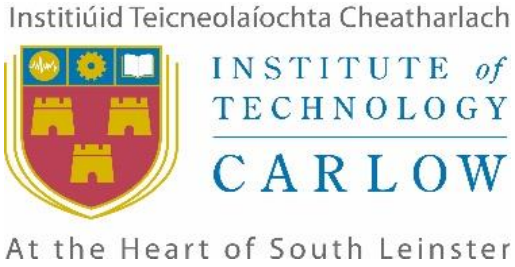
Coordinated Development of Leading Biomass Pretreatment Technologies for the Generation of Bioethanol from Irish Crops



By

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Declaration

I, the undersigned, certify that the information I have provided in this thesis is correct and that I have read and am aware of my responsibilities as detailed in Institute of Technology Carlow's Policy and Procedures for Postgraduate Research Students (Admissions, Registration, Supervision and Examination). I further confirm that I am unaware of any potential conflicts of interest that would compromise the Institute and / or the applicant in pursuit of the level 10 award sought.

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This is original work and all conclusions made are drawn from original work and data gathered by me.

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Abstract

Increased environmental awareness, coupled with increasing global energy demands, is facilitating the emergence of a green economy; a low carbon, resource efficient and socially inclusive economy aimed at reducing polluting emissions, preventing loss of biodiversity and valuing ecosystem services. Irish energy crops have the potential to contribute to the national green economy for the production of second generation biofuels provided improved lignocellulosic deconstruction processes can be identified and developed with the necessary economic and environmental-impact characteristics.

Four dedicated energy crops which can be grown in Ireland were selected for this study; switchgrass (*Panicum virgatum L*), miscanthus (*x-giganteus*), hemp (*Cannabis sativa L*) and willow (*Salix*). The primary aim was to explore, develop and compare biomass pretreatment approaches for these energy crops and to gain an insight into their potential for the production of second generation biofuels. The objectives of this study were to:

- Conduct a comprehensive review of leading biomass pretreatment technologies and select prospective approaches for the bioconversion of the Irish energy crops.
- Perform a comparative analysis of various chemical and enzymatic pretreatment approaches for lignocellulosic hydrolysis and bioethanol production using the four crops.
- Evaluate the economic performance of the targeted pretreatment chemicals.
- Conduct a Life Cycle Assessment (LCA) profile of the pretreatment technologies.

Chemical and enzymatic pretreatment was demonstrated to be crop specific. Pretreatments employing ammonia proved most effective for willow and hemp saccharification with yields of 59% and 35.7%, respectively. Sulphuric acid pretreatment generated highest saccharification yields for miscanthus at 41.5%, while methanol pretreatment generated the highest yields for switchgrass at 69%.

Through a series of process refinements and improvements, including the introduction of simultaneous saccharification and fermentation, these bioconversion yields significantly increased to 97% for switchgrass (methanol pretreatment), 80% for miscanthus (ammonia pretreatment), 98% for hemp (sulphuric acid pretreatment) and 99% for willow (ammonia pretreatment).

Assessment of the cost of switchgrass pretreatment demonstrated that methanol was the most efficient pretreatment chemical at €0.55 kg^{-1} glucose and €0.50 L^{-1} ethanol. This compares to sodium hydroxide at €2.52 kg^{-1} glucose and €1.96 L^{-1} ethanol; sulphuric acid at €2.41 kg^{-1} glucose and €1.83 L^{-1} ethanol; ammonia at €0.92 kg^{-1} glucose and €0.80 L^{-1} ethanol.

An LCA conducted for switchgrass pretreatment processes demonstrated that the environmental receptors are pretreatment-specific and that there is no one leading pretreatment technique. However, it is concluded that methanol generated the lowest emissions output contributing to the lowest Global Warming Potential. This is significant as methanol has the potential to be a leading pretreatment technology with commercial viability.

The research has shown that the pretreatment step can be optimised to increase the yield of ethanol from energy crops grown in Ireland while minimising environmental impact.

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List of Abbreviations

Chemicals

CH_3COOH – Acetic Acid

CH_3OH – Methanol

CMC – Carboxymethyl Cellulose

DNS – 3, 5-Dinitrosalicylic Acid

H_2SO_4 – Sulphuric Acid

KOH – Potassium Hydroxide

Na_2SO_3 – Sodium Sulphate

NaOCl – Sodium Hypochlorite

NaOH - Sodium Hydroxide

NH_3 – Aqueous Ammonia Solution

NH_4OH – Ammonium Hydroxide

$Ca(OH)_2$ – Lime

$CaCO_3$ – Calcium Carbonate

HCL – Hydrochloric Acid

N – Nitrogen

P – Phosphorus

Emissions

CO_2 – Carbon Dioxide

SO_2 – Sulphur Dioxide

CH_4 – Methane

PO_4 – Phosphate

$C_6H_4Cl_6$ – Dichlorobenzene

C_2H_4 – Ethylene

C_3H_6O - Acetone

C_6H_6 – Benzene

C_7H_8 - Toluene

VOC – Volatile Organic Compound

NMVOC – Non-Methane Volatile Organic Compound

NO_x – Nitrogen Oxide

N_2O – Nitrous Oxide

CFC – Chlorofluorocarbons

Methodology

LCA – Life Cycle Assessment

LCI – Life Cycle Inventory

LCIA – Life Cycle Impact Assessment

GHG – Greenhouse Gas

POD – Photochemical Oxidation Demand

GWP – Global Warming Potential

AP – Acidification Potential

EP – Eutrophication Potential

LHW – Liquid Hot Water

AFEX – Ammonia Fibre Explosion

ARP – Ammonia Recycle Percolation

SAA – Soaking Aqueous Ammonia

SSF – Simultaneous Saccharification and Fermentation

SHF – Separate Hydrolysis and Fermentation

SSFF – Simultaneous Saccharification Filtration and Fermentation

CMC – Carboxymethyl Cellulose

ASL – Acid Soluble Lignin

ASIL – Acid Insoluble Lignin

TFA – Trifluroacetic Acid

EMP – Embden – Meyerhof - Parnas

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Chapter 1. Introduction

1.1 General introduction and project objectives

The application of biotechnology to the production of commodity products relevant to the chemicals, food, materials and energy industries (the bio-economy) offers benefits in terms of sustainable resource supply and environmental quality. As discussed in more detail in Section 1.2, plant biomass (and derived waste products) represents both the dominant foreseeable source of feedstocks for biotechnological processes as well as the only foreseeable sustainable source of organic fuels. In the upcoming decades there will be a continuing worldwide shift away from near total dependence on fossil raw materials, towards a bio-economy in which plant-based feedstocks become the sources of fuels, chemicals and many manufactured goods.

The primary objective of this research study was to explore, define, develop and compare biomass pretreatment approaches for selected energy crops - switchgrass (*Panicum virgatum L*), miscanthus (*x-giganteus*), hemp (*Cannabis sativa L*) and willow (*Salix*). With the aim of providing further insight into the challenges for the economic / sustainable bioconversion of dedicated Irish energy crops grown at Teagasc, OakPark, Co. Carlow. Teagasc National Crop Research Centre in Carlow has many years of experience in the evaluation of crop species for bioenergy, examining plant suitability, growth, processing and end use. The Teagasc crops selected for this study are described in Section 1.3.

A review of current biomass conversion processes is presented in Section 1.4. The key challenge to commercialising the generation of biobased products is to reduce processing costs enough to ensure attractive investor returns and eventually compete with fossil-derived products without subsidies. Biomass must be pretreated in order to realise high yields vital to commercial success in biological conversion. Pretreatment is amongst the costliest steps and has a major influence on the cost of various pre- and post-operations. While many pretreatment options have been reported, comparisons are usually difficult due to differences in research methodologies and feedstock use.

The current study describes a co-ordinated approach to the investigation and development of leading biomass pretreatment technologies for dedicated Irish energy crops to yield comparative information on the performance of leading pretreatment options based on

defined process ingredients (including biocatalysts) and advanced bio-analytical methods. The pretreatment and bioconversion technologies targeted for assessment and development should be generally applicable to the generation of a wide range of bioproducts, although the initial focus in this study is on bioethanol production from lignocellulosic materials.

The specific objectives of this study may be summarised as follows:

1. Establishment of a comprehensive review of leading biomass pretreatment technologies and selection of prospective approaches for bioconversion of the Irish energy crops (Section 1.4; Chapter 3 and 4).
2. Performance of a comparative analysis of various chemical and enzymatic pretreatment approaches for lignocellulosic hydrolysis and bioethanol production using the four crops (Chapter 3).
3. Selection of one crop for more detailed investigation and analysis focused on bioconversion yield, cost and environmental impact (Chapter 4).
4. Creation of a Life Cycle Assessment (LCA) profile of the pretreatment technologies (Chapter 5).

Chapter 6 summarises the main conclusions arising from this study and future prospects for the research.

The research outcomes from this study have been presented in three manuscripts and submitted for publication in peer-review journals. These broadly aligned with Chapters 3, 4 and 5. One of these manuscripts has been published (Smullen et al., 2017a) and the remaining two are under review (Smullen et al., 2017b and c).

Preliminary results of the research have been presented at various conferences and these are as follow:

Smullen et al. (2015) A comparative analysis of pretreatment chemicals for the bioconversion of Willow, Miscanthus and Hemp. Aspects of Applied Biology, Biomass and Energy Crops V, 131, 169-173.

Smullen et al. (2015) Pretreatment chemicals for the bioconversion of Willow, Miscanthus and Hemp. Association of Applied Biologists, Biomass and Energy Conference, Brussels, Belgium, 20thth – 22nd October 2015.

Smullen et al. (2015) Bioconversion of energy crops and commercial cellulase preparations. Environment 2015, the 25th Irish Environmental Researchers' Colloquium, Institute of Technology Sligo, 8th – 10th April 2015.

Smullen et al. (2013) A comparative analysis of pretreatment techniques for Willow and Hemp. Environment 2013, the 23rd Irish Environmental Researchers' Colloquium, Ryan Institute, NUI Galway, 30th Jan. - 1st Feb. 2013.

Smullen et al. (2013) Development of bioconversion techniques for Irish grown Willow, Miscanthus and Hemp. EU 21st European Biomass Conference and Exhibition, Copenhagen Denmark, 3rd-7th June 2013.

1.2 The Bioeconomy

Since the late 18th century, the world has been heavily dependent on fossil fuels (coal, gas and oil), consuming them at an ever increasing rate in an attempt to meet energy demands (Escobar et al., 2009). Future fossil resources, however, are predicted to be of limited availability. Depleting resources, coupled with a continuously increasing population have challenged the security of fossil energy supplies (Rabemanolonsoa and Saka, 2016). In addition, environmental, economic and social issues brought on by the extensive use of fossil fuels have raised questions regarding the sustainability of fossil resources and the potential long term effects surrounding its environmental and economic performance (Rabemanolontsoa and Saka, 2016). Consequently, energy agencies and government bodies are encouraging the investigation and development of alternative energy sources which can sustain the ever increasing global population and future energy demand (Hamelinck et al., 2005; PEA, 2005).

Biotechnology offers technological solutions for many of the problems facing the world, resulting in the emergence of the "bioeconomy" (OECD, 2017). An economy founded on biomass instead of fossil fuels represents a significant shift in socioeconomic, agricultural, energy and technical systems (Mc Cormick and Kautto, 2013). Potential benefits from the transition to a bio-based economy include a reduction in greenhouse gas (GHG) emissions, decrease in dependence on fossil resources, wiser management of natural resources, and improved food security (Langeveld and Sanders, 2010). The bioeconomy by 2030 is likely to involve three main elements: advanced knowledge of genes and complex cell processes, renewable biomass, and the integration of

biotechnology applications across different sectors (OECD, 2017). For the bioeconomy to advance in the current energy market, further research, development and innovation is necessary.

In recent years, society has begun to recognise the opportunities offered by a future sustainable economy based on renewable sources and has started to finance research and development (R&D) activities for its implementation (Cherubini et al., 2010). Globally, the three main outputs of the bioeconomy are bioenergy, biofuels and biochemicals (Cherubini et al., 2010), produced using a fundamental technology known as biorefineries (Kamm and Kamm, 2004).

The use of biorefineries is not a totally novel concept, it is a relatively new field, introduced to replace traditional petroleum-based refineries (Mc Cormick and Kautto, 2013). Many attempts have been made to define the biorefinery concept. The International Energy Agency (IEA), has defined the biorefinery process as "the sustainable processing of lignocellulosic biomass, with the creation of various bio-based materials (food, feed, chemicals, and materials) and bioenergy (biofuels, power and/or heat)" (IEA Bioenergy, 2009), providing significant versatility and options in the utilisation of biomass.

The biorefinery process has two main objectives: (1) fractionation of the three main components of lignocellulosic biomass for further conversion to biobased products (chemical building blocks, detergents, pulp and paper etc.), and (2) the production of primary biofuels (bioethanol, biodiesel and biogas) for the transport sector (Hayes, 2013; Kim et al., 2016). It is anticipated that biorefinery technology will play a leading role in creating a new biobased industrial sector with the aim of replacing fossil based fuels, chemicals and oil, subsequently reducing GHG emissions (Park and Kim et al., 2012). The European Commission has estimated that a shift to biological raw materials and biological processing materials could save up to 2.5 billion tons of CO_2 equivalents per year by 2030 (EC, 2016).

The main biobased products are obtained from the conversion of biomass to basic products such as: starch oil, cellulose, lactic acids, adhesives, detergents and dyes (Cherubini et al., 2010). Biobased chemical production (glycerol, sorbitol, levulinic acid, aspartic acid), is challenged by a lack of conversion technology (Bozell and Petersen, 2010). Conversion of renewable carbon is the least developed and most complicated of

all biorefinery operations. Subsequently, as the biorefinery industry has expanded over the years, its focus has been almost exclusively on single product operations producing bioethanol and biodiesel (Bozell and Petersen, 2010).

The efficient production of transportation biofuels is seen as one of the main promoting factors for the future development of biorefineries (Cherubini and Strømman, 2010). For this reason, research at Institute of Technology Carlow focused on the generation of biofuels, specifically bioethanol, from Irish crops. Although, it must also be noted that several related by-products such as lignin can be extracted from the process and used in other biorefinery operations (Bozell and Petersen, 2010). Lignin removed from the lignocellulosic complex has been used in many different industries including the pulp, paper and plastic industries. Vanillin (Bjørsvik and Minisci, 1999), Bakelite (hard plastic used as utensil handles), resins and filter materials are just some of the by-products produced from the resulting lignin (Pandey and Kim, 2011; Matson et al., 2011).

Bio-based transport fuels are generally categorised into three groups (First, second and third generation biofuels) of which there are three main kinds of fuel.

- Bioethanol, the most widely utilised biofuel worldwide, is used as a blending agent in gasoline or as an E85 fuel (ethanol fuel blend of up to 85%, by volume, denatured ethanol fuel, together with gasoline or other hydrocarbons).
- Biodiesel, derived from plant oil (palm, rape, sunflower and soy oil), waste oil, and from tall oil (a by-product of the Kraft process of wood pulp production).
- Biogas, created from the fermentation of organic matter, including domestic, farm and food industry waste (EASAC, 2012).

Other less significant biofuels are also being produced worldwide including bio-methanol, vegetable oils, biosynthetic gas, bio-oil, bio-char, Fischer-Tropsch liquids, and bio-hydrogen (Balat, 2011).

1.2.1 First Generation Biofuels

First generation biofuels are produced from food crops (Mc Cormack and Kautto, 2013); sugarcane ethanol in Brazil, corn ethanol in the United States, oilseed rape biodiesel in Germany and palm oil biodiesel in Malaysia (Sims and Taylor, 2008). Of these, ethanol is the leading biofuel produced around the world and is steadily increasing, with countries such as France, China, and Canada now producing ethanol using feedstocks such as

wheat, cassava, and sorghum juice (Timilsina and Shrestha, 2011; Balan, 2014). However, their ability to achieve targets for oil-product substitution, climate change mitigation and economic growth is limited. In recent years, the sustainability of first generation biofuels has been debated, with reports of:

- Increased food prices due to competition with food crops,
- Increased cost, both in the initial investment (excluding government grants and subsidies) and on the global biofuels market when compared with traditional petroleum based fuels,
- Significant differences in the expected environmental benefits and the actual benefits achieved due to differences in production methodology, and
- The potential negative impact on biodiversity and available water resources (Sims and Taylor, 2008).

The cumulative impacts of these concerns have increased interest in further developing biofuels produced from non-food crops (Sims and Taylor, 2008).

1.2.2 Second Generation Biofuels

Many of the issues associated with first generation technologies can be addressed by the use of second generation technologies. Second generation biofuels are based on non-food crops, such as lignocellulosic biomass (agricultural residues, municipal solid wastes and energy crops) (Melligan et al., 2012; Mc Cormack and Kautto, 2013). Lignocellulosic biomass is advantageous in the production of biofuels as it:

- Does not compete for land and has been demonstrated to grow on poor and degraded soil,
- The energy yield achieved is significantly higher than that of 1st generation biofuels (Sims and Taylor, 2008), and
- 2nd generation biofuels have been shown to have a positive environmental impact as the feedstocks themselves are carbon neutral (Zhang et al., 2013).

