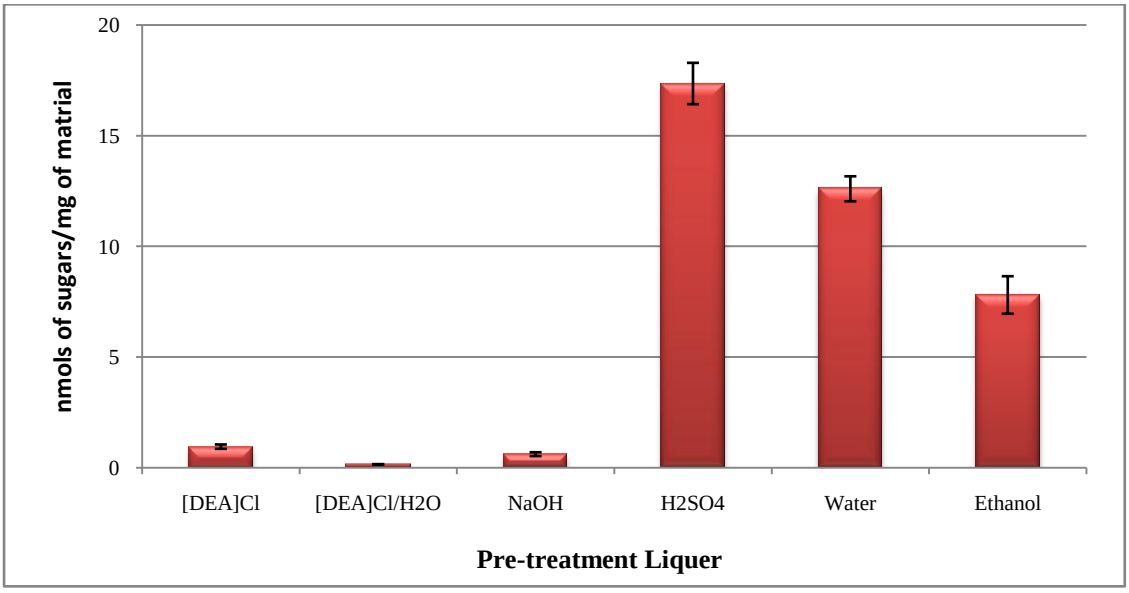


**Figure 90: Sugar release from simultaneous pre-treatment and enzyme hydrolysis using different pre-treatment solvents as stated.**

If this data is compared to the standard separate pre-treatment and saccharification data as Figure 91 shows, the 0.1 M $H_2SO_4$, water and ethanol sugar results all improve slightly but as expected the best results are with [DEA] Cl, [DEA] Cl/$H_2O$ and 0.1 M NaOH.

This experiment has demonstrated two things: firstly one-pot pre-treatment and saccharification is currently not possible using ILs or bases as the solvent. This is because the ILs appear to denature the enzymes. Secondly washing the plant biomass before saccharification is crucial, otherwise the enzymes will be partially inhibited during saccharification, lowering the yield of sugar release, especially with the IL and 0.1 M NaOH examples. It is likely the acid pre-treatment combination worked because the acid was dilute and the enzymes favour a pH of ca. 5.5, hence would not have been denatured as with the IL and base samples.

Therefore, improving enzyme stability in ionic liquids is a very important goal in using ILs on plant biomass pre-treatment, as currently the process pathway cannot be reduced at this step.

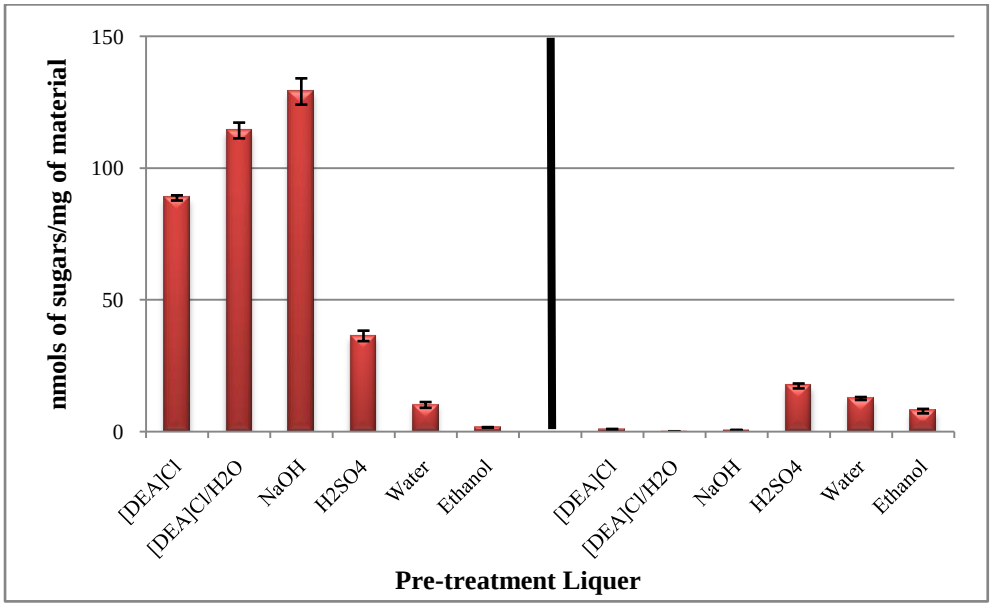


**Figure 91: Comparison of sugar release from standard pre-treatments followed by enzyme hydrolysis (shown on the left hand side of the graph) compared to simultaneous pre-treatments with enzyme hydrolysis (shown on the right hand side of the graph) with solvents as stated.**

Research has stated that the enzymes must be tolerant with > 20 % IL concentration.[178] There is a need for glycoside hydrolases isolated from thermophilic and halophilic microbes, tolerant at up to 30 % IL concentrations. Thermophilic hosts such as *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* have been identified and JTherm is a cellulase cocktail trialled for one pot-pre-treatment and saccharification of biomass.[178]

Specific impacts that ILs have on enzymes is due to their associated interaction of the cations and anions on the surface pH, catalytic mechanism and active site confirmation of each individual enzyme.[123] Therefore understanding of these factors will play a crucial role in developing one-pot pre-treatment and saccharification.

## 2.10 Final Conclusions

In this chapter, the optimal conditions for the use of ILs on pre-treating biomass have been explored through saccharification experiments. To this end, many parameters affecting the efficiency have been optimised one by one initially and then in combination. The factors of improvement are the following:

**Plant Biomass Selection:** It was determined that miscanthus appears to be the most efficient biomass to be pre-treated with ionic liquids [DEA Cl], as it achieves a 12 times improvement on sugar release compared to controls. Poplar shortly follows this with an 8 times improvement compared to ethanol, making these two biomass materials optimum choices. Many other lignocellulosic materials exist which were not tested in this project that could also prove effective with [DEA] Cl.

**Reaction Conditions:** In optimising the best conditions for plant biomass pre-treatment, 24 hours at 100 °C was determined to be the point where most of the biomass had been pre-treated sufficiently ready for digestion. Slightly higher amounts of sugar release were achievable at higher temperatures of 150 °C and at longer time periods of 72 hours, however the rate of improvement at these conditions was insignificant compared to the extra time and energy input required.

**IL Cation Choice:** In determining the best ionic liquid cation, diethanolammonium [DEA], was found to be the best protic choice. The presence of the OH groups off the ammonium centre were found to be crucial in the cation activity. If only one OH group was present on the ammonium centre the pre-treatment active function of the cation was reduced by half and further reductions when no OH groups were present. This decrease when no OH groups were present was comparable to just the ethanol control pre-treatment. Further modifications on the cation structure seemed to play little role in pre-treatment efficiency, however, only ammonium based protic ILs were tested in these experiments.

**IL Anion Choice:** The analysis based on the ionic liquid anion effectiveness on pre-treatment was much simpler then the cation, due to the role of the anion being much more understood in the literature[27]. In all cases the acidic anions of $Cl^-$ and $NO_3^-$ are favoured with double the improvement on sugar release compared to $HSO_4^-$ anion. Acetate and fluorine anion doping comparisons had little effective function on the

pre-treatment of miscanthus. Therefore with [DEA] as the cation, either $Cl^-$ or $NO_3^-$ could be used as an effective pre-treatment solvent. However, as explained in section 2.5, the potential for protic $NO_3^-$ ILs to explode has been noted in the literature[78] and so we suggest $Cl^-$ as the safer alternative.

**Heating Choice:** It was shown that effective pre-treatment with ILs is achievable with conventional heating at 100 °C for 24 hours, however, microwave heating was also shown to be a comparable and useful tool after only 10 minutes of pre-treatment at 100 °C. The energy advantages microwaves offer are significant over conventional heating. A potential caveat with microwave heating is super heating in heterogeneous samples and therefore considerably more research is required to understand the source of some benefits of microwaving IL based plant biomass pre-treatments.

**ILs Versus Industry Standards:** The research has shown that the IL, [DEA] Cl, in hydrothermal bombs is comparable to dilute acid pre-treatment and conventionally is 4 times better then acid pre-treatment for sugar release efficiency. Within error, [DEA] Cl is comparable to dilute base pre-treatment as well. There are some benefits in efficiency of combining these pre-treatments due to each one affecting the plant biomass structure in different ways (re. Chapter 3). Further work is needed to optimise the pre-treatment combinations to achieve the highest efficiency, whilst not increasing the steps of processing pathway significantly.

**Saccharification Conditions:** The enzyme hydrolysis studies demonstrated that 8 hours of incubation was enough time for sufficient biomass digestion, as longer time periods had little increase on sugar release. The current yield of sugar release with full loading of enzyme concentration is 40%, using [DEA] Cl on miscanthus for 24 hours at 100 °C. This can hopefully be improved further by reducing the amount of sugars lost during the pre-treatment.

Currently the cellulase enzymes used for saccharification are not compatible or stable with simultaneous pre-treatment/enzyme hydrolysis one-pot reaction, as the enzymes are denatured in the IL and base. Further work is needed on these mixtures with tolerant enzymes.

**Recyclability and Reduction of IL:** The research has demonstrated that [DEA] Cl can be used repeatedly for miscanthus pre-treatment without the need for purification and still maintain the same function and activity with high amounts of sugar release after hydrolysis. This makes [DEA] Cl a promising solvent for plant biomass pre-treatments in industry.

Water and $H_2SO_4$ have also been used with [DEA] Cl as examples of IL mixtures and in both cases have shown positive increases on the effects in efficiency on pre-treatment. This reduces the amount of IL and hence the cost needed in the pre-treatment steps on plant biomass.

**Finally:** In summary, for the optimised pre-treatment of plant material in this research, [DEA] Cl should be used to pre-treat miscanthus at 100 °C for 24 hours. This is followed by the IL being washed three times with ethanol and the material then hydrolysed with enzymes for 8 hours. Chapter 3 will explain why this optimised pre-treatment and the other results from chapter 2 are occurring in the process pathway. Using structural and chemical analysis techniques to characterise the plant material before and after pre-treatments, a detailed understanding of crystallinity, thermal stability, morphology and chemical polymer content can be achieved.

# 2.11 Future Work

This section details potential future work based on chapter 2 and how the project could be developed further.

## *2.11.1 Alternative Lignocellulosic Materials as a Biofuel Source for IL Pre-treatment*

This project tested 5 different lignocellulosic materials for investigating the effectiveness of IL pre-treatments. As was observed, even with the most effective IL pre-treatment significant variations were observed. There are still, many more potential choices including more waste biomass examples which could be tested as materials to be pre-treated with [DEA] Cl.

Bio-energy crops such as poplar, switchgrass and miscanthus[52] are important in current research, and switchgrass has not been tested in his project. Switchgrass is a perennial grass from North America,[31] beneficial due to its;

- Low chemical and water requirements[179]
- Marginal land required for production
- Self-regeneration per crop cycle. [31]

Therefore this would be an excellent biomass choice to be tested with these protic ILs.

In order to gain greater structural understanding of biomass breakdown, some researchers are using model plant compounds such as *Brachypodium distachyon,* as a simple analogy to grass species. Using this method the overall problem over understanding the breakdown pathways is more achievable. *Brachypodium distachyon* is a good choice as it is a small, temperate and easily propagated grass, with a rapid life cycle and small genome. This makes it an excellent species for cell wall and biomass research and could make understanding the degradation of plant cell walls with [DEA] Cl easier to understand. [180]

### *2.11.2 Ionic Liquid Investigations for Optimum Plant Biomass Pre-treatments*

Ionic liquids are the main focus of this investigation and already a wide range of protic ILs have been screened for potential biomass breakdown. However, a full understanding of the structure of the ILs required for biomass processing is still unknown, especially for cations. Hence further structural-activity insight is required to test how effective ILs can be at biomass pre-treatment and include the following research;

1. Analysis of more cation structures based on the current structure-activity study from chapter 2. Including careful modification of the OH functionality on the ammonium centre with further examples.
2. Phosphonium based cations. A whole new area which could prove effective, based on cations from chapter 2, which are also relatively cheap to synthesise.
3. Anion choice, insights into the effects of H-bonded ions. Other examples of H-bonding anions including dicyanamide which has shown positive effects at dissolving sugars, and improvements for polysaccharide dissolution.[122]
4. Other pre-treatment comparisons to ILs could be incorporated including concentrated acids, ammonia fibre explosion and biological pre-treatments.
5. Pre-treatment combination experiments could be further expanded, a current method has looked at the possibility of grinding biomass in mechanical pre-treatment whilst the biomass is soaked in an IL. Imidazolium based ILs with pine wood have shown how the swelling of the material due to the IL has significantly reduced the energy input and increased the size reductions for pre-treatment. This is not a chemical modification but physical modification and [DEA] Cl could also be used with this same combination but as a cheaper IL.[181]
6. Use of the ChemSpeed for optimisation of further IL solvent mixtures for pre-treatment on miscanthus to understand more about this water/IL interactions on the plant biomass.
7. Use of more Choline IL derivatives, or use of amino acid anions which are non-toxic, biodegradable and a cheap cost. Promise of these ILs at reducing cellulose crystallintiy and removal of lignin has already been shown in the

literature with aprotic IL cations.[156] Table 17 shows the use of amino acid anions and the effects on lignin solubility. They are shown to extract lignin from wood at just 60 °C and have the benefits of the ILs being halogen free, natural and safe amino acids.[182] This is a new area which could be researched with the $[DEA]^+$ as a comparison. Also the use of reversible IL's like butadiene sulfone have shown promise in this area of research and have the ability to access the starting materials when the IL needs to be recycled.[148]

**Table 17: Results of the dissolution test of lignin in various types of *N*-methyl-*N*-(2-methoxyethyl)pyrolidin-1-ium salts from reference.[182]**

| **Entry** | **IL($[P_{1ME}][X]$)** | **Solubility (wt% *vs.* solvent)** | | |
|---|---|---|---|---|
| | **Anion (X)** | **60 °C** | **100 °C** | **Total** |
| **1** | Glycine | 20 | 0 | 20 |
| **2** | Alanine | 20 | 15 | 35 |
| **3** | Valine | 5 | 15 | 20 |
| **4** | Leucine | 25 | 10 | 35 |
| **5** | Isoleucine | 15 | 0 | 15 |
| **6** | Methionine | 35 | 15 | 50 |
| **7** | Proline | 30 | 10 | 40 |
| **8** | Phenylalanine | 15 | 35 | 50 |
| **9** | Tryptophan | 0 | 20 | 20 |
| **10** | Serine | 30 | 15 | 45 |
| **11** | Threonine | 20 | 25 | 45 |
| **12** | Asparagine | 15 | 25 | 40 |
| **13** | Glutamine | 25 | 25 | 50 |
| **14** | Aspartic acid | 20 | 5 | 25 |
| **15** | Glutamic acid | 15 | 30 | 45 |
| **16** | Cysteine | 5 | 5 | 10 |
| **17** | Tyrosine | 35 | 15 | 50 |
| **18** | Histidine | 35 | 5 | 40 |
| **19** | Lysine | 40 | 15 | 55 |
| **20** | Arginine | 35 | 20 | 55 |

Finally, Figure 92 shows other potential IL cation and anion possibilities based on hydrogen bond capabilities. These IL designs could be tested a long side [DEA] Cl analogues for their effectiveness at plant biomass pre-treatment.

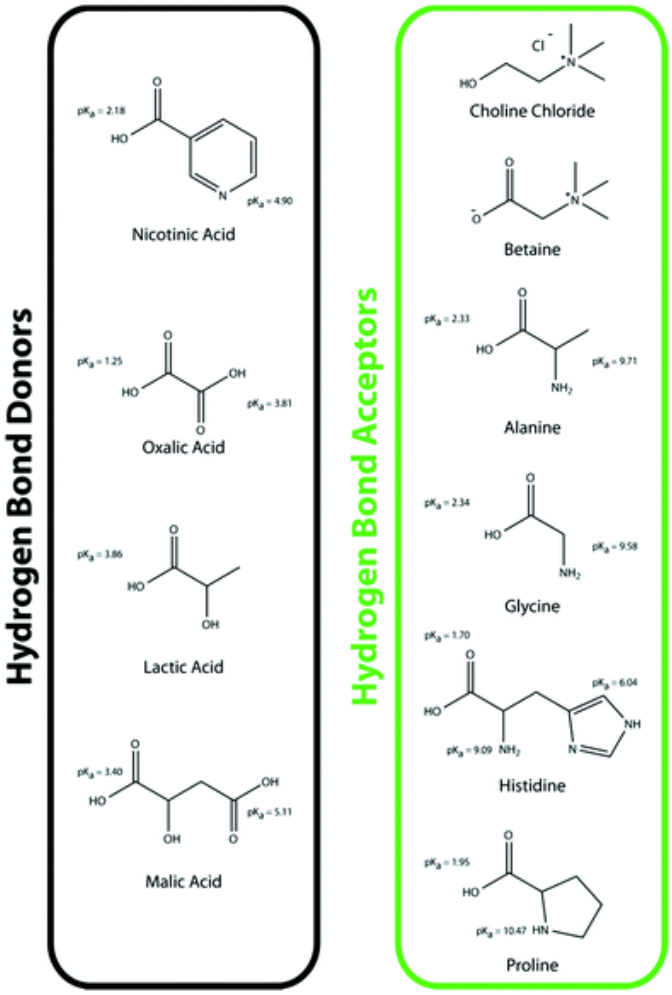


**Figure 92: Hydrogen bond donors and acceptors as potentials for IL designs for plant biomass pre-treatment, modified from reference 183.**

# Chapter 3

# Plant Biomass Structural and Chemical Studies

In the previous chapter, pre-treatment and saccharification processes have been optimised with ionic liquids. A wide range in efficiencies have been reported depending on the IL cations and anions for the pre-treatment; Plant biomass choice; Heating method; Pre-treatment time period. All of these variables produced a wide variety of saccharification efficiencies. However, the question must be asked, what is the basis of such considerable variation in saccharification? Why are certain pre-treatments more efficient than others?

To answer these questions, structural and chemical changes in the plant biomass before and after pre-treatment will be studied. In terms of crystallinity, thermal stability and cohesive forces, morphology, and chemical composition of the biomass material.

## 3.1 Structural Techniques

In order to investigate these chemical and physical changes in the plant biomass after IL pre-treatment, the following range of techniques have been chosen to probe any modifications during pre-treatment:[184]

1. Powder X-ray Diffraction
2. Thermogravimetric Analysis
3. Thermogravimetric Analysis and Infra Red Tandem
4. Scanning Electron Microscopy
5. Infra Red
6. Elemental Analysis
7. Polymer Compositional Analysis

The goal in this chapter is to compare all of the results from chapter 2 by studying changes in the biomass before and after pre-treatment. Key changes that could be causing this improvement in saccharification are:

- Changes in cellulose crystallinity to a more amorphous polymer
- Morpholgy changes and accessible surface area changes
- Particle size variation
- IL incorporation into the biomass
- Chemical content and modification
- Removal/dissolution of hemicellulose or lignin polymers

### *3.1.1 Powder X-Ray Diffraction*

The structure of biomass polymers, namely cellulose, have been studied previously using rheological measurements and scattering methods, such as small angle X-ray scattering, powder X-ray diffraction, light scattering and small angle neutron scattering.[185] These techniques give us an insight into the bulk material and can be used to assess the crystallinty of cellulose but are statistically averaged data. In this thesis powder X-ray diffraction, (XRD), was used to probe changes in cellulose crystallinity from native cellulose I to amorphous cellulose II.

### *3.1.2 Thermogravimetric Analysis*

Thermal analysis contains multiple techniques including thermogravimetric analysis (TGA), which can be used to obtain both qualitative and quantitative information.[186] These techniques have previously shown to be effective in determining any analysis or characterisation of lignocellulosic materials based on reaction rates or volatility of components during processes.[186] During thermal decomposition information regarding the biomass thermal stability can be generated, giving insight into the structural composition of biomass before and after IL pre-treatment.

### *3.1.3 Scanning Electron Microscopy*

Morphology of these plant biomass macromolecular systems, can be obtained using techniques such as SEM and TEM.[185] In this chapter SEM is used to probe surface morphology and surface area of fibres or fibrils of plant biomass polymers before and after IL pre-treatment.

### *3.1.4 FT-IR*

FTIR spectroscopy is a powerful tool in characterising the physico-chemical and conformational properties of polysaccharides.[186] Lignocellulosic materials contain alcohols, alkanes, aromatics, esters, ketones and other oxygen based functional groups which can be probed by FT-IR.[186] In this chapter FT-IR is used to characterise the qualitative amounts of each of the biomass polymers present in the material before and after IL-pre-treatment.

### *3.1.5 Polymer Compositional Analysis*

The use of chemical tests with UV-assays to quantify polymer content will be employed and section 6.9 details the methods employed for cellulose, hemicellulose and lignin. With this data a full understanding of which plant polymers are effected through dissolution or other chemical modifications during the IL pre-treatment, will be further understood.

## 3.2 Determining Cellulose Crystallinity using Powder XRD

Why are some biomass materials better at releasing sugars after IL pre-treatment compared to others? The structure of biomass itself, which consists mainly of cellulose, hemicellulose and lignin, should provide a key towards answering this question. The molecular basis of an effective pre-treatment has been postulated to be as follows: ions from the IL interact with the cellulose polymer to de-crystallise the cellulose.[132] These ions are mostly the anions but in some cases cations will also interact.

Figure 93 shows the powder XRD pattern of crystalline α-cellulose. This is the typical structure for native cellulose I, with a primary 002 lattice peak plane and a secondary overlapped 101 peak.[132] Characteristic peaks at 2θ = 14.9°, 16.3° and 22.5°can be seen in the figure.[43,143] 22.5° corresponds to the distance between hydrogen-bonded sheets in cellulose, 16.3° and 14.9° are peaks from $I_\alpha$ and $I_\beta$.[187]

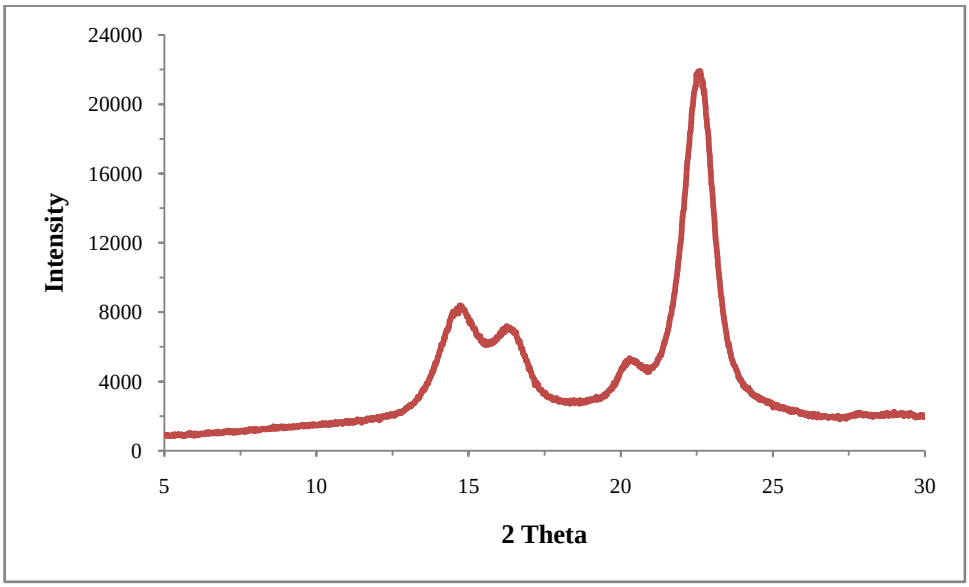


**Figure 93: Cellulose powder XRD diffraction pattern.**

Cellulose is present in biomass as cellulose I or α-cellulose,[143] which is the most recalcitrant form. Studies have shown by using powder XRD, that the highly crystalline cellulose polymer becomes amorphous after some IL pre-treatments, namely [BMIM] based ILs.

It is believed that ILs can interact *via* hydrogen bonding with the cellulose polymer and that the intra- and intermolecular hydrogen bonds in cellulose, (shown in Figure 94), are broken.

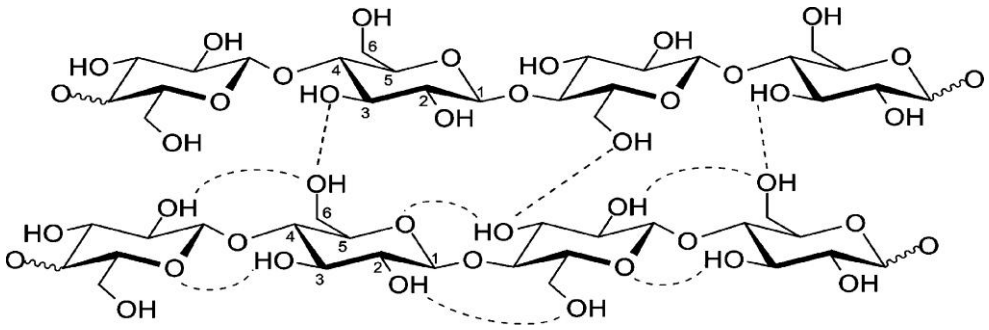

**Figure 94: Extensive intra-and intermolecular hydrogen bonds in cellulose modified from reference 38.**

New hydrogen bonds are formed between individual cellulose chains and the IL chosen, (shown in Figure 95). These new hydrogen bonds are usually formed with the anion although the cation can play an equal, but as of yet, unknown role.

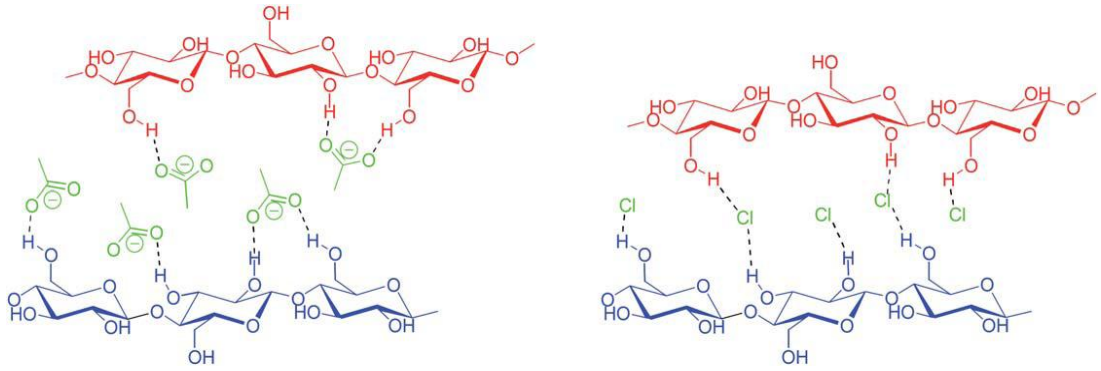

**Figure 95: IL anions hydrogen bonding with individual cellulose strands as modified from reference 38.**

To test this hypothesis, miscanthus before and after pre-treatment with [DEA] Cl and [BMIM] Cl was analysed using powder X-ray diffraction to determine the crystallinity of the cellulose. Hemicellulose is a branched polymer and is hence un-crystalline, and lignin is also relatively disordered at a molecular level so only cellulose will be detected by XRD.[64]

Figure 96 shows the diffraction pattern for pure miscanthus, in blue. It is clear to see the two characteristic peaks in the diffraction pattern, due to crystalline cellulose as shown before. The red line shows miscanthus after it has been pre-treated for 24 hours at 100 °C with [DEA] Cl and the diffraction pattern remains essentially unchanged. The green line shows miscanthus after it has been pre-treated for 24 hours at 100 °C with [BMIM] Cl and again the diffraction pattern remains essentially unchanged.

Therefore, this indicates that the bulk crystallinity of the cellulose polymer in miscanthus remains unchanged after pre-treatment with both ILs, and that the cellulose I form is present, not amorphous cellulose II.[188]

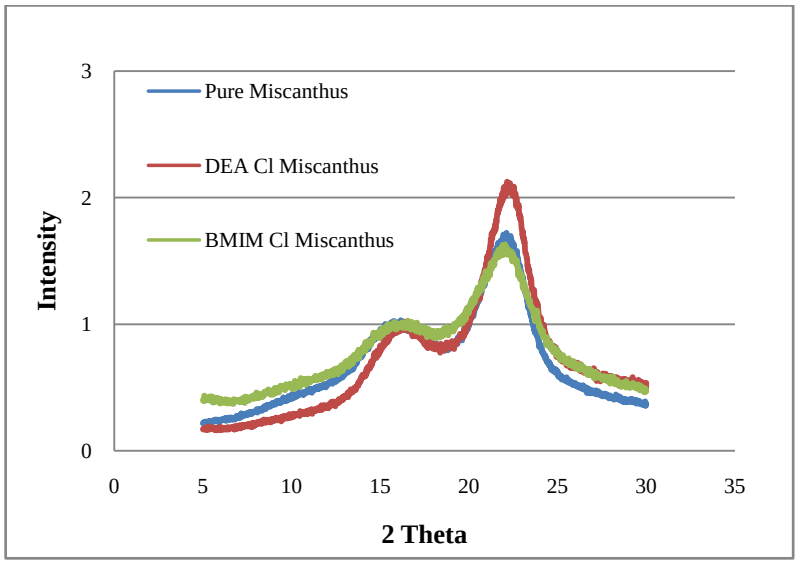


**Figure 96: Powder XRD patterns of pure miscanthus in comparison to the [DEA] Cl and [BMIM] Cl pre-treated miscanthus for 24 hours at 100 °C.**

By analysing other samples of miscanthus pre-treated for 24 hours at 100 °C with different successful solvents for comparisons from chapter 2, it can be determined if cellulose crystallinity plays a role in successful biomass breakdown for miscanthus.

Figure 97 shows the powder XRD patterns for [DEA] Cl/water percentage weight mixtures, dilute acid and $sCO_2$, all of which were shown to be effective pre-treatment solvents in chapter 2. The results show that in all cases the diffraction patterns are essentially the same, hence we can conclude that none of the IL or other pre-treatments tested on miscanthus in this project, affect the crystallinity of the cellulose and this essentially remained unchanged in all cases.

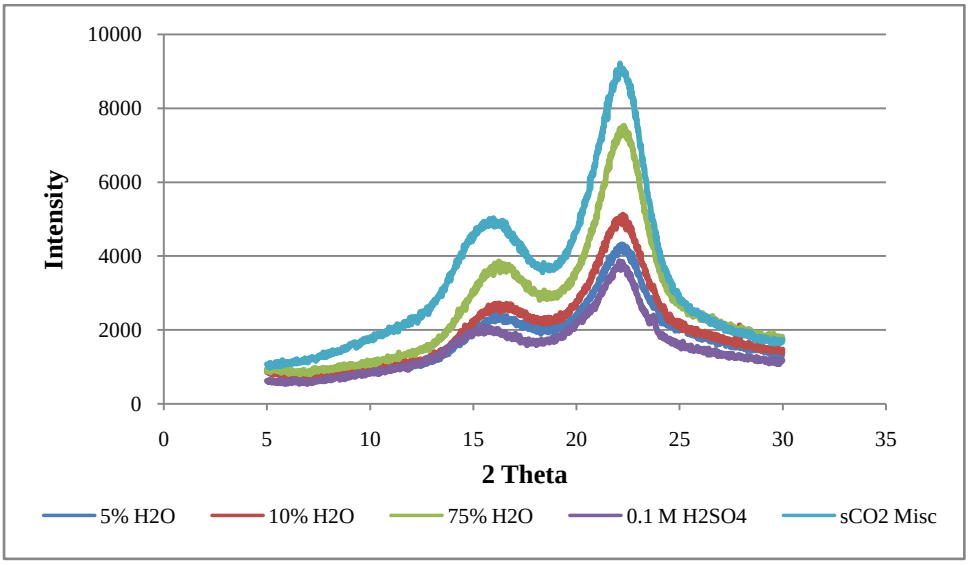


**Figure 97: Powder XRD patterns of miscanthus pre-treated with water and [DEA] Cl mixtures (wt %) as shown, in comparison to 0.1 M $H_2SO_4$ and $sCO_2$ pre-treated miscanthus for 24 hours at 100 °C.**

Based on recent literature reviews, this lack of change in crystallinity is unexpected, as it is believed that a key role in lignocellulosic hydrolysis is cellulose crystallinity.[38,109] When the question is asked why are ILs more successful at cellulose dissolution? The answer that is given is due to the hydrogen bonds between anions of ILs and hydroxyl groups on cellulose being three times stronger than hydrogen bonding energy from either methanol or water solvents.[189] Also the possibility of the IL imidazolium rings and Van der Waals forces forming between the cation and sugar rings. These hydrogen bonds cause changes in the confirmation of cellulose and hence reduce its crystallinity for effective dissolution on ILs.[189]

Figure 98 from Kilpeläinen and co-workers[190] shows cellulose crystallinity in spruce sawdust after pre-treatment with [AMIM] Cl and [BMIM] Cl changes in form, from cellulose I to cellulose II. With our ILs and miscanthus this does not happen. However, other studies have shown that effective biomass pre-treatment is not always linked to cellulose solubility. [EMIM] [OTf] does not dissolve cellulose, yet was effective on digestion of milled Norway spruce wood. This was due to swelling and fractionation of the lignin-rich material, allowing for reactive dissolution.[134]

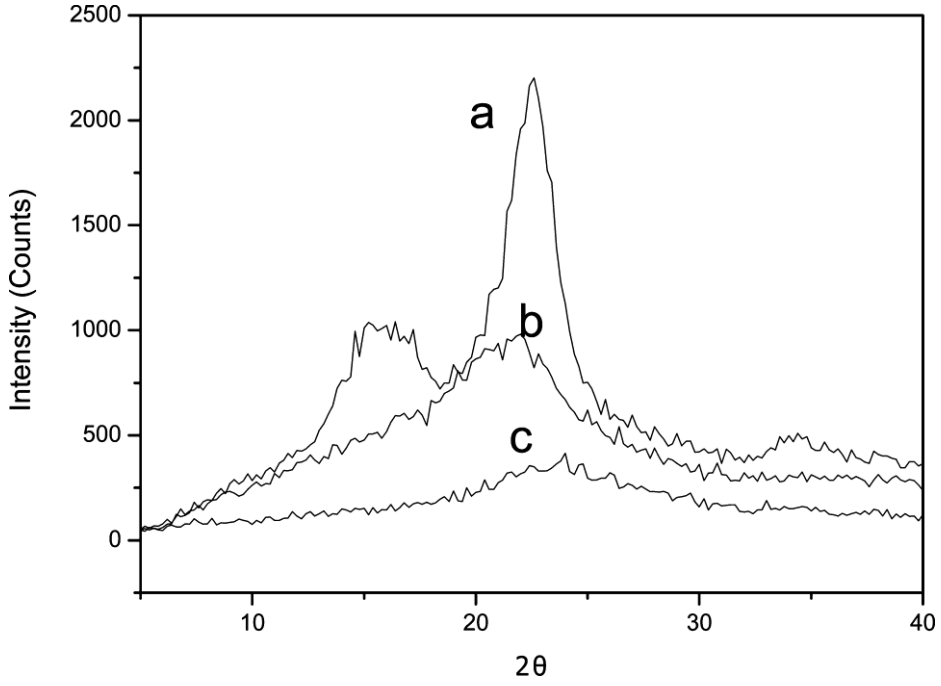


**Figure 98: Powder X-ray spectra of (a) spruce sawdust, (b) regenerated spruce from [AMIM]Cl using $H_2O$ as the anti-solvent, (c) 8% wt spruce sawdust in [BMIM]Cl solution. Spectra modified from reference [190].**

In summary, contrary to what was potentially expected based on current research, the cellulose crystallinity is essentially unaffected, after the IL, dilute acid and $sCO_2$ pre-treatments. However, this means there are other physical/morphology or chemical changes occurring which make the IL pre-treatment successful at improving sugar release from plant biomass.