Biofuels produced using 2nd generation technologies face certain technical barriers in their commercialisation (Zabed et al., 2016). These include the necessity of energy consuming pretreatment processes, diversity in the nature and composition of lignocellulosic biomass, the inability of natural microorganisms to ferment the resulting

monomeric sugars and the formation of inhibitors (Taha et al., 2016; Tye et al., 2016; Paulová et al., 2013).

Numerous efforts have been made in recent years to overcome these barriers and attain sustainability in lignocellulosic biofuel production. While 2nd generation biofuels are relatively immature, they have the potential for further investigation and development (Sims and Taylor, 2008). Significant investment in pilot and demonstration facilities in both the United States and Europe have given rise to the expectation that, in the near future, 2nd generation biofuels will reach full commercialisation (Zabed et al., 2016; Balan, 2014; Sims and Taylor, 2008).

1.2.3 Third Generation Biofuels

Third generation biofuels are derived from microalgae. Considered an ideal biofuel feedstock because of their rapid growth rate, greenhouse gas fixation ability and high production capacity of lipids, microalgae production is seen as a feasible alternative to 1st and 2nd generation biofuels (Nigam and Singh, 2011; Dragone et al., 2010).

Microalgae can provide several different types of renewable biofuels, including methane (Spolaore et al., 2006), biodiesel (Gavrilescu and Chisti, 2005), and bio-hydrogen (Kapdan and Kargi, 2006). However, disadvantageously, microalgae are unable to produce bioethanol, which is the main biofuel produced and consumed globally (Limayem and Ricke, 2012).

Production of microalgae biofuels have both advantages and disadvantages, for example, microalgae can produce 15-300 times more biodiesel than traditional crops on area basis (Dragone et al., 2010). However, microalgae biomass production is more expensive and technologically more challenging than growing crops, with successful production of microalgae relying on strict temperature control as well as freely available sunlight and natural light intensities (Christi, 2007). The commercial viability of 3rd generation biofuels is unlikely in the near future due to the numerous challenges that face this extremely expensive process (Alam et al., 2015; Lardon et al., 2009).

It is evident from the literature that second generation biofuels will be the main driver of the transition from a petro-economy towards the worldwide bioeconomy. For the immediate future, and up to the EU target date of 2020, it is likely that 1st generation biofuels will play a major part in biofuel supply (EASAC, 2012). However, recent

revisions approved by the EU of the 2009/28/EC directive will soon see an enforced production cap of 7% on 1st generation biofuels to allow for the development and expansion in the 2nd generation biofuel market (Sebastião et al., 2016).

1.3 Lignocellulosic Biomass for 2nd Generation Biofuel Production

Lignocellulosic biomass is the most abundant organic material in nature. Relatively distributed worldwide, approximately 10-50 billion dry tons are produced annually (Zhao et al., 2009). In 2012, the total amount of feedstock available for liquid biofuels was 341 million tons, of which 70% came from agricultural residues and 30% from forest residues (Balan, 2014).

Lignocellulosic biomass can be categorised into four major groups based on its source:

- Woody biomass,
- Agricultural residues (wheat and barley straw, corn stover, sugarcane bagasse),
- Energy crops (willow, poplar, switchgrass, miscanthus, canary reed grass and hemp),
- Municipal solid wastes (Kim et al., 2016).

It is anticipated that an estimated 422 billion litres of bioethanol can be produced each year using lignocellulosic biomass if total crop residues and wasted crops are considered (Kim and Dale, 2005; Sarkar et al., 2012).

The use of energy crops for second generation biofuel production was explored in this research study. Energy crops are a "novel" source of lignocellulosic biomass which remain relatively unexplored compared to more traditional feedstocks such as wheat and barley. Switchgrass (*Panicum virgatum L*), miscanthus (*x-giganteus)*, hemp (*Cannabis sativa L),* and willow (*Salix*) a woody hardwood crop, have received a lot of attention in recent years and subsequently were selected for investigation in the current study.

1.3.1 Switchgrass (Panicum virgatum L)

Switchgrass (*Panicum virgatum L*) (Figure 1.1) is a summer perennial grass native to North America (Hadar, 2013). It is a natural component of the tall grass prairie, rich in hollocellulose (cellulose and hemicellulose), capable of growing throughout the country in a range of climates, resistance to drought, pest and plant diseases. It can thrive well on

degraded or contaminated soils, while also helping to reduce soil erosion (Nlewen and Thrash, 2010). Switchgrass is self-seeding and self-regenerating, and can produce high yields of approximately 27.4 ton ha^{-1} (Mc Laughlin and Kszos, 2005), with low applications of fertiliser and other chemicals (Parrish and Fike, 2009). More importantly, switchgrass is considered an environmentally beneficial feedstock, trapping CO_2 in the ground (Pimentel and Patzek, 2005) resulting in carbon neutrality.

Figure 1.1: Switchgrass (*Panicum virgatum L*) grown at Knockbeg, Co. Laois.

1.3.2 Miscanthus (x-giganteus)

Miscanthus (*x-giganteus*) is a typical C4 perennial grass species with lignified stems resembling bamboo and which has great potential as a leading sustainable energy crop (Lewandowski et al., 2003; Figure 1.2).

Figure 1.2: Miscanthus (*x-giganteus*) grown at Teagasc Oakpark Carlow.

Miscanthus originates from East Asia and can grow up to 3.5 metres tall with little water or fertiliser inputs. It is similar to switchgrass with respect to cold and drought tolerance and water use efficiency (Ng et al., 2010). Demonstrated to produce yields of 38 ton ha^{-1} per annum in Europe with an approximate cellulose content of 40%, it has an estimated life time of 20-25 years (Lewandowski et al., 2003). Miscanthus is commonly used as a raw material in building materials, geotextiles and paper and packaging industries (Visser, 2001). Identified as an ideal feedstock for energy production, miscanthus offers many environmental benefits including; climate, soil, biodiversity and bioremediation (Chandel et al, 2011).

1.3.3 Hemp (Cannabis sativa L)

Hemp (*Cannabis sativa L*) is one of the oldest annual crops in the world, traditionally grown for its long bast fibres (Karus, 2002). After revoking the hemp prohibition in the European Union, cultivation of industrial hemp for energy purposes was approved (EC, 2003), subject to strict regulations. Hemp fibres are currently being used in many industries including the textile and paper industries (Harris et al., 2008). Cultivated in various climates, hemp is drought tolerant, and can reach high biomass yields, up to 20-23 dry ton ha^{-1} (Struik et al., 2000) (Figure 1.3). Chemical inputs for the cultivation of hemp are significantly reduced as it can be grown in nutrient deficient soils and has the ability to over grow weeds. In addition, hemp also acts as an excellent break crop as its extensive root system improves the soil structure (Bosca and Karus, 1997).

Figure 1.3: Hemp (*Cannabis sativa L)* grown at Teagasc Oakpark Carlow.

1.3.4 Willow (Salix)

Fast growing short rotation feedstocks such as willow can play an important role in bioenergy production. Willow (*Salix*) is a hardwood species which originates in the Northern hemisphere (Perlack et al., 2005) (Fig. 1.4). It is not a demanding species, willow will flourish on a wide range of soils and environmental conditions. As an annual crop, willow may be harvested 6 to 8 times on a 3 year cycle, giving a plantation life of 19-25 years (Caslin et al., 2010), and is generally a high yielding crop. A wide range of yields can be expected depending on site and weather conditions, but an annual yield ranging from 7 to 12 ton dry matter ha^{-1} can be expected (Caslin et al., 2010). In addition, willow is virtually carbon neutral, sequestering significant quantities of carbon in their roots (Caslin et al., 2010).

Figure 1.4: Willow (*Salix*) grown at Teagasc Oakpark Carlow.

Similar to other sources of lignocellulosic biomass energy crops are typically comprised of cellulose (38-50%), hemicellulose (23-32%), and lignin (15-25%), as well as small amounts of extractives (Mc Kendry, 2002) (Fig. 1.5). Cellulose and hemicellulose are chain polysaccharides, with similar polysaccharide structures to starch (Sun et al., 2016). They typically make up two-thirds of cell wall dry matter, and can be hydrolysed to sugars and then fermented to bioethanol (Balat, 2011). Lignin is also heterogeneous, and is a cross-linked three-dimensional phenyl-propane polymer, which closely associates with cellulose and hemicellulose (Mussatto et al., 2008). These components are strongly inter-meshed and bonded through covalent or non-covalent bonds forming the lignocellulosic

matrix (Sun et al., 2016), which is naturally recalcitrant (Mosier et al., 2005). Unfortunately, lignin cannot be used for bioethanol production (Balat, 2011).

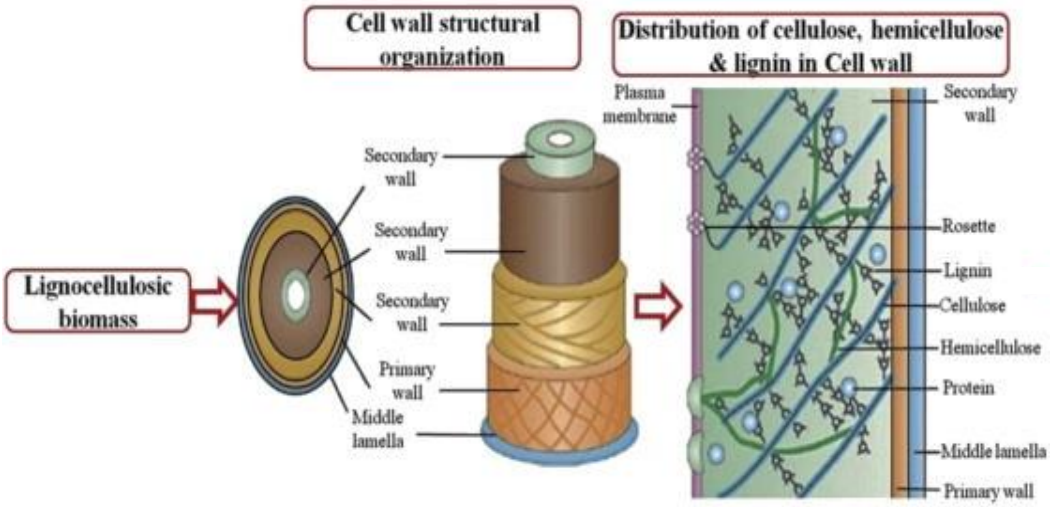


Figure 1.5: The structural composition of lignocellulosic biomass (Anwar et al., 2014, adapted by Menon and Rao, 2012).

1.3.5 Cellulose

Cellulose is composed of a linear chain of β-1, 4 linked D-glucose units with a degree of polymerisation (n) ranged from several hundreds to over ten thousand (Fig. 1.6). Consisting of carbon (44.44%), hydrogen (6.17%) and oxygen (49.39%), it is the most abundant organic polymer on the earth (Chen, 2014; Sun et al., 2016), and the main component of the plant cell wall (Agbor et al., 2011). Because of the polysaccharide structure of cellulose, a large amount of hydroxyl groups exists along the cellulose backbone. Every glucosyl ring of cellulose has three active hydroxyls: one primary hydroxyl group and two secondary hydroxyl groups (Chen, 2014). These hydroxyl groups can form well-ordered hydrogen bonding networks, which enforce the linear integrity and rigidity of the cellulose molecule, resulting in a packed crystalline structure (Chen, 2014; Sun et al., 2016). The repeating unit of the cellulose chain is the disaccharide cellobiose as opposed to glucose in other glucan polymers (Desvaux, 2005). Additionally, partial cellulose chains are arrayed irregularly, resulting in the amorphous region of the cellulose,

forming cellulose fibres. This specific structure makes it water insoluble and resistant to depolymerisation (Mosier et al., 2005). Subsequently, acid, microbial and/or alkaline degradation is necessary for the production of cellulose products and by-products (Chen, 2014).

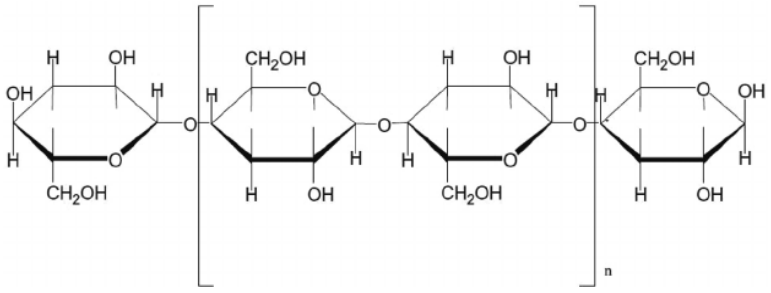


Figure 1.6: Schematic structure of cellulose with cellobiose as repeating unit (Akil et al., 2011).

1.3.6 Hemicellulose

Compared to cellulose, hemicellulose is a heteropolymer consisting of short linear and highly branched chains of several monomers (Zabed et al., 2016), and is the second most abundant polymer (Agbor et al., 2011). The content and composition of hemicelluloses can vary with different plants and their parts. In most grasses and hardwoods, xylan is the primary hemicellulose (polymer of xylose), which mainly contains β-D-xylopyranosyl residues linked by 1, 4 glycosidic bonds (Sun et al., 2005). The major monomers in hemicellulose include hexoses (β-D-glucose, α-D-galactose and β-D-mannose), and pentoses (β-D- xylose and α-L-arabinose). Certain sugar acids, namely uronic acids may also be present in a typical hemicellulose molecule. Sometimes other sugars including α-L-rhamnose and α-L-fructose are present in small quantities when the hydroxyl groups of sugars is partially substituted with acetyl groups (Gírío et al., 2010). Hemicelluloses are more readily hydrolysed compared to cellulose because of its branched, amorphous nature (Lee et al., 2007). The dominant sugars in hemicelluloses are mannose found in softwoods and xylose the main hemicellulose in hardwoods, forest wastes, agricultural residues and municipal and industrial wastes (Limayem and Ricke, 2012; Gírío et al., 2010; Taherzadeh and Karimi, 2008) (Fig. 1.7).

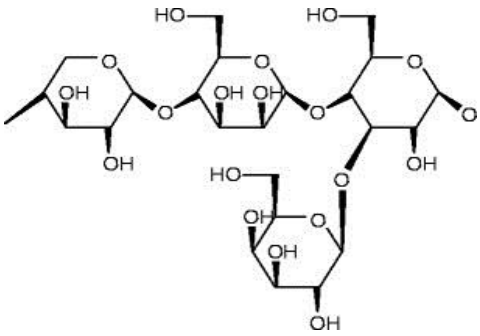


Figure 1.7: Schematic structure of hemicellulose (Kaith, 2011)

1.3.7 Lignin

Lignin, the third most abundant polymer in nature, is a heterogeneous three-dimensional network macromolecule mainly constructed from the oxidative combinatorial coupling of *p*-hydroxycinnamyl alcohol monolignols (Ralph et al., 2004) (Fig. 1.8). Lignin is a highly branched macro-nuclear aromatic polymer, present in the cell wall of certain biomass, particularly woody biomass, and is frequently adjacent to cellulose fibres to form a lignocellulosic complex (Drummond and Drummond, 1996). Because of its close association with cellulose microfibrils, lignin has been identified as a major deterrent to enzymatic and microbial hydrolysis of lignocellulosic biomass (Avgerinos and Wang, 1983). It has been shown that the removal of lignin prior to hydrolysis can significantly enhance the digestibility of the biomass (Chang and Holtzapple, 2000; Alvira et al., 2010). Different feedstocks contain different amounts of lignin. Softwood barks have the highest level of lignin (30-60%) followed closely by the hardwood barks (30-55%), while grasses and agricultural residues contain the lowest level of lignin (10-30% and 3-15%, respectively) (Limayem and Ricke, 2012).

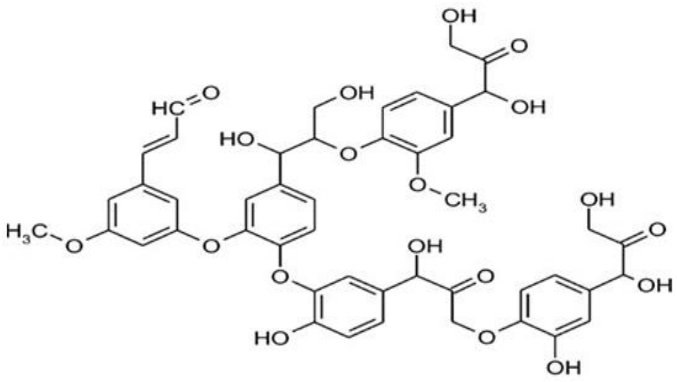


Figure 1.8: Schematic structure of lignin (Rangaswani and Bagyaraja, 1993)

1.4 Bioconversion of Lignocellulosic Biomass for Ethanol Production

The cell wall of lignocellulose is highly resistant towards chemical and biological degradation (Malinovsky et al., 2014; Hayes et al., 2015), and subsequently requires a series of conversion steps to disrupt its primary goal, protecting the cell wall from microbial attack (Malinovsky et al., 2014). Its complex structure is related to the presence of lignin (Grabber et al., 2008), the degree of crystallinity (Park et al., 2010), the degree the polymerisation of the polysaccharides (Merino and Cherry, 2007), available surface area and moisture (Hendriks and Zeeman, 2009). It is not only the presence of lignin that dictates the recalcitrance, the amount of ferulate cross-linking in lignin also has an impact on recalcitrance, as well as the subunit composition of the lignin and the degree of ester linkages between the lignin and carbohydrates (Chandra et al., 2007; Grabber et al., 2008). Furthermore, challenges faced by enzymes to act on an insoluble substrate and inhibitors generated during the conversion process may contribute to the recalcitrance of lignocellulosic biomass to enzymes (Himmel, 2007).

Overcoming the recalcitrant nature of lignocellulosic biomass requires a stringent and logical process of conversion, either biochemical or thermo-chemical to break down the lignin structure and disrupt the hemicellulose / cellulose matrix. It is the necessity for an effective and efficient conversion process which lead to this research study. In general, the process involves three stages: pretreatment, hydrolysis and fermentation. In this study, a particular focus was placed on the development and optimisation of the pretreatment process which has a significant impact in the economics of the overall bioconversion process and all subsequent downstream processes tailored to the pretreatment results (Kim et al., 2013).

1.4.1 Pretreatment of Lignocellulosic Biomass

Pretreatment is the most widely researched parameter of the bioconversion process and can be retraced back as far as 1819 (Braconnot, 1819). It was regularly employed in wood science for pulp and paper fabrication, and also in agricultural and crop science to increase the digestibility of forage by ruminants (Braconnot, 1819; Rabemanolontsoa and Saka, 2016). Since then, various pretreatment technologies (physical, chemical and biological), have been proposed and developed (Hayes, 2009), with the aim of challenging the complexity of the biomass structure and the formation of potential degradation products (Rabemanolontsoa and Saka, 2016).

In recent years, numerous research and review articles have been written, focusing on the identification, evaluation and demonstration of promising approaches. Research attention in these studies (Sassner et al., 2008; Gupta and lee, 2010; Yu et al, 2013; Zhang et al., 2016), have been particularly focussed on the enhancement of lignocellulose digestibility for efficient conversion of cellulose to ethanol (Menon and Rao, 2012). These and other related studies have highlighted the necessity for knowledge and understanding of the complex nature of lignocellulosic biomass in order to design a suitable pretreatment technique, while also taking into consideration the selection and optimisation of chemical parameters for the pretreatment technology, which can affect the configuration of the process, cost and global application too (Rabemanolonsoa and Saka, 2016).

One objective of this study was to explore potential pretreatment technologies available, and to create a comprehensive review of established techniques; allowing for the unique approach to the selection and ease of choice for different pretreatment techniques for a given application. This review synthesises the technical evolution and recent developments of the most promising pretreatment technologies, providing a comprehensive assessment of each chemical, biological and physicochemical procedure. Factors affecting the pretreatment process, including effects on feedstock physicochemical structure, potential degradation product formation, known energy, economic and environmental inputs and outputs, as well as the advantages and disadvantages of different procedures were evaluated. Furthermore, to enhance the exclusivity of this work, the chosen pretreatment technologies for investigation in this thesis were further assessed for their effects on selected dedicated energy crops, willow, miscanthus, hemp and switchgrass.

1.4.1.1 Selection of Pretreatment Technologies

The quality of biofuel produced is majorly dependent on the production routes (Aditiya et al., 2016). Selected methods from available technologies will give different outcomes, have varying advantages and disadvantages, while also having substantial differences among their effects on the physicochemical structure of the feedstock. Choice of pretreatment technology is critical to the successful conversion of cellulose to fermentable monomeric sugars. There are several key features for the effective pretreatment of lignocellulosic biomass which must be considered:

- Improvement in the enzymatic hydrolysis reaction,

- Production of minimal / no inhibitory compounds,
- Minimising the need for sample preparation prior to pretreatment, for example size reduction,
- Low energy demand, cost and consumable input (Chandra et al., 2007; Kim et al., 2013).

Additionally, key properties of the pretreatment process must also be considered:

- High yields for multiple crops, sites ages and harvesting times. Various pretreatments have been shown to be suited to different feedstocks. Our own studies (Chapter 3 and 4) have demonstrated that pretreatment is crop specific.
- Highly digestible pretreated solid; the cellulose content of the chosen feedstock should be significantly high, thus pretreatment of the cellulose should exhibit yields greater than 90% (Yang and Wyman, 2008). While the concentration of monomeric sugars succeeding pretreatment and enzymatic hydrolysis should be above 10% to ensure that ethanol concentrations are adequate to keep recovery and other downstream costs manageable (Yang and Wyman, 2008).
- Non-production of waste products / residues; chemical, feedstock and waste water are all substantial contributors to the cost of waste disposal for 2nd generation technologies (Alvira et al., 2010). The ability to reduce, recover and reuse inputs and outputs of the system is necessary for economic and environmental viability (Bensah and Mensah, 2013).
- Effectiveness at low moisture content; the moisture content of different feedstocks can vary sizeably depending on the feedstock type, cultivation inputs and environmental conditions during production (Chapter 3).
- Fermentation compatibility; the distribution of sugar recovery between pretreatment and subsequent enzymatic hydrolysis should be compatible with the choice of microorganism chosen for fermentation to ethanol (Alvira et al., 2010).
- Lignin recovery; in recent years, lignin and other usable constituents have been recovered from the system, for conversion into valuable by-products (see Section 1.2) and for the simplification of downstream processing (Yang and Wyman, 2008).

1.4.1.2 Pretreatment Technologies

Pretreatment can be classified into several categories: physical (milling, grinding and irradiation), biological (microorganisms and fungi), chemical (acid, alkaline, organic solvents and ozonolysis), and physico-chemical (steam explosion / autohydrolysis and wet oxidation) (Balat et al., 2011).

1.4.1.2.1 Physical Pretreatment

Aim: To reduce biomass particle size and crystallinity of lignocellulosic biomass in order to increase the specific surface area and reduce the degree of polymerisation (Hendriks and Zeeman, 2009; Bhutto et al., 2017).

Classification of Physical Operations: Mechanical operations (chipping, grinding and milling), and irradiation (gamma rays, electron beams and microwaves) (Taherzadeh and Karimi, 2008).

Effect on Feedstock Physicochemical Properties: Mechanical comminution such as chipping, shredding, grinding and milling have been used to enhance the digestibility of lignocellulosic biomass (Palmowski and Muller, 2000; Taherzadeh and Karimi, 2008; Kumar et al., 2009; Balat, 2011). These treatments have been employed to increase the available specific surface area and reduce both the degree of polymerisation (DP) and cellulose crystallinity (Sun and Cheng, 2002) of the cellulosic biomass. Resulting in a reduction of biomass particle size, the substrate is rendered more amenable to subsequent enzymatic hydrolysis (Balat, 2011).

Among these mechanical comminution processes, chipping and milling are commonly applied to hardwood lignocellulosic biomass (Taherzadeh and Karimi, 2008). Chipping reduces particles to 10-30 mm, while milling has the potential to reduce particle size to 0.2-2 mm (Kumar et al., 2009; Leustean, 2009) through employing various milling methods (Taherzadeh and Karimi, 2008). Milling can often be demonstrated as a more effective process of reducing particle size and crystallinity compared to chipping due to the shear force generated during milling (Agbor et al., 2011).

Irradiation is another process employed as a method of physical pretreatment, utilised in the treatment of biomass with high energy radiation including gamma rays, ultrasound,

electron beam, pulsed electrical field, UV and microwave heating (Alvira et al., 2010; Elgharbawy et al., 2016). Irradiation is limited in its ability to remove hemicellulose or lignin from lignocellulosic materials or reduce their particle size. Therefore is employed solely for the assistance of other pretreatment techniques (da Sousza Moretti et al., 2014).

Degradation Products: There are no known degradation products reported in the literature as a result of physical pretreatment (mechanical and irradiation).

Known Energy Requirements: The most challenging characteristic of mechanical comminution is its vast power consumption. Physical reduction has been reported as using one-third of the total energy consumption of the entire bioethanol production process (Aden et al, 2002). For hardwood species, 0.14 kW h kg^{-1} of biomass is required to reduce the particle size to 1.6 mm. To reduce the size of corn stover to 1.6 mm, 0.02 kW h kg^{-1} of energy is required (Ruffell, 2008). Power consumption for mechanical comminution can be controlled by adjusting the sizes of the initial feedstock input and desired substrate output. Some studies have shown irradiation to be too energy intensive (Zheng et al., 2009) for commercial viability and so has remained largely unutilised with specific process parameters unknown.

Process Economics: Operation / maintenance costs of physical pretreatment are significantly high due to the increased energy demand of the process. Hendriks and Zeeman (2009), have reported that it is unlikely that mechanical comminution will be economically feasible for commercial application. Irradiation has also been reported as being far too expensive for wide application (Zheng et al., 2009).

Environmental Evaluation: Input energy for a selected pretreatment technology accounts for the highest contribution to its environmental impact (Passos et al., 2014). Physical pretreatments operate solely on large energy inputs to the system with little energy output. Based on lab-scale pretreatments, physical pretreatment results in negative energy balances, partly due to low solid concentrations in the lignocellulosic biomass (Choi et al., 2013; Passos et al., 2013; Passos et al., 2014). Some techniques such as irradiation have been described by researchers as non-environmentally friendly and commercially unfeasible (Zheng et al., 2009; Alvira et al., 2010; Elgharbawy et al., 2016).

Selection of Suitable Feedstock: Physical pretreatment is suitable for all hardwood and softwood feedstocks.

Advantages and Disadvantages: There are several advantages and disadvantages to physical pretreatment. These have been widely investigated in many studies over the years (Balat, 2011; Zabed et al., 2016; Sun et al., 2016), and are summarised in Table 1.1.

Table 1.1 A comparative summary of the advantages and disadvantages of physical pretreatment.

Physical Pretreatments	Advantages	Disadvantages
Mechanical Comminution	Reduces size and degree of cellulose crystallinity (1,2)	Power consumption usually higher than inherent biomass energy (1,2)
	Increase in surface area (2-4)	No lignin removal (2-4)
	Reduction in degree of polymerisation (DP) (2-4)	
Irradiation	Increases the rate of enzymatic hydrolysis (5)	Excessive irradiation dose reduces the glucose yield (5)
	Partial lignin degradation (2-4)	Expensive (2-4)
	Increased surface area (2-4)	Long residence time – slow rate of reaction (2-4)
	Reduction in cellulose crystallinity and degree of polymerisation (2-4)	Increases degradation of polysaccharides which leads to loss of yield (5)
		Not environmentally friendly (2-4)

(1) Kumar et al., 2009, (2) Alvira et al., 2010, (3) Elgharbawy et al., 2016, (4) Zheng et al., 2009, (5) Butto et al., 2017.

1.4.1.2.2 Biological Pretreatment

Aim: To employ microorganisms found in nature to degrade the lignin and hemicellulose in lignocellulosic biomass, with partial degradation of cellulose (Sun and Cheng, 2002; Sindhu et al., 2016).

Classification of Microorganisms: *Phanerochaete chrysosporium* (Potumarthi et al., 2013), *Pleurotus ostreatus* (Castoldi et al., 2014), *Ceriporiopsis subvermispora*

(Cianchetta et al., 2014), *Irpex lacteus* (Du et al., 2011), *Punctualaria sp.* TUFC20056 (Suhara et al., 2012), *Cyathus stercolerus* and *Pleurotus ostreaus* (Kumar et al., 2009), *Ceriporia lacerate* (Alvira et al., 2010).

Effect on Feedstock Physicochemical Properties: Biological pretreatments employ microorganisms, mainly brown, white and soft-rot fungi, which alter the structure of lignin and hemicellulose and separate them from the lignocellulosic matrix. This results in their degradation with minimal disruption to cellulose, which is more resistant than any other component (Sánchez, 2009). Brown-rot fungi primarily attack cellulose, whereas white and soft-rot fungi are more effective on lignin and hemicellulose via the production of enzymes (lignin peroxidases, polyphenol oxidases, and laccases which degrade lignin) (Sun and Cheng, 2002; Agbor et al., 2011). Fungi breakdown lignin anaerobically using a family of extracellular enzymes collectively termed *lignases*. In addition to the nature and composition of the biomass, other process parameters such as microorganism type, incubation time, incubation temperature and pH and aeration rate affect the performance of biological pretreatment (Sindhu et al., 2016). Many researchers (Magnusson et al., 2008; Agbor et al., 2011; Shirkavand et al., 2016) have suggested developing an efficient and effective combined pretreatment technique to aid in the optimisation of the process and eliminate some of the potential drawbacks of the process.

Degradation Products: There are no known degradation products reported in the literature as a result of biological pretreatment.

Known Energy Requirements: Important process parameters affecting biological pretreatment include:

- Incubation time: 10-14 days (Agbor et al, 2011)
- Incubation temperature: 39^{o}C (White-rot fungi) (Sindhu et al., 2016)
- Incubation pH: 4.0-5.0 (Sindhu et al., 2016)

Pretreatment Economics: Biological pretreatment is a cost competitive technique which has been demonstrated as a low cost method (compared to other processing methods) with no significant process inputs or capital funding required (Paulová et al., 2013; Zabed et al., 2016). Large scale operations have the potential for increased operational costs since pretreatment is performed under sterile conditions (Chaturvedi and Verma, 2013). Using techno-economic modelling software (SuperPro Designer), Barel and Shah (2017), it has

been estimated that sugar production utilising biological pretreatment would cost an estimated €1.20 kg^{-1}.

Environmental Evaluation: Biological pretreatment is considered an environmentally friendly or natural process, with little or no release of toxic compounds (SO_2, CH_4 or CO_2) to the environment (Sindhu et al., 2016).

Selection of Suitable Feedstocks: Biological pretreatment has been demonstrated to be effective on various types of lignocellulosic biomass including corn stalks (Du et al., 2011), wheat straw (Cianchetta et al., 2014), corn stover (Song et al., 2013), bamboo (Suhara et al., 2012), and plant biomass (Dhiman et al., 2015).

Advantages and Disadvantages: There are several advantages and disadvantages to biological pretreatment. These have been widely investigated in many studies including Limayem and Ricke, (2012), Sun et al. (2016) and Aditiya et al. (2016), and are summarised in Table 1.2.

Table 1.2. A comparative summary of the advantages and disadvantages of biological pretreatment.

Biological Pretreatment	Advantages	Disadvantages
Microorganisms	Low energy demand (1,2)	A relatively time consuming process (4)
	An environmentally friendly process. No release of toxic compounds to the environment (3). Low severity (9)	Significant amount of space required which can increase cost (5)
		Delignification rates are dependent on the microbial strains (6-8)
	No generation of fermentation inhibitors (3)	Loss of carbohydrates as consumed by microbes (6-8)
	No chemical input required (6-8)	

(1) Sun and Cheng, 2002, (2) Shi et al., 2008, (3) Sindhu et al., 2016, (4) Chaturvedi and Verma, 2013, (5) Bhutto et al., 2017, (6) Paulová et al., 2013, (7) Alvira et al., 2010, (8) Dashtban et al., 2014, (9) De carvalho et al., 2015.

1.4.1.2.3 Chemical - Acid Pretreatment

Aim: Pretreatment using acids at ambient temperatures enhance the anaerobic digestibility of lignocellulosic biomass. Subsequently solubilising hemicellulose, and by this, increasing the accessibility of cellulose to enzymatic hydrolysis (Hendrik and Zeeman, 2009; Alvira et al., 2010; Sun et al., 2016).

Classification of Acids for Pretreatment: Pretreatment employing acids can be performed using both concentrated and dilute acids. There are several common acids used including hydrochloric acid, acetic acid, sulphuric acid, nitric acid and phosphoric acid (Aditiya et al., 2016).

Effects on the Feedstocks Physicochemical Properties: During acid pretreatment, hemicellulose is partially solubilised from lignocellulosic materials, since the glucosidic bonds of hemicellulose and cellulose are susceptible to acid (Alvira et al., 2010; Sun et al., 2016). The acid in dilute acid pretreatment releases oligomers and monomeric sugars by affecting the reactivity of the biomass carbohydrate polymers. Depending on the combined severity of the pretreatment the sugars can be converted to aldehydes such as furfural and hydroxymethyl furfural (HMF) (Agbor et al., 2011). This pretreatment method gives high reaction rates and significantly improves cellulose hydrolysis (Karimi et al., 2006a; Karimi et al., 2006b).