## 3.3 Thermal Stability of Pre-treated Plant Biomass

Thermogravimetric analysis (TGA), can be used to determine several basic characteristics of plant biomass; (1) Water Content; (2) Volatile matter potential; (3) Ash content and residual carbon.[191]

A useful insight into how stable the cohesion of the plant polymers in biomass are, can be obtained through the recalcitrance of the material, hence the stability of the material against thermal degradation. The purpose of this section is two-fold: (1) to gain insight into changes in miscanthus recalcitrance through thermogravimetric analysis; (2) to identify the resistant and non-resistant chemical bond/biomass polymers in miscanthus through TG-IR spectroscopic analysis of the released volatile material.

### *3.3.1 Thermogravimetric Analysis on Miscanthus*

Thermogravimetric analysis (TGA) was used in order to investigate the qualitative strength of interaction within miscanthus, both in the original material and after IL pre-treatment for comparison. A slight alteration in the composition of the plant biomass would affect the thermal behaviour and hence the TGA curve, offering the TGA curve as a fingerprint for biomass materials.[191]

It was hoped that any changes in the decomposition temperature or profile, would allow further understanding of the benefits of ILs for effective pre-treatment on miscanthus. A higher degree of crystallinity which has been observed in section 3.1, would usually indicate enhanced thermal properties.[43] Based on cellulose alone, there should be no change in the thermal stability of IL pre-treated biomass.

Figure 99 shows the pure miscanthus TGA profile and the temperature it starts to decompose at in blue, and the [DEA] Cl and [BMIM] Cl pre-treated miscanthus profiles are shown in red and green respectively. The data shows that the pure miscanthus decomposition profile is between ca. 310 °C to ca. 390 °C, with a fairly smooth downward curve resulting in approximately 75% loss in the weight of the sample. This single-stage degradation is due to a complex sequence of reactions until the material remains in a solid state.[192]

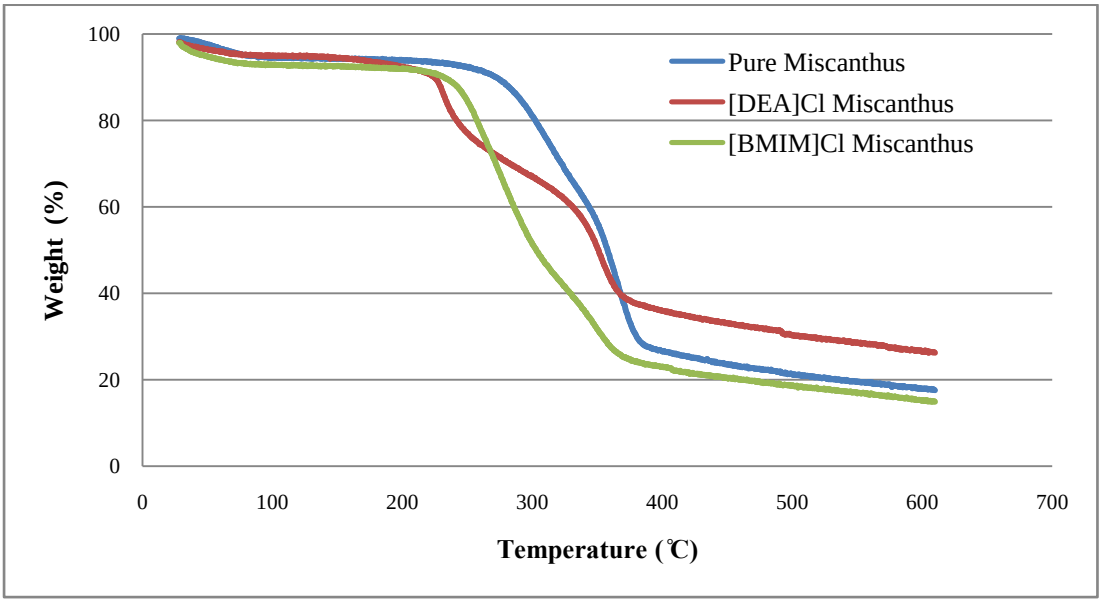


**Figure 99: TGA of pure miscanthus and miscanthus pre-treated in [DEA] Cl and [BMIM] Cl for 24 hours at 100 °C.**

The IL pre-treated miscanthus profiles, both show interesting decomposition pathways and mass losses.

Firstly, both IL pre-treated samples decompose at ca. 50 °C lower than the pure miscanthus at ca. 260 °C. This implies a change in either crystallinity or morphology has occurred reducing the thermal stability of the biomass.[143] As section 3.1 showed crystallinity remains unchanged, this reduction in thermal stability must be due to morphology reasons or chemical content of the biomass. Another reason for this loss could be decomposition of [DEA] Cl remaining in the miscanthus. However, based on the amount of material lost, this loss cannot be just due to the IL, but also the miscanthus itself.

[DEA] Cl pre-treated miscanthus starts to decompose at ca. 250 °C with a temperature range to ca. 390 °C. The final decomposition temperature remains the same at ca. 390 °C, however, the final char content is higher.

The second observation in Figure 99 is the curve patterns, In the [BMIM] Cl pre-treated miscanthus, the curve is as smooth as the pure miscanthus profile, with the only change being the reduced initial decomposition temperature. However, with the [DEA] Cl pre-treated miscanthus, as well as the initial decomposition temperature changing, the curve has two distinctive parts. The first part of the curve is different to the pure miscanthus profile until ca. 370 °C, whereas the second part of the curve overlaps with the pure miscanthus profile with 60 wt % of the original miscanthus remaining.

This could indicate that part of the miscanthus chemical structure is effected differently by [DEA] Cl but not all components, for example, the lignin polymer may be effected and partially removed but not the cellulose part during this pre-treatment.

Another explanation could be that thermal cross-linking reactions are occurring during the initial decomposition step, which then decompose in the second step. Cross-linking reactions during thermal degradation have been reported for epoxide groups, hydroxylterminated siloxanes, polyamides, and between hydroxyl groups and carboxylic acid side chains.[193] Lignin can exhibit multi-step degradation processes due to its complex structure, and so if it is being partially removed by [DEA] Cl, this could account for the two stage TGA decomposition profile.[194]

In order to understand the decomposition of the pre-treated miscanthus in more detail, TG-IR was used to see if further analysis of the volatile products being removed during decomposition could be characterised.

### *3.3.2 Thermogravimetric Analysis-Infrared Tandem*

Here the relative stability of cohesive bonds in the biomass can be clarified through complementing thermogravimetric measurements with structural information on the biomass attained through IR.

[DEA] Cl pre-treated miscanthus was analysed using TG-IR to determine and identify which compounds/polymers or products are being decomposed and removed at which temperature profile on the TGA curve. As shown in section 3.3.1, the TGA profile for [DEA] Cl has two decomposition parts to the curve. Figure 100 shows the IR spectrum from both parts of the TGA curve. The part shown in blue is from the first decomposition step and the part shown in red is from the second decomposition step.

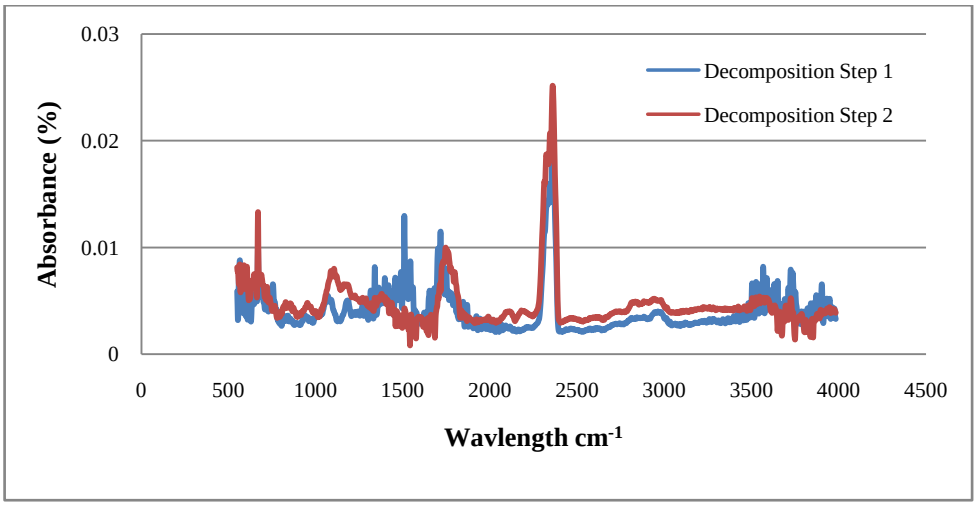


**Figure 100: TG-IR spectrum of miscanthus pre-treated in DEA Cl, analysing the two decomposition steps of the biomass using IR.**

It was discovered that from the first decomposition step, ca. 210-260 °C, there is evidence of lignin functional groups present as C=O and C-O stretches are present, in which C=O most likely could be due to the lignin polymer but also the decomposition product $CO_2$.

The second decomposition step, ca. 270-360 °C, shows less of these groups present, hence this could imply cellulose and other simple sugars are being decomposed at this step. Overall that data is hard to analyse as multiple products could be being produced during the breakdown.

This reduction in thermal stability of the miscanthus after IL pre-treatment currently does not fully explain why the IL pre-treatment is more effective. This is because judging from Figure 99, the [BMIM] Cl pre-treated miscanthus decomposes at a lower temperature along with the [DEA] Cl pre-treated miscanthus, yet from chapter 2 we see [DEA] Cl is significantly more effective as a pre-treatment solvent then [BMIM] Cl.

Therefore, thermal stability is NOT an indirect indicator of saccharification improvements in structural modifications beneficial to hydrolysis of the biomass, and there must be other factors based on morphology and chemical composition playing a key role in the pre-treatment improvements.

## 3.4 Studying Morphology Changes After Plant Biomass Pre-Treatment Using Scanning Electron Microscopy

Besides the thermal stability of the pre-treated biomass which has been investigated by TGA, there is an additional factor which contributes to effective hydrolysis: namely the morphology of biomass samples pre-treated. Numerous literature studies[27,195] have used electron microscopy to probe this effect.

As an example, the larger the surface area, the more ILs are able to access the plant cell wall polymers, as well as being able to pull apart the main fibres into fibrils and strands. This simple consideration illuminates the need for studying the morphology of the biomass samples in order to understand their digestibility better.

To this end, scanning electron microscopy (SEM) was used to analyse the surface, structure and morphology of the biomass materials before and after pre-treatment. The aim was to determine whether the improvement in digestibility was due to significant surface area modifications or because of chemical content modifications.

Figure 101 shows a SEM image of un-treated miscanthus. It is quite clear to see the fibre and cell wall are completely intact.

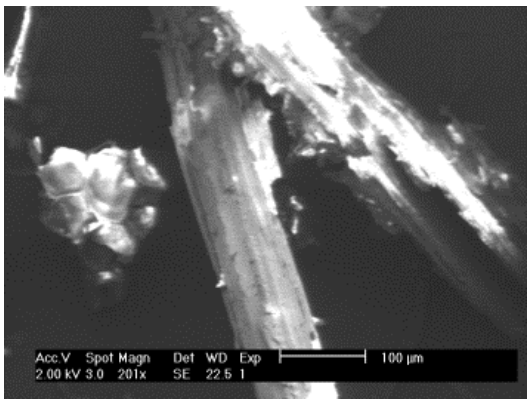


**Figure 101: SEM of Un-treated Miscanthus.**

Figure 102 shows SEM images of miscanthus pre-treated with [DEA] Cl, and now it is quite clear to see there are significant differences in the particle morphology. The fibres have now been pulled completely apart into much thinner and finer strands/fibrils which are more homogeneous.[196] The SEMs show how the surface area of the miscanthus has greatly increased and a significant reduction in particle size has allowed for increased enzyme digestibility. The swelling is caused due to hydrogen bonds and other electrostatic interactions being broken and the IL being allowed to access and saturate the biomass sample.[196]

In each image in Figure 102, it is clear to see the smaller fibril strands of miscanthus. The cellulosic skeleton of fibre bundles remains intact but the strands have been shredded by the IL.[197] The most obvious change from the original fibre can be noted in the 3rd image of Figure 102, as here some of the original fibre still remains intact, but shows swelling down each side of the strands, some of which have started to break away from the main fibre.

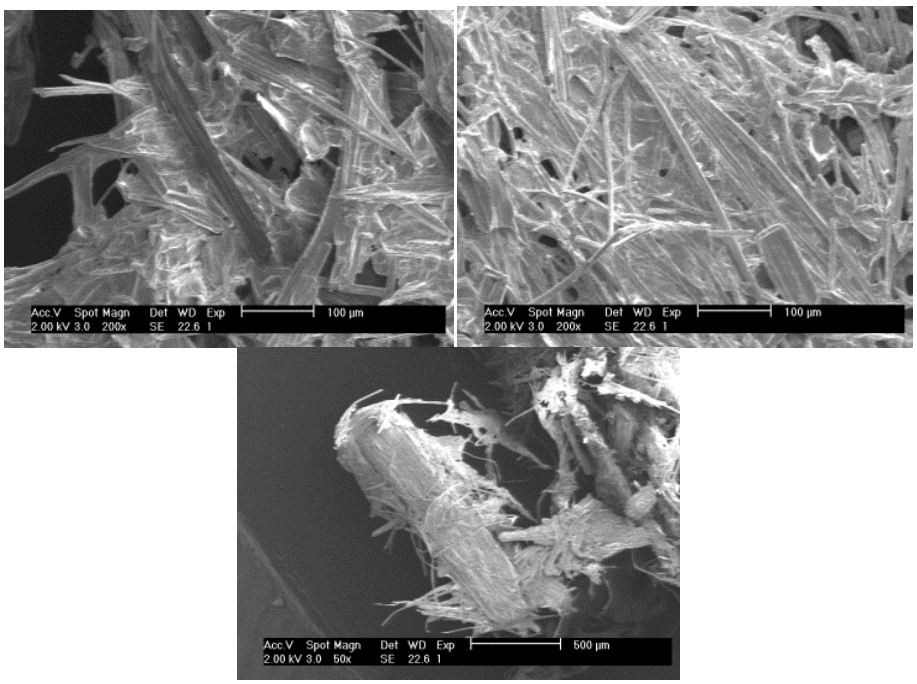


**Figure 102: SEM images of [DEA] Cl pre-treated miscanthus.**

Figure 103 shows a SEM image of [BMIM] Cl pre-treated miscanthus, and although there are some surface modifications, including fragmentation of cellulosic structure being removed from the main fibre, most of the structure remains significantly attached. This is the first notably difference between the [DEA] versus [BMIM] pre-treated miscanthus, and could help to explain why the saccharification efficiency greatly improves for the [DEA] IL on sugar release from miscanthus.

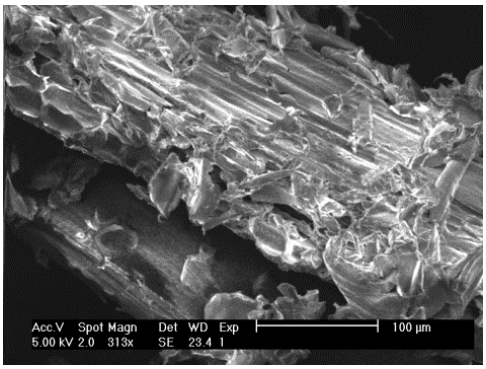


**Figure 103: SEM image of [BMIM] Cl pre-treated miscanthus.**

Further proof that the modifications to miscanthus after pre-treatment with [DEA] Cl shown in Figure 102 are significant can be seen in the SEM images taken of the ethanol-control pre-treated miscanthus. This demonstrated that the alterations in morphology and surface area were not just due to mechanical stirring and heat pre-treatment but the IL of choice. As can be seen in Figure 104, the ethanol control has modified the surface of the miscanthus, as the cell wall structure is more visible compared to un-treated miscanthus. However, the fibres still remain essentially intact, which further proves this change in surface area and morphology is due to the IL interaction of [DEA] Cl.

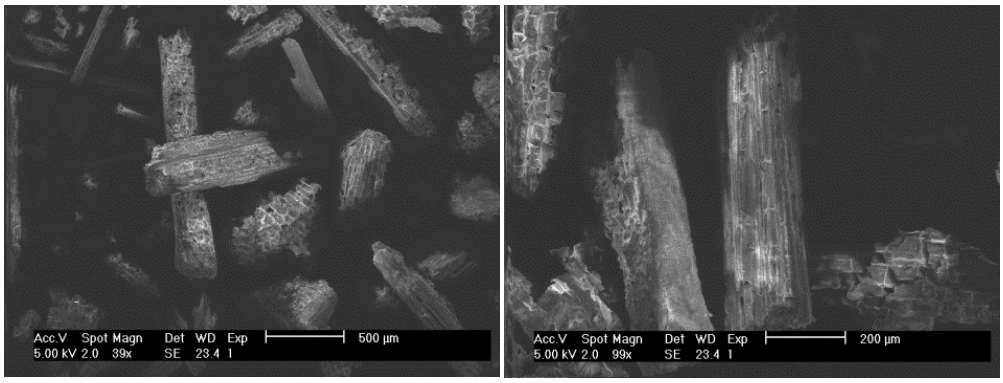


**Figure 104: SEM images of ethanol pre-treated miscanthus.**

A further comparison to Figure 102 and the [DEA] Cl pre-treated miscanthus, is Figure 105, showing [DPA] Cl pre-treated miscanthus. [DPA] Cl is as a protic IL which was shown not to be effective at pre-treating plant biomass in chapter 2 compared to [DEA] Cl. As can be seen from the SEM image, again few changes have occurred in the surface morphology or surface area of the miscanthus.

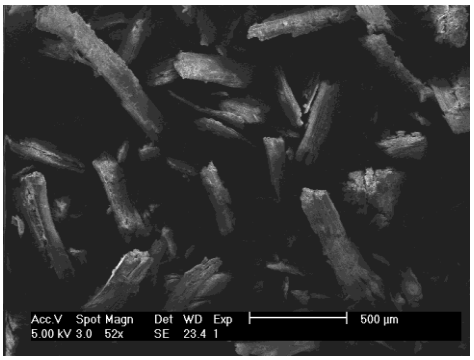


**Figure 105: SEM image of [DPA] Cl pre-treated miscanthus.**

Figure 106 shows dilute acid, (0.1 M $H_2SO_4$), pre-treated miscanthus for comparison and again it can be seen there are slight surface changes taking place with significant improvements on the visibility of the plant cell wall structure. However, unlike [DEA] Cl pre-treated miscanthus, there is no significant fibrils or stands being swollen or pulled apart, concluding why dilute acid pre-treatment was shown to be 4 times more effective then no pre-treatment but [DEA] Cl was shown to be 12 times more effective at pre-treating miscanthus then no pre-treatment.

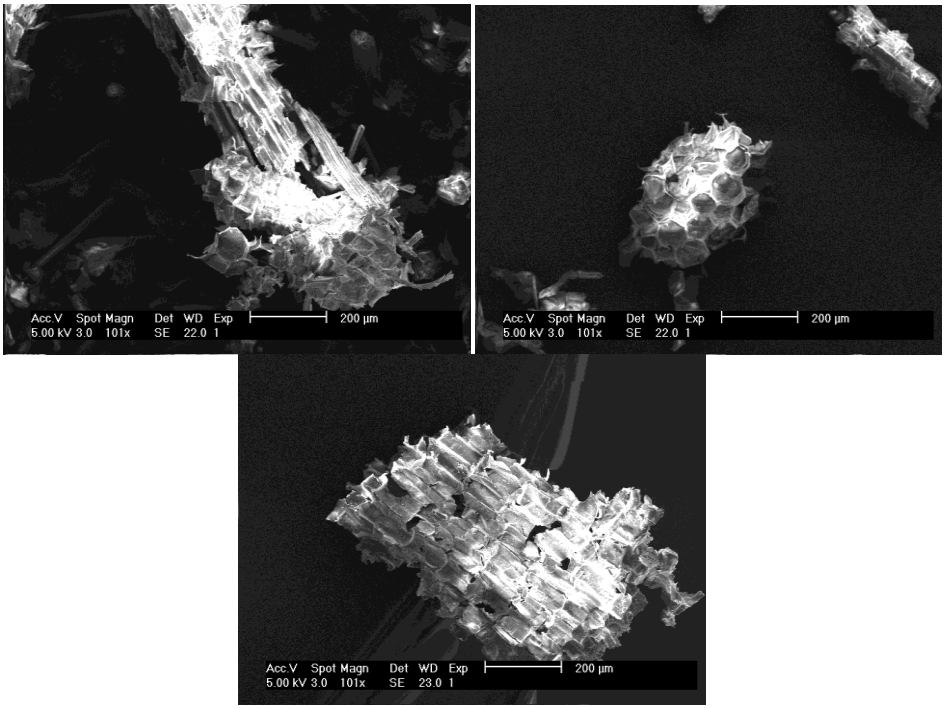


**Figure 106: SEM images of 0.1 M $H_2SO_4$ pre-treated miscanthus.**

In conclusion the SEM data has shown that the particle morphology is an important factor for the improved enzyme hydrolysis of miscanthus. The images show the more the surface and fibre structure are modified and the greater the surface area of the fibrils, the better the digestibility of miscanthus and the higher the sugar release after hydrolysis.

However, the previous 3 sections have currently not addressed the question or analysed the impact of the chemical content of the miscanthus after pre-treatment to see how this factor could play a role in the improved hydrolysis of the biomass.

## 3.5 Elemental Analysis of Miscanthus for Residual Ionic Liquid Content

Ionic liquid remaining in the biomass after the ethanol washing procedure, could affect the enzyme hydrolysis results through enzyme de-naturation, although the precise effects of [DEA] Cl on *Trichoderma reesei* is still unknown. This is underscored by the observation in Section 2.8.3, which has shown that when the enzyme hydrolysis mixture was added directly to the IL pre-treatment mixture, the enzyme activity was reduced to zero. Hence determining the content of residual ILs is essential in understanding saccharification.

It may be a factor that other ILs are successful at biomass pre-treatment that have previously been screened for pre-treatment activity, but the residual IL present in the biomass after the ethanol wash denatures the enzymes, making the IL a useless pre-treatment solvent. To this end, elemental analysis has been carried out on miscanthus after [DEA] Cl and [BMIM] Cl pre-treatment.

This data was calculated based on the nitrogen content in the ionic liquid cation by mathematically modelling the amount of IL in the sample compared to the CHN data received from the experiment. It was determined that for miscanthus after 24 hours pre-treatment in [DEA] Cl, ca. 3.5 wt % residual IL was found. For miscanthus pre-treated in [BMIM] Cl for 24 hours, this number was slightly higher at ca. 3.7 wt %.

With ca. 3.5 wt % of IL in both miscanthus samples, this could affect the ability of the enzyme to function efficiently and therefore, removing the residual IL would prove more beneficial because: (1) Enzyme activity can further be increased; (2) a reduction in the loss of ILs over pre-treatment reduces the cost of the whole process.

Nevertheless, these saccharification enzymes *Trichoderma reesei,* do function with small amounts of these ILs present in the biomass, as has been shown here and literature has shown that it is common to have residual [BMIM] Cl in cellulose of ca. 5 wt % being reported, of which our data is slightly better. [160] Reports suggest that at 20 wt% of [BMIM] Cl remaining in the sample, only 15% of the *Trichoderma reesei* cellulase activity remains.[160] However, IL-tolerant enzymes for cellulose hydrolysis would increase this efficiency even further.

## 3.6 FT-IR on IL Pre-treated Miscanthus

The analysis so far in chapter 3 has not really fully answered the original question: What kind of structural or chemical changes occur in the biomass in order to improve hydrolysis efficiency?

Understanding of cellulose crystallinity, thermal stability and morphology of the biomass polymers have been addressed, but what about the chemical content of plant biomass? To answer this question, the chemical composition will first be analysed qualitatively with FT-IR and then in detail with compositional analysis of the cell-wall polymers, both before and after IL pre-treatment will be comparatively analysed to shed light to the IL-induced compositional changes.

Pure miscanthus and various IL pre-treated miscanthus samples were analysed using FT-IR to see if different polymer functional groups were altered during the pre-treatment process.[198] Table 18 shows the IL pre-treated samples of miscanthus and qualitatively shows which polymers fractions are altered. The stretching frequencies used to identify each component from the IR spectra were obtained from[119,140] and these are quoted in the table headings. The analysis was based on a reduction or increase in the intensity of the absorption bands for each corresponding polymer for the pre-treatments and hence the content had been reduced or stayed the same.

**Table 18: Observations of polymer content after pre-treatment based on changes in the infrared spectrum for each sample. The qualitative observation is based on pure miscanthus and is labelled as having similar or less content of each polymer.**

| Biomass Pre-treatment | Cellulose 1100-1200 $cm^{-1}$ | Hemicellulose 1743 $cm^{-1}$ | Lignin 1034 $cm^{-1}$ (Also 916-835 $cm^{-1}$) |
|---|---|---|---|
| Pure Miscanthus | - | - | - |
| [DEA] Cl | Less | Similar | Less |
| [BMIM] Cl | Similar | Less | Less |
| [BMPip] Cl | Same | Less | Same |
| Microwave [DEA] Cl | Similar | Similar | Less |
| 1 M $H_2SO_4$ | Same | None | Same |
| 1 M NaOH | Same | Less | Less |
| [DEA Cl]/water – 75:25 | Similar | Less | Less |
| [DEA Cl]/DMSO – 50:50 | Similar | Less | Less |

The band at 1160 $cm^{-1}$ represents the antisymmetric bridge stretching of C-O-C groups predominantly in cellulose. At 1318 $cm^{-1}$ the $CH_2$ wagging vibrations occur indicative to both cellulose and hemicellulose.[119] The band at 870 $cm^{-1}$ is the stretch bands for benzene rings in lignin.[199]

As can be seen from the table, there are differences in the relative polymer contents, depending on the IL used and this will affect the digestibility of the miscanthus. Analysing the table we can see that the samples which perform best at releasing sugars after hydrolysis are the ones where the lignin content is reduced. However, as this is only qualitative results a complete picture is absent and a quantitative approach is required, as explained in section 3.7 using total polymer compositional assays.

# 3.7 Cell Wall Total Polymer Compositional Analysis

There are three kinds of polymers in biomass as previously introduced: cellulose, hemicellulose and lignin. Are any one of these species affected preferentially by the IL pre-treatment? To answer this question, a systematic and quantitative analysis to evaluate the loss of each individual polymer has been completed and compared.

## *3.7.1 UV-assay introduction*

In order to determine the total polymer content of the plant biomass after pre-treatment, various chemical procedures and UV assays were performed.

The cellulose content was determined using anthrone as started in section 6.9.2 where the Updegraff method[200] was employed using nitric acid followed by sulfuric acid to quantify the cellulose.

Hemicellulose content was determined using dinitrosalicyclic acid to quantify the sugars from hemicellulose as stated in section 6.9.1.

Lignin content was determined using the Foster, Fukushima and Hatfield method[201-202] involving acetyl bromide as stated in section 6.9.3.

### *3.7.2 Total Polymer Content of Miscanthus Pre-treated in [DEA] Cl and [BMIM] Cl Over a Time Range*

In section 2.3.2 miscanthus was pre-treated with [DEA] Cl at 100 °C over a range of time periods. In order to see how each of the plant polymers were individually affected during the pre-treatment, the corresponding polymer compositional analysis was performed and the results shown below.

The results indicated that time periods 72 hrs ≥ 48 hrs > 24 hrs ≥ 5 hrs > 60 mins for pre-treatment effectiveness based on sugar released from miscanthus for the IL [DEA] Cl. 5 hrs, 24 hrs and 72 hrs were analysed for the total polymer content.

**1) Digestibility versus losing cellulose content**

Table 19 shows the cellulose content of miscanthus before pre-treatment, after ethanol pre-treatment, and after a range of increasing time periods with [DEA] Cl pre-treatment. As the table shows, when you pre-treat miscanthus with ethanol there is loss of some of the cellulose content, as the residual acid can hydrolyse some sugars which are soluble and are then removed with the ethanol after the pre-treatment. As the time period of pre-treatment with [DEA] Cl was increased, the amount of cellulose continues to reduce until at 72 hours of [DEA] Cl pre-treatment there is now half the amount of the original cellulose remaining.

The reasons for these reductions are most likely due to acid hydrolysis of the cellulose polymer during the pre-treatment. There are two places where an acid source is available; (1) from degradation of the plant polymer compounds including levulinic acid and HMF are produced from the dehydration of sugar rings; (2) from the protic [DEA] Cl equilibrium back to the free acid and base as shown in section 1.4.

**Table 19: Cellulose content of miscanthus pre-treated at different time periods in [DEA] Cl.**

| **Treatment (Time-IL)** | **Cellulose (wt %)** Error ± 0.5 % |
|---|---|
| Un-treated Miscanthus | 34.0 |
| 24 hr Ethanol Control Miscanthus | 27.1 |
| 5 hr [DEA] Cl Miscanthus | 20.8 |
| 24 hr [DEA] Cl Miscanthus | 19.0 |
| 72 hr [DEA] Cl Miscanthus | 17.3 |

After 5 hours of [DEA] Cl pre-treatment on miscanthus there are significant losses of the cellulose polymer in comparison to the un-treated miscanthus and the ethanol control, and this continues to decline as time for pre-treatment increases. This leaves the IL pre-treatment in a complicated situation, as the pre-treatment increases digestibility by in-part, hydrolysing the cellulose polymer, but by doing this the cellulose is partially lost and hence maximum available sugars is reduced. The goal is a balance on the situation to minimise cellulose loss while maximising digestibility efficiency.

The conclusion that cellulose hydrolysis during pre-treatment has been observed to improve digestibility, but at the expense of some cellulose content, the source of sugars for bio-ethanol production, has occurred for the IL [DEA] Cl. The question is, is this dilemma inevitable?

To answer this question, [BMIM] Cl was compared to [DEA] Cl as a pre-treatment solvent over time. From chapter 2 [BMIM] Cl is shown to be a poor pre-treatment solvent for miscanthus with an improvement ratio of ca. 1.5. Hence the hypothesis would be, that if overtime with [BMIM] Cl pre-treatment the amount of cellulose remains similar, we can assume that part of the reason for [DEA] Cl being a good pre-treatment solvent is because it hydrolyses and removes cellulose. As is shown in Table 20, at 24 hours the amount of cellulose present in the miscanthus after [BMIM] Cl pre-treatment is comparable to [DEA] Cl pre-treatment. This reduction in cellulose can therefore, only play a small role in the improvement of hydrolysis, as the [BMIM] Cl pre-treated samples should have the same high sugar release rates as [DEA] Cl pre-treated samples, if cellulose removal was the only key.

**Table 20: Cellulose content of miscanthus pre-treated at different time periods in [DEA] Cl and [BMIM] Cl.**

| **Treatment (Time-IL)** | **Cellulose (wt %)** Error ± 0.5 % |
|---|---|
| Un-treated Miscanthus | 34.0 |
| 24 hr Ethanol Control Miscanthus | 27.1 |
| 24 hr [DEA] Cl Miscanthus | 19.0 |
| 24 hr [BMIM] Cl Miscanthus | 18.5 |
| 72 hr [DEA] Cl Miscanthus | 17.3 |
| 72 hr [BMIM] Cl Miscanthus | 13.5 |

Analysing the longer pre-treatment time of 72 hours, the [BMIM] Cl pre-treated miscanthus continues to lose more cellulose then the [DEA] Cl material. Hence this effect is now worse with [BMIM] Cl, as the loss of cellulose is higher and hence the amount available to be digested by the enzymes is less.

This secondary effect of [BMIM] Cl over time could explain the reduction in sugar release after enzyme hydrolysis, however, this concept of hydrolysing cellulose during the IL pre-treatment still does not completely answer the question of why there is increased sugar release. If it did, [BMIM] Cl would be as effective as [DEA] Cl at miscanthus pre-treatment and this is not the case.

## 2) Hemicellulose - Removal from Miscanthus

Following on from cellulose analysis, hemicellulose analysis was completed on the time periods for [DEA] Cl and [BMIM] Cl. Table 21 shows the hemicellulose content of miscanthus during [DEA] Cl and [BMIM] Cl pre-treatments over time. After ethanol pre-treatment there is a general reduction of hemicellulose, as seen previously with the cellulose content. Interestingly, with [BMIM] Cl, some hemicellulose is removed over time, however, this quickly plateau's off, after a 25 % reduction in the hemicellulose content.

[DEA] Cl pre-treatment on the other hand, continues to remove hemicellulose at the longer time periods, and nearly 50 % of the hemicellulose content is removed after 72 hours pre-treatment. This difference could explain the improvement of digestibility after pre-treatment with [DEA] Cl compared to [BMIM] Cl, however, after 24 hours pre-treatment time, the amount of hemicellulose content is similar with both ILs, so again, hemicellulose removal is an important, but potentially not the main factor involved in the improvement of miscanthus digestibility.

**Table 21: Hemicellulose content of miscanthus pre-treated at different time periods in [DEA] Cl and [BMIM] Cl.**

| **Treatment (Time-Solvent-Biomass)** | **Hemicellulose (wt %)** Error ± 0.8 % |
|---|---|
| Un-treated Miscanthus | 39.0 |
| 24 hr Ethanol Control Miscanthus | 34.9 |
| 5 hr [DEA] Cl Misc | 29.0 |
| 24 hr [DEA] Cl Misc | 29.5 |
| 24 hr [BMIM] Cl Misc | 31.0 |
| 72 hr [DEA] Cl Misc | 24.0 |
| 72 hr [BMIM] Cl Misc | 30.0 |

### 3) Lignin - Completing the picture

Even though no sugars can be extracted from lignin, it plays a crucial role in the structural integrity of the plant biomass. As stated in section 1.2, the recalcitrant nature of lignocellulosic materials is most likely due to the presence of lignin. However, lignin can also act as an inhibitor of cellulase enzymes during saccharification, as lignin can bind non-specifically to the enzymes reducing the hydrolysis of the material as active sites are blocked and cellulose cannot bind effectively.[203]

Another major issue with lignin during hydrolysis is the reduction of the surface area available for cellulose to interact with the enzymes as any lignin that remains bound to cellulose reduces the cellulose available for saccharification. Finally some aromatic phenols and aldehydes derived from lignin can denature enzymes, and further down the line kill yeast, by destroying the membrane during hydrolysis/fermentation, hence reducing the sugar/ethanol yield. Therefore, the effect of pre-treatment on lignin in biomass is a crucial factor in the efficiency of the subsequent hydrolysis.

The lignin content of the miscanthus pre-treated with the ILs over time was analysed and shown in Table 22. After the ethanol control pre-treatment, the lignin content remained essentially un-changed. Conversely, after the IL pre-treatments the lignin content reduced significantly as the time period increased. This was to be expected and the amount of lignin removed from miscanthus was more significant with [DEA] Cl pre-treatment compared to [BMIM] Cl pre-treatment.

**Table 22: Lignin content of miscanthus pre-treated at different time periods in [DEA] Cl and [BMIM] Cl**

| **Pre-treatment (Time-Solvent-Biomass)** | **Lignin (wt %)** Error ± 0.6 % |
|---|---|
| Un-treated Miscanthus | 29.9 |
| 24 hr Ethanol Control Miscanthus | 29.0 |
| 5 hr [DEA] Cl Miscanthus | 26.7 |
| 24 hr [DEA] Cl Miscanthus | 16.0 |
| 24 hr [BMIM] Cl Miscanthus | 21.7 |
| 72 hr [DEA] Cl Miscanthus | 13.9 |
| 72 hr [BMIM] Cl Miscanthus | 19.8 |

After 24 hours of pre-treatment, the amount of lignin in miscanthus dropped from ca. 29.9 wt % to ca. 16.0 wt % with [DEA] Cl, compared to ca. 21.7 wt % with [BMIM] Cl pre-treatment. After 72 hours of pre-treatment the lignin amount with [DEA] Cl reduces further to ca. 13.9 wt %, whereas [BMIM] Cl pre-treatment remains similar at ca. 19.8 wt % lignin.

This removal of lignin during the IL pre-treatment will play an important, if not crucial, role in improving the digestibility of the sugar based polymers for saccharification.

### 4) In summary

The total polymer compositional content losses over IL pre-treatment time, that affect the enzyme hydrolysis can be summarised as follows:

1. All three main polymers reduce notably in content over time
2. The loss of cellulose over time is not essential for effective hydrolysis
3. Hemicellulose removal seems beneficial but not essential to saccharification
4. Lignin removal plays the most significant part over time in efficient saccharification

These observations are summarised on Figure 107 showing all 3 polymer content amounts of cellulose, hemicellulose and lignin, from miscanthus, over the IL time periods of pre-treatment. As can be seen, all 3 polymers reduces in content in an average decline over pre-treatment time and the biggest difference is noted with lignin, the green bar, which shows the more lignin removed from a sample, the better the hydrolysis results.

The results of loosing cellulose content over IL pre-treatment time are not uncommon and have been reported with other ILs, including [BMIM] $HSO_4$ where more effective removal of lignin and hemicellulose has consequently resulted in further removal of cellulose.[170]

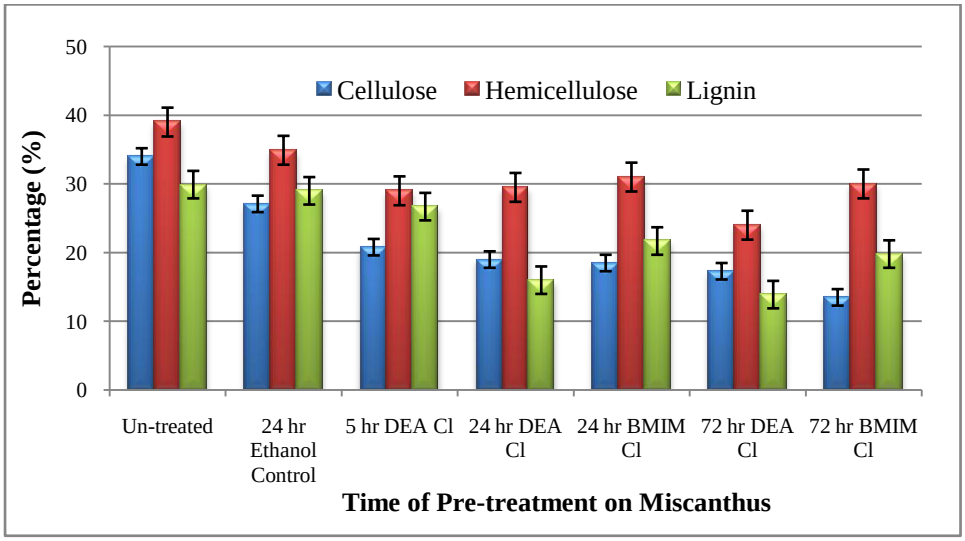


**Figure 107: Comparison of cellulose, hemicellulose and lignin content in miscanthus pre-treated in a range of time periods with [DEA] Cl or [BMIM] Cl.**

### *3.7.3 Total Polymer Content of Miscanthus Pre-treated in a variety of IL cations and anions for 24 hours*

Optimal choices of cations and anions have been explored in chapter 2, sections 2.4 and 2.5 in terms of pre-treating miscanthus for increased sugar release during hydrolysis.

The order of improvement on sugar release from miscanthus for the IL cations was; [DEA] > [PA] > [EA] > [DPA] ≥ [EthylA] = [BMPip] = [BPy] = [DiethylA] = [BMPyr] ≥ [BMIM] = [B-2-MEA] = [DMEA] = [Choline] = [BMMorph] > [TriethylA] > [BMP].

The order of improvement on sugar release from miscanthus for the IL anions was; Cl ≥ $NO_3$ > $HSO_4$ > [OAc].

The ion species dependence on pre-treatment will now be elucidated through the change in polymer composition that the pre-treatment induces. Table 23 shows the cellulose content of a selection of the ILs from sections 2.4, 2.5 that worked efficiently versus those that did not, and there corresponding amounts of cellulose.

**Table 23: Cellulose content of miscanthus pre-treated with different ILs for 24 hours at 100 °C.**

| **Pre-treatment (Time-IL-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % |
|---|---|
| Un-treated Miscanthus | 34.0 |
| 24 hr Ethanol Control Miscanthus | 27.1 |
| 24 hr [DEA] Cl Miscanthus | 19.0 |
| 24 hr [DEA] [OAc] Miscanthus | 17.7 |
| 24 hr [DEA] $NO_3$ Miscanthus | 16.4 |
| 24 hr [DPA] Cl Miscanthus | 24.9 |
| 24 hr [BMPip] Cl Miscanthus | 22.2 |
| 24 hr [BMPyr] Cl Miscanthus | 33.2 |
| 24 hr [BMMorph] Cl Miscanthus | 34.4 |

**1) Cellulose loss and pre-treatment efficiency do not correlate:**

As Table 23 shows, in some cases after pre-treatment the amount of cellulose from the ILs tested remains essentially unchanged for cation alterations. For example, [BMPyr] and [BMMorph] cations have essentially had no effect on the value of cellulose compared to un-treated miscanthus.

[DPA] Cl has had a reduction from 34 wt % to 24 wt % of the cellulose content, and compared to [DEA] Cl at 19 wt % cellulose remaining, is less of a reduction but still a significant amount removed. However, [DPA] Cl has an improvement ratio of 2 on miscanthus sugar release compared to [DEA] Cl with an improvement ratio of 12. Hence, the IL pre-treatments that don't remove cellulose from the miscanthus are ineffective at pre-treatment as shown by enzyme hydrolysis, but also are some ILs that remove significant amounts of the cellulose polymer during pre-treatment. This would imply that cellulose lose during pre-treatment is not an essential factor for improved enzyme hydrolysis.

For the anions, Cl, $NO_3$ and [OAc], all have a similar effect on the cellulose composition, with amounts of 19 wt %, 16.4 wt % and 17.7 wt % respectively. This implies that the cellulose loss could be due to the cation [DEA] as this cation removes more cellulose than any other IL cation. However, further studies would needed to confirm that effect and as cellulose seems to play little role in hydrolysis improvement for these protic ILs it was not followed up.

### 2) Hemicellulose loss correlates to pre-treatment efficiency

In the hemicellulose data from section 3.5.2 studying the effect of pre-treatment time, it was determined that hemicellulose does play an important role in improving saccharification efficiency, but to what extent is currently unknown. Table 24 shows how the hemicellulose data has changed with the different IL cations/anions on pre-treatments of miscanthus.

Using [DEA] Cl as a base line, we can see from un-treated miscanthus at 39 wt % to [DEA] Cl pre-treated miscanthus at 29.5 wt %, this is a 25 wt % reduction. All of the other cation modifications do not drop this significantly, hence this would imply there hasn't been as much hemicellulose hydrolysis and dissolution during those pre-treatments.

**Table 24: Hemicellulose content of miscanthus pre-treated with different ILs for 24 hours at 100 °C.**

| **Pre-treatment (Time-IL-Biomass)** | **Hemicellulose (wt %)** Error ± 0.8 % |
|---|---|
| Un-treated Miscanthus | 39.0 |
| 24 hr Ethanol Control Miscanthus | 28.9 |
| 24 hr [DEA] Cl Miscanthus | 29.5 |
| 25 hr [DEA] [OAc] Miscanthus | 16.7 |
| 24 hr [DEA] $NO_3$ Miscanthus | 16.6 |
| 24 hr [DPA] Cl Miscanthus | 36.2 |
| 24 hr [BMPip] Cl Miscanthus | 40.4 |
| 24 hr [BMPyr] Cl Miscanthus | 32.3 |
| 24 hr [BMMorph] Cl Miscanthus | 34.5 |

The anion change from Cl to $NO_3$ and [OAc], shows an even further reduction of hemicellulose to 16.6 wt % and 16.7 wt % respectively, which is over a 50 wt % drop. However, with this increased reduction from the anions, this does not come with higher saccharification yields, demonstrating that removing more hemicellulose does not necessarily aid further improvements in sugar release and that hemicellulose is only one factor in the saccharification improvements.

### 3) Lignin loss and enzyme hydrolysis efficiency strongly correlate

Analysing the lignin content of the miscanthus pre-treated with different IL cations/anions shows the most interesting result in this subsection. Table 25 shows the lignin data from the pre-treated miscanthus and it is clear to see that in most cases the lignin content either remains unchanged or reduces slightly for the poor pre-treatment ILs. In the case of the ILs which function efficiently for pre-treatment, [DEA] Cl and [DEA] $NO_3$, the reduction in the amount of lignin is significant (ca. 50 wt %). This significant removal of lignin suggests it is the most important factor so far, as to determining which ionic liquids function more effectively as a pre-treatment, as there is a distinct trend present for both cation and anion variations of the IL and the effect they have on lignin removal.

**Table 25: Lignin content of miscanthus pre-treated with different ILs for 24 hours at 100 °C.**

| **Pre-treatment (Time-IL-Biomass)** | **Lignin (wt %)** Error ± 0.6 % |
|---|---|
| Un-treated Miscanthus | 29.9 |
| 24 hr Ethanol Control Miscanthus | 29.0 |
| 24 hr [DEA] Cl Miscanthus | 16.0 |
| 24 hr [DEA] [OAc] Miscanthus | 22.5 |
| 24 hr [DEA] $NO_3$ Miscanthus | 15.7 |
| 24 hr [DPA] Cl Miscanthus | 27.8 |
| 24 hr [BMPip] Cl Miscanthus | 30.6 |
| 24 hr [BMPyr] Cl Miscanthus | 24.8 |
| 24 hr [BMMorph] Cl Miscanthus | 29.1 |

### 4) IL summary

The total polymer compositional losses against IL cations and anions that affect the IL pre-treatment of miscanthus can be summarised as follows:

1. Lignin removal is the most important determining factor of hydrolysis efficiency.
2. Cellulose removal is not an indicator of how effective hydrolysis can be.
3. Hemicellulose removal is important with different IL pre-treatments but does not always indicate an efficient IL for pre-treatment as [DPA] Cl demonstrated.

Figure 108 shows all 3 polymer compositional results of cellulose, hemicellulose and lignin from the IL pre-treated miscanthus in this section. As the graph confirms, lignin is the main reason why an IL structure is effective at miscanthus pre-treatment, with [DEA] $NO_3$ and [DEA] Cl showing significant reduction in lignin and hemicellulose.

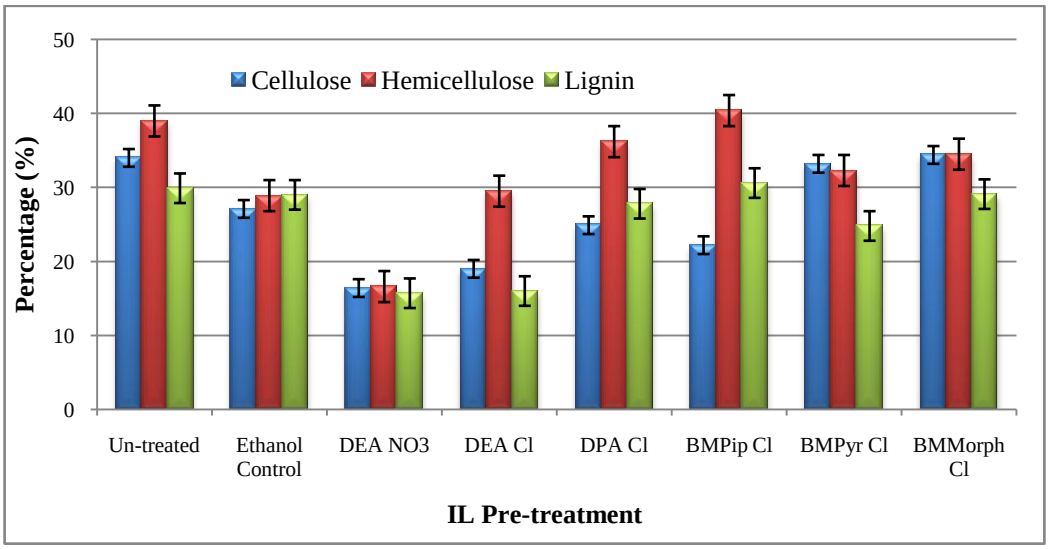


**Figure 108: Comparison of cellulose, hemicellulose and lignin content of miscanthus pre-treated with different ILs for 24 hours at 100 °C.**

### *3.7.4 Polymer Content of Miscanthus Pre-treated for 24 hours in [DEA] Cl and Water Mixtures*

In chapter 2, section 2.8, water and [DEA] Cl mixtures were tested for pre-treatment activity on miscanthus. It was shown that with 2.5 wt % to 10 wt % water, there was a slight reduction in the improvement factor of the IL pre-treatment for sugar released. However, between 20 wt % to 75 wt % water, there was an increase comparable to pure [DEA] Cl on the hydrolysis, before a final reduction above 75 wt % water. What is causing this improvement of sugar release from miscanthus, even at high water-[DEA] Cl ratios?

**1) Cellulose removal is important?**

Table 26 shows the cellulose content data for the water and [DEA] Cl mixtures. The trend here does seem to correlate that the more cellulose that is removed from miscanthus, the better the hydrolysis results. Although as stated previously we do not want to remove cellulose in our process, this partial removal seems to help digest the rest of the material potentially by reducing the degree of polymerisation due to hydrolysis of the cellulose chains. The addition of water may aid this processes as the sugars hydrolysed are readily soluble in water, and unlike the pure IL, the viscosity is significantly reduced allowing for faster mixing times.

**Table 26: Cellulose content of miscanthus pre-treated for 24 hours at 100 °C with [DEA] Cl mixed with varying percentages of co-solvent water.**

| **Pre-treatment (Time-Solvent-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % |
|---|---|
| 24 hr Water Miscanthus | 23.9 |
| 24 hr 75 % water: 25 % [DEA] Cl Miscanthus | 21.2 |
| 24 hr 50 % water: 50 % [DEA] Cl Miscanthus | 21.9 |
| 24 hr 25 % water: 75 % [DEA] Cl Miscanthus | 24.8 |
| 24 hr 10 % water: 90 % [DEA] Cl Miscanthus | 24.6 |
| 24 hr 7.5 % water: 92.5 % [DEA] Cl Miscanthus | 28.3 |
| 24 hr 5 % water: 95 % [DEA] Cl Miscanthus | 23.1 |
| 24 hr [DEA] Cl Miscanthus | 19.0 |

## 2) Hemicellulose removal - still partially a mystery

In Table 27 the effect of hemicellulose content during the different mixture pre-treatments is recorded. There does not seem to be a comparable trend between [DEA] Cl and the addition of water, in fact at higher additions of water there is less hemicellulose which could assist the digestibility. However, [DEA] Cl functions as a pre-treatment solvent without losing much hemicellulose so once again the role of hemicellulose removal for these protic IL mixtures is unclear.

**Table 27: Hemicellulose content of miscanthus pre-treated for 24 hours at 100 °C with [DEA] Cl mixed with varying percentages of co-solvent water.**

| **Pre-treatment (Time-Solvent wt %-Biomass)** | **Hemicellulose (wt %)** Error ± 0.8 % |
|---|---|
| 24 hr 100 % water Miscanthus | 22.8 |
| 24 hr 75 % water: 25 % [DEA] Cl Miscanthus | 17.6 |
| 24 hr 50 % water: 50 % [DEA] Cl Miscanthus | 17.8 |
| 24 hr 25 % water: 75 % [DEA] Cl Miscanthus | 20.1 |
| 24 hr 10 % water: 90 % [DEA] Cl Miscanthus | 20.1 |
| 24 hr 7.5 % water: 92.5 % [DEA] Cl Miscanthus | 30.8 |
| 24 hr 5 % water: 95 % [DEA] Cl Miscanthus | 22.2 |
| 24 hr [DEA] Cl Miscanthus | 29.5 |

### 3) Lignin content - most important factor again!

Finally the lignin content of the pre-treated miscanthus after water and [DEA] Cl mixture pre-treatments was analysed. Here, the significant trend that the lower the lignin content the better the pre-treatment is again observed. Both 100 % water and low weight percentage water mixtures have more lignin present after the pre-treatment compared to pure [DEA] Cl or the higher and more successful weight percentage water mixtures.

**Table 28: Lignin content of miscanthus pre-treated for 24 hours at 100 °C with [DEA] Cl mixed with varying percentages of co-solvent water.**

| **Treatment (Time-Solvent-Biomass)** | **Lignin (wt %)** Error ± 0.6 % |
|---|---|
| 24 hr 100 % water Miscanthus | 29.1 |
| 24 hr 75 % water: 25 % [DEA] Cl Miscanthus | 15.8 |
| 24 hr 50 % water: 50 % [DEA] Cl Miscanthus | 13.6 |
| 24 hr 25 % water: 75 % [DEA] Cl Miscanthus | 12.8 |
| 24 hr 10 % water: 90 % [DEA] Cl Miscanthus | 14.6 |
| 24 hr 7.5 % water: 92.5 % [DEA] Cl Miscanthus | 17.3 |
| 24 hr 5 % water: 95 % [DEA] Cl Miscanthus | 19.2 |
| 24 hr [DEA] Cl Miscanthus | 16.0 |

### 4) In summary

The total polymer compositional losses against water and [DEA] Cl mixtures that are effective for the pre-treatment of miscanthus can be summarised as follows:

1. Lignin removal is crucial for improved sugar release after pre-treatment.
2. Cellulose removal for the mixtures appears to play a role in the improvement of digestion after pre-treatment.
3. Hemicellulose has no trend on the improvement of pre-treatment for the aqueous IL mixtures.

Figure 109 compares the cellulose, hemicellulose and lignin content from the [DEA] Cl and water mixture pre-treatments. It is clear from this graph that once again it is the lignin content that plays the greatest role on improvement in digestibility.

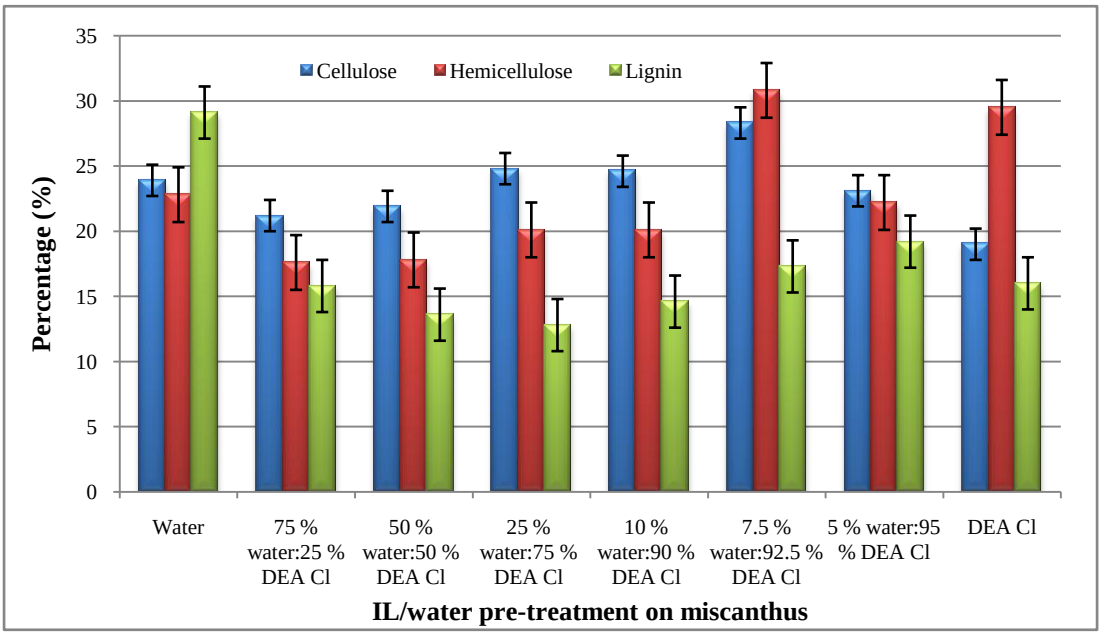


**Figure 109: Comparison of cellulose, hemicelluloses and lignin from miscanthus after pre-treatment with [DEA] Cl/water mixtures.**

## *3.7.5 Total Polymer Content of Plant Biomass Variation Pre-treated in [DEA] Cl*

In chapter 2, section 2.3, 5 different plant biomass materials were pre-treated with [DEA] Cl over a range of time periods to analyse their ability to be used in biofuel production.

The improvement in enzyme hydrolysis after pre-treatment with [DEA] Cl was as follows; miscanthus > poplar > wheat > sugarcane bagasse ≥ maize stover.

This section will analyse the polymer content of those 5 plant materials to see if removal of polymers during the IL pre-treatment is the cause of why each material is hydrolysed differently.

**1) Cellulose - Is back to no correlation between loss and improvement of biomass material digestibility**

Table 29 shows miscanthus, poplar, sugarcane bagasse and wheat samples, untreated and after [DEA] Cl pre-treatment for 24 and 72 hours respectively. Maize stover was not analysed as the results were comparable to sugarcane bagasse.

**Table 29: Cellulose content of various pre-treated lignocellulosic materials at different time periods in [DEA] Cl.**

| **Pre-treatment (Time-IL-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % |
|---|---|
| Un-treated Miscanthus | 34 |
| 24 hr [DEA] Cl Miscanthus | 19 |
| 72 hr [DEA] Cl Miscanthus | 17.3 |
| Un-treated Poplar | 40.5 |
| 24 hr [DEA] Cl Poplar | 25.5 |
| 72 hr [DEA] Cl Poplar | 13.5 |
| Un-treated Sugarcane Bagasse | 28.0 |
| 24 hr [DEA] Cl Sugarcane Bagasse | 24.0 |
| 72 hr [DEA] Cl Sugarcane Bagasse | 17.0 |
| Un-treated Wheat | 38.5 |
| 24 hr [DEA] Cl Wheat | 15.0 |
| 72 hr [DEA] Cl Wheat | 10.5 |

Table 29 shows firstly, how much the amount of available cellulose varies greatly in the different biomass materials. For miscanthus there was 34 wt % cellulose available at the start, for poplar this is 40.5 wt % and wheat 38.5 wt %, hence much more cellulose available for bioethanol production. Sugarcane bagasse on the other hand has only 28 wt % cellulose from the total material available, this low amount of cellulose could make using sugarcane bagasse as a bioethanol source less feasible.

After pre-treatment with [DEA] Cl, all the amounts of cellulose in each material drop as expected, however for sugarcane bagasse this is a much smaller drop of only a few percent between each time period finishing with 17 wt % cellulose after 72 hours.

Poplar and wheat are very similar in behaviour, both reduce dramatically and after 72 hours pre-treatment with [DEA] Cl have ca. 30 wt % of the original cellulose left. Relating this data back to section 2.3, the enzyme hydrolysis data showed us that the improvement ratio was 8 for poplar and ca. 2.5 for both sugarcane bagasse and wheat. As both poplar and wheat share the same trend with regards to cellulose loss, this would suggest that cellulose loss during pre-treatment plays little role on the improvements in the amounts of sugars released during hydrolysis. Once again this loss is negative as less sugar can be produced from the material, unless it is recovered from the IL mixture.

**2) Hemicellulose - no trend observed with loss versus saccharification improvement**

Analysing the hemicellulose variation within the plant polymers, is shown in Table 30. In this case the observations are all similar. In all samples the amount of hemicellulose during IL pre-treatment dramatically reduces in each biomass sample. In miscanthus there is ca. 40 % reduction in hemicellulose after [DEA] Cl pre-treatment. In sugarcane bagasse and wheat this figure is higher than ca. 50 % removal of hemicellulose and in poplar it is ca. 75 % loss over time.

Yet referring back to the hydrolysis improvement results of; miscanthus > poplar > wheat > sugarcane bagasse ≥ maize stover, this data does not match. Both miscanthus and poplar appear on top of the improvement ratios, but are both at opposite sides of the spectrum with regards to hemicellulose loss. So as with cellulose loss for biomass pre-treatment, there is no trend with hemicellulose loss either.

**Table 30: Hemicellulose content of various pre-treated lignocellulosic materials at different time periods in [DEA] Cl.**

| **Pre-treatment (Time-IL-Biomass)** | **Hemicellulose (wt %)** Error ± 0.8 % |
|---|---|
| Un-treated Miscanthus | 39 |
| 24 hr [DEA] Cl Miscanthus | 29.5 |
| 72 hr [DEA] Cl Miscanthus | 24 |
| Un-treated Poplar | 32.5 |
| 24 hr [DEA] Cl Poplar | 9.6 |
| 72 hr [DEA] Cl Poplar | 11.7 |
| Un-treated Sugarcane Bagasse | 20.8 |
| 24 hr [DEA] Cl Sugarcane Bagasse | 11.0 |
| 72 hr [DEA] Cl Sugarcane Bagasse | 7.1 |
| Un-treated Wheat | 22.5 |
| 24 hr [DEA] Cl Wheat | 10.6 |
| 72 hr [DEA] Cl Wheat | 8.8 |

### 3) Lignin - Still no effect on lignin loss versus saccharification improvement for the different biomass materials

The final results shown in Table 31 are the lignin composition of the original materials versus [DEA] Cl pre-treated samples. As can be seen by the untreated materials, the amount on lignin in each type of biomass varies significantly as with the cellulose content, with poplar having ca. 35.8 wt % followed by sugarcane bagasse at ca. 25.7 wt % and wheat with only ca. 20.8 wt %. However, when analysing the lignin content after [DEA] Cl pre-treatment it can be seen in all samples that the amount drops dramatically to 50 % or less. This is what is expected when the biomass material of choice is successful after IL pre-treatment, as in the previous three sections 3.7.2, 3.7.3 and 3.7.4. However, this is not expected when the biomass is only slightly improved for enzyme hydrolysis.

**Table 31: Lignin content of various pre-treated lignocellulosic materials at different time periods in [DEA] Cl.**

| **Pre-treatment (Time-IL-Biomass)** | **Lignin (wt %)** Error ± 0.6 % |
|---|---|
| Un-treated Miscanthus | 29.9 |
| 24 hr [DEA] Cl Miscanthus | 16.0 |
| 72 hr [DEA] Cl Miscanthus | 13.9 |
| Un-treated Poplar | 35.8 |
| 24 hr [DEA] Cl Poplar | 15.9 |
| 72 hr [DEA] Cl Poplar | 16.2 |
| Un-treated Sugarcane Bagasse | 25.7 |
| 24 hr [DEA] Cl Sugarcane Bagasse | 12.3 |
| 72 hr [DEA] Cl Sugarcane Bagasse | 10.6 |
| Un-treated Wheat | 20.8 |
| 24 hr [DEA] Cl Wheat | 11.0 |
| 72 hr [DEA] Cl Wheat | 11.4 |

Therefore, these results do not fit the previous trends observed. Comparing with previous literature research, these results were potentially expected and not a surprise. Research has shown that biomass recalcitrance is very complex and it is difficult to devise a strategy for all feedstocks when different compositional changes has very different effects on each individual species.[151] It has been recorded that no specific component of the cell wall was the key determinant in biomass digestibility

improvement when cross-comparing samples of switchgrass and poplar.[151] In some cases hemicellulose was determined to be the most important and in other cases lignin was, both were biomass choice dependent.

This clearly relates to the data directly observed here, as for example, removing lignin content from one biomass material, wheat, may not prove to be as important as removing it from miscanthus and hence the efficiency of saccharification is notably less.

## 4) In summary

The total polymer compositional losses for the different biomass materials pre-treated with [DEA] Cl and there comparison for effective pre-treatment and increased sugar release can be summarised as follows:

1. No correlation between cellulose loss and saccharification improvement.
2. No correlation between hemicellulose loss and saccharification improvement.
3. No correlation between lignin loss and saccharification improvement.

Figure 110 shows the comparison of cellulose, hemicellulose and lignin polymer contents over time pre-treated in [DEA] Cl, from the 5 biomass materials used. As can be seen here when comparing poplar, as being a successful material pre-treated with [DEA] Cl, compared to sugarcane bagasse or wheat which were much less effective at sugar release after the pre-treatment, it can be seen that the relative distribution in polymer content over time remains similar between each biomass type. This indicates, understanding why certain biomass materials are pre-treated more effectively with [DEA] Cl is far more complicated than just the total polymer content and relates to the biosynthetic production of the plant cell walls from the each species plant growth and environment.

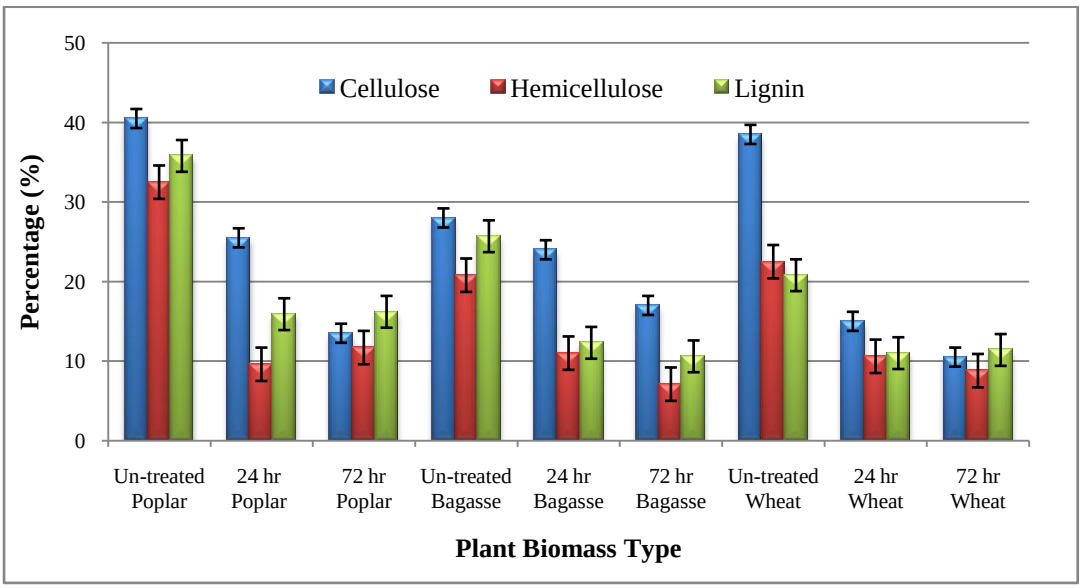


**Figure 110: Comparison of cellulose, hemicellulose and lignin content with different lignocellulosic materials pre-treated in a range of time periods and [DEA] Cl.**

### *3.7.6 Polymer Content of Miscanthus Pre-treated with Ionic Liquid and Dilute Acid/Base Comparisons*

In chapter 2, section 2.7, dilute acid and dilute base pre-treatments were analysed to compare to IL pre-treatment of miscanthus. Table 32 shows the comparison of [DEA] Cl with 0.1 M $H_2SO_4$, 0.1 M NaOH and [DEA] Cl/water mixtures.

**Cellulose Content:** As the data shows, both the acid and base do not remove much cellulose from the biomass. This is important and a benefit over [DEA] Cl pre-treatments which unfortunately do remove some cellulose during the pre-treatment.

**Lignin Content:** The removal of lignin is much less significant with the dilute acid and the dilute base pre-treatments. This could explain the difference in the hydrolysis results after pre-treatment and explain why multiple pre-treatments, although costly, improve the biomass digestion.

**Hemicellulose Content:** In dilute acid and dilute base pre-treatments, hemicellulose is considerably stripped from the miscanthus compared to [DEA] Cl, making way for increased surface area of cellulose to be digestible.

**In summary:** The significant difference with dilute acid and dilute base pre-treatment of miscanthus, is the higher removal of hemicellulose, versus lower removal of lignin and cellulose.

**Table 32: Comparison of cellulose, hemicellulose and lignin content of miscanthus pre-treated with [DEA] Cl, 0.1 M NaOH, 0.1 M $H_2SO_4$ and [DEA] Cl/water mixture for 24 hours at 100 °C.**

| **Pre-treatment (Time-Solvent-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % | **Hemicellulose (wt %)** Error ± 0.8 % | **Lignin (wt %)** Error ± 0.6 % |
|---|---|---|---|
| Un-treated Miscanthus | 34.0 | 39.0 | 29.9 |
| 24 hr [DEA] Cl Miscanthus | 19.0 | 29.5 | 16.0 |
| 24 hr 0.1 M NaOH Miscanthus | 30.4 | 22.9 | 24.5 |
| 24 hr 0.1 M $H_2SO_4$ Miscanthus | 36.3 | 16.6 | 26.2 |
| 24 hr 50 % water: 50 % [DEA] Cl Miscanthus | 21.9 | 17.8 | 13.6 |

### *3.7.7 Polymer Content of Miscanthus Pre-treated in Ionic Liquids with Conventional Heating Versus Microwave Heating*

Results from chapter 2, section 2.6, showed the effectiveness of microwaves for 10 minutes at pre-treating miscanthus with ILs compared to conventional pre-treatment. Table 33 shows the comparison of un-treated miscanthus with [DEA] Cl pre-treated miscanthus conventionally, compared to [DEA] Cl and [BMIM] Cl pre-treatments in the microwave. As the results show, there are some noticeable differences compared to the conventional results previously shown.

**Lignin Content:** The amount removed from the microwave samples has been reduced compared to previous results, which would imply a negative effect on pre-treatment. However, as the improvement ratios for microwaves are significantly high, other changes must occur with hemicellulose and cellulose to counter act this reduction.

**Cellulose Content:** The amount of cellulose removed in the microwave pre-treatments is less compared to conventional pre-treatments. This result is favoured, as higher amounts of cellulose will remain in miscanthus and should result in higher amounts of sugars being released.

**Hemicellulose Content:** The content here is significantly less than conventional IL pre-treatment and hence this could account for the overall benefits of the microwave pre-treatment over the conventional heating.

**Table 33: Cellulose, hemicellulose and lignin content of miscanthus pre-treated with ILs with conventional heat and microwave heat.**

| **Pre-treatment (Time-Solvent-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % | **Hemicellulose (wt %)** Error ± 0.8 % | **Lignin (wt %)** Error ± 0.6 % |
|---|---|---|---|
| Un-treated Miscanthus | 34.0 | 39.0 | 29.9 |
| 24 hr [DEA] Cl Miscanthus | 19.0 | 29.5 | 16.0 |
| 10 min Microwave [DEA] Cl Miscanthus | 32.9 | 23.3 | 21.7 |
| 10 min Microwave [BMIM] Cl Miscanthus | 32.1 | 26.3 | 22.7 |

**In summary:** The polymer content data for IL microwave pre-treatment of miscanthus is very similar to that of dilute acid/base analysis. In this method lignin is only partially removed but hemicellulose is significantly removed. Why this change occurs in the microwave with the same ILs, potentially could be due to any changes in viscosity on super heating of the microwave samples. Further research is required to fully understand this phenomena.

## 3.8 Conclusions

Chapter 3 has aimed to analyse the plant biomass materials, as a means to explaining why the improved hydrolysis in chapter 2 is observed from a structural and chemical content perspective.

What is the chemical basis of an efficient sugar release from biomass upon pre-treatment by ILs? This is the question central to the chapter. There are several important factors which have been discovered and are summarised below in the order of importance.

The most important single factor is the degree of lignin removal; The more lignin removed from the sample, the more efficient sugar release becomes. This conclusion, which has been shared amongst the previously-proposed methods,[27] still holds true for our protic ammonium IL pre-treatments.

The next important factor, hemicellulose, which automatically follows lignin removal, as significant parts of hemicellulose are cross-linked and covalently bound to lignin. This factor has also been noted as important for pre-treatment methods[26] and remains important for the protic ILs in this study.

Morphological change of biomass also plays an important role in the efficiency of sugar release. Roughly speaking, the more exposed the surface, the more efficient the sugar release becomes. Achieving a homogeneous sample where the biomass has become swollen in size and the fibrils pulled away from the main fibre has been shown to occur for the protic ILs. This is one of the reasons why [DEA] Cl is the best performing pre-treatment solvent. However, morphological studies still remain largely observational; quantifying morphology will be necessary for a true quantitative comparison.