Degradation Products: Depending on the process temperature, some sugar degradation compounds such as furfural, hydroxymethyl furfural (HMF), acetic acid, vanillin and aromatic lignin degradation compounds can be produced (Saha et al., 2000). At high temperatures, the produced inhibitors such as hydroxymethylfurfural could also degrade into other degradation products such as formic and levulinic acids (Larsson et al., 1999). Inhibitors can be removed by filtration of hydroxylate liquor followed by washing and drying of cellulose-rich residues (Saha et al., 2000).

Known Energy Requirements: In concentrated acid pretreatment, shorter residence times and milder temperatures are employed (Iranmahboob et al., 2002) compared to that of dilute acid which requires longer residence times and lower temperatures or shorter residence times and higher temperatures (Alvira et al., 2010; Agbor et al., 2011).

- Reaction time: 1-90 min (Alvira et al., 2010; Agbor et al., 2011)

- Reaction temperature: 140ºC – 215ºC (Alvira et al., 2010; Agbor et al., 2011)
- Chemical concentration: 0.2-2.5 % w/w (Dilute acid) 41-86 % w/w (concentrated acid) (Tao et al., 2011; Liu et al., 2012)

Pretreatment Economics: Using concentrated acid is more economic as the process can be performed at low temperatures (Gírío et al., 2010). However, additional funding is required for acid recovery, specialist equipment and other process related requirements (Sun and Cheng, 2002), which can significantly drive up the cost of the process. At concentrations below 4 % dilute acid pretreatment employing sulphuric acid has been shown to be inexpensive and effective (Kumar et al., 2009). Using techno-economic modelling software (SuperPro Designer), Barel and Shah (2017) estimated that the cost of producing sugar using acid pretreatment was approximately €0.49 kg^{-1}.

Environmental Evaluation: The use of large quantities of chemicals is an environmental concern. Concentrated chemicals have been shown in many studies (Alvira et al., 2010; Paulová et al., 2013) to have a negative impact on both product formation and downstream processing. The application of acids such as sulphuric acid for the pretreatment of lignocellulosic biomass has potential for a higher environmental impact compared to non-chemical pretreatment technologies such as liquid hot water (LHW) (Guo et al., 2014). Environmental parameters of acidification, eutrophication and eco-toxicity are significantly greater due to the additional chemical inputs and induced emissions for acidic processes (Guo et al., 2014). da Costa Sousa et al. (2009) reported that a less corrosive chemical with low toxicity would contribute to reducing cost as well as increasing safety and environmental benefits.

Chapter 5 provides an assessment of the environmental impacts of acid pretreatment. Using life cycle assessment (LCA) the environmental inputs and outputs of the system were evaluated and potential emissions calculated.

Selection of Suitable Feedstock: Lignocellulosic feedstocks which have been shown to benefit from this method of pretreatment include switchgrass (Digman et al., 2010; Li et al., 2010a), corn stover (Du et al., 2010; Xu et al., 2009), spruce (Shuai et al., 2010) and poplar (Wyman et al., 2009; Kumar et al., 2009).

Advantages and Disadvantages: There are several advantages and disadvantages to acid (concentrated and diluted) pretreatment. These have been widely investigated in many

studies including Agbor et al, (2011), Sun et al. (2016) and Aditiya et al. (2016), and are summarised in Table 1.3.

Table 1.3. A comparative summary of the advantages and disadvantages of acid pretreatment.

Acid Pretreatment	Advantages	Disadvantages
Concentrated Acid	Complete removal of cellulose crystalline structure (1,3)	Corrosion of equipment, need for acid recovery (1,3)
	Achievement of amorphous cellulose (1,3)	
	Increased accessible area (1,3)	High operational and maintenance costs (1,3)
	Can achieve high reaction rates and significantly improve hemicellulose (4)	Little lignin removal (1,3)
Dilute Acid	Increased accessible surface area (1,3)	Requirement of neutralisation (1,3)
	Alteration of the lignin structure, with the removal of hemicellulose (1,3)	Formation of inhibitors (1,3)

(1) Paulová et al., 2013, (2) Moiser et al., 2005, (3) Alvira et al., 2010, (4) Bhutto et al., 2017.

1.4.1.2.4 Chemical - Alkaline Pretreatment

Aim: To increase cellulose digestibility and improve the effectiveness of lignin solubilisation, exhibiting minor cellulose and hemicellulose solubilisation (Carvalheiro et al., 2008).

Classification of alkaline reagents: Most commonly applied alkali chemicals include sodium hydroxide, potassium hydroxide, aqueous ammonia, calcium hydroxide and oxidative alkali (Rabemanolontoa and Saka, 2016).

Effects on the Feedstocks Physicochemical Properties: Alkaline pretreatment causes swelling of the lignocellulosic cell wall, increasing the surface area and decreasing the

degree of polymerisation and crystallinity, which provokes disruption of the lignin structure (Taherzadeh and Karimi, 2008; Kim, 2013). The alkali reagent employed is believed to saponify the uronic ester linkages of 4-O-methyl-*D*-glucuronic acids attached to the xylan backbone, producing a charged carboxyl group and cleaving the linkages of lignin and other hemicelluloses (Rabemanolontoa and Saka, 2016). The breakdown in the lignocellulosic structure, results in the removal of lignin and hemicellulose, thereby, making the feedstock more accessible to enzymatic hydrolysis (Taherzadeh and Karimi, 2008). A neutralising step to remove lignin and inhibitors is required before subsequent enzymatic hydrolysis (Bhutto et al., 2017).

Degradation Products: The formation of inhibitors (salts, phenolic acids, furfurals and aldehydes) during alkaline pretreatment is dependent on several factors including alkali reagent severity, residence time, temperature and even choice of pretreatment chemical (Bhutto et al., 2017). The neutralisation of some reagents such as lime reduces the potential for inhibitor formation (Mathew, 2011). Washing of solids following pretreatment removes enzyme inhibitors and residual unreacted reagents (Bensah and Mensah, 2013).

Known Energy Requirements: Alkaline pretreatment is advantageous as it can be performed at low temperatures and pressures over long or short residence times. The process itself has not been demonstrated to be energy intensive. However, the recovery of alkali reagents from the system can increase the energy demand significantly (Rabemanolontoa and Saka, 2016).

- Residence time: 5 - 60 min (Kim et al., 2016), hrs – days (Mosier et al., 2005)
- Reaction temperature: 60 - 180^{o}C (Kim et al., 2016)
- Reaction pressure: Standard vapour pressure
- Chemical concentration: < 4 % w/w (dilute alkali) 6-20 % w/w (concentrated alkali) (Mirahmadi et al., 2010; Bensah and Mensah, 2013)

Pretreatment Economics: The cost of alkaline pretreatment can vary significantly with different alkali reagents. Some reagents such as aqueous ammonia have been found to be inexpensive compared to other reagents as they are currently used in the production of fertilisers (Kim et al., 2016). In addition, ammonia can be recovered and reused because of its high volatility, subsequently reducing processing costs (Kim, 2013). Sodium and

calcium hydroxide can also be easily recovered when reacted with carbon dioxide (Carvalheiro et al., 2008; Alvira et al., 2010) and have a lower cost.

Environmental Evaluation: Alkaline pretreatment is similar to acid pretreatment with respect to its environmental impacts related to equipment corrosion, the handling of concentrated chemicals and the need for acid/alkaline removal. Alkaline processes suffer from silica scaling during chemical recovery because many agricultural feedstocks, such as rice and wheat straw, have a very high silica content. The scaling problem prohibits the recovery of alkaline chemicals from pretreatment liquor (Zhu et al., 2009). Neutralisation of alkaline reagents, however, could be an alternative option. Neutralisation of reagents would minimise the handling and expose of reagents to the environment which Guo et al. (2014) has reported as an important contributor to environmental impacts accounting for 20-50% of burdens on eutrophication and toxicity 20-30%. Dilute acid / alkaline reagents showed environmental advantages over other pretreatment techniques such as LHW in abiotic depletion, GWP, and POD impact categories (Guo et al., 2014).

Chapter 5 provides an assessment of the environmental impacts of alkaline pretreatment. Using LCA the environmental inputs and outputs of the system were evaluated and potential emissions calculated.

Selection of Suitable Feedstocks: Lignocellulosic feedstocks that have been shown to benefit from this method of pretreatment include corn stover, switchgrass, bagasse, and wheat and rice straw (Liang et al., 2010; Park et al., 2010).

Advantages and Disadvantages: There are several advantages and disadvantages to alkaline pretreatment. These have been widely investigated in many studies including Balat, (2011), Kim et al. (2016) and Aditiya et al. (2016), and are summarised in Table 1.4.

Table 1.4. A comparative summary of the advantages and disadvantages of alkaline pretreatment.

	Advantages	Disadvantages
Alkaline Pretreatment	High digestibility – Significant removal of hemicellulose and lignin (1)	Longer residence times required (4)
	Lower degradation of sugars compared to acid pretreatment (2,3)	Conversion of alkali reagent into irrecoverable salts (2,3)
	Increased surface area (2,3)	pH adjustments required for subsequent processes (2,3)
	Can be performed at lower temperatures and pressures (4)	Potential for inhibitor formation (5)

(1) Refaat, 2012, (2) Mosier et al., 2005, (3) Alvira et al., 2010, (4) Rabemonolontsoa and Saka, 2016, (5) Balat et al., 2011.

1.4.1.2.5 Chemical - Ozonolysis

Aim: To degrade the lignin polymer and solubilise the hemicellulose content of the lignocellulosic biomass (Sun and Cheng, 2002) without the formation of inhibitory products.

Classification of Process Operations: Ozonolysis can be performed using ozone in a single step process or can be combined with different solvents such as ethanol or physical pretreatment to aid the depolymerisation of lignin (Travaini et al., 2016). Ozonolysis has also been used for other applications, like enzyme production (Rodriguez-Gomez et al., 2012).

Effects on the Feedstocks Physicochemical Properties: Ozone pretreatment has placed a particular focus on the delignification of lignocellulosic biomass as reactions with cellulose and hemicellulose are inefficient (Travaini et al., 2016). Ozonolysis works using several different mechanisms: selective reaction with carbon-carbon double bonds, attack on aromatic centres and glycosidic bond cleavage (Bule et al., 2013). Ozone preferentially

reacts with olefinic, aromatic and phenolic compounds, degrading lignin and solubilising hemicellulose slightly, depending on the system parameters.

Degradation Products: Sugar degradation generates inhibitory compounds during ozonolysis including oxalic acid, formic acid, acetic acid and levulinic acid (Travaini et al., 2013). Lignin degradation products can also be produced including a wide range of aromatic compounds and polyaromatic compounds which are subsequently converted to carboxylic acids (Travaini et al., 2013). Some studies have shown water washing of ozonated samples can detoxify the sample, removing the inhibitory compound (Schultz-Jensen et al., 2011).

Known Energy Requirements: The energy requirements for this pretreatment can vary significantly. In general, the most commonly employed process conditions include:

- Reaction time: 30-90 mins (Silverstein et al., 2007; Zabed et al., 2016)
- Reaction temperature: Room temperature (Sun and Cheng, 2002; Travaini et al., 2016)
- Reaction pressure: Standard vapour pressure (Sun and Cheng, 2002; Travaini et al., 2016)
- Chemical concentration: 4 % w/w (Silverstein et al., 2007)

Pretreatment Economics: Despite extensive laboratory research, full scale biomass pretreatment with ozone has not yet been developed. Ozone is extremely expensive to generate and large amounts are required (Bensah and Mensah, 2013). Schultz-Jensen et al. (2011) have demonstrated how technological advances are steadily reducing the cost of producing ozone, with an estimated 30 % decrease in the last four years.

Environmental Evaluation: Ozonolysis is similar to physical pretreatment with respect to the high energy demand for process operation performance. The generation of ozone is energy intensive and has a significant effect on both the environmental and economic impact of the system (Travaini et al., 2016; Balat, 2011). Ozonolysis also requires energy for cooling (Travaini et al., 2016).

Selection of Suitable Feedstocks: There are several published reports on ozonolysis employing different feedstocks including wheat straw (Schultz-Jensen et al., 2011; Kádár et al., 2015), rye straw (García-Cubero et al., 2009), sugar bagasse (Travaini et al., 2013),

energy grass (Panneerselvam et al., 2013), coastal Bermuda grass (Lee et al., 2010), and maize stover (Li et al., 2015).

Advantages and Disadvantages: There are several advantages and disadvantages to ozonolysis. These have been widely investigated in many studies including Haghighi Mood et al. (2013), Zabed et al. (2016) and Travaini et al. (2016), and are summarised in Table 1.5.

Table 1.5. A comparative summary of the advantages and disadvantages of ozonolysis.

	Advantages	**Disadvantages**
Ozonolysis	Selective lignin degradation with minimal effects on cellulose and hemicellulose (1)	Highly reactive, flammable, corrosive and toxic properties of ozone make it a dangerous process (1)
	Low inhibitor formation, with no furfural or HMF generation (1)	Significant energy demand with high ozone generation costs (1,2)
	Potential for on-site ozone generation and direct utilisation (1)	Exothermic characteristics of ozonolysis may require cooling system (1)
	Ozonolysis forms a negligible amount of inhibitors (3,4)	Solvents employed need to be separated (3)

(1) Travaini et al., 2016, (2) Balat, 2011, (3) Zheng et al., 2014, (4) Refaat, 2012

1.4.1.2.6 Chemical - Organosolvent Pretreatment

Aim: To solubilise lignin, which can increase the pore volume and accessible surface area of lignocellulosic materials and significantly reduce their lignin contents (Sun et al., 2016).

Classification of Organosolvents: Various organic solvents have been utilised in the pretreatment of lignocellulosic feedstocks. These include ethanol, methanol, acetone, ethylene glycol, organic peracid and tetrahydrofurfuryl alcohol (Zhao et al., 2009) with ethanol being the most favourable solvent. In some studies, mixtures are combined with acid catalysts (Alvira et al., 2010).

Effects on the Feedstocks Physicochemical Properties: Organosolvent pretreatment is primarily a delignification process and can be carried out using alcohol or organic solvents (El Hage et al., 2010). Lignin can be extensively removed using alcohol, while hemicellulose is almost completely solubilised. On the application of alcohols, internal lignin bonds are hydrolysed, as well as the ether and 4-O-methylglucuronic acid ester bonds between lignin and hemicellulose (Zhao et al., 2009). In addition, glycosidic bonds in hemicellulose and partially in cellulose are hydrolysed (Zhao et al., 2009).

Pretreatment with organic acids varies considerably, proceeding via the dissociation of partial hydrogen ion to accelerate delignification / hydrolysis of cellulose and dissolution of the lignin fragments (Mc Donagh, 1993). Delignification kinetics vary with the solvent used during pretreatment.

Degradation Products: Side reactions such as acid catalysed degradation of monosaccharides can produce inhibitory compounds such as furfurals and HMF which can inhibit the fermentation by microorganisms (Agbor et al., 2011). Bensah and Mensah (2013) reported that filtrated and washed solid residues of the pretreatment hydrolysates can remove solvent, thus reduce the potential for inhibitor formation, which may possess inhibitory characteristics to downstream process.

Known Energy Requirements: Different process conditions are employed based on the solvent (alcohol or organic solvent) utilised.

- Reaction time: 30-90 mins (alcohols) 2-5 hrs (organic solvents) (Zhang et al., 2016)
- Reaction temperature: 180-195^{o}C (alcohols) 60-145^{o}C (organic solvents) (Zhang et al., 2016)
- Reaction pressure: Atmospheric pressure (alcohols and organic solvents) (Zhang et al., 2016)
- Chemical concentration: 30-75 % w/w (alcohols) 10-100 % w/v (organic solvents) (Zhang et al., 2016)

Pretreatment Economics: The commercial price of solvents can be quite high as observed in Chapter 4, and must be taken into consideration. For economic reasons, among all possible solvents, the low molecular weight alcohols with lower boiling points such as ethanol and methanol are more favourable (Alvira et al., 2010). Several studies (Giarola et al., 2014; Kautto et al., 2014; Laure et al., 2014) have conducted a techno-

economic assessment of the organosolvent pretreatment process, reporting on the monitory benefits as well as the high value by-product generation benefits of the process. In 2014, Laure et al. (2014) conducted an LCA based on aspen process simulation of an industrial production plant in Germany. In this study, an economic assessment of the process was performed, evaluating the cost of glucose production. Laure et al. (2014) reported that producing 1 kg of glucose cost approximately €0.24 kg^{-1}.

Environmental Evaluation: The environmental impact of pretreatment utilising organic solvents can vary significantly depending on the process parameters employed (chemical concentration, feedstock type, reaction time, temperature, and pressure, and method of solvent recovery, recycling and reuse) (see Chapter 5). Many studies (Spatari et al., 2010; González-García et al., 2010; González-García et al., 2012a; González-García et al., 2012b ; Nguyen and Hermansen, 2015) have been commissioned over the years, solely to investigate the environmental impacts associated with the bioconversion of lignocellulosic feedstocks. Michels and Wagemann (2010) reported that lignocellulose feedstock biorefinery that uses organosolvent pretreatment is characterised by lower emissions of CO_2 and SO_2 equivalents compared to more traditional methods which have been previously employed. However, the use of mineral acids in the organosolvent process is an environmental concern (Bensah and Mensah, 2013).

Chapter 5 provides an assessment of the environmental impacts of organosolvent pretreatment. Using LCA the environmental inputs and outputs of the system were evaluated and potential emissions calculated.

Selection of Suitable Feedstocks: Lignocellulosic feedstocks that have been shown to benefit from this method of pretreatment include wheat straw (Sun et al., 2004; Pan and Sano, 2005), corn stover (Qin et al., 2012), miscanthus (Wang et al., 2011), sugarcane bagasse (Singh et al., 2010), bamboo (Li et al., 2013), Sitka spruce (Bouxin et al., 2014) and horticultural waste (Geng et al., 2012).

Advantages and Disadvantages: There are several advantages and disadvantages to organosolvent pretreatment. These have been widely investigated in many studies including Paulová et al. (2013), Seidl and Goulart. (2016) and Elgharbawy et al. (2016), and are summarised in Table 1.6.

Table 1.6. A comparative summary of the advantages and disadvantages of organosolvent pretreatment.

	Advantages	Disadvantages
Organosolvent Pretreatment	Pure lignin recovery with minimum cellulose loss (less than 2%) (1)	Formation of potential inhibitors (2,3)
	High digestibility resulting in high pretreated material yield (2,3)	Requirement for removal of solvents (2,3)
	Low sugar degradation (2,3)	High chemical and handling cost (2,3,5)
	Solvent removal easily performed with distillation (4)	High energy costs (2)
	Low environmental impact (2,3)	

(1) Zhao et al., 2009, (2) Elgharbawy et al., 2016, (3) Paulová et al., 2013, (4) Pan et al., 2006, (5) Sun et al., 2016

1.4.1.2.7 Physicochemical – Liquid Hot Water Pretreatment

Aim: To solubilise mainly hemicellulose, to make the cellulose more accessible and to avoid the formation of inhibitors (Alvira et al., 2010).

Classification of Pretreatment Operations: Liquid hot water (LHW) pretreatment (high or low severity) can be performed with or without a catalyst (acid or alkali). Catalytic hydrothermal pretreatment has been developed to assist in the removal of hemicellulose and lignin and to optimise the recovery of both hemicellulosic and cellulosic sugars (Sun et al., 2014).

Effects on the Feedstock Physicochemical Properties: LHW pretreatment is employed to hydrolyse hemicellulose (>80 %) (Laser et al., 2002), and remove lignin, subsequently rendering the cellulose more accessible (Yang and Wyman, 2004). The hot water cleaves hemiacetal linkages thus liberating acids during biomass hydrolysis, which facilitates the breakages of ether linkages in biomass (Antal, 1996). The release of these acids can help or hinder the formation and removal of oligosaccharides, or further hydrolyse hemicellulose to monomeric sugars, which can be subsequently degraded to aldehydes

(Mosier et al., 2005). Hemicellulose is mostly depolymerised, and its degradation products are dissolved in the liquid phase, while cellulose is retained completely in the solid phase. Lignin undergoes significant depolymerisation (Ko et al., 2015), with approximately 20-30% removed from the biomass during pretreatment (Yu et al., 2013).

Degradation Products: Many studies have cited LHW pretreatment as being advantageous in that little to no inhibitors are formed (Alvira et al., 2010; Paulová et al., 2013). However, sugars produced from hemicellulose during this process can potentially be converted to aldehydes (furfurals and HMF) which can inhibit microbial fermentation (Palmqvist and Hahn-Hägerdal 2000). Several methods including simultaneous saccharification and fermentation (SSF) have been adopted to relieve the end product inhibition (Zhuang et al., 2016).

Known Energy Requirements: Energy requirements are based on the severity of the process which is divided into two categories: high severity and low severity.

- Reaction temperature: 160-240^{o}C (Cao et al., 2014; Sun et al., 2014). Above 240^{o}C and severe cellulose degradation can occur.
- Reaction time: 10-120 mins (Xiao et al., 2014)
- Reaction pH: 4-7 (Mosier et al., 2005; Hayes, 2009)
- Low severity: 230^{o}C 10 MPa^{-1} 15mins^{-1}
- High severity: 270-280^{o}C 10 MPa^{-1} 15mins^{-1} (Ogura et al., 2013)

Pretreatment Economics: The cost of LHW pretreatment is relatively low compared to other pretreatment techniques (acid, alkali, mechanical) as the process can be performed without the need for chemicals or catalysts (Alvira et al., 2010). No rapid decomposition or expansion is required and the utilisation of pressure is only for maintaining water in its liquid state, preventing evaporation (Haghighi Mood et al., 2013). Although a large amount of water and energy is required (Alvira et al., 2010; Paulová et al., 2013), these can be recovered and reused to help elevate some of the potential costs.

Environmental Evaluation: For pretreatment employing liquid hot water to be an environmentally sustainable process, the use of fresh water, the amount of waste water and the energy consumption need to be minimised (Chandel et al., 2007). LHW pretreatment has been reported in many studies as an environmentally friendly process as there is no release of toxic compounds to the environment (Alvira et al., 2010).

Selection of Suitable Feedstocks: Lignocellulosic feedstocks that have been shown to benefit from this method of pretreatment include sugarcane bagasse (Laser et al., 2002), wheat straw (Pérez et al., 2008), corn cob (Garrote et al., 2001), and rye straw (Rogalinski et al., 2008). In general, LHW is applicable to a wide range of biomass species including softwood (Rabemanolontsoa and Saka, 2016).

Advantages and Disadvantages: There are several advantages and disadvantages to liquid hot water pretreatment. These have been widely investigated in many studies including Agbor et al. (2011), Zabed et al. (2016) and Zhuang et al. (2016), and are summarised in Table 1.7.

Table 1.7. A comparative summary of the advantages and disadvantages of liquid hot water pretreatment.

	Advantages	**Disadvantages**
LHW Pretreatment	Does not require any catalyst or chemicals (1-3)	High water demand (1-3)
	Significant increase in pore volume and specific surface area (4)	High energy requirement (1-3)
	Direct utilisation of wet or fresh lignocellulosic materials (5)	Hemicellulose degradation (1-3)
	Little or no inhibitor formation (1-3)	Potential for inhibitor formation (1-3)
	No rapid decompression or expansion required (6)	Not successful with softwood feedstocks (7)
	Removal of hemicellulose (1-3)	
	Structural and chemical alteration in lignin (1-3)	

(1) Paulová et al., 2013, (2) Moiser et al., 2005, (3) Alvira et al., 2010, (4) Nitsos et al., 2013, (5) Sun et al., 2016, (6) Haghighi Mood et al., 2013, and (7) Bunnell et al., 2010

1.4.1.2.8 Physicochemical - Steam Explosion Pretreatment

Aim: To solubilise the hemicellulose and lignin components of lignocellulosic biomass to make the cellulose more accessible to enzymatic hydrolysis, while also avoiding the formation of inhibitors (Sun and Cheng, 2002).

Classification of Pretreatment Operations: Steam explosion pretreatment is a three stage process known as a thermos-mechanochemical method which involves the breakdown of structural components by steam-heating (thermo), shearing (meachano), and auto-hydrolysis (chemical) of glycosidic bonds (Haghighi Mood et al., 2013).

Effect on the Feedstocks Physicochemical Properties: Steam pretreatment is the most commonly used physicochemical pretreatment method to pretreat lignocellulosic materials (Sun et al., 2016). During steam pretreatment, chipped biomass is subjected to high pressure saturated steam at high temperatures. The material is exposed and separated into fibres resulting in the decomposition of hemicellulose and lignin which is subsequently removed from the lignocellulosic material in different degrees (Pan et al., 2005). Removal of the lignin and hemicellulose, consequently exposes the surface of the cellulose and increases enzyme accessibility (Alvira et al., 2010). Balat (2010), summarises steam pretreatment using three simple points: (1) increase in cellulose crystallinity, (2) hydrolysis of hemicellulose and (3) delignification.

Degradation Products: Potential for inhibitor formation due to acidic conditions in the reaction chamber, sugar degradation can occur resulting in the production of furfural and HMF (Garcia-Aparicio et al., 2006).

Known Energy Requirements: Parameters affecting steam explosion efficiency are particle size, temperature and residence time (Tomás-Pejó et al., 2008).

- Reaction temperature: 160-170^{o}C (Haghighi Mood et al., 2013)
- Reaction time: secs-mins (Zabed et al., 2016)
- Reaction pressure: 0.69-4.83 MPa (Rabemanolontsoa and Saka et al., 2016)

The addition of a catalyst (H_2SO_4, CO_2 or SO_2) can potentially decrease the reaction time and temperature required, effectively improving the rate of hydrolysis and the risk of inhibitor formation (Kumar et al., 2009).

Pretreatment Economics: Pretreatment utilising steam has been demonstrated to be a cost effective process due to its low capital investment requirements and higher energy efficiency (Tomás-Pejó et al., 2008). Prasad et al. (2007) reported that steam is especially cost efficient for hardwood and agricultural residues but less effective for softwood. The most significant input for this pretreatment is energy, required to maintain reaction pressure. Using techno-economic modelling software (SuperPro Designer) the cost of

sugar production using steam pretreatment was estimated to be €0.37 kg $^{-1}$ (Barel and Shah, 2017).

Environmental Evaluation: Compared to other pretreatments steam offers a significantly lower environmental impact, more potential for energy efficiency, less hazardous process chemicals and conditions and complete sugar recovery (Tomás-Pejó et al., 2008). Similar to LHW pretreatment, steam explosion is considered by many researchers (Sun and Cheng, 2002) as an environmentally friendly process as there is no release of toxic compounds.

Selection of Suitable Feedstocks: Various different biomass species have shown positive effect on pretreatment with steam such as poplar wood, pine chips, wheat straw, sugarcane bagasse and miscanthus (Jacquet et al., 2012).

Advantages and Disadvantages: There are several advantages and disadvantages to steam pretreatment. These have been widely investigated in many studies including Rabemanolontsoa and Saka et al. (2016), Zabed et al. (2016) and Aditiya et al. (2016), and are summarised in Table 1.8.

Table 1.8. A comparative summary of the advantages and disadvantages of steam pretreatment.

	Advantages	**Disadvantages**
Steam Pretreatment	Cost effective (1)	Potential for inhibitor formation (1)
	High glucose and hemicellulose yield (1)	Incomplete disruption and solubilisation of the lignin (1)
	Improved enzyme accessibility – reduction in particle size (2-4)	Partial hydrolysis of the hemicellulose component (2-4)
	Increased pore size (2-4)	

(1) Aditiya et al., 2016, (2) Paulová et al., 2013, (3) Moiser et al., 2005, (4) Sun and Cheng, 2002

1.4.1.2.9 Physicochemical - Ammonia Fibre Explosion Pretreatment

Aim: To rapidly solubilise lignocellulosic materials, disrupting the lignin component (Alvira et al., 2010), increasing surface area and pore size, and subsequently modifying the hemicellulose / cellulose structure (Kim et al., 2009; Kim et al., 2016) to enhance enzymatic hydrolysis.

Classification of Pretreatment Operations: Ammonia fibre/freeze explosion (AFEX), ammonia recycle percolation (ARP) and soaking aqueous ammonia (SAA) are various pretreatment options employing ammonia (Agbor et al., 2011). As a physicochemical method, AFEX is similar to steam explosion operating at high pressures but conducted at ambient temperatures. ARP is performed at higher temperatures (Kim and Lee, 2005) compared to those employed for AFEX. Soaking aqueous ammonia (SAA) is a modified version of AFEX performed at moderate temperatures (Kim and Lee, 2005).

Effects on the Feedstocks Physicochemical Properties: AFEX is a physicochemical pretreatment where the biomass is exposed to ammonia at higher temperature and pressure for a limited period of time (Balat et al., 2008; Behara et al., 2014). The application of liquid ammonia to lignocellulosic biomass causes swelling and the crystal structure of cellulose to change from native cellulose I to cellulose III (Sun et al., 2016). Little lignin or hemicellulose is removed from this complex matrix (Sun et al., 2016). Exposed at a given temperature and high pressure, the reactivity of the remaining carbohydrates is significantly increased, making the lignocellulosic components easily hydrolysable (Foster et al., 2001; Kim and Lee, 2002). The lignin distribution remains comparably the same after AFEX pretreatment, however, the structure is rigorously altered resulting in increased water-holding capacity and digestibility (Agbor et al., 2011). Mosier et al. (2005) summarised that AFEX de-crystallises cellulose, hydrolyses hemicellulose, depolymerises lignin and increases the micropore structure of the cell wall. The pore size of AFEX pretreated biomass is larger than 10nm (Chundawat et al., 2011).

Degradation Products: AFEX is advantageous for having no sugar loss or degradation of sugars into inhibitors (Lau and Dale, 2009; Mathew et al., 2016). Ammonia recovery and recycling has been demonstrated to be very effective in the reduction of potential inhibitors formed in downstream processes (Teymouri et al., 2005; Alvira et al., 2010).

Known Energy Requirements: Energy requirements can vary significantly depending on feedstock employed. In general, conditions are:

- Reaction time: < 30 mins (variable times) (Kumar et al., 2009)
- Reaction temperature: 60-120^{o}C (Kumar et al., 2009; Alvira et al., 2010)
- Reaction pressure: 1.72-2.06 MPa (Kumar et al., 2009)

Pretreatment Economics: The overall cost of ammonia pretreatment is difficult to calculate due to the varying methodologies published in the literature (Balat et al., 2008; Sánchez and Cardona, 2008). Using techno-economic modelling software, Barel and Shah (2017) estimated the cost of sugar production to be € 0.55 kg^{-1} for AFEX pretreated lignocellulosic biomass. In order to reduce the high operational costs which result from the high chemical cost, ammonia must be recovered, recycled and reused (Holtzapple et al., 1992). The overall cost required to recycle ammonia has been estimated by da Costa Sousa et al. (2016), as €0.53 - €0.56 kg^{-1} depending on the enzyme loading rate. Taking all process inputs (ammonia make up costs, energy costs, for ammonia recycling and enzyme costs) into account da Costa Sousa et al. (2016), estimated that employing AFEX pretreatment in the production of 2nd generation ethanol would cost approximately € 3.12 kg^{-1} of ethanol.

Environmental Evaluation: The strong smell of ammonia can have a negative influence on the utilisation of AFEX pretreatment (Balat et al., 2008). It is critical that all residual ammonia is recovered from the reaction. Ammonia is a highly volatile substance and similar to acid and alkali pretreatment extreme caution must be taken to avoid exposure to human, aquatic and terrestrial ecosystems (Sun and Cheng, 2002; Balat et al., 2008). Chapter 5 provides an assessment of the environmental impacts associated with the employment of pretreatment utilising ammonia. Using LCA the environmental inputs and outputs of the system were evaluated and potential emissions calculated. In general, AFEX pretreatment has been described as having the potential to enhance environmental benefits beyond the direct impact of the pretreatment (da Costa Sousa et al., 2016).

Selection of Suitable Feedstock: AFEX is most effective on agricultural residues and herbaceous crops, with limited effectiveness demonstrated on woody biomass and other high lignin feedstocks (Wyman et al., 2005). More specifically, AFEX has been shown to have a positive effect on switchgrass (Bals et al., 2010), rice straw (Zhong et al., 2009) and corn stover (Gao et al., 2014; Uppugundla et al., 2014).

Advantages and Disadvantages: There are several advantages and disadvantages to AFEX pretreatment. These have been widely investigated in many studies including Mathew et al. (2016), Zabed et al. (2016) and Aditiya et al. (2016), and are summarised in Table 1.9.

Table 1.9. A comparative summary of the advantages and disadvantages of AFEX pretreatment.

	Advantages	Disadvantages
AFEX Pretreatment	Ammonia can be recovered and recycled (1)	Ineffective if lignin content is high (1)
	Energy efficient – moderate temperature and residence time (1)	Cost of ammonia can be quite high (2)
	A high selectivity for reaction with lignin (1)	Environmental concern for the strong stench of ammonia (2)
	Low formation of inhibitors (4)	No significant solubilisation of hemicellulose (1)
	Reduction in cellulose crystallinity (5,6)	Recovery of ammonia from the reaction can be expensive (3)

(1) Balat, 2011, (2) Alvira et al., 2010, (3) Brodeur et al., 2011, (4) Refaat, 2012, (5) Paulová et al., 2013, (6) Moiser et al., 2005

1.4.1.3 The Coordinated Development of Leading Biomass Pretreatment Technologies for the Generation of Bioethanol from Irish Crops

Varying methodologies, insufficient and / or unreproducible process information has challenged the investigation, development and evaluation of pretreatment technologies for many years. The overview of current pretreatment technologies presented in Section 1.4.1.2 can assist researchers in the optimisation of established techniques and further promote their development.