Cellulose crystallinity and thermal stability do not play a significant role in pre-treatment efficiency. Crystallinity has previously been considered to be an important factor behind the efficient sugar release after pre-treatment of plant biomass in general.[132] In stark contrast, this factor, in the case of the protic ammonium ILs, does not play a significant role. These above factors successfully rationalised why [DEA] Cl is the best-performing pre-treatment solvent and why the other ILs or variables were not as effective

# 3.9 Future Work

The continuation of structural and chemical analysis using powder XRD, TGA, SEM, TG-IR on any new pre-treatment experiments or IL comparisons should be characterised. Also further polymer analysis of new biomass samples pre-treated using chemical UV assays for total polymer content compositional analysis.

Chapter 3 has concluded that the main difference causing the improvement on plant biomass pre-treatment with [DEA] Cl is the lignin content removal, hence probing lignin and its interaction with ILs, as well as lignin degradation mechanisms, would be the next important step for this work.

## *3.9.1 Lignin Studies*

Detailed studies on lignin with the IL [EMIM][OAc] have shown numerous important factors for lignin transformations during IL pre-treatment. In summary; (1) An increase in phenolic OH due to β-O-4' linkages being cleaved, but reduction in aliphatic OH due to dehydration reactions; (2) Degradation of β-β' and β-5' at higher temperatures; (3) Selective degradation of G-type units, and condensation at S-type units; (4) Demethoxylation at G-type units.[168] Figure 111 shows a summary of these lignin transformations possible in the IL. An important note for consideration is the lignin composition of S and G type units because this has been known to affect enzyme hydrolysis.[204]

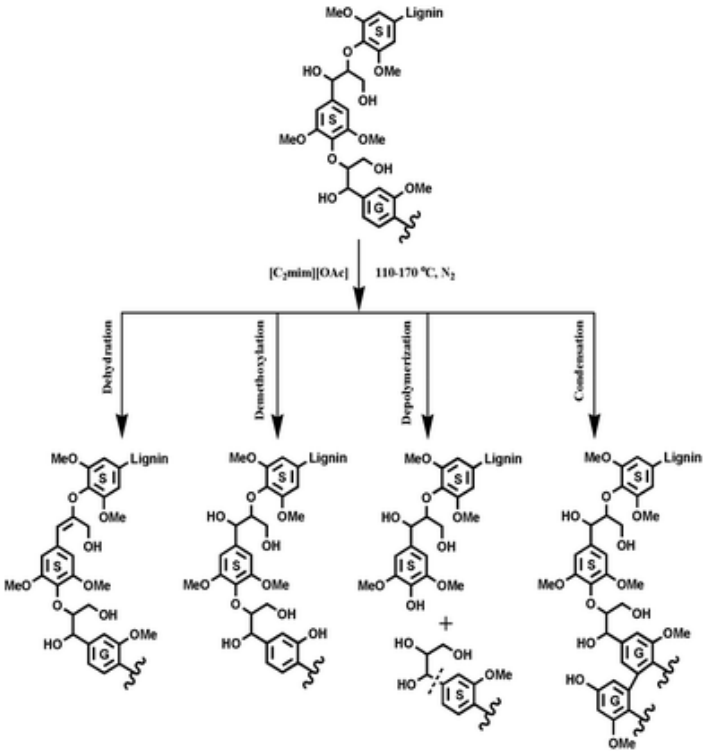


**Figure 111: [EMIM] [OAc] and potential pre-treatment reactions on lignin modified from reference 204.**

Secondly, literature has shown IL anion plays a more significant role in reducing lignin molecular weight compared to the cation.[205] Fragmentation hierarchy of lignin and IL is sulfates > lactate > acetate > chloride > phosphates in reducing molecular weights.[205] Different anions cause different cleavages and this opens the door from combining ILs usage. This research work has only studied acetate and chloride from the above list and hence the other anions could be studied with emphasis on lignin removal.

Separate studies into looking at enzymes that degrade lignin could be added to IL pre-treatments to further improve the digestibility of the biomass. Most studies into lignin degradation include some research into ligninolytic enzymes from fungal secretome. Lignin peroxidase, manganese peroxidase and laccases are all enzymes being investigated for lignin degrading enzymes during pre-treatment.[203]

Current research has shown the use of NMR in determining lignin structure modification after pre-treatment. HSQC NMR has shown condensed lignin structures being formed after pre-treatment, determined in the protonated aromatic

region.[65] This method could be used to determine the lignin based structures being formed during the IL pre-treatments in this research.

### *3.9.2 New methods*

There are other methods in the literature which have been used to study plant biomass pre-treatments, which could also be employed for this research. Firstly, a method using TEM to study IL-biomass mixtures in real time can be used, due to the benefits of ILs being non-volatile, hence can be used with high vacuum techniques without affecting the experiment.[185]

Secondly, the use of microwaves as a pre-treatment heat source has shown great promise in reducing the time and energy efficiency of pre-treatment. However, during this pre-treatment it is possible that some super heating of the biomass in the IL occurs, because it is a heterogeneous sample. Tests of any volatiles released during the pre-treatment and useful chemical complexes formed can be undertaken using Infrared or GC tandem techniques.

Challenges with viscosity issues, still pose a significant problem when using ILs for pre-treating plant biomass. Research has shown that using a Bohlin Gemini cone-and-plate rheometer, *in situ* measurements can be made on the kintetics of cellulose dissolution.[153] Measuring the sheer rate of the IL/cellulose slurry, until a homogeneous solution is formed, allows further testing of structure-activity relationships for numerous ILs. Also to monitor the DP of cellulose by viscosimetry analysis as a function of the pre-treatment.[55]

Another method to be explored is the Gutmann accepter number. As this method probes the strength of protonation of a weak base by the acid being studied, found to be useful in studying protic ILs potential at catalysts.[206]

ILs are also ideal for use with MS studies due to their greater spectral peak intensities and lower limits of detection, and so analysing the components remaining in the ILs could be obtained using this method.[176]

Finally, an important point which keeps re-occurring, is a need to study biomass recalcitrance in detail in order to combat it. As our data shows, the results vary greatly between each species, even if the same reactions/pre-treatments are taking

place. One research group are incorporating deuterium into the plant cell wall via enriched hydroponic species.[207] This allows NMR and neutron studies of thermochemical and enzyme degradation of plant biomass. The process is relatively easy to incorporate $^{2}H$ *via* photosynthesis from the environment using hydroponic cultures.[207] Kale samples were enriched and after IL treatment it was shown that 33% of deuterium was incorporated into mostly the carbohydrate component of the plant.[207] The IL used for these studies was a pyridinium based chloride IL with DMSO, but this could be extended for studies with [DEA] Cl.[207] In connection, biomass variation $^{13}C$ enrichment has been used to study the cell wall of plants as well. This is relatively easy and not expensive *via* growth in a $^{13}C$ enriched $CO_2$ and cell incorporation by photosynthesis.[32] These methods will help understand species dependent on pre-treatment, which in turn, will improve the designs of ILs for plant biomass pre-treatment.

# Chapter 4

# Cellulose Pre-Treatment and Saccharification: Towards Unification of Structural and Chemical Studies Using a Model Compound

## 4.1 Introduction

Cellulose is the most important polymer in plant biomass, when considering bioethanol production. In the previous chapter, biomass pre-treatment efficiency has been shown to depend mainly on two points; (1) Lignin removal; (2) Morphological changes. A factor which was shown not to be important was cellulose crystallinity.

The saccharification study in this chapter indeed underscores this view (section 4.2). However, the efficiency of lignin removal has some downfalls, the loss of cellulose in the process. This is because cellulose in biomass is covalently linked to lignin. How can we remove lignin while reducing the cellulose loss in the process? To what extent do the ILs, especially [DEA] Cl, effect cellulose pre-treatment?

Morphological changes, which have been identified as an important factor in the previous chapter, are very hard to disseminate. Two important factors seem to be essential from our observations: (1) Particle size; (2) Porosity. In order to disseminate these two factors, we have analysed cellulose with distinctive particle sizes to eliminate the factor (1), to see if there is any significance from contribution (2).

Studying the effect of pre-treatment of the crystalline form of cellulose is another important feature. This chapter will aim to examine and confirm, whether cellulose crystallinity really is a factor irrelevant to sugar release with these protic ILs. This is the conclusion from chapter 3, which is in stark contrast to the previous studies in this field, which conclude that cellulose crystallinity plays a crucial role.[38] Hence, it is necessary to settle this difference through systematic decomposition studies on cellulose crystals.

Avicel cellulose was chosen as the model compound for these studies on this system, due to avicels well-reported, highly microcrystalline structure and characteristics.

Avicel is also commonly used in research as a material for cellulose studies and is a suitable comparison to lignocellulosic materials.[132]

By the end of this chapter the following goals will have been achieved:

1. The effect of ILs on pure cellulose versus plant biomass.
2. Particle size studies on IL pre-treatment of cellulose.
3. The contribution of morphology to cellulose pre-treatment and hydrolysis.
4. Changes in cellulose crystallinity recorded where they differ from chapter 3.

## 4.2 Saccharification Studies on Cellulose as a Model Compound for IL Pre-treatment

To analyse the effect of ionic liquids on cellulose, saccharification analysis was completed to determine the quantity of glucose released after pre-treatment.

For these experiments the standard 100 °C was used as the pre-treatment temperature as although studies have shown the [EMIM] [(Meo)(R)$PO_2$] series of ionic liquids to dissolve microcrystalline (MC) cellulose at room temperature,[112] the weight percentage of cellulose dissolved is significantly small. The same conditions and procedures were followed as in chapter 2 and as described in section 6.2 and 6.8.

Figure 112 shows the saccharification results obtained using cellulose pre-treated with [DEA] Cl over a range of time periods, from 30 minutes to 72 hours. The controls used in these experiments were the standard cellulose pre-treated in ethanol at reflux for 24 hours. The aim, as in chapter 2, was to compare the cellulose pre-treated in ethanol over time and at the same temperature, to ensure any differences in sugar release during hydrolysis were not just due to thermal or mechanical modification, but chemical IL pre-treatment.

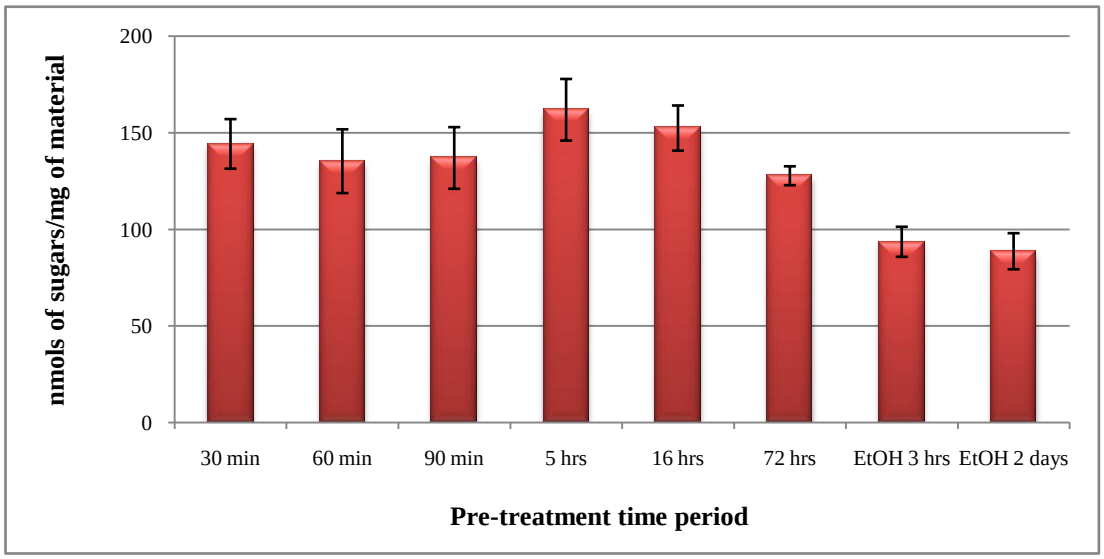


**Figure 112: Cellulose pre-treated with [DEA] Cl over a range of time periods at 100 °C.**

Figure 112 clearly shows that after saccharification the ethanol control produced the lowest amount of sugars, ca. 90 nmols of sugars/mg of material, compared to [DEA] Cl. The [DEA] Cl pre-treated cellulose had ca. double the amount of sugar release at ca. 160 nmols of sugars/mg of material released, from 30 minutes to 16 hours. After

16 hours of pre-treatment with [DEA] Cl, there is a slight decline in the amount of sugars released from cellulose, with a minimum at 72 hours of approximately 130 nmols of sugars/mg of material.

This increase over time is as expected and matches the same trend observed with biomass in chapter 2. However, the overall reduction in the pre-treatment effectiveness, shows how cellulose is only marginally effected during IL pre-treatment and that in chapter 2 and 3 it is the other polymers of hemicellulose and lignin that play the key role. In the best examples, an improvement of between 8-12 times more sugar release compared to the control pre-treatment was possible, however, with pure cellulose only ca. double the sugar release occurred. Hence this would confirm that the major effect of IL pre-treatment on biomass is not on cellulose and backs up the analysis that cellulose removal does not play a key role in pre-treatment efficiency.

The reduction in the amount of sugars released after 16 hours of IL pre-treatment is a new observation not present in chapter 2. This could potentially be due to a few factors; (1) Firstly, the sugar monomer units released after hydrolysis, could be dehydrated, catalysed by the $Cl^-$ anion.[208] The $Cl^-$ is a fairly strong nucleophilic anion and depending on the amount of water present to solvate the [DEA] Cl, the $Cl^-$ could attack the carbon atoms attached to OH groups and break down the sugar ring into furfurals and carboxylic acids as explained in chapter 1.

However, after NMR analysis, no resonances were observed in the 8-12 ppm chemical shift region, expectant due to the aldehyde functional group present in HMF and furfurals. This could imply that no, or very little HMF was formed during the pre-treatment, however, with the difficulty of removing the IL from the ethanol and sugar solution, this dominates the NMR signal and so a clear answer using NMR is not possible.

(2) Secondly, the other possibility, is after the cellulose has increased time pre-treated in the ionic liquid, more of the [DEA] Cl becomes bound (*via* H-bonding) to the cellulose chains and this could potentially deactivate or decrease the enzymes efficiency. [DEA] Cl is very viscous and through hydrogen bonding could remain intact in the cellulose material after pre-treatment and hence be present in the enzyme mixture. It is known in the literature that currently most enzymes are

currently denatured in IL solutions[109], so it is reasonable to expect that the more time the cellulose is pre-treated with the IL, the more likely the concentration of residual IL will increase and hence more likely to decrease the enzymes activity. This trend was observed also in chapter 3.

Figure 113 shows photos of the cellulose samples after pre-treatment in [DEA] Cl. In Figure 113 from left to right the time period of cellulose pre-treated in [DEA] Cl decreases from 72 hours to 30 minutes. As can be seen, on the far right the samples resemble the white starting powder, conversely, on the far left the samples are flaky, clumpy, and have a faint yellow tint, potentially due to optical impurities in the IL after the longer time periods and indicating IL is present in the cellulose.

**Figure 113: Cellulose samples treated in DEA Cl. From left to right pre-treatment time periods are 72 hrs, 48 hrs, 16 hrs, 5 hrs, 90 mins, 60 mins and 30 mins.**

## 4.3 Cellulose Particle Size Saccharification Studies

It is known and has been stated that smaller particle sizes for cellulose solubility in ionic liquids are beneficial.[129,209]Avicel microcrystalline cellulose is available in many different particles sizes, particle shapes and moisture content, making it easy to test the effects of ILs on cellulose pre-treatment.[209] Some literature demonstrates that decrystallising cellulose during pre-treatment is not an inescapable step, as this work has also discovered, and that it is not necessary prior to bio-catalytic experiments. The consideration of particle size of the biomass is a determining factor for effective plant polymer removal.[57]

In order to demonstrate the effect of the particle size of our model compound cellulose compared to the improvement of sugars released after pre-treatment with ILs, different particle sizes of cellulose were tested. 20 µm, 50 µm and the standard heterogeneous cellulose particle sizes, were used in an experiment using the previous time periods of choice, pre-treated with the IL [DEA] Cl.

Figure 114 shows the experimental results, with 20 µm cellulose shown in red, 50 µm cellulose shown in purple and heterogeneous cellulose shown in blue. Ethanol controls are shown at the end of the figure and [BMIM] Cl has been added as a comparison with 20 µm cellulose.

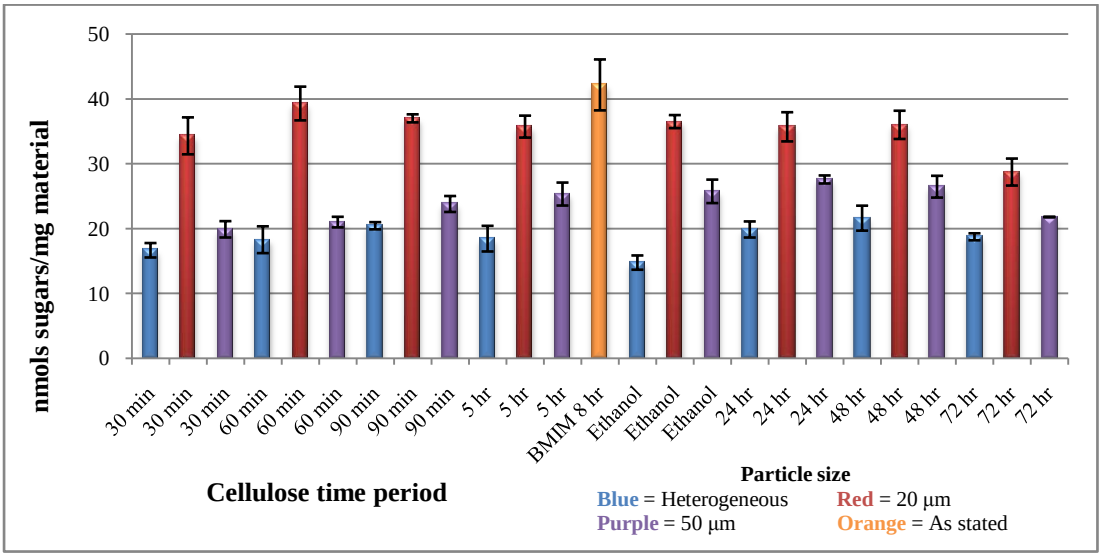


**Figure 114: Pre-treating cellulose with varied particle sizes with [DEA] Cl over a range of time periods at 100 °C.**

The data shows that the smaller the particle size of cellulose, the more effective the IL pre-treatment is, as more sugars are released after enzyme hydrolysis. This was expected and makes chemical sense, as with the smaller cellulose particle sizes, a larger surface area of the cellulose is able to interact, *via* electrostatic interactions/Hydrogen-bonding/Van der Waals interactions with the IL.

Hence the most effective pre-treatment with the highest sugar release is from 20 μm cellulose, followed by 50 μm cellulose, followed by heterogeneous cellulose. The general increase over time pre-treated in the IL is present, however as stated in section 4.2 this effect is less with just cellulose as the model compound over biomass.

The [BMIM] Cl example shows a comparable sugar release rate to [DEA] Cl, this was tested as a comparison from the literature to further highlight the importance of particle size when pre-treating cellulose. This section has demonstrated how much the affectivity of IL pre-treatments can vary depending on particle size, and hence this is an important consideration when designing IL pre-treatment refineries for lignocellulosic materials.

## 4.4 TGA Studies on Pre-treated Cellulose

In order to investigate any changes in the chemical interactions between cellulose polymer chains, TGA was used to investigate thermal stability of the cellulose polymer after IL pre-treatment, which may indirectly relate to the strength of interaction between polymer chains. TGA was used to see if intermolecular interactions, mostly hydrogen bonds in cellulose, had been reduced in any way.

Figure 115 shows pure MC cellulose in red, compared to [DEA] Cl and [BMIM] Cl pre-treated MC cellulose in green and purple respectively. The data shows a small change in the onset of thermal decomposition temperature of ca. 30 °C sooner for the IL treated MC cellulose samples. However, as the polymer starts to decompose, the temperature required for complete decomposition is higher for the two IL pre-treated samples then pure MC cellulose.

This implies that although some change in the interactions of the cellulose polymers have occurred, little overall effect is observed. In chapter 3 a significant change was present in the biomass materials after IL pre-treatment. The reduction in thermal stability could be therefore, due to weakening and removal of the hemicellulose and lignin polymers predominantly, with a small input from the reduction in cellulose interactions.

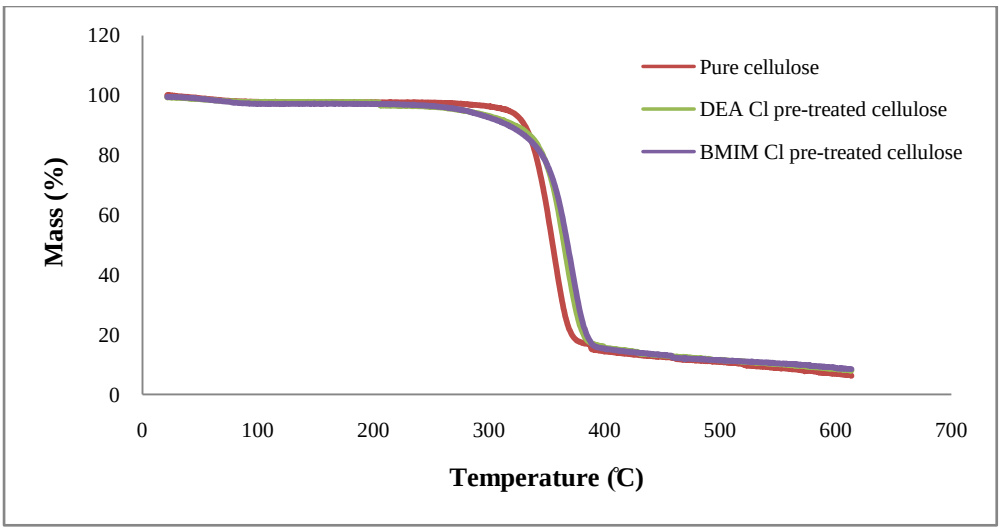


**Figure 115: TGA of pure cellulose and cellulose pre-treated in DEA Cl and BMIM Cl for 24 hours at 100 °C.**

Figure 116 shows the difference in TGA data of the change in particle size of cellulose experiment, in section 4.3. As expected the smaller the particle size of cellulose the lower the decomposition temperature and weaker the intra- and inter-molecular interactions after pre-treatment. Also the longer the MC cellulose is pre-treated with IL, the lower the decomposition temperature. This implies that more changes occur, the longer the MC cellulose is pre-treated, hence explaining why longer pre-treatment times can release more sugars after enzyme hydrolysis.

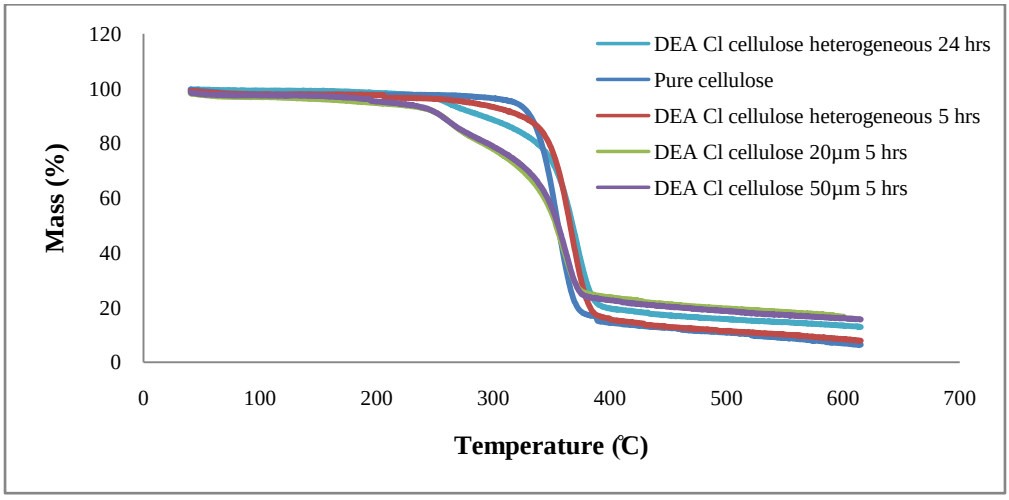


**Figure 116: TGA of variation in cellulose particle sizes, all pre-treated in [DEA] Cl for 24 hours at 100 °C.**

It may not just be structural changes, as chemical changes will also affect the decomposition temperatures, as some of the cellulose chains could be hydrolysed and chain length reduced during IL pre-treatment.

# 4.5 Optical Microscopy on Cellulose

In order to analyse the polymer surface morphology, optical microscopy was used on cellulose before and after IL pre-treatment.

Figure 117 shows pure cellulose, [DEA] Cl pre-treated cellulose and [BMIM] Cl pre-treated cellulose images respectively. The red scale bar is 50 μm and pure cellulose comprises of a mixture of different length fibres, mostly 200 μm or longer.

For [DEA] Cl pre-treated cellulose, there isn't much change apart from some of the fibres looking more swollen after the IL pre-treatment, which could explain increased effectiveness of enzyme hydrolysis. However, [BMIM] Cl pre-treated cellulose, instead of fibres, is now more like aggregates of particles as shown. This may imply that [BMIM] Cl does interact more with the cellulose polymer upon pre-treatment, more than [DEA] Cl does, however, upon precipitation the material clumps together in different shapes making it harder to hydrolyse by enzymes. There is no evidence in these experiments of significant cellulose solubility with [BMIM] Cl.

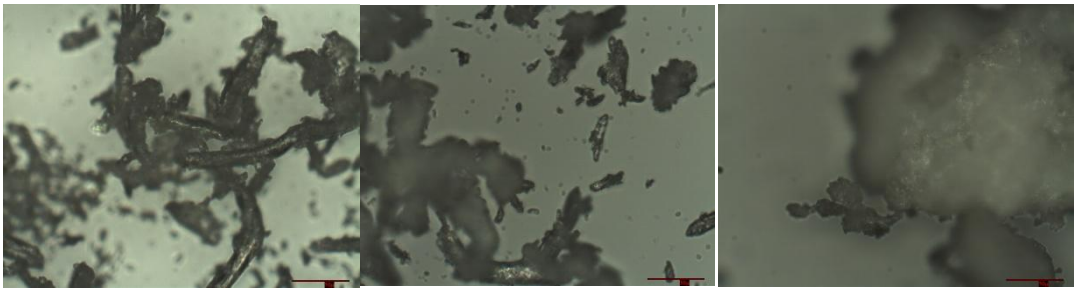

**Figure 117: Images of A) pure cellulose B) [DEA] Cl pre-treated cellulose for 24 hours at 100 °C C) [BMIM] Cl pre-treated cellulose for 24 hours at 100 °C.**

## 4.6 Scanning Electron Microscopy on Cellulose

Finally, in order to further analyse the surface morphology of pure cellulose compared to [DEA] Cl and [BMIM] Cl pre-treated cellulose, SEM was used to take a closer look at the cellulose fibres.

Figures 118-120 show the SEM images and how the morphology changes from pure cellulose to [DEA] Cl and [BMIM] Cl pre-treated cellulose respectively. It can be seen, that in pure cellulose in Figure 118, the outside fibres are visible and a heterogeneous texture is present. In Figure 119 it can be seen that some of the cellulose fibres are smaller with more 'bumps' on the outside of the fibres, where the IL has caused the polymer to swell. In Figure 120 the same observations can be seen with some swelling and structure change.

The outcome is that there are minimal changes with surface morphology of the cellulose polymer with some emphasis on changes in particle size to improve digestion to sugar monomers and potential imperfections on the outside of the fibres.

This could explain why some improvement is observed with increased effectiveness of sugar release but it is not to the extent of plant biomass in chapter 3, as significantly less physical modifications are observed, potentially observed due to the removal of lignin and hemicellulose from the plant biomass.

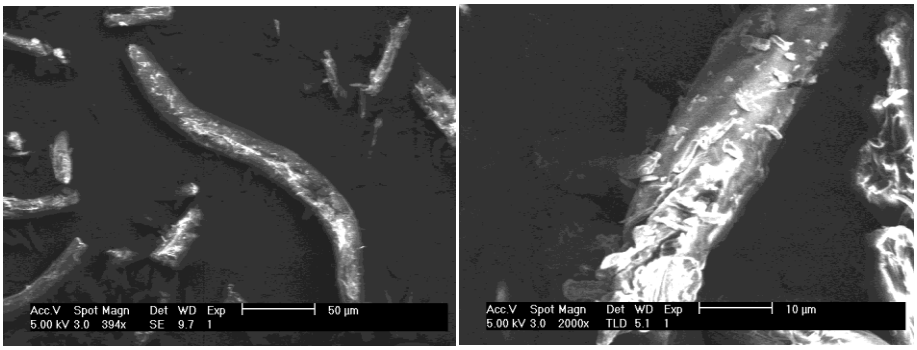


**Figure 118: SEM images of pure cellulose.**

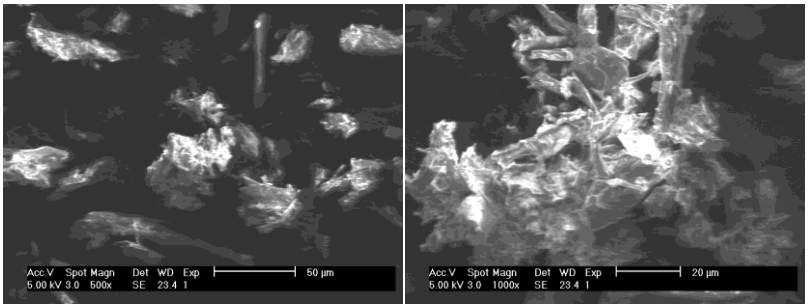


**Figure 119: SEM images of cellulose pre-treated with [DEA] Cl showing change in morphology.**

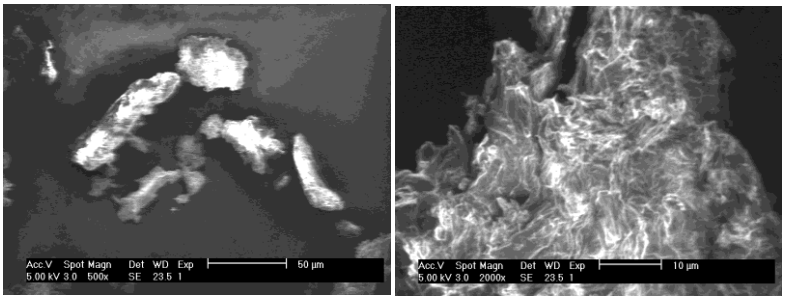


**Figure 120: SEM images of cellulose pre-treated in [BMIM] Cl showing change in morphology.**

# 4.7 Ionic Liquid Variation for Cellulose Saccharification

As section 4.2 showed, although only a small improvement was noted, there was a improvement on saccharification of cellulose after [DEA] Cl pre-treatment. In an attempt to investigate how important the cation and anion combination was in pre-treating cellulose as a model compound compared to biomass, the cation and anion were altered separately to see the effect this had on pre-treatment and sugar release. [DEA] [OAc], [DMEA] Cl and [BMIM] Cl were chosen as initial examples based on results from chapter 2 and the project aims.

## *4.7.1 IL Anion Change to [OAc] on Cellulose Pre-treatment*

The [DEA] [OAc] pre-treatment of cellulose showed little improvement, if any, on pre-treatment of cellulose time, as shown in Figure 121. The ethanol control releases the same amount of sugars as the [DEA] [OAc] releases.

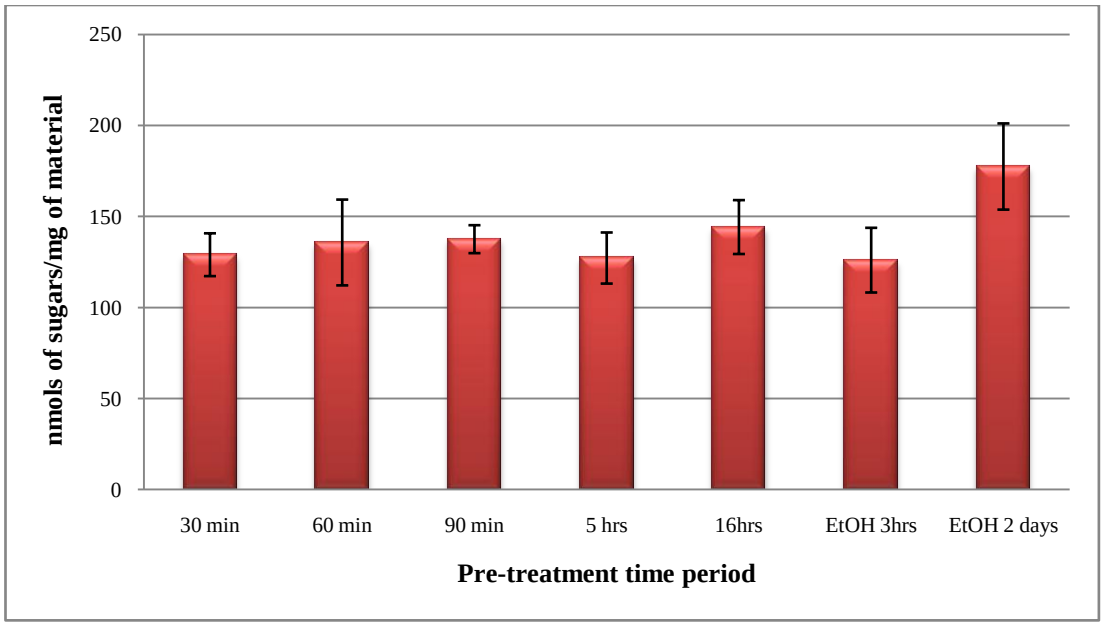


**Figure 121: Cellulose pre-treated with [DEA] Ac over a range of time periods at 100 °C.**

## *4.7.2 IL Cation Change to [DMEA] for Cellulose Pre-treatment*

Saccharification results from cellulose pre-treated in [DMEA] Cl are shown in Figure 122 and from the data, within error, it is clear to see there is no overall improvement in the amount of sugar released over the time periods 30 minutes to 72 hours. However, the amounts of sugars released after the pre-treatment in [DEA] Cl at a maximum, are nearly double compared to the ethanol control. This would imply that when changing the cation to from [DEA] to [DMEA] we maintain some improvement in pre-treatment with the Cl anion just not as much as [DEA].

This is compared to changing the anion from Cl to [OAc] but keeping the cation [DEA] the same, where all functionality of the effectiveness of the IL pre-treatment was lost. Therefore, the chloride anion must play the most significant role in the pre-treatment of cellulose compared to the cation as literature states[38]. Nevertheless, choosing appropriate cations is essential in getting the maximum benefit for pre-treatment and hence maximum sugar release. This data matches the trends we observe in chapter 2 with IL design.

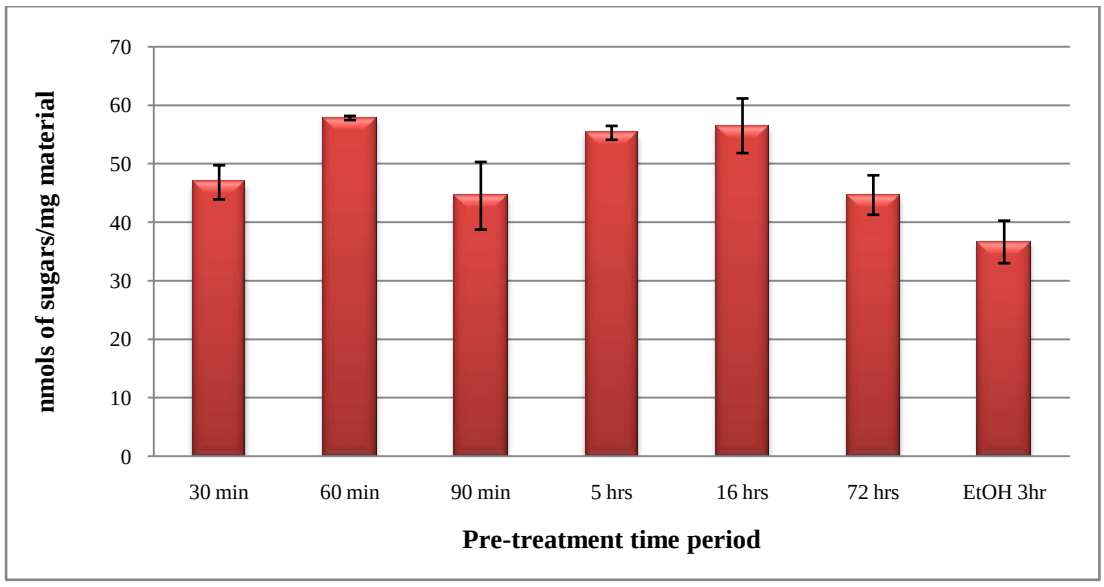


**Figure 122: Cellulose pre-treated with [DMEA] Cl over a range of time periods at 100 °C.**

So the question presented here is; Why in the pre-treatment experiments is the chloride anion better than acetate anion at 'opening up' the structure of cellulose for improved enzyme digestion?