The review of pretreatments carried out in this study was central to the selection of potential pretreatment techniques which can be applied to the four dedicated Irish grown energy crops (willow (*Salix*), miscanthus (*x-giganteus*), hemp (*Cannabis satvia L*) and switchgrass (*Panicum virgatum L*)) selected for assessment in this research study.

Following an extensive review of the literature, a chemical pretreatment approach employing sulphuric acid, sodium hydroxide, ammonia and methanol was chosen. Consequently, a detailed assessment of these four selected pretreatment technologies was performed, to characterise the targeted feedstocks, analyse the reaction and recovery of sugars and solvents, and the fate of the biomass constituents.

1.4.1.3.1 Characteristics of Targeted Feedstocks

Willow (*Salix*)

Willow (*Salix*) has two remarkable characteristics that promote its cultivation: (1) it can be adapted to thrive in flooded environments and (2) their easy vegetative propagation (Kord and Kord, 2011). Willow, specifically the *Salix* spp. has a chemically uniform composition with few contaminants and undesirable components (Stolarski et al., 2013).

The physicochemical composition of willow is presented in Table 1.10. The results of an investigation of the chemical composition of the willow employed in this study is presented in Chapter 3.

Table 1.10. Chemical and Physical Properties of Willow (Salix).

Physicochemical Composition	% dry matter
Cellulose	48.0 – 51.0 (1)
Glucan	33.8 (2) 41.5 (3)
Xylan	10.4 (3) 15.0 (2)
Galactan	2.1 (3) 2.1 (2)
Arabinan	1.2 (2) 2.1 (3)
Mannan	2.2 (3) 3.2 (2)
Lignin	22.0 – 24.3 (1)
Acid -soluble	2.2 (2)
Acid-insoluble	24.2 (2)
Moisture	20 (4)
Ash	0.9 – 1.2 (1,2)
Porosity	0.76 – 0.78 (1)
Dry density	325 – 357 kg m^{-3} (1)
Potential Energy	172 GJ kg^{-1} ha^{-1} at 20% moisture (4)

(1) Kord and Kord, 2011, (2) Sassner et al., 2008, (3) Ali and Tschirner, 2010, (4) Caslin et al., 2010

Miscanthus (*x-giganteus*)

Miscanthus (*x-giganteus*) has been widely investigated in many research studies and is regularly regarded as a favourable alternative source of bioenergy (Parajuli et al., 2015). The physicochemical composition of miscanthus is presented in Table 1.11. The results of an investigation of the chemical composition of the miscanthus employed in this study is presented in Chapter 3.

Table 1.11. Chemical and Physical Properties of Miscanthus (*x-giganteus*).

Physicochemical Composition	% dry matter
Cellulose	39.9 (1) 37.1 (3)
Glucan	40.9 (2)
Xylan	19.9 (2)
Galactan	0.6 (2)
Arabinan	1.7(2)
Mannan	0.1 (2)
Lignin	22.4 (2) 23.3 (1)
Acid -soluble	2.8 (1)
Acid-insoluble	20.4 (1)
Moisture	7.0 (1) 8.8 (3)
Ash	2.8 (1) 3.5 (3)
Hemicellulose	22.1 (1)

(1) Han et al., 2011, (2) Scordia et al., 2013, (3) Melligan et al., 2012

Hemp (Cannabis sativa L)

The cellulose content of hemp is quite high compared to other lignocellulosic feedstocks (Öhgren et al., 2005; Linde et al., 2008) making hemp a good potential crop for ethanol production (Sipos et al., 2010). The hemp stem consists of blast fibres and a woody core. It is these blast fibres that are rich in cellulose, while the woody core contains a significant proportion of lignin (Garcia-Jaldon et al., 1998).

The physicochemical composition of hemp is presented in Table 1.12. The results of an investigation of the chemical composition of the hemp employed in this study is presented in Chapter 3.

Table 1.12. Chemical and Physical Properties of Hemp (*Cannabis sativa L*).

Physicochemical Composition	% dry matter
Cellulose	44 (1)
Glucan	39.8 (2)
Xylan	14.4 (2)
Galactan	2.5 (2)
Arabinan	0.9 (2)
Mannan	2.9 (3)
Lignin	15 (2)
Moisture	20 (3)
Ash	5.8 (2)
Potential Energy	296 GJ kg^{-1} ha^{-1} at 20% H_2O (3)
Density	60-70 kg m^{-3} (3)
Calorific value	0.02 GJ kg^{-1} (3)

(1) Sipos et al., 2010, (2) Kuglarz et al., 2014, (3) Rice, 2008

Switchgrass (Panicum Virgatum L)

There are various factors affecting switchgrass's biomass yield and composition: genotype, ecotype, harvest time, fertiliser application, precipitation, storage method, and other environmental and cultivation conditions (Kim et al., 2011).

The physicochemical composition of switchgrass is presented in Table 1.13. The results of an investigation of the chemical composition of the hemp employed in this study is presented in Chapter 3.

Table 1.13. Chemical and Physical Properties of Switchgrass (*Panicum Virgatum L*).

Physicochemical Composition	% dry matter
Cellulose	31.4 – 45 (3)
Glucan	29.9 – 35.5 (1)
Xylan	20.5 -22.5 (1)
Galactan	0.7 – 1.9 (3)
Arabinan	2.7 – 3.4 (1)
Mannan	0.3 – 0.4 (3)
Lignin	18.8 – 22.6 (1)
Acid-soluble lignin	3.3 – 3.7 (3)
Acid-insoluble lignin	15.8 – 16.5 (3)
Hemicellulose	22 – 35.1 (3)
Ash	3.3 – 4.3 (1)

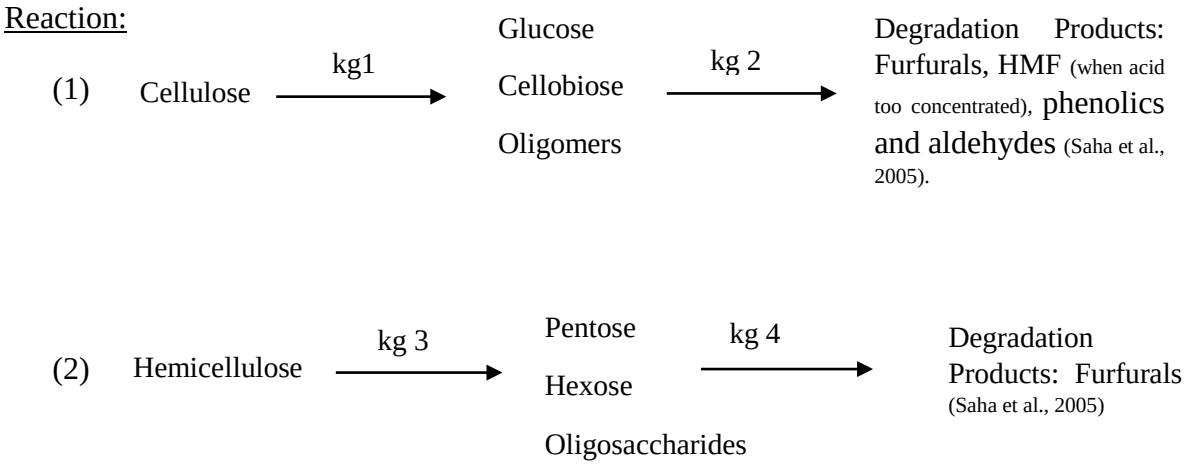
(1) Kim et al., 2011, (2) Hu et al., 2010, (3) Sun and Cheng, 2002

1.4.1.3.2 Analysis of Reaction Mechanisms

The reaction that lignocellulosic biomass undergoes with different pretreatment chemicals can vary significantly depending on several factors: process parameters (time, temperature, pH and concentration), complexity of the lignocellulosic biomass (degree of polymerisation, cellulose crystallinity, available surface area and lignin content), the addition of a catalyst, and the formation of inhibitory compounds (Hendriks and Zeeman, 2009; Alvira et al., 2010). For this reason, each pretreatment chemical is individually evaluated, assessing the reaction mode and other contributory factors.

Acid pretreatment

Pretreatment employing acids can produce high reaction rates and improved cellulose hydrolysis (Sun and Cheng, 2002). There are two reaction modes that have been observed when acid has been applied to lignocellulosic biomass, decreasing the degree of polymerisation, disrupting cellulose crystallinity and causing significant delignification.



Both acid-catalysed reactions have Arrhenius's temperature dependencies (kg 1 and kg 3), and the reactions have first order dependencies on the effective acid concentration. At high temperatures the H_2SO_4 employed in this study dissociates to $[HSO_4]^-$ and provides 1 mol of acid equivalent. With knowledge of activation energies and rate constants, the optimal time and temperature can be found to maximise production of the intermediate, the desired saccharides (Sun and Cheng, 2002).

In a typical process, biomass is ground to facilitate the permeation of acid into the biomass. Hemicellulose can be completely removed by pre-hydrolysing the biomass. Dilute acid pretreatment can increase the rate of cellulose depolymerisation, more than any other pretreatment technique (Kumer et al., 2009; Alvira et al., 2010).

Alkaline Pretreatment

During alkaline pretreatment, lignocellulosic biomass undergoes two reactions, solvation and saponification (Hendriks and Zeeman, 2009).

Solvation – Is the interaction between a solute (solid) and solvent (liquid), which leads to the stabilisation of the solute species in the solution (Lai et al., 1991).

The intermolecular ester bonds cross-linking xylan hemicellulose, other hemicellulose, and lignin are saponified (Rabmanolontoa and Saka, 2016). The resulting lignocellulosic material is swollen, increasing surface area, decreasing crystallinity and disrupting the lignin structure (Sun and Cheng, 2002; Rabmanolontoa and Saka, 2016).

Saponification – A process by which triglycerides are reacted with sodium or potassium hydroxide to produce glycerol and a fatty acid salt (Sun and Cheng, 2002). Lipids that contain fatty acid ester linkages undergo subsequent hydrolysis (Rabmanolontoa and Saka, 2016).

Alkaline pretreatment is predominantly a delignification process. Studies have shown that pretreatment employing sodium hydroxide can increase the rate of delignification by approximately 75 % (Zhao et al., 2008). Unlike acid-catalysed pretreatments, alkaline pretreatments are limited by their potential production of irrecoverable salts and/or incorporated salts in the lignocellulosic biomass (Hendriks and Zeeman, 2009).

Organosolvent pretreatment

The organosolvent reaction mechanism requires the addition of an (aqueous) organic solvent mixture with/without a catalyst-such as an acid, base or salt – to the biomass under specific temperatures and pressures (Chum et al., 1985; Sun and Cheng, 2002; Alriols et al., 2009). During pretreatment, internal lignin bonds, lignin-hemicellulose bonds, and glycosidic bonds in hemicellulose react with the organic solvent (Alriols et al., 2009), resulting in the formation of lignin droplets on the surface of the pretreated biomass.

Without the addition of a catalyst, organosolvent pretreatment begins with the auto-ionization of water. The resulting hydronium ions and acetic acid released from hemicellulose serve as catalysts that promote the hydrolytic cleavage of both α- and β-aryl ether linkages in lignin. It is the cleavage of these ether linkages that is primarily responsible for lignin breakdown prior to dissolution of the fragments (El Hage et al., 2010).

1.4.1.3.3 Analysis of Recovery Mechanisms

The necessity for acid, solvent and sugar recovery from the reaction mixture is critical not only for the successful conversion of fermentable sugars to ethanol but for the economic and environmental commercial viability of the process. Each pretreatment process demonstrates the necessity for recovery and the individual recovery requirements which must be examined.

Acid Pretreatment

During acid pretreatment both the chemicals and sugars produced require recovery to reduce potential inhibitor formation and feedback inhibition (see Chapter 3) (Alvira et al., 2010; Paulová et al., 2013; Guo et al., 2014; Sindhu et al., 2016).

Acid Recovery – The use of acids requires special corrosion-resistant reactors, additional personnel and special handling procedures due to the hazardous corrosive nature of the pretreatment technique (Sun and Cheng, 2002; Banerjee et al., 2010; Alvira et al., 2010). Removal of acids from the reaction mixture is essential due to high operational costs as a consequent of its additional requirements (Conde-Mejía et al., 2012), compared to other pretreatments. Some studies have investigated the neutralisation of pretreatment acids. Cara et al. (2007) reports that this can lead to an increase in solid waste. The use of low acid concentrations will reduce the necessity for acid recovery from the reaction mixture, with dilute acids simply being removed using a separation and filtration process. (Alvira et al., 2010; Paulová et al., 2013).

Sugar Recovery – The application of a three stage conversion process (pretreatment, hydrolysis and fermentation), otherwise known as separate hydrolysis and fermentation (SHF) requires the implementation of a sugar recovery process. Production of sugars

utilising SHF has the potential for feedback inhibition (Kumar et al., 2015), as discussed in Chapter 3. Consequently, sugars produced using this process must be recovered.

Recovery is found to have different optimal conditions with regards to temperature. A high temperature is desirable to maximise glucose yields, while a lower temperature is advantageous in the liberation of xylose (Bensah and Mensah, 2013). Sugar recovery is a two stage process. A low severity (low temperature and low acid concentration) process required to promote hemicellulose hydrolysis and a high severity (high temperature and high acid concentration) process is used to hydrolyse the remaining proportion of cellulose to glucose (Nguyen et al., 2000; Bensah and Mensah, 2013).

Alkaline Pretreatment

The use of alkaline chemicals has been demonstrated to be very effective in the removal of lignin and the increased digestibility of cellulose. However, the use of sodium hydroxide and other alkaline chemicals is very expensive and for economic reasons must be recovered from the reaction mixture (Bensah and Mensah, 2013). Two of the most commonly recovered reagents are ammonia and lime, while the hydroxides are generally neutralised (Kim et al., 2003; Bensah and Mensah, 2013).

Ammonia recovery – Ammonia Recycle Percolation (ARP) is a process whereby the biomass is pretreated with aqueous ammonia in a flow-through column reactor. The ammonia flows at high temperatures through the column which has been packed with biomass (Kim et al., 2003). The solid and liquid fractions are subsequently separated, with the liquid fraction sent into a steam-heated evaporator for ammonia recovery. The aqueous ammonia can then be recycled to the reactor inlet (Kim et al., 2003).

Lime recovery – Lime ($Ca(OH)_2$) is advantageous in its ease of recovery compared to other alkali pretreatments such as sodium, potassium and ammonium hydroxide (Sharmas et al., 2002). Lime is recoverable from water as insoluble calcium carbonate ($CaCO_3$) by reaction with CO_2 to form precipitates of $CaCO_3$ or bicarbonate (pH dependant), and thus allowing the wash water to be reused. The carbonate can be converted to lime using established lime kiln technology (Chang et al., 1998).

Organosolvent Pretreatment

Organosolvent pretreatment is considered to be an expensive process. The solvents however, due to their volatility and low boiling points are easily recovered and recycled, making the process an attractive alternative to conventional pretreatment techniques (Zhao et al., 2009). The chemical recovery process can isolate lignin as a solid material and carbohydrates as a syrup (Lora and Aziz, 1985; Aziz and Sarkanen, 1989).

The advantage of employing solvents of low boiling points, is the ease at which they can be recovered from the reaction mixture by simple distillation with concomitant low energy requirement for their recovery (Zhao et al., 2009). Ethanol losses during pretreatment can be readily replenished from fermentation of the dissolved sugars (Yawalata, 2001).