An answer could be due to Cl being slightly smaller in ion volume compared to [OAc], but it is more likely that having more hydrogen bond capabilities is the main factor, shown in Figure 123.

The [OAc] has a 'pincer' type approach to hydrogen bonding, on the one face of the molecule, whereas Cl has a spherical approach forming multiple directional hydrogen bonds.[38] This will increase the ability of the cellulose chains to be solublised during pre-treatment.

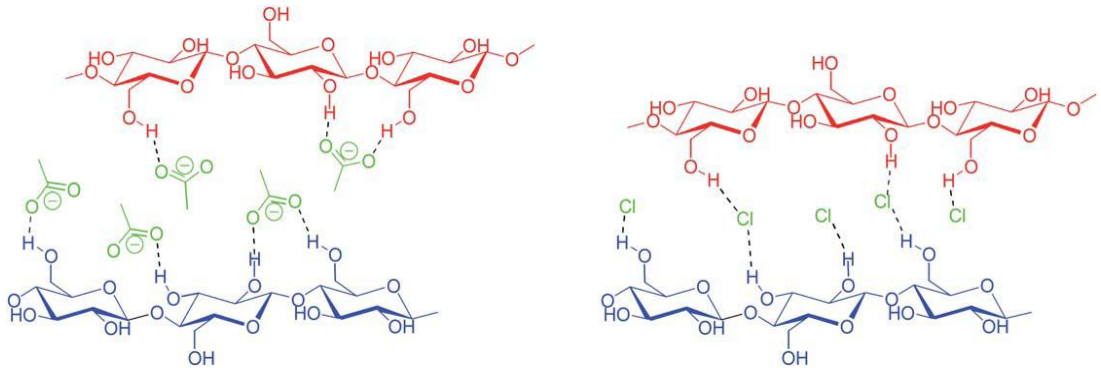

**Figure 123: Schematic showing chloride and acetate anions hydrogen bonding to cellulose modified from reference 38.**

Comparing the two cations used currently in the cellulose pre-treatment experiments, [DEA] has 2 hydroxyl groups compared to [DMEA] which has only one. This could imply having OH groups on the cation is important in the cellulose pre-treatment, as determined in chapter 2. However, there could be other reasons, for example the interaction between the cation and anion in the bulk IL and how this could differ between the individual ion pairs or neighbours pairs could also play a role.

### *4.2.3 IL Cation Change to Aprotic [BMIM] on Cellulose Pre-treatment*

To evaluate the difference between protic and aprotic cations and the affect they have on cellulose pre-treatment, [BMIM] Cl was used as a standard aprotic IL comparison. Aprotic imidazolium ILs are considerably more expensive then the protic ammonium ILs, although recent literature[109] shows their potential at dissolving these lignocellulosic materials and the successes so far.

[BMIM] Cl is currently one of the best ILs determined for dissolving lignocellulosic materials with 20% solubilisation of cellulose[109]. Figure 124 shows cellulose pre-treated with [BMIM] Cl and the outcome after 90 minutes and above shows pre-treatment has made a 30% improvement compared to the ethanol control.

This amount of sugars released is considerably less than the protic ammonium chloride IL pre-treatment with [DEA] Cl, further proving that in this case the cation and anion combination are important for effective pre-treatment. The slight reduction, within error, of sugars released after longer time periods of cellulose pre-treated in [BMIM] Cl, could be due to dehydration of sugar units or contamination of enzyme hydrolysis and explained previously.

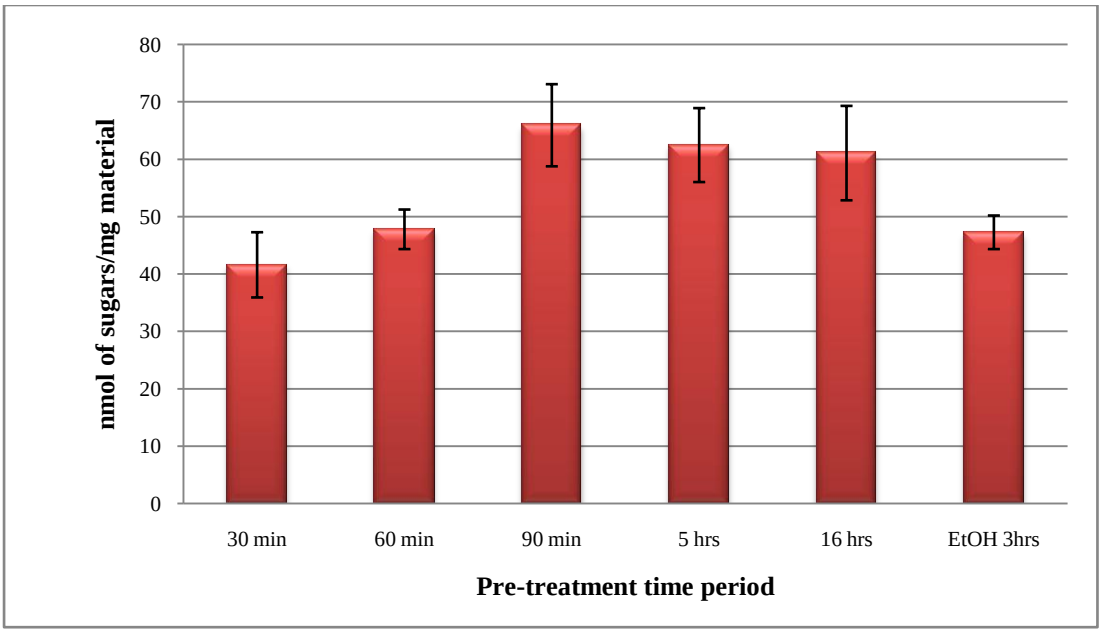


**Figure 124: Cellulose pre-treated with [BMIM] Cl over a range of time periods at 100 °C.**

### *4.7.4 Saccharification Studies Summary*

From the data in section 4.7, it is clear to see an improvement on cellulose pre-treatment is achieved after pre-treatment with ILs, however, as expected this is significantly smaller then the impact seen for biomass pre-treatment. The question must be asked, can we find an IL which pre-treats cellulose effectively, that does not remove cellulose as shown in chapter 3? This would give the advantage of high sugar release rates without the loss of cellulose during the IL pre-treatment.

To this end, it is necessary to study which ILs are important for this process, by comparing the different IL cations as systematically modified in chapter 2.

# 4.8 Ionic Liquid Cation Systematic Alteration for the Pre-treatment of Cellulose

In order to further explore the benefits in IL design from section 4.7, the IL cations were altered in the aim of studying the structure-activity relationship between the IL and cellulose during pre-treatment, as in chapter 2, sections 2.4 and 2.5.

## *4.8.1 Protic IL Cations*

Figure 125 shows the saccharification results based on the different cations as shown in chapter 2, figures 52-55. The ethanol control is in green.

A major difference between the experiments here and in chapter 2 is that some of the solid IL samples tested in chapter 2, were previously just added to the biomass, as with the liquid samples (explained in 6.2) and a solid-solid pre-treatment method took place. Consequently the solid IL samples never functioned as effectively as the liquid IL samples, which could have been due to the cation and anion combination, but also to do with lack of solvation issues.

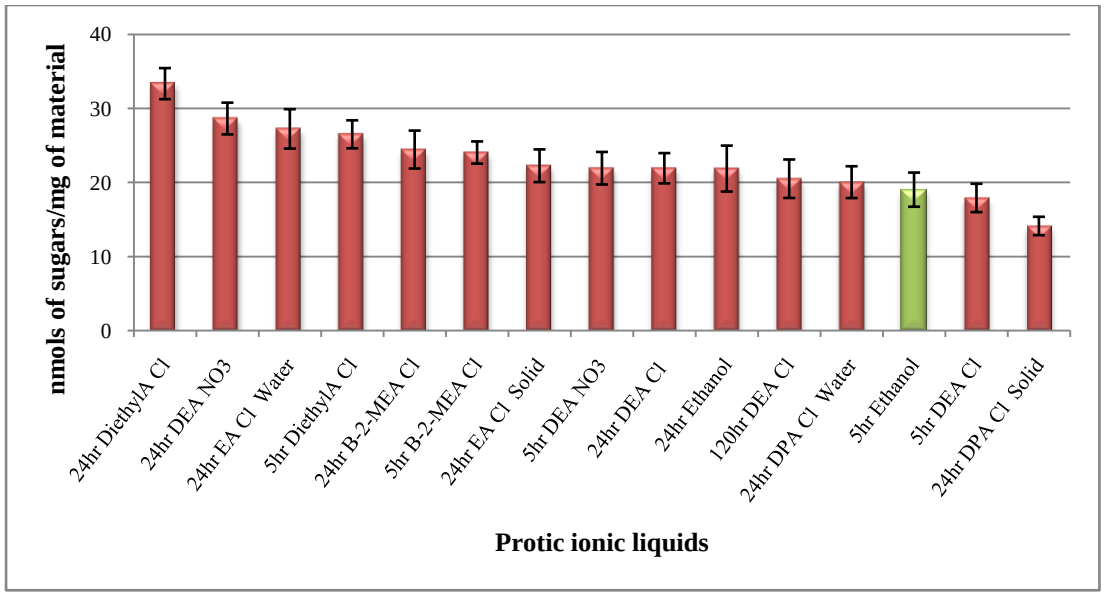


**Figure 125: Cellulose pre-treated with a variety of protic ionic liquids for 24 hours at 100 °C.**

However, studies from chapter 2, section 2.8, showed how adding water to the IL did not prevent the biomass pre-treatment from being efficient and in some cases improved the efficiency. Hence for the solid IL samples as well as still using them as solids a duplicate run was used with the IL dissolved in a minimum amount of water.

The aim was to hopefully see some changes with regards to the improvement on cellulose digestion after IL pre-treatment, from some of these IL/water solutions.

The protic IL data shows in all cases, the 24 hours pre-treatment is more efficient than 5 hours pre-treatment, and the solid ILs dissolved in water are more effective than the solid ILs on their own for pre-treatment. In Figure 125 the $NO_3^-$ anion is also shown with [DEA] as the cation, the improvement ratio of using [DEA] $NO_3$ is 1.5.

As for cations diethylammonium, diethanolammonium and ethanolammonium were the best choices, with improvement ratios from 1.4 to 1.75. Whereas, *bis*-2-methoxyethylammonium and dipropylammonium showed no improvement overall.

Some of the same trends from chapter 2 are present with the saccharification data, with potentially a few more ILs being more effective due to them being dissolved in water. However, unfortunately the enzyme hydrolysis results showed that overall there was no trend on the IL cation structure, as with chapter 2, and that also as the cellulose model compound is only slightly improved for digestion after IL pre-treatment, it is hard to tell how important the effects observed are on the pre-treatment process overall.

## *4.8.2 Aprotic IL Cations*

Figure 126 shows the aprotic structure trend with the ethanol control in green. In this case the use of water to dissolve solid IL salts before using them for pre-treatment for cellulose, has in some cases improved the IL pre-treatment. This has occurred with the [BMP] and [BPy] cations but not with the [BMPyr] cation.

However, the improvement on cellulose pre-treatment is small that it is hard to make firm conclusions based on this data.

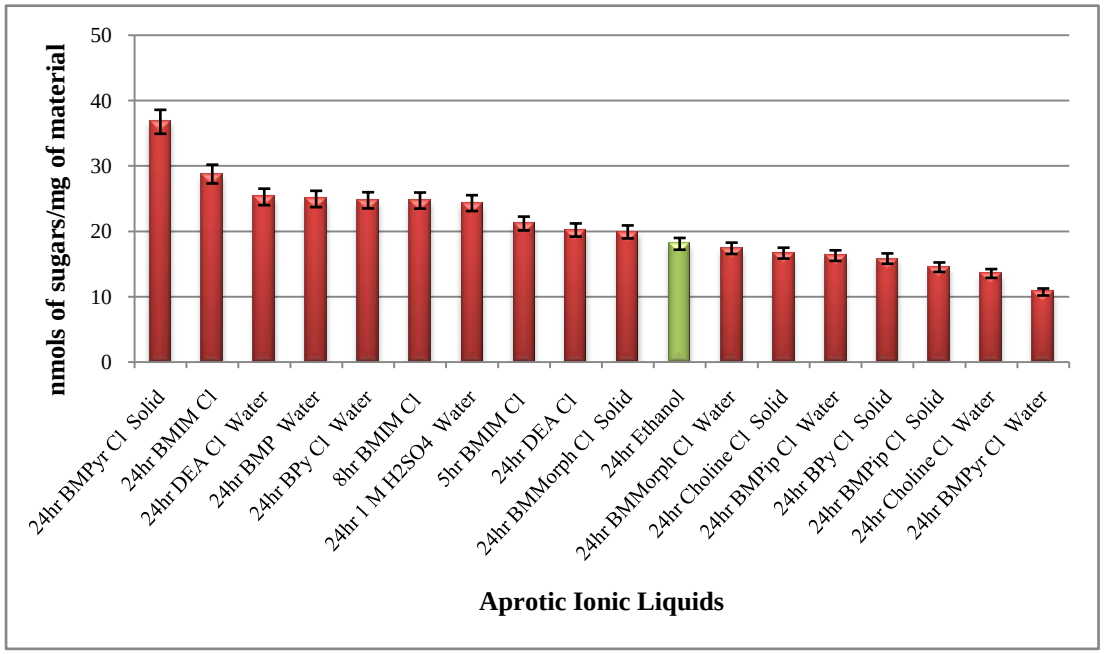


**Figure 126: Cellulose pre-treated with a variety of aprotic ionic liquids for 24 hours at 100 °C.**

## 4.9 HPLC Study on Sugars After Saccharification from IL Pre-treatment

In order to determine what sugars were being released from the saccharification of cellulose after IL pre-treatment, HPLC analysis was performed on the samples over the standard pre-treatment time periods. Figure 127 shows the HPLC data of the 9 common sugars released from cellulose (as in chapter 2), over the different time periods pre-treated in [DEA] Cl. The hypothesis was to identify which common sugars were present in this material (comparing to standards) and to see if there was an effect or change over time.

Cellulose is comprised of glucose, however from the HPLC data we can see a significant composition of xylose, most likely from cross linking of cellulose to hemicellulose from the source of the material of cellulose. Over time the composition of xylose decreases, this would imply that the [DEA] Cl effects hemicellulose and xylose units before cellulose and backs up data observed in chapter 2. This is most likely due to hemicellulose being more amorphous so it is easier for the IL to penetrate the polymer chains and solubilise the sugars. Overall this could explain why the composition of glucose collected increases over time.

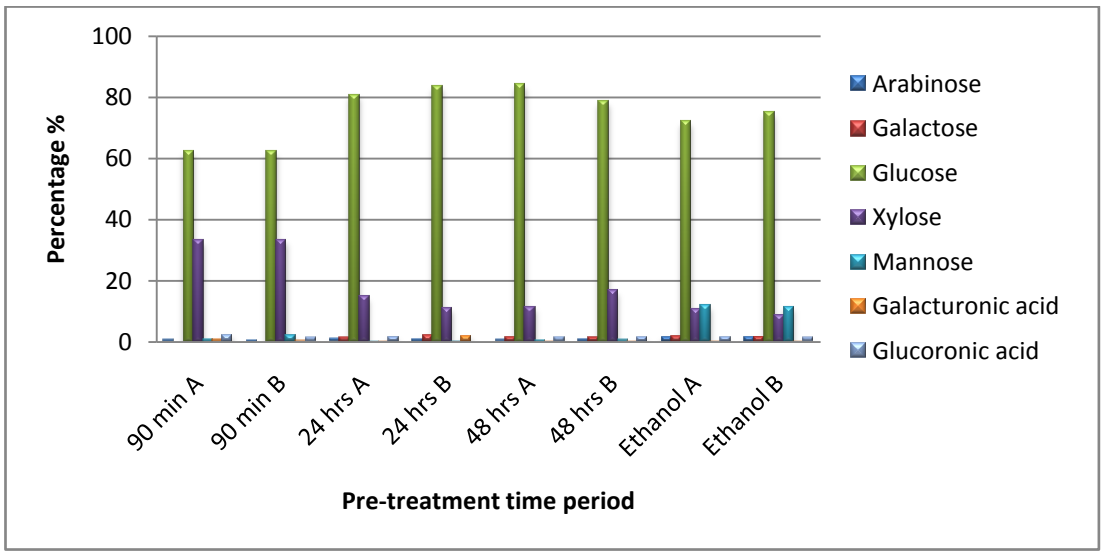


**Figure 127: HPLC of sugars released after pre-treatment of cellulose in [DEA] Cl over range of time periods at 100 °C.**

## 4.10 Cellulose Content Analysis

In order to examine the effect the [DEA] Cl was having on the cellulose polymer during pre-treatment, cellulose and hemicellulose determination was carried out. Hemicellulose determination was carried out due to content of xylose present as shown in the HPLC results in section 4.5.

Table 34 shows the cellulose and hemicellulose polymer results. For pure cellulose the percentage of cellulose was 94.3%. After pre-treating the cellulose with [DEA] Cl for 72 hours, the cellulose content was determined at 82.14%. This result indicates a loss of 10% of the cellulose polymer during pre-treatment.

The most likely cause of the cellulose loss is acid hydrolysis of the individual cellulose chains into glucose, which is soluble in [DEA] Cl. It is difficult to remove this sugar as the affinity of glucose for the IL is stronger than for any other organic solvent, hence simply separations are not possible.

Due to the nature of the ILs it is not possible, or very difficult, to distil them as they have negligible vapour pressures and hence further separations this way are not possible. However, current research indicates that some protic ILs can be removed at high temperatures under reduced pressure to leave the original acid and base.[210] For future research, reducing the loss of cellulose is important and this section further confirms, that [DEA] Cl does hydrolyse the polymer during the pre-treatment.

**Table 34: Cellulose and hemicellulose content of cellulose before any IL pre-treatment and after 72 hour pre-treatment with [DEA] Cl.**

| **Pre-treatment (Time-Solvent-Biomass)** | **Cellulose (wt %)** Error ± 0.5 % | **Hemicellulose (wt %)** Error ± 0.8 % |
|---|---|---|
| Pure Cellulose | 94.67 | 2.56 |
| 72 hr [DEA] Cl Cellulose | 82.14 | 0.57 |

# 4.11 Determining Cellulose Crystallinity

## *4.11.1 Powder XRD Measurements of Cellulose Crystallinity*

In order to determine the crystallinty of cellulose after IL pre-treatment, powder XRD was used, as in chapter 3. In chapter 3 the cellulose crystallinity of the plant biomass samples did not change upon IL pre-treatment.

Figure 13 shows the diffraction pattern for pure cellulose and the definitive peaks associated with the polymer in cellulose I form, α-cellulose. This is the typical structure for native cellulose I, with a primary 002 lattice peak plane and a secondary overlapped 101 peak.[132] As in section 3.2, the key observations were peak shifts and broadening from the cellulose samples after IL pre-treatment.

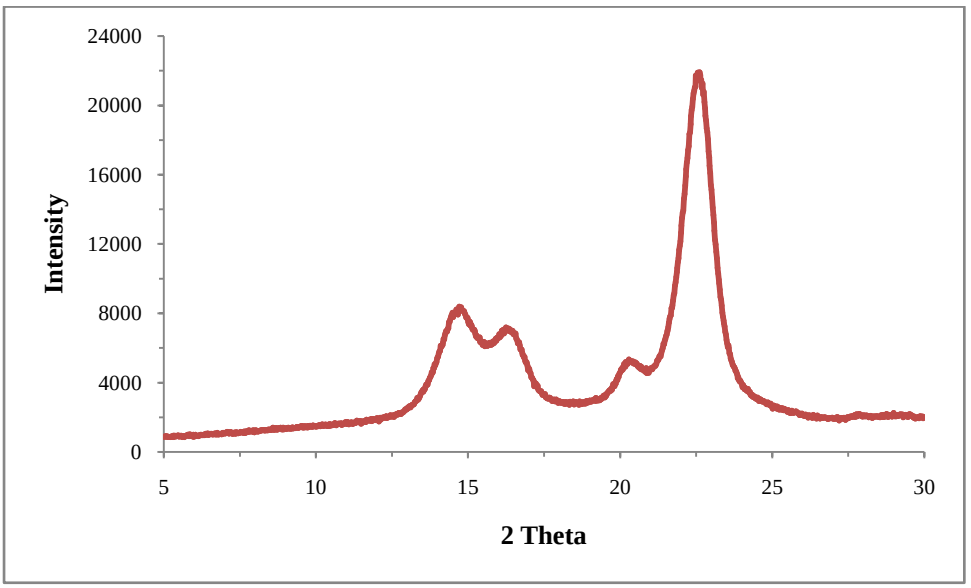


**Figure 128: Powder XRD pattern of pure cellulose. All observed peaks can be assigned to JCPDS database number 00-050-2241.**

Figure 14 shows the powder XRD analysis for pure cellulose compared to [DEA] Cl and [BMIM] Cl pre-treated samples. As can be seen from the diffraction patterns there is essentially no change in the crystallinty of cellulose after either of the IL pre-treatments. This was expected, as we see the same observations in chapter 3, however, using just cellulose as the pre-treatment sample may have changed this outcome.

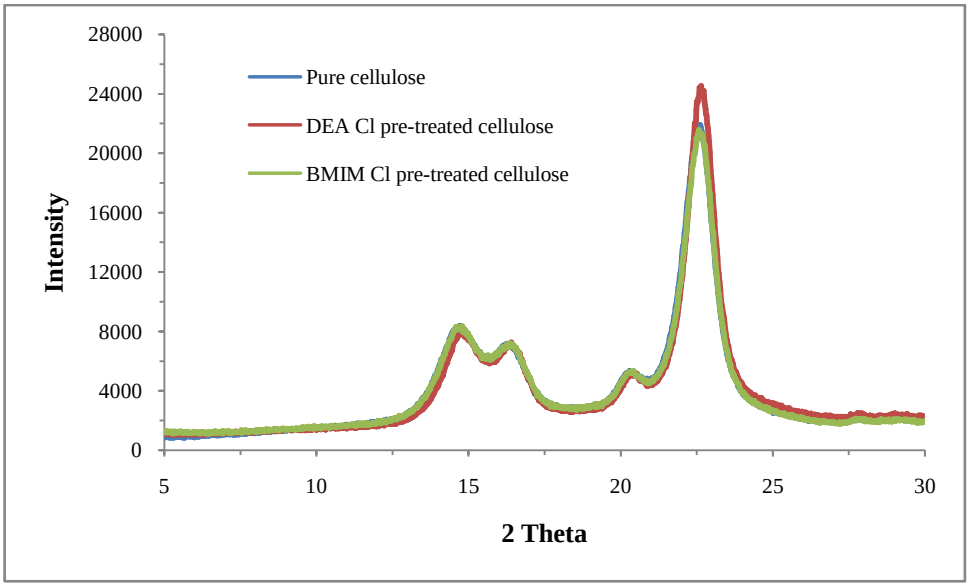


**Figure 129: Powder XRD patterns of pure cellulose in comparison to the [DEA] Cl and [BMIM] Cl pre-treated cellulose for 24 hours at 100 °C.**

Figure 15 shows further IL pre-treatments of cellulose, (as labelled), to determine if other aprotic IL samples or other successful protic IL had an effect on cellulose crystallinity. Again, in all cases the crystallinity remains essentially the same, as the diffraction patterns remain essentially unchanged. The powder XRD patterns will not account for a reduction in the DP of the cellulose polymer chains due to potential hydrolysis, however, the data does imply the bulk crystallinity remains the same in all IL pre-treated cellulose examples.

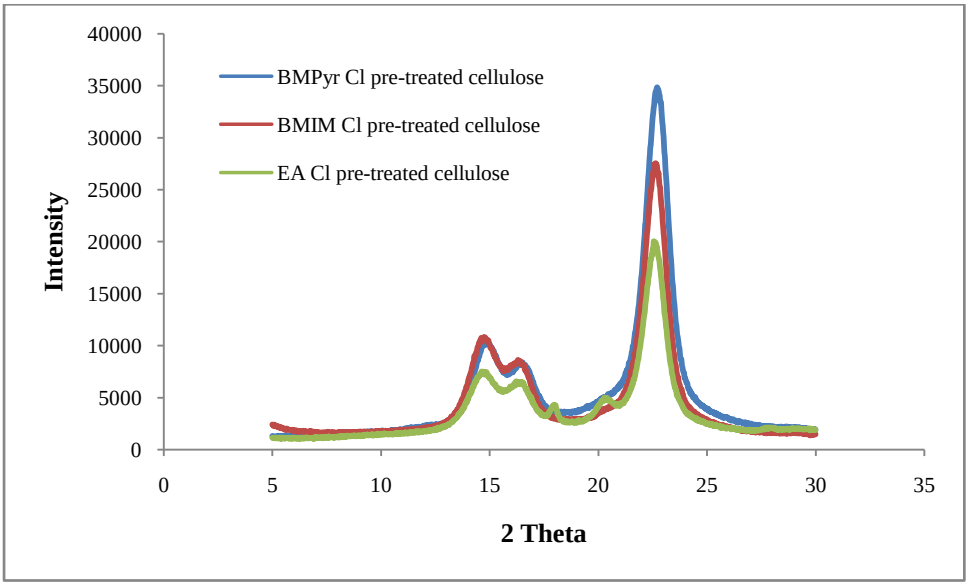


**Figure 130: Powder XRD patterns of cellulose pre-treated in [BMPyr] Cl, [BMIM] Cl and [EA] Cl for 24 hours at 100 °C**

[BMIM] Cl is reported in the literature as reducing the crystallinty of cellulose[38], so the question must be asked, why do we not see this reduction in our experiments? One answer may be that the cellulose crystallinity is changed during the pre-treatment but this effect is not significant to the bulk material. Another factor to take into account, as shown in section 4.3, is the particle size of the cellulose chosen. In all of the other figures and experiments so far a heterogeneous sample of cellulose was used.

Figure 16 shows comparisons between pure cellulose of 20 µm size, [DEA] Cl pre-treated 20 µm cellulose and [BMIM] Cl pre-treated 20 µm cellulose. Interestingly the data shows how now the [BMIM] Cl pre-treated cellulose sample becomes more amorphous with a reduction in the crystallinity, but not the [DEA] Cl pre-treated sample. The [BMIM] Cl pre-treated cellulose is no longer cellulose I but in fact cellulose II. Cellulose I fibrils exhibit an overall polarity due to the parallel orientations in cellulose chains, however cellulose II chains are antiparallel hence no polarity, resulting in a broadening of the XRD peaks.[132]

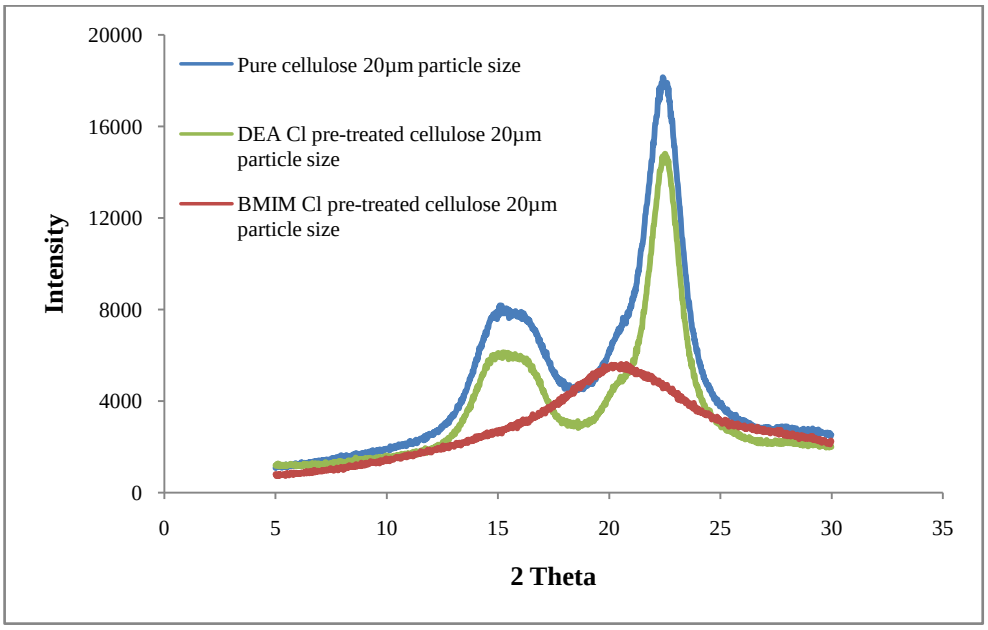


**Figure 131: Powder XRD pattern of pure 20 µm cellulose particles compared to [DEA] Cl and [BMIM] Cl pre-treated 20 µm cellulose for 24 hours at 100 °C.**

Recent studies have shown wood has been pre-treaded with the removal of lignin without dissolving or removing cellulose. This has been shown with the IL imidazolium acesulfamate.[142]

## *4.11.2 Solid State NMR to Investigate Cellulose Crystallinity*

Sources in the literature show determining cellulose crystallinity is a complicated process due to; (1) A reference sample of amorphous cellulose not being possible; (2) Cellulose reflections are broad and ill defined; (3) Peak broadening is strongly dependent on particle size.[39] Therefore combining XRD with $^{13}$C NMR or FT-IR is advised, to clarify crystallinity of samples.

To confirm the crystallinity remains constant throughout the standard IL pre-treatments, as shown by powder XRD, the same 4 key samples were analysed using solid state $^{13}$C CPMAS NMR. Pure cellulose, ethanol-control pre-treated cellulose, [DEA] Cl pre-treated cellulose and [BMIM] Cl pre-treated cellulose were analysed using this technique. $^{13}$C CPMAS NMR is a good technique in determining crystallinity, but could not be used previously for plant biomass pre-treatment due to paramagnetic metal impurities being present in the plant material, which would broaden and shift the NMR signals. Figure 132 shows the standard $^{13}$C NMR peaks for cellulose.

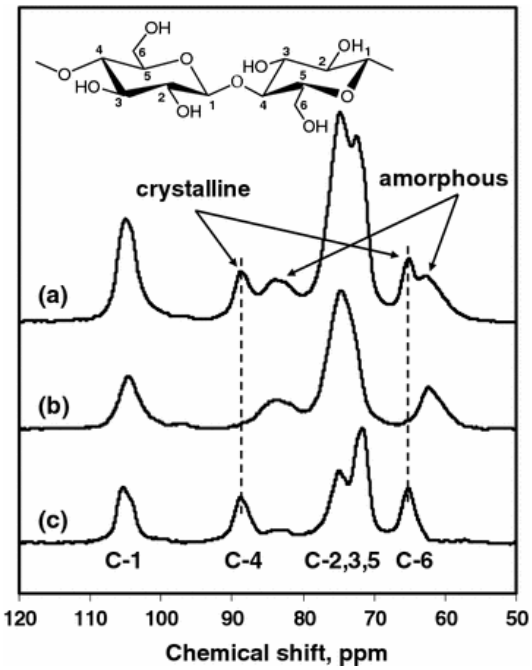


**Figure 132: Example of subtraction procedure to determine the crystallinity index of cellulose. (a) solid state $^{13}$C NMR spectra of Sigma α-cellulose, (b) amorphous cellulose, (c) "crystalline fraction" determined by subtracting (b) from (a)[211]**

As can be seen in Figure 133, there is no line broadening, compared to pure cellulose, in any of the carbon environments, therefore indicating the crystallinity remains unchanged.

**$^{13}$C MAS NMR Spectra Harman-Hahn cross polarisation (CP) used MAS frequency 8 kHz**

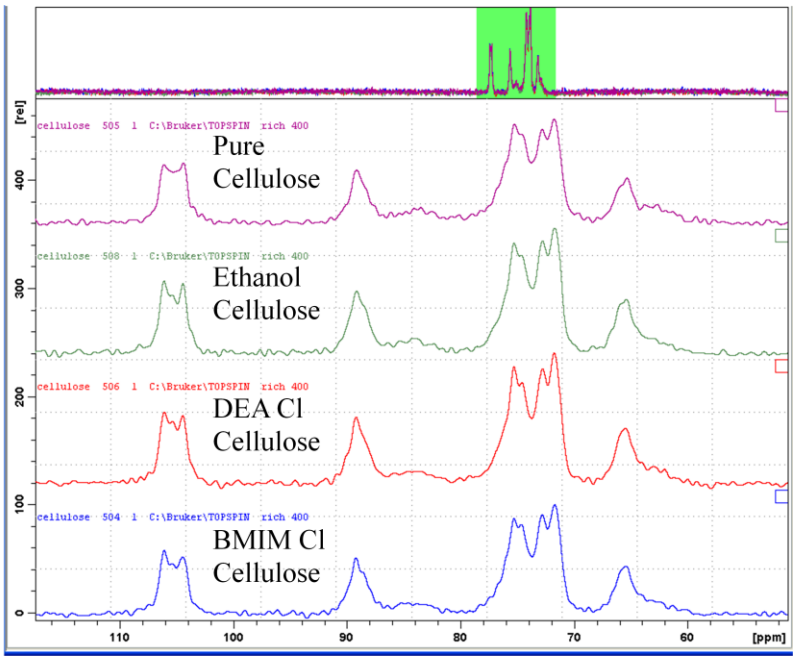


**Figure 133: Solid state NMR of Cellulose treated samples.**

Figure 134 confirms this by showing that all the $^{13}C$ CPMAS NMR traces are overlapped, with no change in broadening of the line width. Hence, solid state NMR data confirms the powder XRD data that no change in bulk crystallinity is observed in cellulose after IL pre-treatment and the changes in improvement of sugar release are due to different factors. What neither of these techniques shows so far, is what is happening 'real time' during the experiment, only the before and after pictures.

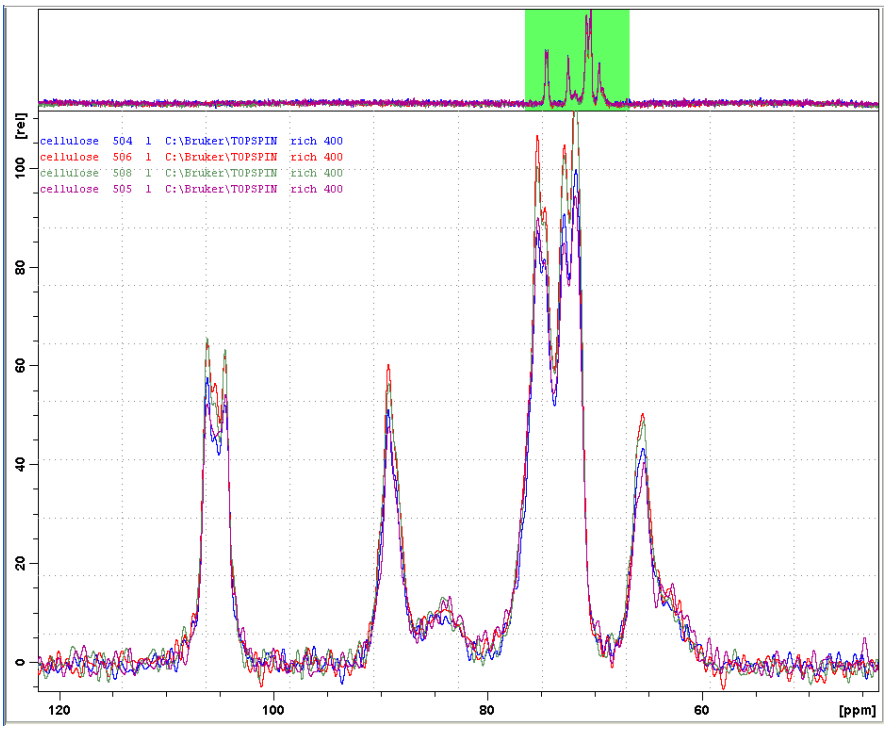


**Figure 134: Solid state $^{13}C$ CPMAS NMR overlay of all 4 cellulose samples.**

In conclusion, celluloses with highly amorphous regions are usually hydrolysed by enzymes at a much higher rate, it is unclear the exact extent of the role crystallinity plays. Accessibility of plant cell walls to cellulase enzymes will dominate the hydrolysis results, and this can be achieved through; (1) Lignin content; (2) Hemicellulose content; (3) Particle size; (4) Porosity of cell wall; (5) Surface area; (6) Degree of polymerisation. Hence crystallinity is only one of many factors.[42]

Additional problems with understanding crystallinity have recently been reported and include that fact that as well as crystalline and amorphous regions, there is a paracrystalline form which is a transition period between the other two.[42] This makes studying crystallinity of cellulose more complicated and less reliable to use.

## 4.12 Conclusions and Future Work

In conclusion the cellulose model compound studies have shown that [DEA] Cl has a 2 times improvement ratio on the digestibility of cellulose after pre-treatment. The overall effects of pre-treatment were determined to be much lower for pure cellulose compared to miscanthus and poplar pre-treatments, indicating the role of the IL in pre-treatment is on the other polymers, hemicellulose and lignin, compared to cellulose.

**Saccharification:** Hydrolysis analysis shows $Cl^-$ to be again the best anion paired with the cation [DEA]. The cation studies, however, did show some different ILs functioning at a higher efficiency of pre-treatment when the solid ILs were dissolved in water. However, due to the overall improvement being small, it is hard to get significant conclusions from the data. This section demonstrated the importance of lignin and hemicellulose removal from plant biomass is order to see significant improvements in the hydrolysis of cellulose.

**Particle size**: The size of the cellulose chosen for pre-treatment was shown to be important, with smaller particle sizes of 20 μm realising more sugars after hydrolysis. This compares to the literature research with [BMIM] Cl, as this IL de-crystallises cellulose whereas [DEA Cl] doesn't. However, [DEA] Cl pre-treated cellulose releases more sugars after hydrolysis than the [BMIM] Cl pre-treated cellulose.

**Cellulose crystallinty:** This is not effected in any other case, but there are changes in morphology, thermal stability and cellulose polymer reductions during IL pre-treatment. All of these factors contribute to improving the enzyme hydrolysis of the cellulose.

### *4.12.1 Hemicellulose and Lignin Model Compound Studies into Structural Changes with Ionic Liquid Pre-treatment*

A problem still remaining from chapters 3 and 4 is separating the sugars from the IL solution. A method to separate the cellulose degradation products could be capillary electrophoresis based on the p$K_a$ of products, as have been demonstrated in recent research.[212]

Cellulose model compound studies were completed in chapter 4, and this could be extended to hemicellulose and especially lignin model compound studies, to further probe the methods of degradation. Lignin depolymerisation is a significant goal in successful plant biomass pre-treatment and an obstacle that is needed to be crossed. Often after removal, the lignin reforms and repolymerisation or condensation reactions occur.[213] ILs have been shown to be capable or tuning which lignin products are produced. Using a lignin model compound with the ILs in this thesis could be used to explore potential structures released and characterised using NMR as the tool.[213]

# Chapter 5

# Simultaneous Saccharification Fermentation and Project Conclusion

## 5.1 Background

Bio-ethanol is made by fermenting the sugars produced from biomass material after enzyme hydrolysis. Fermentation is the process of converting this sugar using yeast or bacteria to ethanol and carbon dioxide. Sterile conditions are required but conditions can be aerobic as some yeast strands favour fermentation over respiration, even in the presence of oxygen. Figure 135 shows the different fermentation pathways and some of the possible end products.

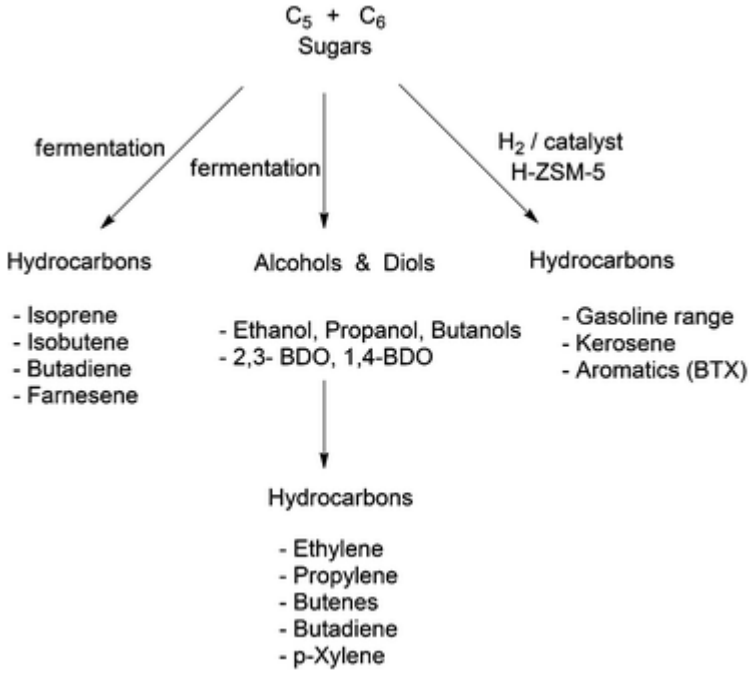


**Figure 135: Potential fermentation pathways and possible products formed modified from reference 8.**

Fermentation requires three steps; (1) Sugars from a biomass source in solution; (2) Fermentation with yeast converts sugars to ethanol; (3) Separation and purification of ethanol by distillation or sometimes filtration.[60]

The yeast of choice for this research is *Saccharomyces cerevisiae.* This was chosen as it ferments six membered sugar rings, such as glucose, into bio-ethanol.

*Saccharomycs cerevisiae* is also the most well studied eukaryotic organism and is well characterised bio-chemically and physiologically.[214] This yeast is known as 'bakers' yeast as it is used in the baking industry.[60] *Saccharomyces cerevisiae* does not ferment xylose as it lacks enzymes which convert xylose to xylulose.[16]

There are 3 types of fermentation techniques; (1) Batch; (2) Fed-batch; (3) Continuous.[16] For small scale research labs, batch is the simplest and does not require further nutrients to be added during the process. However, no modifications, depending on the experiment, can therefore be made during the process.

There are also 3 types of hydrolysis and fermentation strategies; (1) Separate hydrolysis and fermentation, SHF; (2) Simultaneous saccharification and fermentation, SSF; (3) Direct microbial conversion, DMC.[16] Strategy 1, SHF, requires low solid loadings to high enzyme loading due to inhibitor compounds produced. Strategy 3, DMC, combines cellulase production with hydrolysis and fermentation, however, a low bio-ethanol yield occurs due to by-products formed like acetate lactate and low tolerance of microorganisms to ethanol. Strategy 2, SSF, is the most common process and has the advantages of higher hydrolysis rates as sugars are converted in process, lower enzyme concentration required, higher yields and shorter time periods of reaction.[16]

Simultaneous saccharification and fermentation (SSF) is a useful combination technique, as it reduces the processing time of the biomass into a one step procedure and hence will be used for these experiments. This reduction in time leads to an increase in the amount of ethanol from the process, as the hydrolysed polymers release glucose which is quickly converted into ethanol by yeast. Another benefit is during the process less end-product inhibitors are produced, prevent the yeast from being killed during the process.[215]

Figure 136 shows the overall process pathway from plant biomass to bioethanol and shows the combined SSF step.

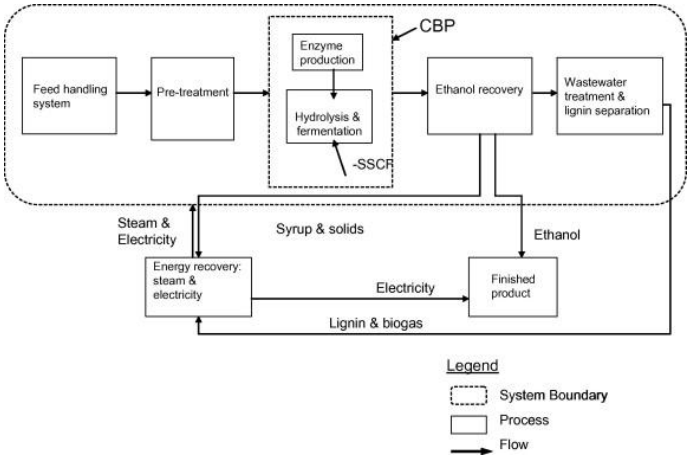


**Figure 136: Overall plant biomass to bioethanol process showing SSF step, modified from reference 216.**

The aims of this chapter are to attempt SSF on IL pre-treated miscanthus to see if bioethanol can be produced and in what possible yield. To compare this IL pre-treated miscanthus to control and dilute acid pre-treated samples.

## 5.2 Simultaneous Saccharification and Fermentation Experiments

Fermentation experiments were carried out in one-pot reactions of simultaneous saccharification and fermentation (SSF) as shown in Figure 137. All pre-treatments were carried out before SSF and included IL pre-treatments varying the cation, dilute acid/base pre-treatments and ethanol controls.

Figure 137 shows the SSF setup containing pre-treated plant biomass with enzyme and yeast broth. The broth contains water, sodium acetate buffer, yeast stock and cellulases as detailed in chapter 6, section 6.11. Due to the amount of biomass required for the experiment, the scale of the biomass pre-treatment experiments were increased from 400 mg to 2 g of material. With 2 g of biomass available, the SSF experiments could be carried out with samples taken over time to monitor the sugar release versus ethanol production.

The pre-treatment solvents analysed in the SSF experiments were:

1. DEA Cl
2. BMIM Cl
3. DPA Cl
4. Ethanol
5. 0.1 M $H_2SO_4$

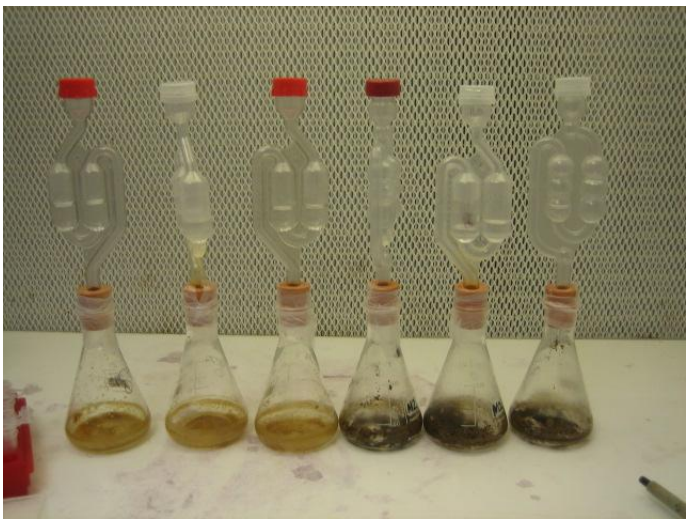

**Figure 137: Simultaneous saccharification and fermentation flasks of miscanthus with the enzyme/yeast broth.**

### *5.2.1 Miscanthus Saccharification Results of SSF Experiment*

Miscanthus was chosen for SSF as the plant biomass due to the success in IL pre-treatment efficiencies as shown in chapters 2 and 3. The experimental set up required the enzyme mixture to be added to the miscanthus and incubated at 50 °C for 24 hours. This step is the saccharification step and an initial sugar release check was taken and analysed with MBTH before any yeast was added.

The yeast stock solution was added after 24 hours and the temperature of the incubator was pre-reduced to 37.5 °C. Samples were taken at 6, 24, 48 and 72 hours after the yeast stock solution was added to the broth. Figure 138 shows the MBTH results after 24 hours of enzyme incubation (labelled as 0) and hence over time with the yeast present, up to 72 hours with yeast incubation.

In theory the expected result would be that 0 hours the largest bar, shown in blue, as this is the main saccharification step where just the enzyme mixture is present and incubated at the standard optimum temperature. Figure 138 confirms this as all the pre-treatment types tested on miscanthus, release the largest amount of sugar at 0 hours on the yeast time scale.

After this result we would expect similar amounts of sugars still being produced but potentially then being converted to ethanol and $CO_2$ before eventually a complete drop off and reduction as all of the sugars are converted to ethanol.

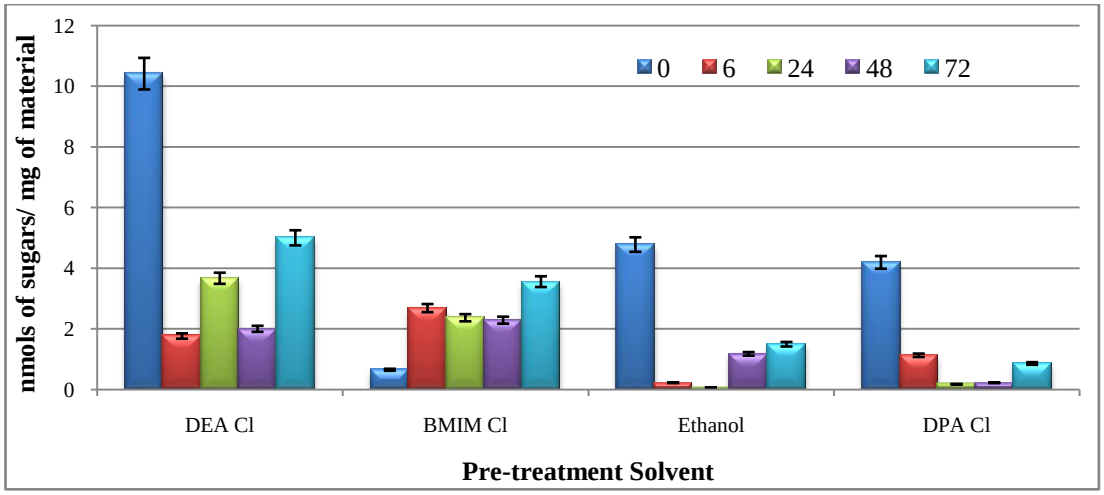


**Figure 138: Sugars released from enzyme hydrolysis over time as determined using MBTH method, shown from addition of yeast stock solution.**

However, Figure 138 shows this is not the case with this experiment. In most of the pre-treatments, the lowest result is the 6 hour result just after the yeast has been added. This may imply that ethanol is being produced and because of the lower temperature of incubation less sugar is still being produced. In contrary though as time increases so does the amount of sugars, hence does this imply the yeast is not successfully converting much sugar to ethanol, as we see accumulation of sugars?

This leaves us with a few questions initially not expected. If we start by assuming the fermentation part of SSF has not functioned as planned, we would expect to see the amounts of sugars rise and then plateau off, however, this does not occur which suggests fermentation is, in part, working, just not as efficient as it should. Perhaps a problem with the yeast incubation, or in the experimental setup.

A second observation to note is the amounts of sugars released in this experiment are very low based on other pre-treatment results in chapter 2, to be precise, by an order of magnitude of 10. As stated before the experiment cannot be judged on the exact numbers alone, due to variations in the enzyme composition, however, you would expect to see similar amounts to experiments in previous chapters. In the SSF experiments the significant difference from previous experiments is the scale up and hence the amount of biomass and pre-treatment solvents used.

The improvement ratio in SSF, comparing [DEA] Cl to ethanol, is just 2 based off the 0 yeast time bar. This is unexpected and actually would imply it is the enzyme hydrolysis step which has not worked as well as normally, not the fermentation step. The question must be asked, why?

In Figure 138 the $H_2SO_4$ MBTH data was omitted, and the reason why is shown in Figure 139. As the data shows, the 0.1 M $H_2SO_4$ pre-treated miscanthus performs as well as in previous chapters with regards to the high amounts of sugar release. This rules out there being something wrong with the SSF setup, but indeed confirms it is the chosen pre-treatments to analyse which are now not efficient. Hence, why has the IL pre-treated samples not released large quantities of sugars as in chapter 2, when [DEA] Cl is known to be a successful pre-treatment solvent?

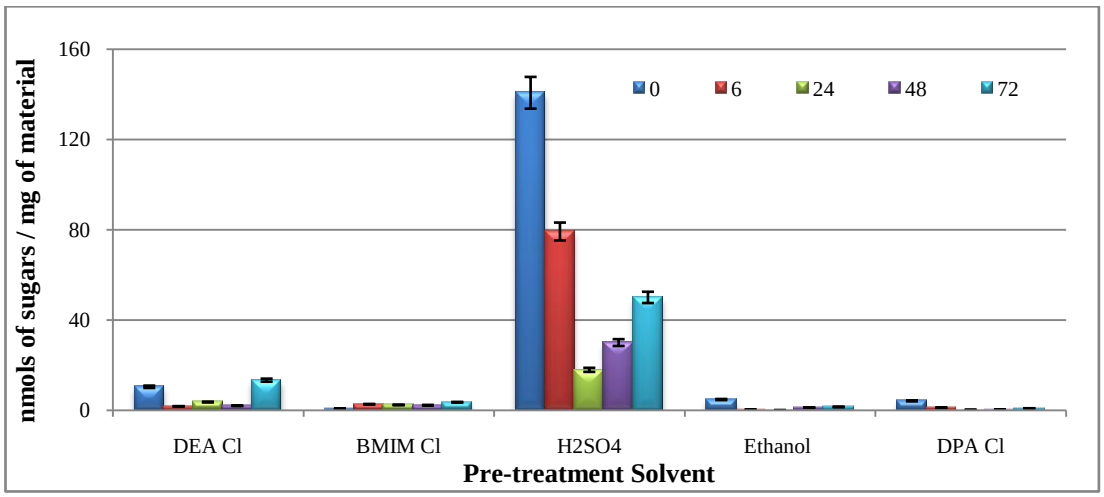


**Figure 139: Sugars released from enzyme hydrolysis over time as determined using MBTH method, shown from addition of yeast stock solution, with the $H_2SO_4$ pre-treatment included.**

The reason for this change therefore has to be the effects from the scale up of the pre-treatment and the consequences this has had on the pH of the biomass mixtures in SSF. We know that residual IL remains in the biomass after ethanol washing, and most likely imidazole impurities from the IL, hence it would make sense that the more biomass you have the more residual IL and impurities are present. Currently only small amounts of biomass ca. 10 mg have been used for saccharification, except for SSF where 2 g has been used, a 2000 times increase.

The enzyme mixture of cellulase in the experiments in this thesis, are buffered at slightly acidic conditions of pH ca. 5.5, where the enzymes function most effectively. However, when the pH was recorded for the 3 IL biomass samples for SSF, it was between 8-10. This significant change in pH, due to the scale up of the experiment, has obviously effected the enzyme efficiency and hence sugar release results from SSF. In future in SSF experiments this significant factor of pH control

will need to be modified and buffered individually for each IL pre-treated sample in order for SSF to work effectively.

Even with this poor saccharification performance after IL pre-treatment, there is still interesting observations from the MBTH data and observing the samples during the experiment, more liquid was produced, implying that fermenting of some sugars to ethanol was taking place. Therefore in order to determine how successful the SSF was, analysis of the sugar data using HPLC to quantify the glucose content was performed.

Figure 140 shows the HPLC results of the pre-treated miscanthus at both 0 yeast start time and 72 hours so that a closer look into how the sugar content was changing during the experiment and to quantify the glucose content. It is clear to see that in all cases there is not significant quantities of glucose present, (shown in purple), in any of the mixtures, which will affect the bio-ethanol outcome. However, the data shows that after 72 hours, further reductions in the amount of glucose are present. This is showing us that some ethanol must be being produced in the SSF experiments. The HPLC data will be needed in order to calculate the ethanol yields which have been achieved.

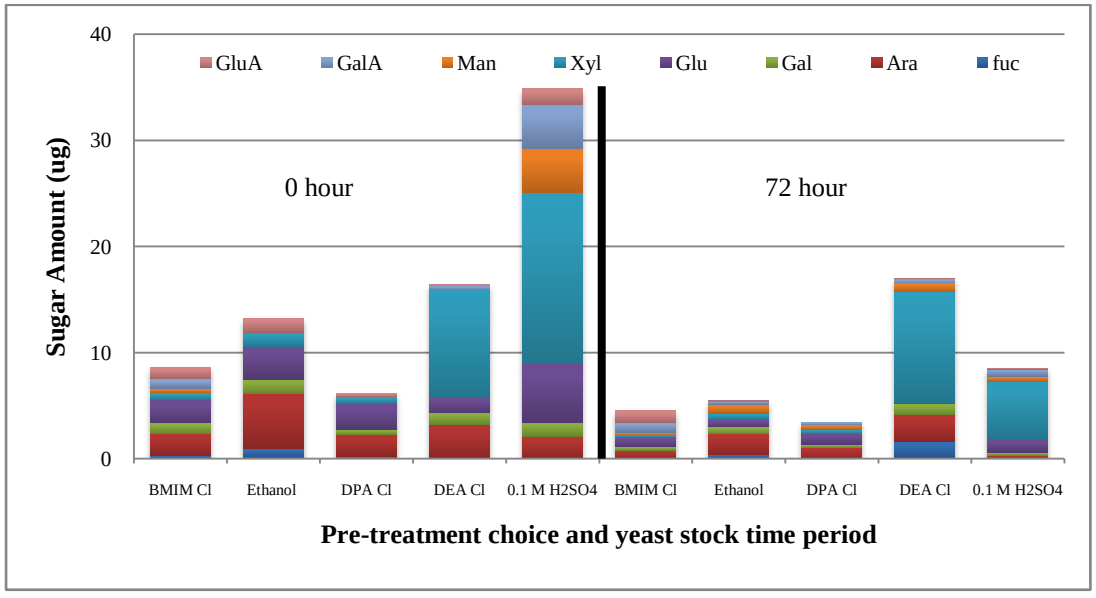


**Figure 140: HPLC analysis of sugars at the start when the yeast stock solution was added (0 on the left) and after 72 hours (on the right).**

### *5.2.2 Ethanol Yields from SSF on Miscanthus*

To calculate how successfully the yeast was at fermenting sugars into bio-ethanol, GC analysis was completed on filtered samples after 72 hours of SSF. Using the GC results and the amounts of sugars released determined by the HPLC results, a yield could be calculated using glucose standards of 0.1 % to 5 %. This theoretical yield is not based on the maximum yield from the un-treated miscanthus because only some sugars were released in the hydrolysis step, but instead is based on those sugars released after hydrolysis potentially being converted to ethanol. Also in theory a yield of 100% conversion from a yeast based process is unachievable as the yeast use some of the glucose to grow during the fermentation.[60]

Table 35 shows the ethanol yield from each pre-treatment type tested in SSF. The interesting result is [DEA] Cl pre-treated miscanthus has an ethanol yield of 30.2 %. The 0.1 M $H_2SO_4$ has a lower yield of 23.7 %, but it is know in the literature that acid based pre-treatments can cause fermentation inhibitors like HMF to form from glucose. The other ILs which have lower yields, had a higher pH of 10 in the SSF mixture, compared to [DEA] Cl with a pH of 8.5. This further proves how sensitive the yeast and enzymes are and will be effected by the pH changes, due to the yeast functioning best at pH 7 and the enzymes ca. 5.5.

**Table 35: Ethanol yield from different pre-treated miscanthus samples from SSF experiment.**

| **Pre-treatment Choice** | **Ethanol Yield (%)** |
|---|---|
| **[DEA] Cl** | 30.2 |
| **[BMIM] Cl** | 8.3 |
| **[DPA] Cl** | 6.1 |
| **Ethanol** | 5.8 |
| **0.1 M $H_2SO_4$** | 23.7 |

In summary it has been shown how SSF can be used on miscanthus pre-treated with ILs and produces a bio-ethanol yield comparable with dilute acid pre-treatment. However, the work has a very long way to go before its fully successful and addressing issues of pH per individual pre-treatment solvent and scale up problems is required. Addressing these could ensure bio-ethanol is produced from [DEA] Cl

pre-treated miscanthus in the future and then could be extended to other successfully IL pre-treated plant biomass.

A final note is temperature studies have shown ethanol productivity severely declines above 40 °C and virtually stops at 45 °C,[120] however, glucose concentration increase at the higher temperatures of 50 °C and above so compatibility issues of SSF still need consideration for maximum efficiency, if not then inhibition can occur.[16]

## 5.3 Chapter Summaries and Fermentation Future Work

### *5.3.1 Chapters Summary and Outcomes*

**Chapter 2:** Here we investigated many variables all associated with lignocellulosic materials and optimising the pre-treatment of them using ionic liquids as the key. These variables included (1) plant biomass choice, (2) ionic liquid cation, (3) ionic liquid anion, (4) pre-treatment heating conventional versus microwave, (5) Pre-treatment time periods, (6) Acid/base comparisons, (7) IL mixtures for pre-treatment, (8) Enzyme hydrolysis concentration and time period and (9) Recycling IL's. [DEA] Cl pre-treatment of miscanthus was most successful, with microwave heating significantly reducing the pre-treatment time required. [DEA] Cl was comparable with dilute acid and base studies and could be used as an aqueous mixture, up to 75 wt % water.

**Chapter 3:** The focus here was analysing the structural and chemical differences taking place within the plant biomass during IL pre-treatment with the hope of maximising these changes. Using (1) powder X-ray diffraction to determine cellulose crystallinity essentially remained unchanged; (2) Thermogravimetric analysis confirmed a reduction in thermal stability of the biomass; (3) Scanning electron microscopy probed the importance of the structural morphology changes during IL pre-treatment; (4) IR, and more significantly chemical UV-assays for total polymer compositional analysis, discovered lignin removal was the key to success, with hemicellulose removal close behind.

**Chapter 4:** This compared the pre-treatment effects from chapters 2 and 3 on studying the model biomass compound, cellulose, and how this polymer was altered during IL pre-treatments. The loss of cellulose in chapter 3 was a negative effect and using the following methods we tried to understand the scope of this effect. Varying (1) IL cation/anion choice; (2) Cellulose of different particle sizes; (3) Structural studies including, powder X-ray diffraction, thermogravimetric analysis, optical and scanning electron microscopy were used to gather chemical insight. The outcomes proving cellulose crystallinity does not change upon pre-treatment, but surface morphology still improved IL and cellulose pre-treatment. The significant reduction in effectiveness, was due to lignin not being present.

**Chapter 5**: This chapter offered an insight into how simultaneous saccharification and fermentation (SSF) could be employed for producing ethanol from lignocellulosic materials after IL pre-treatment. Results showed comparable yields to dilute acid pre-treatment. However, as was shown, there is still a long research journey to be had before this work is fully optimised and then tested on larger scales with careful pH modification.

### *5.3.2 Simple Metrics on Economic Progress of Project*

The aims of this project were to produce sugar from glucose from plant biomass materials using ionic liquids. In order to see how economically feasible this process currently is, simple metrics were calculated based on 1 kg of product being produced. Currently, to buy 1 kg of glucose would cost £25.60 from Sigma Aldrich. The cost of the IL [DEA] Cl is currently £128 for 1 kg. The plant biomass for this purpose was assumed to be 'free' from a waste source, however, this does not take into consideration transport and storage costs, it makes the comparison simpler. For the production of 1 kg of glucose from our process, 2.2 kg of plant biomass is needed, based on the current yield conversion. To pre-treat 2.2 kg of plant biomass, 22 kg of IL [DEA] Cl is required. Hence, basing the cost of the process on the IL, to produce 1 kg of glucose from our process would cost £2,816. This is somewhat greater than the current £25.60 for glucose. The main improvements noted from these simple metrics is to improve the sugar loss during the IL pre-treatment and to somehow reduce the cost of the IL. These, as already stated, are goals for future project work.

### *5.3.3 Future Work for Simultaneous Processes on Pre-Treatment, Saccharification and Fermentation*

Research has shown that the growth and ethanol production from the yeast *Saccharomyces cerevisiae* is strongly affected by residual IL in biomass after pre-treatment.[139] These results are IL dependent on the cation and anion choice. Water has been shown to be more effective at removing more hydrophilic ILs from plant biomass, reducing any inhibitory effects, however this induces significant costs of removal from the IL after processing. [139] Hence, further tests into ILs effect on SSF are needed as well as using water as an anti-solvent to wash current ILs, including [DEA] Cl from the miscanthus. Testing poplar for SSF is also an immediate next step.

A long term goal is to combine all three steps of pre-treatment, hydrolysis and fermentation to significantly reduce time and inhibitors produced. Further goals to combine all 4 steps from grinding, pre-treatment, enzyme hydrolysis and fermentation are even more energy efficient, but at this stage incompatible with catalysts/enzymes used, however, there is preliminary evidence of one pot reactions with IL resistant yeast and enzymes in progress.[120] JTherm enzymes, have been used in one-pot reactions.[174] Currently this is achievable with 10-20 % IL [EMIM] [OAc] with standard cellulase enzymes.[174]

IL continuous pre-treatment processes are also a potential goal. Examples include using 25 wt % biomass with the IL [EMIM] [OAc].[130] This decreases the amount of IL needed and the reaction time, which hence increases the amount of biomass processed. However, it was noted there was a reduction in pre-treatment activity.[130] Trialling this process with [DEA] Cl is feasible.

Finally, issues with pH are the dominating negative factor at this stage. Studies have shown, lowering the pH from 6 to 4 improves biomass delignification with imidazolium ILs.[217] Choosing ILs where pH adjustment is possible is a benefit for the future, reducing corrosive and unstable anions.

Table 36 shows an example of how the pH effects the effective pre-treatment on biomass.[217] This is the most important step to be considered for [DEA] Cl SSF.

**Table 36: Effect of solution pH and IL type on biomass composition and recovery [217].**

| Ionic liquid | pH | Content in pretreated bagasse (%) | | | Recovery from pretreated bagasse (%) | | |
|---|---|---|---|---|---|---|---|
| | | Glucan | Xylan | Lignin | Glucan | Xylan | Lignin |
| **BMIMCl** | 5.9 (unadjusted) | 43.5 | 20.3 | 27.0 | 99.0 | 98.8 | 96.5 |
| | 3.4 | 46.3 | 21.2 | 26.2 | 96.5 | 94.0 | 85.2 |
| | 1.9 | 49.5 | 21.1 | 23.6 | 96.7 | 87.5 | 71.9 |
| | 1.5 | 60.2 | 15.3 | 17.3 | 94.8 | 51.2 | 42.5 |
| | 1.1 | 77.1 | 9.1 | 9.7 | 93.4 | 23.4 | 18.3 |
| | 0.9 | 81.4 | 7.4 | 7.2 | 91.7 | 17.7 | 12.7 |
| | 0.4 | 88.0 | 3.1 | 4.7 | 90.8 | 6.8 | 7.6 |
| **$BMIMCH_3SO_3$** | 3.4 | 46.1 | 21.3 | 26.5 | 96.5 | 94.7 | 86.5 |
| | 0.9 (unadjusted) | 79.3 | 9.1 | 6.5 | 92.1 | 22.5 | 11.8 |
| | 0.4 | 87.3 | 4.1 | 4.6 | 91.4 | 9.1 | 7.5 |
| **$BMIMCH_3SO_4$** | 3.4 (unadjusted) | 45.7 | 21.5 | 26.8 | 96.8 | 96.8 | 88.6 |
| | 0.4 | 87.4 | 3.6 | 4.6 | 90.3 | 7.9 | 7.4 |
| **EMIMCl** | 6.0 (unadjusted) | 43.8 | 20.5 | 26.9 | 98.7 | 98.1 | 94.6 |
| | 0.9 | 82.3 | 7.2 | 6.4 | 91.2 | 16.9 | 11.1 |
| | 0.4 | 88.8 | 2.8 | 4.3 | 90.4 | 6.1 | 6.8 |
| **HCl solution** | 0.4 | 59.8 | 9.5 | 26.5 | 96.1 | 27.5 | 66.4 |
| **Untreated bagasse** | — | 43.2 | 20.2 | 27.5 | 100.0 | 100.0 | 100.0 |

# Concluding Remarks: Is there a Future for Ionic Liquids to Pre-treat Lignocellulosic Materials?

The shortages in energy, as well as increased environmental pollution occurring worldwide, drives the need for renewable chemicals sourced from lignocellulosic materials. ILs can play an important role here, due to their 'designer' nature, and certain cation and anion combinations have shown promise at dissolving/separating lignocellulosic polymers.[198] Challenges include further understanding of IL cation/anion design on biomass and recovery and recycle of ILs after the process.

Green and functional solvents are therefore needed, and combinations including IL/IL, water/IL, $sCO_2$/IL, are sought after for cheap and environmentally friendly usage.[20] However, the separation of all components afterwards is currently ineffective.[20]A further challenge is a large scale application of feedstocks, as its technically feasible, but not economically, with current technologies.[20]

Overall, the research work presented in this thesis has demonstrated how ILs can be used to effectively pre-treat plant biomass. Miscanthus was discovered to be the best biomass choice with the ionic liquid [DEA] Cl. The yield of sugars released was 40% and was comparable or better then dilute base and dilute acid pre-treatment. Microwave pre-treatment produced excellent amounts of sugars released, after just 10 minutes pre-treatment. Other anions including $NO_3$ were highly effective at pre-treatment when paired with [DEA]. Lignocellulosic materials, including poplar, can also be used for the pre-treatment with ILs for sugars.

This work showed how important lignin removal is from plant biomass for efficient saccharification, in agreement with current literature. Hemicellulose also ranked highly as its removal compared to improved sugar release. Morphology and particle size are still important but cellulose crystallinity played no role with this work.

These results have shown how a cheap, readily available, bio-degradable ionic liquid, can be used to effectively pre-treat plant biomass, and hence not only has a future, but also an economic driving force in this research field and industry. It is green, can be recycled and when current technologies become available, can be used on a large scale. This paves the way for a future involving IL pre-treatment on lignocellulosic materials, where [DEA] Cl could play a vital role.

# Chapter 6
# Experimental and Methodology

## 6.1 General

The Chemical suppliers and standard initial laboratory conditions of solvents and equipment is listed here, as well as the origins of the biomass samples.

### *6.1.1 Chemicals and Biomass Samples*

All reagents and solvents used in this research project were sourced from Sigma-Aldrich, Fluorochem and Alfa Aesar and, unless stated, used without further purification.

The protic ionic liquids were synthesised in open atmospheric conditions whereas the aprotic based ionic liquids were synthesised using Schlenk line techniques under a nitrogen atmosphere. The ionic liquid dissolution experiments took place in sealed round bottom flasks and were heated using standard stirrer hot plates with oil baths and digital temperature probes. The temperature was controlled using separate thermometers in the oil bath and maintained at 100 °C unless stated otherwise. The aqueous/ethanol dissolution experiments took place under reflux.

Solvents used in synthesis were purified with the aid of an Innovative Technologies anhydrous solvent engineering system for use in air-sensitive synthesis and all of the ionic liquids were dried thoroughly on a Schlenk line at ca. $10^{-2}$ mbar at 60 °C for 5 hours, before being stored in a desiccator.

Characterisation of ionic liquids was carried out using the following array of analytical techniques: $^{1}H$ Nuclear magnetic resonance (NMR) spectroscopy, performed on a Jeol EXC400 at 400 MHz and the spectra were analysed using Delta NMR software. Karl Fischer Colometer Mettler Toledo DL 32 was used to assess the water content of the ionic liquids. Elemental analysis (CHN) was performed on an Exeter Analytical Inc. CE-440 Analyser.

Biomass samples were sourced from the following locations;

1. Miscanthus from Holland (Water content 5 wt%)
2. Poplar from a Yorkshire Farm (Water content 4 wt%)
3. Maize Stover from France (Water content 7 wt%)
4. Sugarcane bagasse from Germany (Water content 5 wt%)
5. Wheat from Germany (Water content 3 wt%)

All biomass samples were washed before use as stated in section 6.1.2.