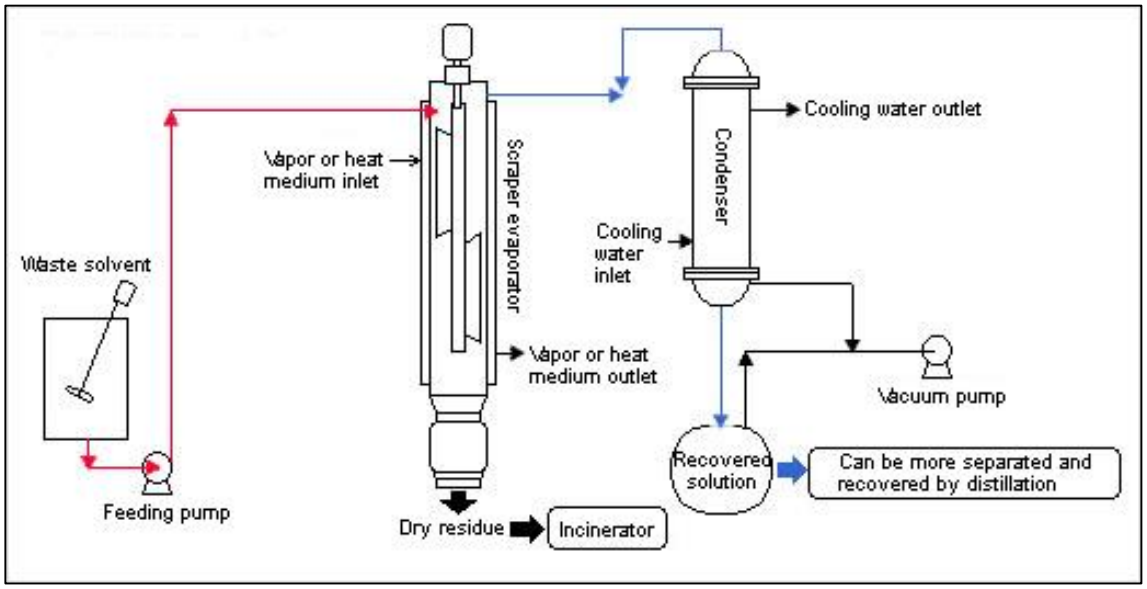


Figure 1.9: Solvent Recovery System (JCEM Vietam, 2012)

1.4.2 Hydrolysis of Lignocellulosic Biomass

Hydrolysis of pretreated lignocellulosic biomass is thought to be the second most important stage of the bioconversion process. It has been shown that effective pretreatment is fundamental for optimal hydrolysis and downstream operations (Wyman, 1994; Gamage et al., 2010). During the hydrolysis of lignocellulosic biomass, the released polymer sugars, cellulose and hemicellulose are hydrolysed into free monomer molecules

readily available for fermentation conversion to bioethanol (Chandel et al., 2007). In general, conversion of the hemicellulose and cellulose fractions into their monomeric sugars involves either chemical (dilute and concentrated acid hydrolysis) or enzymatic hydrolysis (El-Zawawy et al., 2011; Limayem and Ricke, 2012; Zabed et al., 2016). There are some hydrolysis methods in which no chemicals or enzymes are applied. For instance, lignocellulose may be hydrolysed by gamma-ray or electron-beam irradiation, or microwave irradiation. However, these processes are commercially unimportant (Balat et al., 2011).

1.4.2.1 Chemical Hydrolysis of Lignocellulosic Biomass

Chemical hydrolysis involves exposure of pretreated lignocellulose materials to a chemical for a long period of time at specific temperatures (Taherzadeh and Karimi, 2007) and often results in the conversion of cellulose and hemicellulose to monomeric sugars. In the chemical hydrolysis of lignocellulosic biomass either dilute or concentrated acid is applied, each with variations (Balat et al., 2011; Limayem and Ricke, 2012). Dilute acid hydrolysis is one of the oldest technologies for converting cellulose to bioethanol. This process is carried out using high temperatures ($200^{o}C$-$240^{o}C$), low concentrations (1-3%), and high pressure (El-Zawawy et al., 2011). Most dilute acid hydrolysis processes are limited to a sugar recovery efficiency of approximately 50% (Badger, 2002). Concentrated acid hydrolysis, the more prevalent method, has been considered to be the most practical method (Melligan et al., 2012). Unlike dilute acid hydrolysis, concentrated acid hydrolysis is not followed by high concentrations of inhibitors and produces a high yield (90%) of monomeric sugars; however, it requires large-quantities of acid as well as costly acid recycling, which makes it commercially less attractive (Hamelinck et al., 2005). Concentrated acid hydrolysis employs acid concentrations in the range of 10-30% (Iranmahboob et al., 2002), and longer residence times, to complete conversion of cellulose to glucose. In comparison to dilute acid hydrolysis, concentrated acid hydrolysis leads to little sugar degradation and gives sugar yields of 100% (Yu et al., 2008). However, environment and corrosion problems in addition to high acid consumption and recovery costs has resulted in major barriers for economic success (Yu et al., 2008). This particular issue has driven the research and development into enzymatic hydrolysis and cellulolytic enzymes which were investigated in the present study.

1.4.2.2 Enzymatic Hydrolysis of Lignocellulosic Biomass

Enzymatic hydrolysis is a natural and environmentally friendly alternative to acid or alkali hydrolysis, employing carbohydrate degrading enzymes (cellulases and hemicellulases) to hydrolyse lignocellulose into fermentable sugars (Keshwani and Cheng, 2009). Enzymatic hydrolysis was the focus of the conversion processes explored in this study.

Enzymatic hydrolysis is usually performed using either commercially available enzyme preparations or by using enzyme producing microorganisms directly that secrete enzymes during their growth in the media (Zabed et al., 2016). The utility cost of enzymatic hydrolysis is low compared to acid and alkali hydrolysis, however, the cost of producing cellulase and hemicellulose enzymes is still a major challenge for commercial bioethanol production (Koppram and Olsson, 2014).

Cellulose is typically hydrolysed by the enzyme called cellulase. These enzymes are produced by several microorganisms, commonly by bacteria and fungi. *Trichoderma reesei* is one of the most efficient and productive fungi used to produce industrial grade cellulolytic enzymes (Balat, 2011). Cellulase is a mixture of enzymes that act synergistically on cellulose and convert it to glucose. At least three enzymes are required in a typical cellulose mixture, including endo-1-4-β-glucanase or carboxymethylcellulases (EC 3.2.1.4), exoglucanse or cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Taha et al., 2016; Nigam, 2013). An endoglucanase randomly cleaves β-1, 4-glycosidic linkages of D-glucan chains in the amorphous regions of cellulose molecule or the surface of microfibrils and produce free chains that contain both reducing and non-reducing ends. Cellobiohydrase then acts on the reducing and non-reducing ends and cleaves them into cellobiose. β-glucosidase then converts cellubiose into glucose (Bajaj et al., 2009).

Unlike cellulose, hemicellulose (xylan) is chemically quite complex, and its degradation requires more specific and multiple enzyme systems (Zabed et al., 2016). Xylan degrading enzymes are produced by a wide variety of fungi and bacteria such as *Trichoderma* spp. (Wong and Saddler, 1992), *Penicillium* spp. (Jorgensen et al., 2003), *Talaromyces* spp. (Tuohy et al., 1993), *Aspergillus* spp. (Dos Reis et al., 2003), and *Bacillus* spp. (Virupakshi et al., 2005). A typical hemicellulose system includes Endo-1,4-β-xylanase or *endo*xylanase (E.C.3.2.1.8), xylan 1,4-β-xylan esterases, ferulic and

pcoumaric esterases, α-1-arabinofuranosidases, α-glucuronidase (E.C.3.2.1.139), α-arabinofuranosidase (E.C.3.2.1.55), acetylxylan esterase (E.C.3.1.1.72) and α-4-O-methylglucuronosidases xylosidase (E.C.3.2.1.37) (Taha et al., 2016). The endoxylanase hydrolyses the main chains of xylan and β-xylosidase hydrolyses xylooligosaccharides into xylose. The α-arabinofuranosidase and α-glucuronidase act on the xylan backbone and remove arabinose and 4-o-methyl glucuronic acid, respectively (Saha, 2003). Hemicellulolytic esterase include acetyl esterases which hydrolyse the acetyl substitutions on xylose moieties, while feruloyl esterases hydrolyse the ester bonds between arabinose substitutions and ferulic acid. Hemicellulose and lignin are released much easier with the aid of feruloyl esterases (Howard et al., 2003).

1.4.3 Fermentation of Lignocellulosic Biomass

The third and final stage of the bioconversion process is the fermentation of monomeric sugars to ethanol. Pretreatment and hydrolysis processes are designed to optimise the fermentation process (Gamage et al., 2010). Simple sugars produced as a result of the depolymerisation of cellulose and hemicellulose are fermented by microorganisms into biofuels (ethanol, butanol, acetone, diesel, etc.,) or biochemicals (e.g., organic acids), (Chandel et al., 2007; Balan, 2014). Traditionally, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are used. Capable of efficiently fermenting glucose into bioethanol, *S. cerevisiae* is the most frequently used microorganism in the biofuels and the brewing and wine industries (Limayem and Ricke, 2012). *S. cerevisiae* can easily ferment hexose sugars to bioethanol, but have limited ability in the fermentation of xylose, as *S. cerevisiae* lack enzymes that convert xylose to xylulose (Tian et al., 2008). However, this yeast can ferment xylulose (Shi and Jeffries, 1998). Natural xylose-fermenting yeasts, such as *Pichia stipites*, *Candida shehatae*, and *Candida parapsilosis*, can metabolise xylose via the action of xylose reductase to convert xylose to xylitol, and of xylitol dehydrogenase to convert xylitol to xylulose (Katahira et al., 2016).

In recent years, bacteria such as *Scheffersomyces stipitis*, *E. coli* and *Klebsiella oxytoca*, have attracted particular attention, given their rapid fermentation (Hayes, 2009). However, they can often have limitations, which reduce their effectiveness compared to *S. cerevisiae* (Balan, 2014). For instance, *E. coli* cannot tolerate high concentrations of inhibitors, and *S. stipitis* have a low ethanol metabolic yield (Zhong et al., 2009). Meanwhile, *Z. mobilis*, a Gram-negative bacterium, has been recognised for its ability to

efficiently produce bioethanol at high rates from glucose, fructose and sucrose. When compared to *S. cerevisiae*, *Z. mobilis* was shown to produce higher yields of 5% and up to 5-fold higher bioethanol volumetric productivity (Saez-Miranda et al., 2008). Unfortunately, *Z. mobilis* too has little to no effect on the pentose sugars (Hahn-Hägerdal et al., 2006).

Thermophilic anaerobic bacteria including *Clostridium thermohydrosulfuricum* (Cooke and Morgan, 1994), *Thermoanaerobacter ethanolicus* (Avci and Donmez, 2006), *Thermoanaerobacter mathranii* (Larsen et al., 1997), *Clostridium thermosaccharolyticum* (Baskaran et al., 1995), have also been extensively investigated for lignocellulosic ethanol production (Balat, 2011). Thermophilic anaerobic bacteria have a distinct advantage over conventional yeasts in their ability to use a variety of inexpensive biomass feedstocks and withstand temperature extremes (Knutson et al., 1999). However, their use in the biofuels industry is limited by their low bioethanol tolerance (Georgieva et al., 2007) and their production of negative by-products which can create difficulties in the downstream processing of ethanol recovery (Gírío et al., 2010).

In the cellulosic ethanol process, ethanol fermentation can be performed using a variety of process configurations such as separate enzymatic hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and simultaneous saccharification, filtration and fermentation (SSFF) (Hahn-Hägerdahl et al., 2006; Olofsson et al., 2008). Typically, enzymatic hydrolysis and fermentation is carried out separately (SHF). SHF is a conventional two-step process where the lignocellulose is hydrolysed using enzymes to form reducing sugars and the resulting sugars fermented to ethanol using various yeasts (Menon and Rao, 2012), as previously described. The advantage of this process is that each step can be performed using optimum processing conditions (pH 4-6, enzymatic hydrolysis at 45-50^{o}C (318-323K) and fermentation at about 30^{o}C (303K)) (Tengborg et al., 2008), while the major drawback is the risk of inhibitor formation and the inhibition of cellulase and β-glucosidase enzymes by glucose released during hydrolysis (Silverstein, 2004; Kumar et al., 2015).

Simultaneous saccharification and fermentation (SSF) has been demonstrated as the most promising process integration in ethanol production. Effective when combined with dilute acid or high temperature hot-water pretreatment, SSF is widely used in both industrial and laboratory scales (Bertilsson et al., 2009; Balat, 2011). In SSF, cellulases and xylanases are employed to convert carbohydrate polymers to monomeric sugars which

can be easily fermented to bioethanol. These enzymes are notoriously susceptible to feedback inhibition (Jeffries and Jin, 2000). Advantageously, this process has an enhanced rate of hydrolysis, lower enzyme loading rate and results in higher bioethanol yields. Unlike SHF, SSF requires the processing conditions to be similar and compatible with each other. *T. reesei* cellulases, which are the most commonly used commercial enzyme preparations, have optimal activity at pH 4.5 and 55^{o}C (328K). While *S. cerevisiae* are routinely employed at pH 4.5 and 37^{o}C (310K) (Dien et al., 2003). A typical fermentation will take approximately 4-7 days, depending on the feedstock type and pretreatment employed (Schell et al., 1998). More recently, a novel technique known as simultaneous saccharification, filtration and fermentation (SSFF) has been highlighted (Kumar et al., 2015).

In our own research, SSF has been demonstrated to be a necessary part of the bioconversion process (Smullen et al., 2017a; Smullen et al., 2017b), relieving feedback inhibition and inhibitor formation.

1.5 Life Cycle Assessment in the Production of Biofuels

The growing concerns about climate change, rising costs of fossil fuels and the geo-political uncertainty associated with possible interruption of current fossil fuel-based energy supplies, have motivated nations to seek clean and renewable substitutes to reduce their greenhouse gas (GHG) emissions (Roy and Dutta, 2013). A major goal of biofuels is to reduce environmental impacts relative to the fuel source they are displacing. 2nd generation biofuels have been demonstrated as a good source of clean fuel. In fact, ethanol produced using energy crops such as those under investigation in the current study have been proven to reduce CO_2 emissions compared to conventional feedstocks (starches and sugars, and fossil-based resources) and in most cases are carbon neutral (Pimental and Patzek, 2005; Caslin et al., 2010). However, it is the large quantities of chemicals employed during the pretreatment process that still remain a major concern. As a result, emerging technologies must assess their potential environmental impact prior to investigation and development.

Life cycle assessment (LCA) is one such tool to aid in the analysis of environmental performance. A novel technique employed in the biofuels industry and the current study, life cycle assessment (LCA) is a valuable tool used to analyse the environmental

performance of a process and the potential impacts of a product (Borrion et al., 2012). LCA takes into account all resources and energy inputs required to make a product, the wastes and the health and ecological burdens associated with the product (Menon and Rao, 2012). In effect, performing a life cycle assessment of the entire ethanol production system (crop cultivation to ethanol distribution) would quantify the total benefits as well as any drawbacks that the process might contain and identify opportunities for process improvements (Kemppainen and Shonnard, 2005).

LCA was initially designed to examine environmental impacts of historical or current production over short, defined time periods to identify the largest impact reduction potential and improvement strategies without "burden shifting". Traditionally used to inform product development and policy, LCA is now used to enforce such policies and to improve, design and compare both old and current process methodologies (Gerbrandt et al., 2016).

Using a generic framework provided by ISO 14040 and 14044 (ISO, 2006a; ISO, 2006b), LCA can analyse the entire life cycle (cradle-to-grave, i.e. extracting and processing raw materials; manufacturing, transportation and distribution; use, reuse, maintenance; recycling and final disposal) or a specific part of the process (gate-to-gate). By examining the system of interest, quantifying the material and energy inputs and outputs to air, soil and water, LCA can assess not only how the system can potentially impact on the environment, but potential areas of the process which could be improved (Borrion et al., 2012).

An LCA is categorised into four process aims:

- To define the objective and limits of the system,
- To determine the life cycle inventory,
- To quantify the life cycle impact categories,
- Interpretation of the results (ISO, 2006a; ISO, 2006b).

With these aims in mind, an LCA can be constructed using well defined and descriptive process parameters which model the life cycle and calculate potential emissions produced and resources consumed. In addition, emissions and resources can be related to various environmental problems through the act of classification and characterisation (Baumann and Tillman, 2004).

The main phases of the LCA procedure as outlined by ISO 14040 and ISO 14044 include:

(1) The goal and scope,
(2) Inventory analysis,
(3) Impact assessment (ISO, 2006a; ISO, 2006b).

1.5.1 Goal and Scope

The goal and scope of the study is an important component in the LCA. Used to define the product to be studied and the purposes of the study, the goal and scope summarises and communicates the intended application of the study, the reason for carrying out the study and to whom the results are intended for (Baumann and Tillman, 2004). Many other crucial components of the study are formed using the goal and scope, such as the system boundaries (inputs and outputs included in the study, including any limits or exclusions applied), types of environmental impacts being considered, i.e. global warming, eutrophication etc. and the level of detail employed in the study in addition to the data source to be employed (Baumann and Tillman, 2004; Farrell et al., 2006).

1.5.2 Inventory Analysis

Based on the goal and scope of the study, a system model or inventory analysis is developed. A flow model is constructed according to the system boundaries of the study, activities included in the system of interest (production, processes, transports, uses and waste management) and the flow between them (SAIC, 2006). Defining rigorous system boundaries reduces subjectivity, increasing repeatability and minimising unreliable results (Raynolds et al., 2000). In addition, data for all activities is collected including inputs and outputs of all activities (raw materials, products and solid wastes), while the amount of resources used and emissions emitted to air, soil and water are calculated in relation to the functional unit (dependant on the aim of the study, expressed in terms of per unit output i.e. production of 1 L of ethanol) (Baumann and Tillman, 2004; Gnansounou et al., 2005; Power and Murphy, 2009). The functional unit corresponds to a reference flow to which all other modelled flows of the system are related (Baumann and Tillman, 2004). Inventory results are often presented as bar charts and other types of graphic presentations.