### *6.1.2 Initial Biomass Preparation (AIR treatment)*

Solid biomass samples were ground using a Bohr mill to 100-400 μm particle size,[14] except poplar which was sourced and pre-ground up to 1-2 mm particle size.

All biomass samples were washed with ethanol 3 times in the ratio of 20 % weight of biomass to ethanol, to remove simple sugars in solution and leave the alcohol insoluble residue (AIR). To obtain AIR, the biomass material was subjected to 30 minutes incubation with shaking at 50 °C, followed by centrifugation at 3000 rpm at 4 °C for 10 min with removal of the supernatant. This AIR material was dried on a Schlenk line at ~ $10^{-2}$ mbar at 60 °C for 5 hours before being used [218] .

The same initial batch of biomass materials were used throughout the whole project ensuring source variation was controlled, although multiple smaller AIR treatments took place to wash this biomass.

# 6.2 Pre-treatment Studies

## *6.2.1 Standard Pre-treatment studies*

Pre-treatment studies were carried out using the following procedure and as shown in Figure 141.[14] A 100 ml round bottom flask with a biomass loading of 10 wt % per ionic liquid sample was used for pre-treatment, the standard amount being 400 mg biomass/ 4 g of ionic liquid. After pre-treatment for the chosen amount of time, 24 hours being standard, 10 ml of ethanol was added to precipitate the biomass.[107] Some of the more viscous ionic liquid samples required the centrifuge, using a Heraeus Megafuge 40R Centrifuge at 4500 rpm or 3500 rpm for 5 minutes at 20 °C. The supernant was filtered under vacuum using Buchner filtration.[107]

The wash with ethanol was repeated 3 times before the solid biomass samples were dried in preparation for saccharification. The IL was recovered *in vacuo* and if deemed necessary analysed using NMR for any changes in the chemical content. A sample of biomass in ethanol (10 wt %) was used each time as a control for the IL experiments.

Saccharification analysis was performed after all pre-treatments as explained in detail in section 6.8.

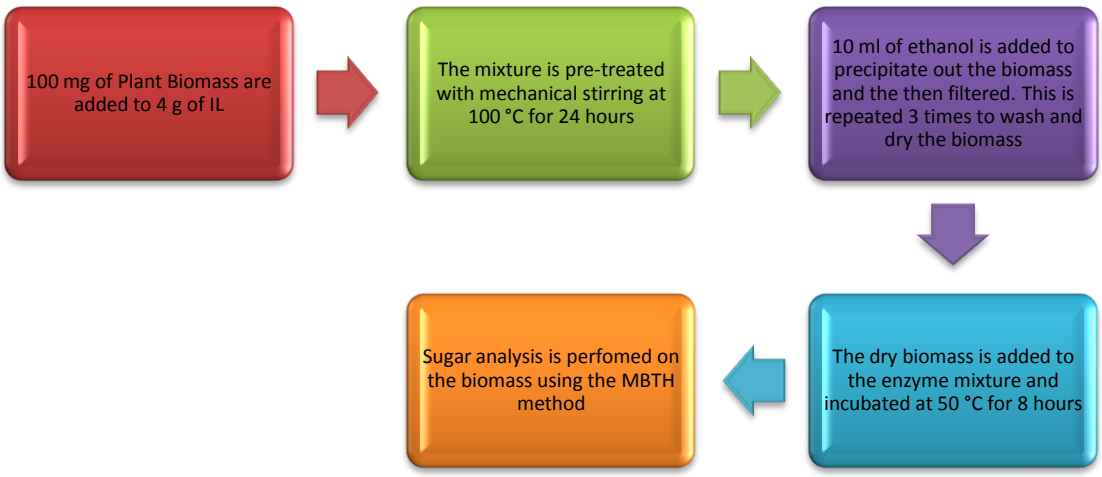


**Figure 141: Scheme showing flow pathway for IL pre-treatments.**

### *6.2.2 Microwave Pre-treatments*

Microwave heating was used as a comparison to the conventional heating pre-treatment experiments. Microwave experiments were performed with a CEM I Discover reactor [2.455 GHz (0.122m) magnetron] with PC control.[14]

A 10 minute to 60 minute time range was used for the experiments. The equipment was operated using a fixed temperature at 100 °C, measured using an inbuilt IR sensor, and the reactions were continuously stirring using a magnetic flea. The power input varied depending on the ionic liquid chosen but was usually in the range of 0-25 W depending on the IL. The maximum pressure was set to 250 psi. The quantities of IL and biomass were kept the same as the standard pre-treatment conditions for comparison of pre-treatment effectiveness.

### *6.2.3 Hydrothermal Bomb Pre-treatments for Industrial Comparison*

For comparison to current industry methods, hydrothermal bombs were used for pre-treatment studies. For these experiments the pre-treatment choice and biomass choice were placed in a hydrothermal bomb and placed in an oven at 110 °C for 60 minutes with no stirring. The same quantities of IL and biomass was used as with standard pre-treatment conditions. After pre-treatment 10 ml of ethanol was added to precipitate the sample. The samples were filtered under vacuum using Buchner filtration.[107] The wash with ethanol was repeated 3 times before the solid biomass sample was dried in preparation for saccharification.

### *6.2.4 High Throughput Robot ChemSpeed*

In order to test the ability to screen a range of IL concentrations with $H_2SO_4$, experiments were run on a fully automated parallel synthesiser, ChemSpeed® Swing-SLT II.[219-220] The synthesiser is equipped with 3 glass block reactors consisting of 16 reaction vessels, each 13 ml in volume. The vessels have thermal jackets connected in series through the reaction blocks to a heating/cooling system Hüber (-90 to 140 °C). The vessels are equipped with coldfinger reflux condensers (~7 °C) and mixing is achieved by vortex agitation (1000 rpm).

$H_2SO_4$ and IL liquid transfers are performed using a gravimetric dispensing unit. Solid biomass samples were dispensed with a solid dispensing unit. Ethanol transfers handled by a 4-needle head capable of 4 simultaneous transfers. The 4-needle head was connected to a reservoir bottle (degassed solvent) for rinsing the needle after removing IL/ethanol mixes. Figure 142 shows some of these key components from the ChemSpeed.

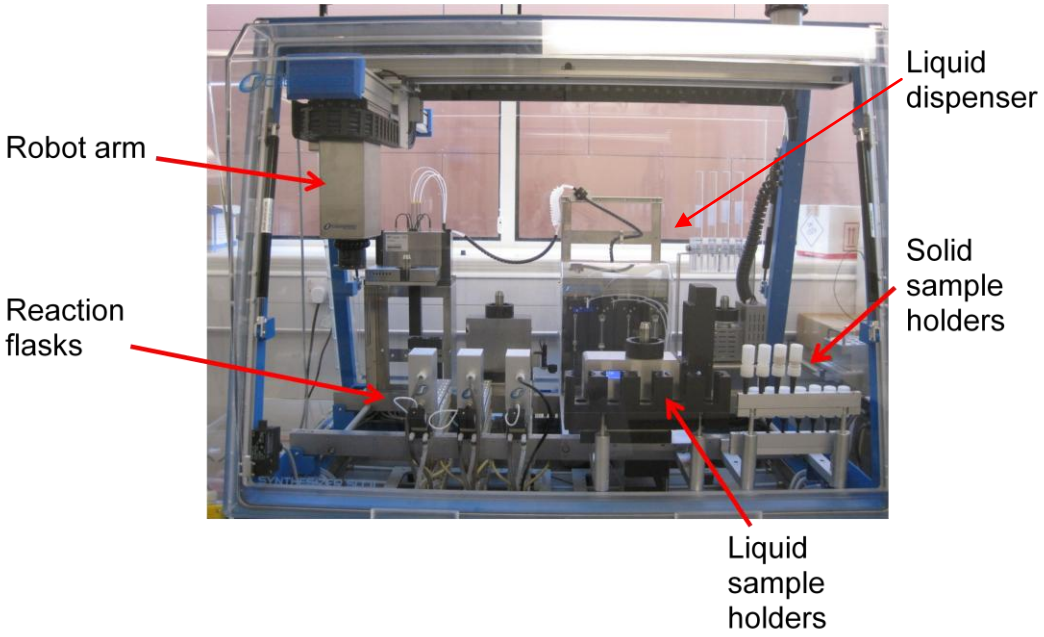


**Figure 142: ChemSpeed with key components labelled.**

When the experiments were carried out, the synthesiser was maintained under an inert atmosphere by supplying a constant flow of nitrogen to the hood of the synthesiser. After the IL and $H_2SO_4$ was added to the reaction vessels, they were heated for 24 hours at 100 °C. The biomass was washed with ethanol and the

IL/ethanol mixture removed by vacuum and transferred to separates flasks. This washing was repeated three times before the biomass was dried and then transferred to vials for saccharification analysis. Figure 143 shows the reaction vessels labelled, which were used in these experiments.

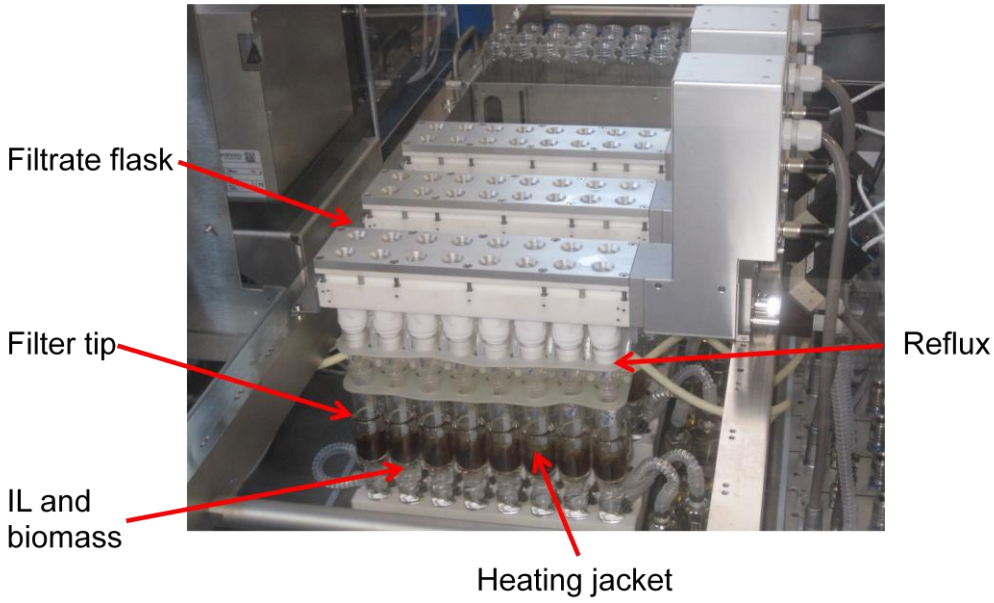


**Figure 143: ChemSpeed reaction vessels used for these experiments.**

### *6.2.5 Supercritical $CO_2$ Pre-treatment*

The supercritical carbon dioxide extractions were carried out using a SFE-500 provided by Thar technologies. Supercritical fluid grade carbon dioxide (99.99%) was used to conduct the extractions. 100 g of milled biomass was placed into the 500 ml extraction vessel and connected to the extraction system. The required temperature and pressure were applied. The reaction vessel was heated to 50 $^{o}$C and 5 minutes were allowed for it to equilibrate. An internal pump was used in order to obtain the required pressure (350 bar). The system was run in dynamic mode, in which the carbon dioxide which contained the epicuticular lipids, was allowed to flow into the collection vessel. A flow rate of 40 g $min^{-1}$ of liquid $CO_2$ was applied and the extraction was carried out for 4 hours.

When the extraction was terminated, depressurisation of the system was carried out over a period of 4 hours. The wax was collected by rinsing the collection vessel twice with approximately 100 ml of DCM. The solvent was removed *in vacuo*. The plant material was removed and a brush was used to clean the extraction vessel. The system was washed in dynamic mode using a combination of supercritical carbon dioxide and ethanol (10%) for 45 minutes at the extraction pressure. The pump supplying the modifier was then turned off and carbon dioxide was allowed to pass through the system for an additional 20 minutes. The pre-treatment studies were performed as the procedure in section 6.2.

# 6.3 General Method for Preparation of Protic Ammonium Ionic Liquids

The synthesis for all protic ILs was carried out under atmospheric conditions following literature preparations. [91,206,221,222,223]

## *6.3.1 Diethanolammonium Chloride*

Diethanolammonium Chloride, [DEA] Cl, was prepared as follows:

To a round bottom flask containing diethanolamine (52.5 g, 0.50 mol) dissolved in dichloromethane (30 ml) solution, concentrated hydrochloric acid (19.5 g, 0.53 mol) was added slowly dropwise over a period of 30 minutes in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator to remove solvent for ca. 1 hour at 30° C and then the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar at 60° C for ca. 5 hours.

The colourless or sometimes faint yellow product was characterised for purity using $^{1}$H NMR, Karl Fischer and CHN analysis and stored in a desiccator.

[DEA] Cl was prepared in batch scales with percentage yields ca. 75% for diethanolammonium chloride. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 3.58 (4H, t, N-C$H_2$), 2.83 (4H, t, O-C$H_2$). Karl Fischer readings ranged between 2 wt% - 5 wt% water content.

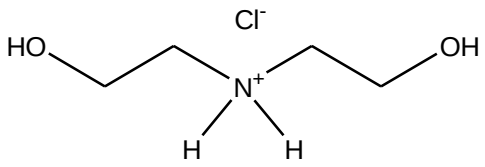


**Figure 144: Chemical structure of [DEA] Cl.**

## *6.3.2 Dimethylethanolammonium Chloride*

Dimethylethanolammonium Chloride, [DMEA] Cl, was prepared as follows:
To a round bottom flask containing dimethylethanolamine (45 g, 0.50 mol) dissolved in dichloromethane (30 ml) solution, concentrated hydrochloric acid (19.5 g, 0.53 mol) was added slowly dropwise over a period of 30 minutes in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator to remove solvent for ca. 1 hour at 30 °C and then the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar at 60° C for ca. 5 hours.

The colourless or sometimes faint yellow product was characterised for purity using $^{1}$H NMR, Karl Fischer and CHN analysis and stored in a desiccator. Percentage yield 95%. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 3.58 (4H, t, N-C$H_2$), 2.83 (4H, t, O-C$H_2$). Karl Fischer reading 4.8 wt% water.

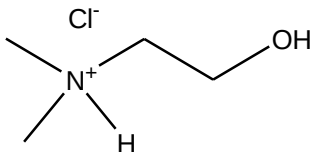


**Figure 145: Chemical structure of [DMEA] Cl.**

### *6.3.3 Diethylammonium Chloride*

Diethylammonium Chloride, [DethylA] Cl, was prepared as follows:

To a round bottom flask containing diethylamine (51 ml, 0.52 mol) and dichloromethane (50 ml), hydrochloric acid (19.5 g, 0.53 mol) was added slowly over a period of 30 minutes in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator to remove solvent for ca. 1 hour at 30° C and then the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar at 60 °C for ca. 5 hours.

The product was a brown solid. Percentage yield 56%. M.p 28-29 °C. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 2.89 (4H, q, N-C$H_2$), 1.10 (6H, t, C$H_3$). Karl Fischer reading 10 wt% water.

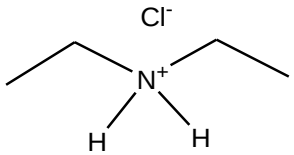


**Figure 146: Chemical structure of [DiethylA] Cl.**

### *6.3.4 Dipropylammonium Chloride*

Dipropylammonium Chloride, [DPA] Cl, was prepared as follows:

To a round bottom flask containing dipropylamine (50 ml 0.5 mol) and dichloromethane (30 ml), hydrochloric acid (22 ml, 0.55 mol) was added *very* slowly over a period of 1 hour in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator at 30 °C, the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar for ca. 5 hours.

The product was a white solid. Percentage yield 62%. M.p 240 °C. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 2.81 (4H, t, N-C*H*$_2$), 1.51 (4H, sextet, N-CH$_2$-C*H*$_2$), 0.80 (6H, t, C*H*$_3$). Karl Fischer reading 1.3 wt% water.

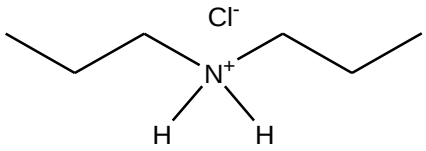


**Figure 147: Chemical structure of [DPA] Cl.**

## *6.3.5 Ethylammonium Chloride*

Ethylammonium Chloride, [EthylA] Cl, was prepared as follows:

To a round bottom flask containing ethylamine (26 ml, 0.3 mol), hydrochloric acid (12 ml, 0.35 mol) was added *very* slowly over a period of 1 hour in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator at 30 °C, the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar for ca. 5 hours.

The product was a white solid. Percentage yield 52%. M.p. 110-111 °C. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 2.86 (2H, t, N-C*H*$_2$), 1.11 (2H, t, N-CH$_2$-C*H*$_2$). Karl Fischer reading 8 wt% water.

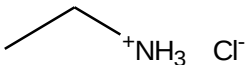


**Figure 148: Chemical structure of [EA] Cl.**

### *6.3.6 Ethanolammonium Chloride*

Ethanolammonium Chloride, [EA] Cl, was prepared as follows:

To a round bottom flask containing ethanolamine (50 ml, 0.5 mol) and dichloromethane (30 ml), hydrochloric acid (22 ml, 0.55 mol) was added *very* slowly over a period of 1 hour in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator at 40 °C, the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar for ca. 5 hours.

The product was a colourless liquid. Percentage yield 90 %. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 3.51 (2H, t, N-C$H_2$), 2.84 (2H, O-C$H_2$). Karl Fischer reading 3.4 wt% water.

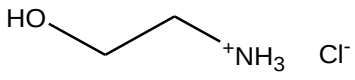


**Figure 149: Chemical structure of [EA] Cl**

### *6.3.7 Propanolammonium Chloride*

Propanolammonium Chloride, [PA] Cl, was prepared as follows:

To a round bottom flask containing 1-propanol-3-amino (50 ml, 0.5 mol) and dichloromethane (30 ml), hydrochloric acid (22 ml, 0.55 mol) was added *very* slowly over a period of 1 hour in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator at 30 °C, the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar for ca. 5 hours.

The product was a colourless liquid. Percentage yield 85%. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 3.55 (2H, t, N-C$H_2$), 2.81 (2H, O-C$H_2$), 1.58 (2H, C$H_2$). Karl Fischer reading 4.6 wt% water.

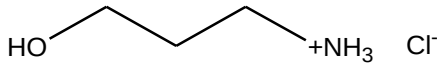


**Figure 150: Chemical structure of [PA] Cl**

## *6.3.8 Triethylammonium Chloride*

Triethylammonium Chloride, [TriethylA] Cl, was prepared as follows:

To a round bottom flask containing triethylamine (50 g, 0.5 mol) and dichloromethane (30 ml), hydrochloric acid (22 ml, 0.55 mol) was added *very* slowly over a period of 1 hour in a 5% stochiometric excess. The resulting mixture was stirred in an ice bath for 2 hours. The mixture was placed on rotary evaporator at 40 °C, the drying process was completed on a Schenk line at ca. $10^{-2}$ mbar for ca. 5 hours.

The product was a white solid. Percentage yield 72%. M.p 240 °C. $^{1}$H NMR $\delta_H$ ppm ($D_2O$): 3.28 (6H, q, 3xN-C*H*$_2$), 1.56 (9H, t, 3xN-CH$_2$-C*H*$_3$). Karl Fischer reading 1 wt % water.

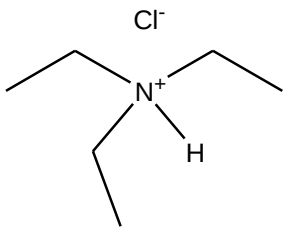


**Figure 151: Chemical structure of [TriethylA] Cl**

### *6.3.9 Diethanolammonium Nitrate*

Diethanolammonium Nitrate, [DEA] $NO_3$, was prepared as follows:
To a round bottom flask containing diethanolamine (62 ml, 0.6 mol), 1 molar nitric acid (100 ml, 1 molar) was slowly added over half an hour. No solvent was used and the mixture was stirred for ~2 hours using an ice bath to control the reaction. Water was removed on the rotary evaporator at 60 °C and also on the Schlenk line at ca.$10^{-2}$ mbar at 70° C for ca. 5 hours.

The product was a colourless liquid. Percentage yield 81%. $^1$H NMR $\delta_H$ ppm ($D_2O$): 3.55 (4H, t, N-C$H_2$), 2.69 (4H, t, O-C$H_2$). Karl Fischer reading 1.7 wt% water.

Diethanolammonium sulfate[171] and acetate were all synthesised in the same manner replacing the 1 M nitric acid with sulfuric and acetic acid respectively.

### *6.3.10 Bis-2-methoxyethylammonium Chloride*

Bis-2-methoxyethylammonium Cl, [B-2-MEA] Cl, was purchased from Bioniq's and was used without further purification. Karl Fischer reading 6.2 wt% water.

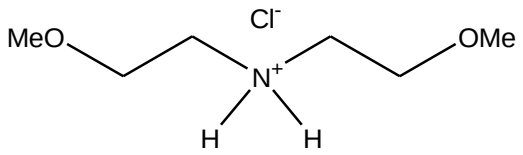


**Figure 152: Chemical structure of [B-2-MEA] Cl**

# 6.4 General Method for Preparation of Imidazolium Ionic Liquids

Imidazolium ionic liquids were synthesised on a Schlenk line under a flow of $N_2$.[91,224,225] The Methylimidazole was first distilled by reduced pressure distillation, at 70 °C for 4 hours at ca. $10^{-2}$ mbar with a percentage recovery of 90%.

## *6.4.1 1-Butyl-1-methylimidazolium Chloride [BMIM] Cl*

Methylimidazole, (25.75 g, 0.31 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[173,226,227] Toluene (50 $cm^3$) and 1-chlorobutane (32.56 g, 0.39 mol) were added to the solution and then the system was at reflux at 110 °C overnight. The sample was removed from the heat and washed with toluene twice and ethyl acetate twice before removing the remaining solvent with the rotary evaporator and Schlenk line evaporation at ca. $10^{-2}$ mbar at 60 °C for 5 hours.[228]

$^1$H NMR $\delta_H$ ppm ($D_2O$): 7.60 (1H, s, top imidazolium-H), 7.40 (1H, s, bottom imidazolium-H), 6.90 (1H, s, bottom imidazolium-H), 4.02 (2H, t, N-C*H*$_2$), 3.72 (3H, s, N-C*H*$_3$), 1.80 (2H, m, C*H*$_2$), 1.30 (2H, m, C*H*$_2$), 0.76 (3H, t, $CH_3$). Karl Fischer reading 0.9 wt% water.

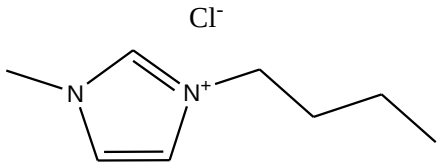


**Figure 153: Chemical structure of [BMIM] Cl**

## *6.4.2 1-Allyl-1-methylimidazolium Chloride [AMIM] Cl*

Methylimidazole, (25.20 g, 0.29 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[226] Toluene (50 $cm^3$) and allyl chloride (31.80 g, 0.37 mol) were added to the solution and then the system was at reflux at 70 °C overnight. The sample was removed from the heat and washed with toluene twice and ethyl acetate twice before removing the remaining solvent with the rotary evaporator and Schlenk line evaporation at ca. $10^{-2}$ mbar at 60° C for 5 hours.[228]

$^1H$ NMR $\delta_H$ ppm ($D_2O$): 8.59 (1H, s, top imidazolium-H), 7.31 (1H, s, bottom imidazolium-H), 7.30 (1H, s, bottom imidazolium-H), 4.02 (2H, t, N-C$H_2$), 3.70 (3H, s, N-C$H_3$), 5.30-5.24 (3H, m, alkene-H). Karl Fischer reading 4 wt% water.

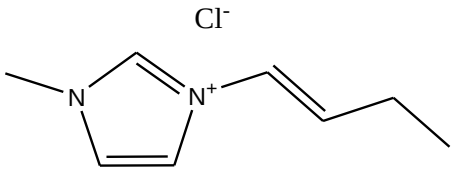


**Figure 154: Chemical structure of [AMIM] Cl**

# 6.5 General Method for Preparation of Aprotic Nitrogen Based Ionic Liquids

All aprotic nitrogen based ionic liquids were synthesised under air sensitive conditions using dry solvents and stored in a desiccator. literature procedures followed.[91]

## *6.5.1 N,N-Butylmethylpiperidinium Chloride [BMPip] Cl*

*N*-Methylpiperidine, (36 ml, 0.2 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.**[226]** Acetonitrile (30 $cm^3$) and 1-chlorobutane (used 31 ml, 0.2 mol) were added to the solution and then the system was stirred at 60 °C for 24 hours. The sample was removed from the heat and a yellow solid crashed out. This was washed with acetonitrile twice and then filtered under vacuum. The solid was dried on the Schlenk line at ca. $10^{-2}$ mbar at 40 °C for ca. 5 hours.**[228]**

The product was a yellow solid. Percentage yield 28%. M.p 245 °C. $^1$H NMR $\delta_H$ ppm ($CDCl_3$): 3.76 (4H, m, Cyclic 2xN-C*H*$_2$), 3.57 (2H, t, N-C*H*$_2$), 3.30 (3H, s, N-C*H*$_3$), 1.76 (6H, broad m, 3xCyclic-C*H*$_2$), 1.63 (2H, q, N-$CH_2$-C*H*$_2$), 1.37 (2H, p, $CH_2$-C*H*$_2$-$CH_3$), 0.93 (3H, t, $CH_2$-C*H*$_3$), 0.76 (3H, t, $CH_3$). Karl Fischer reading 2.4 wt% water.

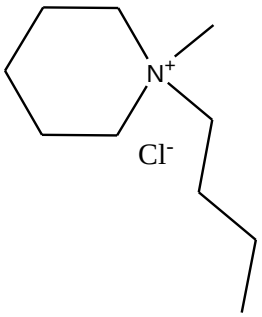


**Figure 155: Chemical structure of [BMPip] Cl**

## *6.5.2 N,N-Buthylmethylmorpholinium Chloride [BMMorph] Cl*

N-Methylmorpholine, (22 ml, 0.2 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[226] Acetonitrile (20 $cm^3$) and 1-chlorobutane (21 ml, 0.2 mol) were added to the solution and then the system was stirred at 75 °C overnight. The sample was removed from the heat and an orange solid crashed out. The solvent was half removed before washing the sample 3 times with ca. 10ml pentane. The pentane was decanted off and the solid dried under vacuum on the Schlenk line at ca. $10^{-2}$ mbar for ca. 5 hours.[228]

Product was a white solid. Percentage yield 24.7%. M.p 192-193 °C. $^1H$ NMR $\delta_H$ ppm ($CDCl_3$): 3.99 (4H, dd, 2x$CH_2$-O), 3.78 (4H, dd, 2x$CH_2$-N ), 3.61 (2H, t, alkyl $CH_2$-N), 3.54 (3H, s, N-$CH_3$), 1.73 (2H, m, N-$CH_2CH_2$), 1.42 (2H, m, $CH_2$-$CH_3$), 0.99 (3H, t, $CH_3$). Karl Fischer reading 12 wt% water.

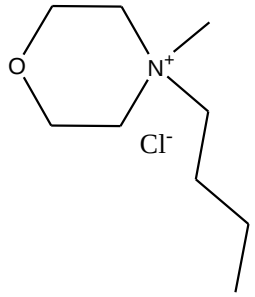


**Figure 156: Chemical structure of [BMMorph] Cl**

### *6.5.3 N-Butylpyridinium Chloride [BPy] Cl*

Pyridine, (32 ml, 0.2 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[226] Acetonitrile (30 $cm^3$) and 1-chlorobutane (42 ml, 0.2 mol) were added to the solution and then the system was stirred at 60 °C for 3 days. The sample was removed from the heat and placed in the freezer where a white solid crashed out. This was washed with acetonitrile twice and then filtered under vacuum. The solid was dried on the Schlenk line at ca. $10^{-2}$ mbar for ca. 5 hours.[228]

The product was a yellow tinted solid. Percentage yield 22%. M.p 34 °C. $^1$H NMR $\delta_H$ ppm ($D_2O$): 9.67 (2H, t, aromatic N-C*H*), 8.46 (1H, t, aromatic-C*H*), 8.11 (2H, dd, aromatic-C*H*), 5.03 (2H, t, N-C$H_2$), 1.99 (2H, m, N-$CH_2$-C$H_2$), 1.37 (2H, m, C$H_2$-$CH_3$), 0.92 (3H, t, C$H_3$). Karl Fischer reading 10 wt% water.

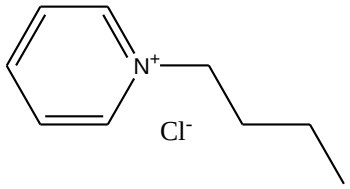


**Figure 157: Chemical structure of [BPy] Cl**

## *6.5.4 N,N-Butylmethylpyrrolidinium Chloride [BMPyr] Cl*

*N*-Methylpyrrolidine, (19 ml, 0.2 mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[226] Toluene (20 $cm^3$) and 1-chlorobutane (21 ml, 0.2 mol) were added to the solution and then the system was stirred at 70 °C for 3 days. The sample was removed from the heat and yellow precipitate was present. This was washed with toluene twice and the solid was dried on the Schlenk line at ca. $10^{-2}$ mbar for ca. 5 hours.[228]

The product was a cream solid. Percentage yield 77%. M.p 138 °C. $^1$H NMR $\delta_H$ ppm ($D_2O$): 3.80 (2H, t, N-$CH_2$ cyclic), 3.70 (2H, t, N-$CH_2$ cyclic), 3.58 (2H, t, N-$CH_2$ allyl), 3.25 (3H, s, N-C$H_3$), 2.23 (4H, t, 2xC$H_2$ cyclic) 1.70 (2H, m, N-$CH_2$-C$H_2$), 1.40 (2H, m, C$H_2$-$CH_3$ ), 0.93 (3H, t, $CH_2$-C$H_3$). Karl Fischer reading 10 wt% water.

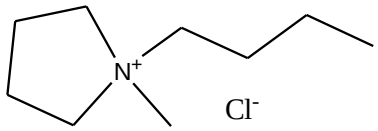


**Figure 158: Chemical structure of [BMPyr] Cl**

## 6.5.5 N,N-Butylmethylpyrrolium Chloride [BMP] Cl

*N*-Methylpyrrole, (16ml g, mol) was added to a sealed round bottom flask under a nitrogen atmosphere and stirred.[226] Acetonitrile (20cm$^3$) and 1-chlorobutane (21ml g, mol) were added to the solution and then the system was stirred at 60 °C for 1 week. The sample was removed from the heat and placed in the freezer for 2 days. The precipitate was washed with acetonitrile twice and the solid was dried on the schlenk line at ca. $10^{-2}$ mbar for ca. 5 hours.[228]

The product was a cream solid. Percentage yield 67%. M.p 148 °C. $^1$H NMR $\delta_H$ ppm ($D_2O$): 6.30 (2H, t, N-$CH_2$ cyclic), 5.10 (2H, t, N-$CH_2$ cyclic), 3.33 (2H, t, N-$CH_2$ allyl), 2.90 (3H, s, N-C$H_3$), 2.03 (2H, q, N-$CH_2$-C$H_2$ ) 1.30 (2H, m, N-$CH_2$-$CH_2$-C$H_2$), 0.96 (3H, t, $CH_2$-C$H_3$). Karl Fischer reading 11 wt% water.

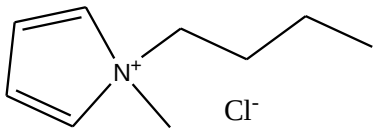


**Figure 159: Chemical structure of [BMP] Cl**

## 6.5.6 Choline Chloride

Choline Cl, was purchased from Alfa Aesar and used without further purification. Karl Fischer reading 2 wt% water.

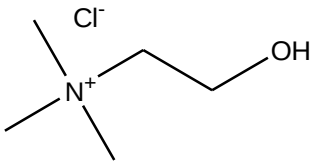


**Figure 160: Chemical structure of Choline Cl**

## 6.6 Structural Analysis of Biomass

To determine the changes occurring in the plant biomass during IL pre-treatment, a number of physical techniques were used to identify the main factors.

### *6.6.1 Powder X-ray diffraction*

Powder XRD patterns were recorded on a Bruker-AXS D8 Advance instrument with Lynx eye detector, using Cu Kα radiation (1.54 Å). Samples were ground to a fine powder and deposited in films on an aluminium sample holder. Typically, data was obtained for a 5-90° 2θ range, with 0.02° 2θ step size and scan speed of 0.1 s.

### *6.6.2 Thermogravimetric Analysis*

Stanton Redcroft Simultaneous Thermogravimetric Analyzer, model: STA 625, equipped with a computer data analyser version C 4.20. The studies were done in nitrogen atmosphere at a flow rate of 50 ml/min and heating rate of 10 °C/min. The weight of the samples used was 10 mg in all cases.

### *6.6.3 FT-IR*

Thermo-Nicolet Avatar 370 FTIR with a film under atmospheric conditions was used to obtain qualitative information about changes in the plant biomass polymers.

### *6.6.4 TG-IR*

A Netzsch 409 TGA coupled via a heated transfer pipe to a Bruker Equinox 55 FT-IR spectrometer was used to analyse the decomposition products from biomass samples.

### *6.6.5 Scanning Electron Microscopy*

SEM images were obtained using an FEI Sirion scanning electron microscope. Samples were prepared by dusting ground biomass powders onto a sticky carbon pad mounted on an aluminium stub, with excess powder removed by gravity. Prior to use, samples were sputter coated with carbon (10 nm layer) using an Agar Auto Carbon Coater to prevent charge build up under the electron beam.

### *6.6.6 Solid State NMR*

Solid-state NMR: $^{13}C$ MAS NMR spectra were recorded on a Bruker Avance II 500 spectrometer, equipped with a standard 2.5 mm double-resonance, double-bearing

CP MAS probe at a Larmor frequency of $\omega 0/2\pi = -125.78$ MHz . Harman-Hahn cross polarisation (CP) was used $^{1}H$ $\pi/2$ pulse duration 4 μs, recycle delay 5 s, and CP contact time 2 ms). MAS frequency was (8 kHz. CW 1H decoupling of 80 kHz was applied during acquisition and 1024 scans accumulated per spectrum. 13C chemical shifts are recorded relative to TMS ($\delta^{13}C = 0$ ppm)

### *6.6.7 Optical Microscopy*

Optical Microscopy was performed using an Olympus BX50 microscope at X100 magnification. Temperature was controlled using a Linkam Scientific LTS 350 heating stage. Samples were held using VWR International borosilicate glass microscope cover slips with a thickness no. 1.

# 6.7 Saccharification Analysis

## *6.7.1 High Throughput Robot for Saccharification Analysis*

Ground biomass material after pre-treatment was weighed into four replicate samples in a 96 deep-well plate using the robotic platform custom-made from Labman Automation, Stokesley, North Yorkshire, UK.[137] It contains the declogging station, where samples are mixed to loosen the particles, and a piercing station, where the sample vials are vibrated to dispense 4mg of sample into the wells. This weight is recorded for later analysis.

Saccharification analysis of this plant biomass was performed using a liquid handling robotic platform Tecan Evo 200; Tecan Group Ltd. Männedorf, Switzerland.[137] Enzymatic digestion was then performed with a 4:1 mixture of Celluclast (Cellulase from *Trichoderma reesei*) and Novozyme 188 (cellobiose from *Aspergillus niger*) and incubated at 50 °C for 8 h with shaking. The Celluclast enzyme is produced by submerged fermentation of the fungus *Trichoderma reesei* and catalyses the breakdown of cellulose into glucose, cellobiose and higher glucose polymers.[135] It has an activity of ≥700 Endoglucanase units (EGU)/g (μmol reducing sugars released per gram of enzyme per minute). The Novozyme 188 enzyme is a cellobioase enzyme preparation obtained by submerged fermentation of an *Aspergillus niger* microorganism and hydrolyses cellobiose to glucose. It has an activity of ≥250 Cellobiase units (CBU)/g (μmol of glucose released per gram of enzyme per minute). The mixture of enzymes is diluted such that 7 FPU (filter paper units) of enzyme is added to each well.

Finally, saccharification potential was determined by measuring the amount of reducing sugars via a colourimetric assay using 3-methyl-2-benzothiazolinone hydrazone (MTBH).[229,230] An aliquot of this digested mix is added to 1M NaOH and MBTH and is heated at 70 °C for 20 minutes in the thermocyclers. Each plate contained standards of 50 nmol, 100 nmol and 150 nmol glucose (three replicates each) and filter paper disks (four replicates) in order to monitor any change in enzyme concentration or activity through time. Change in colour was read with a Tecan Sunrise microplate absorbance reader at 620 nm. Error analysis is completed by repetitions of 4 samples and MBTH reading duplicated with standard error analysis.

Figure 161 shows some of the equipment described previously.

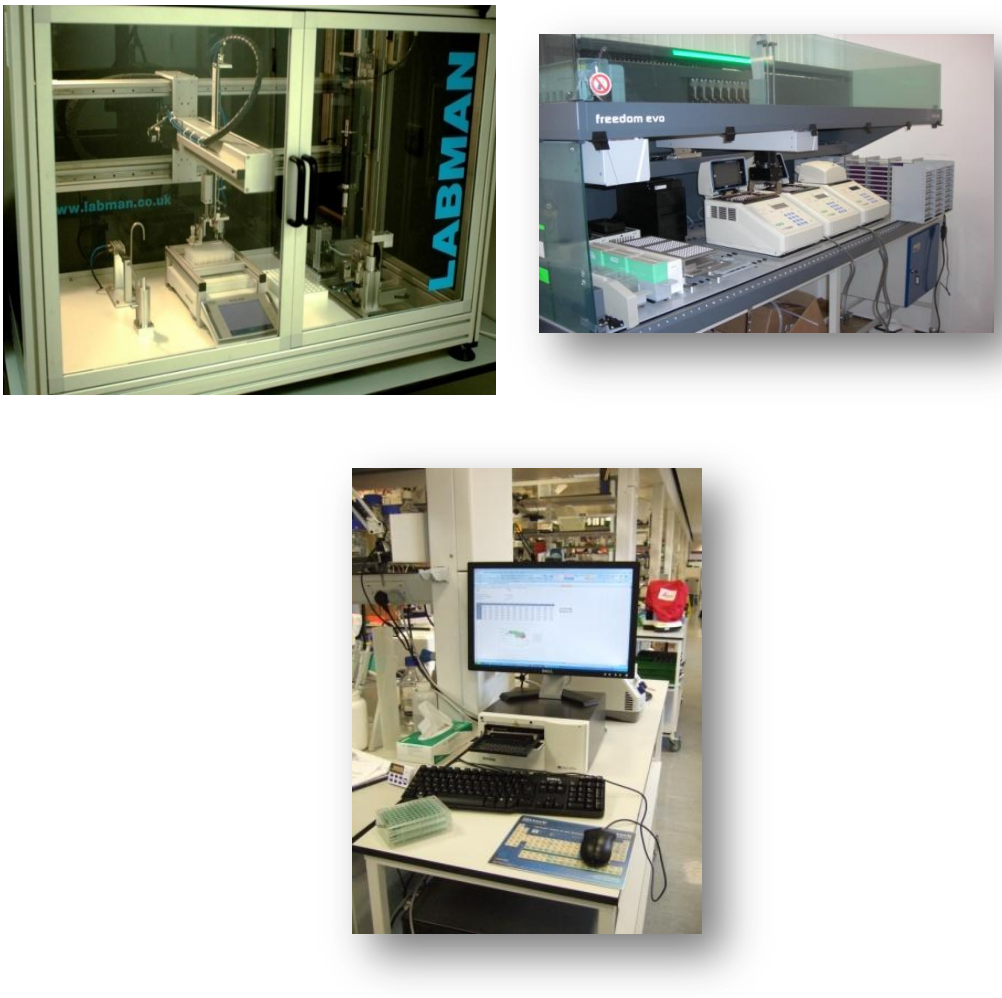


**Figure 161: Shows the declogging station top left, the hydrolysis incubators top right, and the Tecan Sunrise microplate absorbance reader at the bottom. Modified from 137.**

### *6.8.2 Manual Analysis Measuring Reducing Sugars with MBTH*

Hydrolysis performed with 1.6 ml of enzyme mixture in buffer 25mM sodium acetate at pH 4.3 and incubated at 50 °C for 8 hours with shaking. The enzyme mixture is comprised of 30 µl enzyme in 300 µl of buffer.

Preparation of enzyme mixture: Dosage: 10 ug protein/mg dry substance in a ratio of 4:1 of Celluclast/Novozyme 188 (1.27 ml of Celluclast + 0.31 ml of novo188). Filter using Hi-Trap column and use approx 10 FPUs / g of dry material.

Assay for reducing sugars: 300 µl of supernant, with 100 µl of 1 N NaOH and 200 µl of MBTH were heated at 70 °C for 20 minutes in a heating block. MBTH reagent made using equal volumes of 3 mg/ml MBTH and 1mg/ml DTT (stored at 4°C for one week). 400 µl of oxidising reagent (ferric chloride) was added to the 600 µl in the tube. Samples left to develop for 1 hour and the change in colour was read with a Tecan Sunrise microplate absorbance reader at 620 nm. Oxidising reagent made from 0.5 % $FeNH_4(SO_4)_2$, 0.5 % Sulfamic acid and 0.25 N HCl.

### *6.8.3 Time Course Experiments*

Section 6.8.2 is followed apart from the incubation time period is changed. The samples are incubated at first at 50 °C for 8 hours with rotation, then a small aliquot of 30 µl is removed for MBTH analysis. The remainder of the sample is incubated for a further 16 hours before another 30 µl is removed (24 hours after start time). This happens again at 72 hours, collating 3 MBTH readings at 8, 24 and 72 hours. The longer the incubation time the more sugars are released until the enzymes cannot digest anymore of the material.

### *6.8.4 Full Enzyme Loading Analysis*

Procedure followed as in section 6.8.2 except volume of enzyme initially added. In these experiments the manufactures recommended amount of enzyme was added, which is 4 times more concentrated then the standard used in the biology labs. The enzyme mixture is comprised of 120 µl enzyme in 300 µl of buffer.

### *6.8.5 One Step Pre-treatment Followed by Hydrolysis Analysis*

After pre-treatment, usually the pre-treatment solvent is removed and the biomass washed with ethanol three times as in section 6.2. Is this experiment the pre-treatment solvent was not removed and the enzyme mixture was added directly to the solution. For these experiments only 2 g/ 2 ml of IL was used with 10% biomass loading. The saccharification analysis was completed as section 6.8.2.

# 6.9 Cell Wall Polymer Compositional Analysis

All cell wall compositional analysis were carried out in biological triplicates.

## *6.9.1 Hemicellulose Content Determination using DNS (Dinitrosalicyclic acid)*[22- 135]

For a 100 ml assay, DNS 0.75 g, NaOH 1.4 g, sodium potassium tartrate 21.6 g, phenol 0.53 ml and Na metabisulfate 0.59 g were used to make up 100 ml with distilled water and required gently warming to aid dissolution. It was stored at room temperature wrapped in foil.

Assay for reducing sugars: Glucose standards from 4mg/ml stock, as labelled in Table 37 were used in screw cap 2 ml tubes in aliquots of 100µl. 300 ul of DNS reagent was added to all tubes and incubated at 100 °C for 5 minutes in a pre-heated block. The darkening in colour showed the presence of sugars. All samples were cooled to room temperature and aliquots of 100 ul taken into a Corning optical plate. 100 ul water was added to each sample (a dilution by 50%) and read with a Tecan Sunrise microplate absorbance reader at 540 nm.

**Table 37: Glucose concentration for calibration curve for DNS assay.**

| Glucose mg | Glucose nmol | Glucose mg | Water ul |
|---|---|---|---|
| 0.04 | 7.2 | 10 | 90 |
| 0.1 | 18 | 25 | 75 |
| 0.2 | 36 | 50 | 50 |
| 0.3 | 54 | 75 | 25 |
| 0.4 | 72 | 100 | 0 |

### *6.9.2 Crystalline Cellulose Content Determination using Anthrone*

Sample preparation: Using 5 mg plant biomass in 2 ml tubes, 500 µl 2M TFA was added and flushed with Argon. Samples were incubated for 4 hours at 100 °C with shaking every hour, the TFA was evaporated off using a vacuum concentrator (Savant SPD131DDA, Thermo Scientific) with refrigerated vapour trap (Savant RVT4104, Thermo Scientific). The samples were washed with 500 ul distilled water twice without disturbing the pellet and then the pellet was dried in the centrifugal evaporator.

Crystalline cellulose content was analysed using Foster et al.'s method,[231] based on the method reported by Updegraff.[200] 1 ml Updegraff reagent (acetic acid:nitric acid: water 8:1:2 (v/v/v)) was added to 4 mg biomass and heated at 100 °C for 30 minutes. As a result of this treatment only crystalline cellulose remains insoluble in the pellet. Samples were then cooled to RT with an ice bath and centrifuged at 10,000 rpm for 15 minutes. The pellet was then washed with 1.5 ml water and three times with 1.5 ml of acetone. The pellet was air dried and left overnight.

Saeman hydrolysis: The pellet is hydrolysed into glucose using 90 µl 72% Sulfuric acid added to the tube. Incubated at room temperature for 4 hours on a platform rocker, to break up hydrogen bonds. 1890 µl water added and samples vortexed and heated for 4h at 120°C. Samples centrifuged at 10,000 rpm for 5 minutes, leaving some brown insoluble material, being lignin, in the tube.

Finally, the glucose content was quantified using the colourimetric anthrone assay: 10 µl of each sample was added in triplicate to a 96 well polystyrene microtiter plate with 90 µl water and 200 µl anthrone reagent (2 mg anthrone $ml^{-1}$ concentrated $H_2SO_4$). A standard curve for glucose (0, 2, 4, 6, 8 and 10 µg) was made on each plate for quantification, shown in Table 38 overleaf.

The plate was then heated at 80 °C for 30 min, allowed to cool and the absorption read at 620 nm using a Tecan Sunrise microplate absorbance reader.

**Table 38: Glucose standards for crystalline cellulose determination.**

| | Sample (ul) | $H_2O$ (ul) | Anthrone Reagent (ul) |
|---|---|---|---|
| **Blank** | 0 | 400 | 800 |
| **Std 0.5** | 2 | 398 | 800 |
| **Std 1** | 4 | 396 | 800 |
| **Std 2** | 8 | 392 | 800 |
| **Std 4** | 16 | 384 | 800 |
| **Std 6** | 24 | 376 | 800 |
| **Std 8** | 32 | 368 | 800 |
| **Std 10** | 40 | 360 | 800 |
| **Sample** | 40 | 360 | 800 |

### *6.9.3 Lignin Content Determination using Acetyl Bromide*

Lignin content was quantified using Foster et al.'s acetyl bromide method,[201] based on the method reported by Fukushima and Hatfield.[202] Briefly, 3 mg AIR was weighed into a 5 ml volumetric flask and 250 µl freshly prepared acetyl bromide solution (25 % (v/v) acetyl bromide in glacial acetic acid) added. Samples were incubated at 50 °C for 2 hours, followed by a further 1 hour with vortexing every 15 minutes. Samples were then cooled to RT before addition of 1 ml 2 M NaOH and 175 µl freshly prepared 0.5 M hydroxylamine hydrochloride. Samples were then taken to 5 ml with glacial acetic acid, mixed several times and the absorption read using a Shimadzu UV-1800 spectrophotometer at 280 nm. Lignin content (µg $mg^{-1}$ cell wall) was then determined using the following formula:

*((a/b*c) * (d*100))/e*

*a = absorbance, b = coefficient, c = path length, d = total volume, e = biomass weight*

The coefficient is specific to the type of plant that is being analysed and, for grasses a coefficient of 17.75 is used.[202]

## 6.10 Hydrolysis of Polymers to Monosaccharides for Compositional Analysis via HPLC

Monosaccharide analysis was performed using high performance anion exchange chromatography (HPAEC) (Carbopac PA-10; Dionex, Camberley, Surrey, UK).[218] AIR samples of 3 mg were prepared by hydrolysing with 0.5 ml 2 M trifluoroacetic acid (TFA) for 4 h at 100 °C. Samples were then cooled to RT and evaporated completely using a vacuum concentrator (Savant SPD131DDA, Thermo Scientific) with refrigerated vapour trap (Savant RVT4104, Thermo Scientific), rinsed twice with 200 µl isopropanol and once with 500 µl water using the vacuum concentrator. Samples were then re-suspended in 150 µl deionised water, centrifuged at 10,000 rpm for five minutes and the supernatants filtered with 0.45 µm PTFE filters (Millex™, Millipore).

Monosaccharides were separated and quantified by HPAEC using a DionexICS-3000 with integrated amperometry detection. Chromatographic separation was performed on a CarboPac PA20 (3 x 150 mm) column (Thermo) using a gradient elution. The mobile phase consisted of solution A: 100 % water, solution B: 200 mM NaOH, and solution C: 0.1 M Sodium Hydroxide, 0.5 M Sodium Acetate. A flow rate of 0.5 ml $min^{-1}$ was used and the gradient was as follows: 0 minutes: 100 % A; 5 minutes: 99 % A, 1 % B; 15 minutes: 99 % A, 1 % B; 22 minutes: 47.5 % A, 22.5 % B, 30 % C; 30 minutes: 47.5 % A, 22.5 % B, 30 % C. The column was then washed as follows: 30.1 minutes: 100 % B; 37 min: 100 % B; 37.1 minutes: 99 % A, 1 % B; 50 minutes: 100 % A; 55 minutes: 100 % A.

The separated monosaccharides were quantified by using external calibration with a mixture of nine monosaccharide standards at 100 µM (arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose, and xylose) that were subjected to acid hydrolysis in parallel with the samples.

## 6.11 Fermentation Guidelines

Experiments were run for five days in total – One day saccharification, three days fermentation, with collecting samples at 0, 6, 24, 48 and 72 hours. The yeast strand used was *Saccharomyces cerevisiae* and all materials were autoclaved before use in the fermentation reactions. The plate medium was made from: 750 ml of deionised water, with 10 g yeast extract, 20 g Bacto-peptone (source not dependent), 20 g glucose and solution made up to 1 litre. The vessels are split into two 500 ml portions and 10 g Agar (technical) is added to 1 bottle. Agar does not fully dissolve.

Fermentation medium: 0.5 L water with 50 g of yeast and 100 g Bacto-peptone. NO GLUCOSE should be added otherwise the ethanol yield will not be able to be determined. The plates for yeast growth were prepped with agar, using an automated filler with 20 ml of agar on 20 plates. They were allowed to cool and stored in the cold room (3 °C) until required. Yeast was streaked onto plates to achieve single colonies, stored in 30℃ room for 48 hours, checked after 24 hours. Stored in fridge until use, short lifetime only of 1 week.

Liquid culture to develop yeast: 2 x 200ml conical flasks autoclaved before they were needed for the liquid culture. A few colonies of yeast from the cultures were added to the liquid culture and incubated on shaker in 30℃ room. Optical density of yeast should be around 0.5, measured by IR. 50 ml of yeast liquid culture was taken and placed in Vulcan tubes to centrifuge before the supernatant was removed. Sterile water was re-added, tubes shaken and re-read the optical density using IR. When the density was around 2.6 the cultures were used. The excess water was poured away and re-added fresh water to the correct amount ca. 2.3 ml. These cultures were then used in a 100 µl for the fermentation experiment. Amounts used in standard fermentation experiment (10 ml) shown in Table 39:

**Table 39: Fermentation flask components.**

| | |
|---|---|
| Biomass | 2 g |
| Sterile water | 5.375 ml |
| NaOAC | 0.125 ml |
| Enzyme | 0.5 ml (90 µl in 60 ml) |
| Medium | 1 ml |
| Yeast | 100 µl |

# APPENDIX I

**Table 40: Solubility of Cellulose in ILs[108]**

| Carbohydrate | IL | Water content | Solubility | Temperature (°C) |
|---|---|---|---|---|
| **cellulose** | [amim][Cl] | nd | 8−14.5 wt % | 80 |
| | [amim][Cl] | ~0.7% | 5 wt % | 90 |
| | [amim][Cl] | <0.2 wt % | 10 wt % | 100 |
| | [ammim][Br] | 3.2 wt % | 4−12 wt% | 80 |
| | [amim][HCOO] | <0.2 wt % | 10−20 wt% | 60−85 |
| | [bmim][Br] | ~0.7% | <5 wt % | 90 |
| | [bmim][Cl] | ~0.7% | <5 wt % | 90 |
| | [bmim][Cl] | nd | 10 wt % | 100 |
| | [bmim][Cl] | nd | 10 wt % | 110 |
| | [bmim][I] | ~0.7% | 5 wt % | 90 |
| | [bmim][HCOO] | nd | 8 wt % | 110 |
| | [bmim][N(CN)2] | nd | 1 wt % | 110 |
| | [bmim][N(CF3SO2)2] | nd | <0.5 wt % | 110 |
| | [bmpy][Cl] | nd | 12−39% | 105 |
| | [emim][Cl] | ~0.7% | 5 wt % | 90 |
| | [emim][CH3COO] | ~0.7% | 5 wt % | 90 |

| | | | | |
|---|---|---|---|---|
| | [emim][CH3COO] | nd | 15 wt % | 110 |
| | [emim][CH3COO] | nd | 20 g/L | nd |
| | [emim][(CH3O)2PO2] | <1000 ppm | 2% | 45 |
| | [emim][(CH3O)2PO2] | <1000 ppm | 10% | 65 |
| | [hmim][Cl] | ~0.7% | <5 wt % | 90 |
| | [H(OEt)2-mim][CH3COO] | nd | 5 wt % | 110 |
| | [H(OEt)3-mim][CH3COO] | nd | 2 wt % | 110 |
| | [H(OEt)2-mim][Cl] | nd | 1 wt % | 110 |
| | [mmim][(CH3O)2PO2] | ~0.7% | 5 wt % | 90 |
| | [MeOMemim][Br] | 2.5 wt % | 10 g/L | nd |
| | [Me(OEt)2eim][Cl] | nd | 2 wt % | 110 |
| | [Me(OEt)2eim][CH3COO] | nd | 12 wt % | 110 |
| | [Me(OEt)3eim][CH3COO] | nd | 12 wt % | 110 |
| | [Me(OEt)4eim][CH3COO] | nd | 10 wt % | 110 |
| | [Me(OEt)7eim][CH3COO] | nd | 3 wt % | 110 |
| | [Me(OPr)3-eim][CH3COO] | nd | 0.5 wt % | 110 |
| | [Me(OEt)3-bim][CH3COO] | nd | 0.5 wt % | 110 |
| | [omim][CH3COO] | nd | <1 wt % | 110 |
| | [P66614][N(CN)2] | nd | <0.5 wt % | 110 |
| | [pmpy][Cl] | ~0.7% | 5 wt % | 105 |

| | | | | |
|---|---|---|---|---|
| | [tbpm][HCOO] | nd | 6 wt % | 110 |
| | [Bu4N][HCOO] | nd | 1.5 wt % | 110 |
| | [Me(OEt)2-Et3N][CH3COO] | nd | 10 wt % | 110 |
| | [Me(OEt)3-Et3N][CH3COO] | nd | 10 wt % | 110 |
| | [MeMe(EtOH)NH][CH3COO] | 0.29% | <0.5 wt % | 110 |
| | [(MeOEt)2NH2][CH3COO] | 0.13% | <0.5 wt % | 110 |
| | [MeMe(MeOEt)NH][CH3COO] | 1% | <0.5 wt % | 110 |
| | [Me(MeOEt)2NH][CH3COO] | 0.22% | <0.5 wt % | 110 |
| | [Amm110][Cl] | nd | 0.5 wt % | 110 |
| | [Amm110][N(CN)2] | nd | <0.5 wt % | 110 |
| | [Amm110][HCOO] | nd | 0.5 wt % | 110 |
| | [Amm110][CH3COO] | nd | 0.5 wt % | 110 |

**Table 41: Solubility of Polysaccharides in ILs**

| carbohydrate | IL | water content | solubility | temperature (°C) | reference |
|---|---|---|---|---|---|
| **agarose** | [MeOEtmim][Br] | 2.6 wt % | 20 g/L | nd | 27 |
| | [MeOMemim][Br] | 2.5 wt % | 10 g/L | nd | 27 |
| **amylopectin** | [bmim][Cl] | Nd | 5 wt % | 70 | 46 |
| **amylose** | [amim][HCOO] | <0.2 wt % | 4 wt % | 30 | 29 |
| | [amim][HCOO] | <0.2 wt % | 19 wt % | 60 | 29 |
| | [bmim][N(CN)2] | <500 ppm | 4 g/L | 25 | 26 |
| | [MeOEtmim][Br] | 2.6 wt % | 30 g/L | nd | 27 |
| | [MeOMemim][Br] | 2.5 wt % | 30 g/L | nd | 27 |
| **chitin** | [amim][Br] | nd | 10 wt % | 100 | 39 |
| | [bmim][CH3COO] | nd | 3−7 wt % | 110 | 41 |
| | [bmim][Cl] | nd | 10 wt % | 110 | 40 |
| **chitosan** | [amim][Cl] | nd | 8 wt % | 110 | 41 |
| | [bmim][CH3COO] | nd | 12 wt % | 110 | 41 |
| | [bmim][Cl] | nd | 10 wt % | 110 | 40 |
| **inulin** | [amim][HCOO] | <0.2 wt % | 2 wt % | 30 | 29 |
| | [amim][HCOO] | <0.2 wt % | 25 wt % | 55 | 29 |
| **pectin** | [amim][HCOO] | <0.2 wt % | 1.5 wt % | 65 | 29 |
| | [amim][HCOO] | <0.2 wt % | 2.5 wt % | 80 | 29 |

| | | | | | |
|---|---|---|---|---|---|
| **starch** | [amim][Cl] | nd | 15 wt % | 80 | 45 |
| | [amim][Cl] | nd | 20 wt % | 100 | 45 |
| | [bmim][Cl] | nd | 10 wt % | 80 | 44 |
| | [bmim][N(CN)2] | nd | 10 wt % | 90 | 44 |
| **xylan** | [amim][HCOO] | <0.2 wt % | 1.5 wt % | 45 | 29 |
| | [amim][HCOO] | <0.2 wt % | 21 wt % | 95 | 29 |

**Table 42: Solubility of Oligosaccharides in ILs**

| carbohydrate | IL | water content | solubility | temperature (°C) | reference |
|---|---|---|---|---|---|
| **dextrin** | [amim][HCOO] | <0.2 wt % | 3 wt % | 30 | 29 |
| | [amim][HCOO] | <0.2 wt % | 25 wt % | 45 | 29 |
| **α-cyclodextrin** | [bmim][BF4] | nd | <1 wt % | nd | 28 |
| | [bmim][Cl] | nd | 30 wt % | nd | 28 |
| | [bmim][PF6] | nd | <1 wt % | nd | 28 |
| | [MeOEtmim][Br] | 2.6 wt % | 350 g/L | nd | 27 |
| | [MeOMemim][Br] | 2.5 wt % | 350 g/L | nd | 27 |
| **β-cyclodextrin** | [bmim][BF4] | nd | <1 wt % | nd | 28 |
| | [bmim][Br] | nd | 25 wt % | 25 | 47 |
| | [bmim][Cl] | nd | 21 wt % | nd | 28 |
| | [bmim][N(CN)2] | <500 ppm | 750 g/L | 75 | 26 |
| | [bmim][PF6] | nd | <1 wt % | nd | 28 |
| **γ-cyclodextrin** | [bmim][BF4] | nd | <1 wt % | nd | 28 |
| | [bmim][Cl] | nd | 30 wt % | nd | 28 |
| | [bmim][PF6] | nd | <1 wt % | nd | 28 |

**Table 43: Solubility of Disaccharides in ILs**

| carbohydrate | IL | water content | solubility | temperature (°C) | reference |
|---|---|---|---|---|---|
| **sucrose** | [amm110][N(CN)2] | nd | 3.5 wt % | 60 | 31 |
| | [bmmim][Cl] | nd | 14 wt % | 120 | 21 |
| | [bmim][Cl] | nd | 5 wt % | 70 | 46 |
| | [bmim][Cl] | nd | 18 wt % | 110 | 21 |
| | [bmim][BF4] | nd | 0.5 g/L | 25 | 53 |
| | [bmim][BF4] | nd | 0.6 g/L | 60 | 53 |
| | [bmim][CF3SO3] | nd | 2.0 g/L | 25 | 53 |
| | [bmim][CF3SO3] | nd | 5.3 g/L | 60 | 53 |
| | [bmim][N(CN)2] | <500 ppm | 195 g/L | 25 | 26 |
| | [bmim][N(CN)2] | <500 ppm | 282 g/L | 60 | 26 |
| | [emim][BF4] | nd | 0.6 g/L | 25 | 53 |
| | [emim][BF4] | nd | 0.6 g/L | 60 | 53 |
| | [emim][CF3SO3] | nd | 3.1 g/L | 25 | 53 |
| | [emim][CF3SO3] | nd | 7.1 g/L | 60 | 53 |
| | [emim][CH3SO3] | nd | 12.4 g/L | 25 | 53 |
| | [emim][CH3SO3] | nd | ~8 wt % | 75 | 54 |
| | [emim][N(CN)2] | nd | ~10 wt % | 75 | 54 |
| | [hmim][Cl] | nd | 5 g/L | 22 | 54 |

| | | | | | |
|---|---|---|---|---|---|
| | [EtOEtmim][N(CN)2] | <500 ppm | 50 g/L | 25 | 26 |
| | [EtOEtmim][N(CN)2] | <500 ppm | 240 g/L | 60 | 26 |
| | [MeOEtmim][BF4] | <500 ppm | 0.4 g/L | 25 | 26 |
| | [MeOEtmim][N(CN)2] | <500 ppm | 220 g/L | 25 | 26 |
| | [MeOEtmim][PF6] | <500 ppm | 0.7 g/L | 25 | 26 |
| | [MeOEtmim][NTf2] | <500 ppm | 0.13 g/L | 25 | 26 |
| | [MeOEtmim][OTf] | <500 ppm | 2.1 g/L | 25 | 26 |
| | [MeOMemim][N(CN)2] | <500 ppm | 249 g/L | 25 | 26 |
| | [MeOMemim][N(CN)2] | <500 ppm | 352 g/L | 60 | 26 |
| | [Me(OEt)3-Et3N][CH3COO] | nd | 16 wt % | 60 | 31 |
| **lactose** | [bmim][N(CN)2] | <500 ppm | 51 g/L | 25 | 26 |
| | [bmim][N(CN)2] | <500 ppm | 225 g/L | 75 | 26 |
| | [Bt14][CH3SO3] | nd | ~8 wt % | 75 | 54 |
| | [Bt14][N(CN)2] | nd | ~8 wt % | 75 | 54 |
| | [Bt1Bn][CH3SO3] | nd | ~2 wt % | 75 | 54 |
| | [Bt1Bn][N(CN)2] | nd | ~2 wt % | 75 | 54 |

**Table 44: Solubility of Monosaccharides in ILs**

| carbohydrate | IL | water content | solubility | temperature (°C) | reference |
|---|---|---|---|---|---|
| **arabinose** | [bmim][PF6] | nd | 0.25 M | 110−112 | 52 |
| | [omim][Cl] | nd | 0.25 M | RTa | 52 |
| **fructose** | [bmim][Cl] | nd | 5 wt % | 70 | 46 |
| | [bmim][Cl] | nd | 56 wt % | 110 | 21 |
| | [bmmim][Cl] | nd | 40 wt % | 120 | 21 |
| | [bmim][BF4] | nd | 3.3 g/L | 25 | 53 |
| | [bmim][BF4] | nd | 15.9 g/L | 60 | 53 |
| | [bmim][CF3SO3] | nd | 27.0 g/L | 25 | 53 |
| | [bmim][CF3SO3] | nd | 87.5 g/L | 60 | 53 |
| | [emim][BF4] | nd | 7.7 g/L | 25 | 53 |
| | [emim][BF4] | nd | 25.7 g/L | 60 | 53 |
| | [emim][CF3SO3] | nd | 32.8 g/L | 25 | 53 |
| | [emim][CF3SO3] | nd | 123.9 g/L | 60 | 53 |
| | [hmim][Cl] | nd | 62 g/L | 22 | 51 |
| **glucose** | [bmim][Cl] | nd | 5 wt % | 70 | 46 |
| | [bmim][BF4] | nd | 0.9 g/L | 25 | 53 |
| | [bmim][BF4] | nd | 2.7 g/L | 50 | 53 |
| | [bmim][BF4] | nd | 3.5 g/L | 60 | 53 |

| | | | | | |
|---|---|---|---|---|---|
| | [bmim][CF3SO3] | nd | 4.8 g/L | 25 | 53 |
| | [bmim][CF3SO3] | nd | 14.2 g/L | 50 | 53 |
| | [bmim][CF3SO3] | nd | 18.1 g/L | 60 | 53 |
| | [bmim][N(CN)2] | nd | 145 g/L | 25 | 53 |
| | [bmim][PF6] | nd | <0.5 g/L | 25 | 53 |
| | [emim][BF4] | nd | 1.1 g/L | 25 | 53 |
| | [emim][BF4] | nd | 4.8 g/L | 50 | 53 |
| | [emim][CF3SO3] | nd | 6.1 g/L | 25 | 53 |
| | [emim][CF3SO3] | nd | 27.8 g/L | 50 | 53 |
| | [emim][CH3SO3] | nd | 89.6 g/L | 25 | 53 |
| | [emim][CH3SO3] | nd | 133.2 g/L | 50 | 53 |
| | [emim][CH3SO3] | nd | ~10 wt % | 75 | 53 |
| | [emim][N(CN)2] | nd | >10 wt % | 75 | 54 |
| | [hmim][Cl] | nd | 44 g/L | 22 | 51 |
| | [omim][BF4] | nd | 0.7 g/L | 25 | 53 |
| | [omim][BF4] | nd | 1.5 g/L | 50 | 53 |
| | [Bt14][CH3SO3] | nd | ~6 wt % | 75 | 54 |
| | [Bt14][N(CN)2] | nd | >10 wt % | 75 | 54 |
| | [Bt1Bn][N(CF3SO2)2] | nd | ~2 wt % | 75 | 54 |
| | [Bt1Bn][N(CN)2] | nd | ~6 wt % | 75 | 54 |

| | | | | | |
|---|---|---|---|---|---|
| **α-glucose** | [MeOEtmim][Br] | 2.6 wt % | 450 g/L | nd | 27 |
| | [MeOMemim][Br] | 2.5 wt % | 450 g/L | nd | 27 |
| **β-glucose** | [Amm110][CH3COO] | nd | 30 wt % | 60 | 31 |
| | [Amm110][N(CN)2] | nd | 4.5 wt % | 60 | 31 |
| | [bmim][BF4] | <500 ppm | <0.5 g/L | 25 | 26 |
| | [bmim][N(CN)2] | <500 ppm | 145 g/L | 25 | 26 |
| | [bmim][N(CN)2] | <500 ppm | 211 g/L | 40 | 26 |
| | [bmim][N(CN)2] | <500 ppm | 405 g/L | 75 | 26 |
| | [bmim][PF6] | nd | <1 g/L | 55 | 49 |
| | [bmim][PF6] | <500 ppm | <0.5 g/L | 25 | 26 |
| | [bmim][NTf2] | nd | <0.5 g/L | 60 | 31 |
| | [emim][CH3COO] | nd | 60 wt % | 60 | 31 |
| | [EtOEtmim][BF4] | <500 ppm | 2.8 g/L | 25 | 26 |
| | [EtOEtmim][N(CN)2] | <500 ppm | 70 g/L | 25 | 26 |
| | [EtOEtmim][PF6] | <500 ppm | 0.7 g/L | 25 | 26 |
| | [EtOEtmim][NTf2] | <500 ppm | 0.5 g/L | 25 | 26 |
| | [Me(OEt)3eim][CH3COO] | nd | 80 wt % | 60 | 31 |
| | [Me(OEt)3Et3N][CH3COO] | nd | 16 wt % | 60 | 31 |
| | [Me(OEt)7eim][CH3COO] | nd | 26 wt % | 60 | 31 |
| | [Me(OPr)3eim][CH3COO] | nd | 45 wt % | 60 | 31 |

| | | | | | |
|---|---|---|---|---|---|
| | [MeOEtmim][BF4] | nd | 5 g/L | 55 | 49 |
| | [MeOEtmim][BF4] | <500 ppm | 2.8 g/L | 25 | 26 |
| | [MeOEtmim][N(CN)2] | <500 ppm | 91 g/L | 25 | 26 |
| | [MeOEtmim][PF6] | <500 ppm | 2.5 g/L | 25 | 26 |
| | [MeOEtmim][NTf2] | <500 ppm | 0.5 g/L | 25 | 26 |
| | [MeOEtmim][OTf] | <500 ppm | 3.2 g/L | 25 | 26 |
| | [MeOMemim][BF4] | <500 ppm | 4.4 g/L | 25 | 26 |
| | [MeOMemim][N(CN)2] | <500 ppm | 66 g/L | 25 | 26 |
| | [MeOMemim][NTf2] | <500 ppm | 0.5 g/L | 25 | 26 |
| | [MeOMemim][OTf] | <500 ppm | 4.3 g/L | 25 | 26 |
| **mannose** | [bmim][PF6] | nd | 0.25 M | 103−105 | 52 |
| | [omim][Cl] | nd | 0.25 M | RTa | 52 |
| **xylose** | [bmim][PF6] | nd | 0.25 M | 95−97 | 52 |
| | [hmim][Cl] | nd | 50 g/L | 22 | 51 |
| | [omim][Cl] | nd | 0.25 M | RTa | 52 |

# Abbreviations

AIR = Alcohol insoluble residue

AFEX = Ammonia fibre expansion

°C = Centigrade

DCM = Dichloromethane

DMC = Direct microbial conversion

DMSO = Dimethyl sulfoxide

DNS = Dinitrosalicyclic acid

FPU = Filter paper unit

GC = Gas Chromatography

HMF = 5-hydroxymethyl furfural

HPLC = High performance liquid chromatography

ILs = Ionic liquids

IR = Infrared

MBTH = 3-methyl-2-benzothiazolinone hydrazone

MC = Microcrystalline

Me = Methyl

Mins = Minutes

Mg = Milligrams

nmols = nanomoles

NMR = Nuclear magnetic resonance

$sCO_2$ = Supercritical Carbon dioxide

SEM = Scanning electron microscopy

SHF = Separate hydrolysis and fermentation

SSF = Simultaneous saccharification and fermentation

TBAF = Tetra-n-butylammonium fluoride

TEM = Transmission electron microscopy

TGA = Thermogravimetric analysis

W = Watts

Wt = Weight

XRD = X-ray diffraction

µmols = micromoles

**Ionic Liquids**

[AMIM] = Allylmethlyimidazolium

[B-2-MEA] = Bis-2-methoxyethylammonium

[BMIM] = *N*-butyl-*N*-methylimidazolium

[BMP] =*N*-butyl-*N*-methylpyrrolium

[BMPip] = *N*-butyl-*N*-methylpiperidinium

[BMPyr] = *N*-butyl-*N*-methylpyrrolidinium

[BP] = *N*-butylpyridinium

[BMMorph] = *N*-butyl-*N*-methylmorpholinium

[DEA] = Diethanolammonium

[DEP] = Diethylphosphate

[DiethylA] = Diethylammonium

[DMEA] = Dimethylethanolammonium

[DPA] = Dipropylammonium

[EA] = Ethanolammonium

[EMIM] = *N*-ethyl-*N*-methylimidazolium

[EthylA] = Ethylammonium

[HMIM] = *N*-hexyl-*N*-methylimidazolium

[NMM] = *N*-methylmorpholinium

[PA] = Propanolammonium

[TriethylA] = Triethylammonium

[$Tf_2N$] = Bistriflimide

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