1.5.3 Impact Assessment

The final, most conclusive phase of the LCA procedure is the impact assessment. The life cycle impact assessment (LCIA) aims to describe, or at least to indicate, the impacts of the environmental loads quantified in the inventory analysis. In particular, the LCIA aims to transform the inventory results into more environmentally relevant information. This includes information on impacts on the environment rather than just measurements of emissions and resources used (Baumann and Tillman, 2004), translating the environmental loads into environmental impacts, such as acidification, ozone depletion, effect on biodiversity and human toxicity etc. There are several stages to this process:

- Identification and selection of appropriate impact categories, models of cause effect chains and their end-points.
- Classification – assignment of LCIA results parameters to their respective impact categories.
- Characterisation – calculation of the extent of the environmental impact per category.
- Normalisation – relating the characterisation results to a reference value (CML, 2001). The aim is to gain a better understanding of the magnitude of the environmental impacts caused by the system under study.
- Grouping – sorting and ranking of the characterisation indicators into one or more sets, useful for the analysis and presentation of the results.
- Weighting – aggregation of characterisation results across impact categories (not recommended for use in public LCA studies). Weighting is a qualitative and or quantitative process where the relative importance of an environmental impact is weighted against all others (Baumann and Tillman, 2004).

Not all sub-phases are required in an LCIA study. The core, most commonly employed LCIA sub-phases are classification, characterisation and normalisation.

1.5.4 Environmental Performance of Ethanol Production

Emissions from ethanol production can vary significantly depending on the process, design and feedstock employed. Ethanol represents a closed carbon dioxide cycle. The subsequent burning of ethanol releases carbon dioxide which is recycled back into the plant material. The plants then use the carbon dioxide to synthesize cellulose during photosynthesis (Chandel et al., 2007). Additionally, ethanol production processes can

utilise energy from renewable energy sources, as a result no net carbon dioxide is added to the atmosphere, making ethanol an environmentally beneficial energy source (Chandel et al., 2007).

LCA software packages such as SimaPrò and Gabi, and CML methods (described by Guinée et al., 2001) are widely and consensually employed in industry to evaluate and compare the impacts associated with the production of ethanol (Pereira et al., 2015). Using these methods, the use of resources and emissions are categorised in terms of equivalent values according to potential environmental impacts (Pereira et al., 2015):

- *Resource depletion* – the decline in natural resources is seen as both an environmental and societal issue, divided into non-renewable and renewable or abiotic (non-living) and biotic (living) resources (CML, 2002). This category can often be seen as the most difficult as a wide variety of considerations must be included in the assessment and calculation of the impact. Furthermore, existing methods are regularly incomplete as a limited number of resources are assessed (Baumann and Tillman, 2004).
- *Land use* – the use of land as well as changes in land use (transformation) are assessed in this category. Importantly, the extent to which land use and land transformation leads to changes in biodiversity and to life support functions (e.g. Biological functions) is evaluated (CML, 2002).
- *Global warming* – evaluation of climate change based on greenhouse gas (GHG) emissions emitted to the atmosphere and their capacity to absorb infrared radiation, thereby heating the atmosphere. Carbon dioxide (CO_2), methane (CH_4), chlorofluorocarbons (CFCs), nitrous oxide (N_2O) and other trace gases are all detected and measured for their potential contribution to climate change, expressed as its global warming potential GWP. The GWP of a substance is defined by the UN Intergovernmental Panel on Climate Change (IPCC) as the ratio between the increased infrared absorption it causes and the increased infrared absorption caused by 1 kg of CO_2 (ECCC, 2015). As greenhouse gases have different atmospheric life spans, their GWP's are calculated for different time horizons, developed by the IPCC (CML, 2002; Soloman et al., 2007).
- *Acidification* – the acidification potential reflects the maximum acidification a substance can have. Acidification severity can vary depending on where the acidifying pollutants are deposited (Baumann and Tillman, 2004). The major

acidifying pollutants are sulphur dioxide (SO_2), nitrogen oxide (NO_x), hydrochloric acid (HCl) and aqueous ammonia (NH_3). The environmental impact of these pollutants are governed by, for example, the buffering capacity of soil and waters, climatic conditions and the rate of harvesting (Huijbregt, 1999; Baumann and Tillman, 2004).

- *Eutrophication* – results from excessively high levels of nutrients that lead to shifts in species composition and increased biological productivity, for example, algal bloom. In LCA, the eutrophication category covers not only the impact of nutrients, but also those of degradable organic pollution and sometimes waste heat (SETAC-WIA 2, 2001; CML, 2002). Calculation of the eutrophication potential is based on the proportions of nitrogen, phosphorus, carbon and oxygen in the average chemical formula for aquatic biomass formation ($C_{106}H_{263}O_{110}N_{16}P$) (Stumm and Morgan, 1981; Baumann and Tillman, 2004).
- *Photochemical Oxidation Demand* – photo-oxidants are secondary pollutants formed in the lower atmosphere from NO_x and hydrocarbons in the presence of sunlight. Ozone is one of the most important photo-oxidants contributing to the formation of photochemical smog (Harrison, 1990). The photo-oxidant potential of a substance is based in a 5-day trajectory model of pollution transportation over Europe (CML, 2002). Photochemical oxidation is calculated by the estimation of ozone quantity formed photochemically by a given substance (Baumann and Tillman, 2004).
- *Ozone Depletion* – ozone as previously described, is a harmful pollutant formed in the lower atmosphere, damaging plants, human health and the built environment. However, ozone is also an essential substance in the upper atmosphere, the stratosphere, where it screens out more than 99% of the dangerous ultraviolet radiation from the sun (Baumann and Tillman, 2004). Depletion of ozone refers to the thinning of the stratospheric ozone layer, with the formation of large holes, as a result of various chlorinated and bromated substances, such as CFC's and halons (Harrison, 1990). The World Meteorological Organisation (WMO) are responsible for the development of the ozone depletion potentials which are maintained and updated periodically (CML, 2002).
- *Toxicity* – one of the most complicated impact categories, owing to the number of toxicity parameters assessed including human toxicity, aquatic toxicity and ecological toxicity and the many substances (heavy metals, organic solvents etc.)

evaluated. There is no coherent framework for characterising the toxicological impacts of pollutants, but research and methodology development is in progress internationally (CML, 2002; Baumann and Tillman, 2004).

Most importantly toxic substances can be characterised into different groups based on fate, exposure or intake and effect. The USES-LCA model (Guinèe et al., 1995; Huijbregts, 1999), which is very similar to the Ecoindicator'99 method employed by SimaPrò, has been developed and used to produce toxicity indicators for over 200 substances. Using a global fate model that combines the regional, continental and global scales together with physico-chemical property factors of substances, the model can theoretically describe how a substance is dispersed between air, soil and water (Baumann and Tillman, 2004). Subsequently, the model can calculate the predicted environmental concentration of the substance and subsequent degree of impact of the related substance (Baumann and Tillman, 2004).

The environmental harm of these emissions depends on the extent of their dilution in recipient bodies (air, soil and water). Accordingly, it is also possible to calculate the volume of air, soil or water necessary to dilute emissions to a less harmful level. In quantitative LCA studies, the number of result parameters after the inventory calculation could be in the hundreds and so often only a selection of these parameters are presented in a normalised or weighted form to facilitate comparison. Qualitative LCA studies can also be conducted depending on the intended audience, although they often lose to quantitative studies with respect to detail and accuracy but usually gain with respect to speed (Baumann and Tillman, 2004). Qualitative studies are not as conclusive or communicative as quantitative studies and thus are favoured by the author commissioning the study.

1.5.4.1 Water Requirement in the Biorefinery Industry. Necessity for Recycling and Reuse.

Producing biofuels using the sugar platform is a water intensive process. Water is used in almost all of the processing steps, including crop cultivation, the extent of which is dependent on the feedstock type and origin (Wu et al., 2009; Balan, 2014). Ethanol production requires water for feedstock grinding, liquefaction, fermentation, separation and drying. In many cases, water is pumped from the ground, which puts considerable stress on local resources or from surface waters and municipal water supplies (Bernardi

et al., 2013; Yuliani et al., 2013). Reuse and recycling of water after the bioconversion process will help to reduce not only the stress on water resources but the high cost of the process (Balan, 2014).

The impact of large scale production of energy crops on water resources has not yet been fully examined. The amount of water consumed depends on the production process employed and the degree of water reuse and recycling applied. It is estimated that with current technology, producing 3.78 L of cellulosic ethanol via a biochemical conversion process consumes approximately 37 L of water (Aden et al., 2002). It is believed that with the development of new and more efficient technologies and downstream processing systems, this could be reduced considerably to 22.3 L or more, once ethanol yields are increased (Aden et al., 2002). The minimisation of water consumption during each processing step is critical in order to reduce water costs and improve the environmental performance of the process. In some cases, the choice of pretreatment technique can heavily influence the rate of water consumption.

Chapter 2. Materials and Methods

2.1 Cultures, Enzymes and Biochemicals

All materials used in this study were obtained from the following suppliers:

From Biocatalysts (Wales, UK): Cellulase 13L.

From Kerry Ingredients and Flavours (Charleville, Cork): Biocellulase W.

From Novozymes (Bagsvaerd, Denmark): Enzyme Complex.

From Herbs, Gardens and Health (Kent, UK): *Saccharomyces cerevisiae.*

From Sigma Aldrich (Arklow, Wicklow): D-xylose, D-glucose, D-arabinose, D-mannose, D-galactose (h.p.l.c. grade), carboxymethyl methyl cellulose (CMC), oat spelt xylan, cellobiose, glucose assay kit (GO), 4-aminobenzoic acid, benzoic acid, d-biotin, nicotinic acid, vitamin B, d-pantothenic acid (hemicalcium salt), vitamin C and thiamine.

From Fisher Scientific Ltd (Ballycoolin, Dublin 15): Whatman No: 1 filter paper, 0.45µm PTFE syringe filters, sulphuric acid (analytical grade), methanol (analytical grade), sodium hydroxide pellets (analytical grade), aqueous ammonia solution (analytical grade), sodium hypochlorite, sodium sulphite (analytical grade), acetic acid (analytical grade), sodium molybdate, ammonium sulphate, copper (II) sulphate, magnesium sulphate heptahdrate, calcium chloride, iron sulphate, sulphuric acid, manganese sulphate -1-hydrate and potassium dihydrogen phosphate.

From Lennox (Naas, Dublin): Ammonium hydroxide, monoethanolamine, sodium citrate, citric acid, potassium sodium tartarte tetrahydrate, 3, 5-dinitrosalicylic acid.

2.2 Scientific Instrumentation

The instrumentation used in this study was obtained from the following suppliers:

Dionex (Surrey, UK): Ion Chromatograph System 5000 - High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD ICS 5000). Fitted with an electrochemical detector employing a gold electrode, pH probe, reagent free IC-eluent generator (RFIC-EG), and CarboPac column. Metal free and

utilising all PEEK^{tm} flow paths; Chromeleon 7.2 software; Dionex AS-AP autosampler; Dionex SP single gradient pump.

Sanyo Gallenkamp PLC (Leicester, UK): Gallenkamp Orbital Shaker Incubator, variable temperature and speed orbital shaker.

Shimadzu (Tokyo, Japan): Shimadzu UV-VIS 1800 Spectrophotometer.

Shimadzu (Shimadzu UK Ltd, UK): GC-14A Gas Chromatograph fitted with a split / splitless injector port, nitrogen carrier gas, Porapaq Q capillary column and flame ionisation detector.

2.3 Biological / Lignocellulosic Samples

All samples were received from Teagasc Crop Research Centre, Carlow, Ireland. Switchgrass variety Shawnee was harvested at Knockbeg, Co. Laois. Willow, miscanthus and hemp were all harvested on-site at Teagasc Oakpark Carlow.

2.4 Feedstock Selection and Collection

Miscanthus (*x-giganteus*), willow (*Salix)* and hemp (*Cannabis sativa L*) were harvested at the Crops Research Centre, Carlow, Ireland (52.86^{o} N, 6.90^{o} W). Switchgrass (*Panicum virgatum L*) variety Shawnee was harvested at Knockbeg, Co. Laois, approximately 5 km from the Crop Research Centre. All four crops were harvested in 2011.

Miscanthus, willow and hemp were grown on a Eutric cambisol soil (FAO, 2008). Soil pH in the experimental area exceeded 7 during the experiment while levels of phosphorus and potassium were both at the highest index levels (Coulter and Lalor, 2008). Miscanthus (*x giganteus)* sown in 1994 (17 years), was established from microplantlets and a density of 11,188 plants ha^{-1} was measured in October 1994. The crop received no nutrients until 2007 when 70 kg N ha^{-1} were applied to the crop, weeds were controlled periodically by spraying Roundup (glyphosate) during late March before shoot emergence. Willow samples were taken from a crop sown in 2008 (3 years), the crop was sown from 20 cm willow cuttings (*Salix viminalis*) obtained from Seed Technology, Ballymountain, Waterford, Ireland. First year growth from the crop was cut-back in 2008, after which herbicide (41 ha^{-1} Basta (glufosinate-ammonium)) and 100 kg N ha^{-1} were applied to the crop before harvest in 2011. The hemp crop (*Cannabis sativa L*) from which samples

were taken was sown in April 2011 using certified seeds obtained from Cooperative Centrale des Producers de Semences de Chanvre, Le Mans, France. The crop was grown without pesticides and was fertilised with nitrogen, phosphorus, sulphur and potassium to ensure that all major nutrients were non-limiting.

Switchgrass (*Panicum virgatum L*) was sown in May 2008 (3 years), and was grown on a luvisol soil (Finnan et al., 2016), a heavy moisture retentive soil. Upland and lowland ecotypes of switchgrass were sown, at a seeding rate of 20 kg ha^{-1} and rolled immediately afterwards. Plots measured 16.5 m in length and 5 m in width. Trials were managed under a low input regime in order to minimise energy input and greenhouse gas emissions. Applications of 125 kg K ha^{-1} and 30 kg P ha^{-1} were spread on the trial areas in March 2009. While, 70 kg N ha^{-1} was applied in May 2009, 60 kg N ha^{-1} in 2010, and a further 70 kg N ha^{-1} in 2011. The switchgrass crop (*Panicum virgatum L*) from which samples were taken was grown from seeds originating from the United States (Casler et al., 2004). No pesticides were used during the cultivation of the crops, however, a broad leaf herbicide was sprayed on the crop in 2008 (Finnan et al., 2016).

Samples were taken from miscanthus during the winter when most of the leaves had fallen off, all leaf material had fallen from the willow crop, and some leaf material remained on the top of the hemp crop at the time of harvest in September 2011. Switchgrass was harvested in October 2011. However, all leaf material was excluded from the samples of hemp used in this study.

2.5 Compositional Analysis of Willow, Miscanthus, Hemp and Switchgrass

To determine the overall efficiency of processes designed to convert lignocellulosic biomass to ethanol it is necessary to establish a base from which bioconversion yields can be calculated. A particular focus was placed on the production of ethanol from cellulose and so all reported experimental analysis relates solely to the cellulose composition of the crops. As part of the overall study both cellulose and hemicellulose sugars were identified and quantified.

2.5.1 Determination of Total Cellulose using the Monoethanolamine Method

Total cellulose was determined using the monoethanolamine method described by Nelson and Leming (1957), in which 3 g of dry lignocellulosic sample was refluxed at 170^{o}C for 180 min with 100 cm^3 of monoethanolamine. Once the mixture had cooled 100 cm^3 of

water was added. Approximately 100 cm^3 of the supernatant liquor was decanted off into a beaker and the remaining mixture filtered through a weighed sintered glass filtering crucible. Both the decanted liquor and filtrate were re-filtered through the mat of cellulose that had formed in the sintered glass crucible.

200 cm^3 of hot water was used to wash the resulting cellulose, which was subsequently washed into a beaker with 75 cm^3 of water. The washed cellulose was bleached with 10 cm^3 of H_2SO_4 (10% v/v solution) and 10 cm^3 NaOCl (24 g L^{-1} solution) for 5 min at room temperature, before being filtered again with 15 cm^3 of water and 15 cm^3 of sulphurous acid (0.25N). The bleached cellulose mat was washed again with 15 cm^3 of cold water followed by 15 cm^3 of a 3% Na_2SO_3 solution.

The sample was transferred into a 250 cm^3 beaker using less than 50 cm^3 of water, where 50 cm^3 of a 6% Na_2SO_3 solution was added. The beaker was covered and incubated for 20 min in a boiling water bath. (The appearance of a rose colour at this point indicates the presence of lignin, signalling the need for further bleaching). The residue was filter once more. The cellulose residue was washed with 150 cm^3 of boiling water, 50 cm^3 of cold water, 25 cm^3 of cold 10% w/v CH_3COOH, 50 cm^3 cold water, 150 cm^3 of boiling water, 75 cm^3 of cold water containing 2 drops of NH_4OH and finally 200 cm^3 of boiling water. The residue was dried at 105ºC and weighed. The cellulose concentration was calculated based on the initial weight of the sample used (Foyle, 2007).

2.5.2 Quantification of Monomeric Sugars using the Liquid Hot Water Method

Quantification of monomeric sugars was conducted using a modified method of the liquid hot water method (Pérez et al., 2007). 5 g of dried biomass sample was refluxed with 100 cm^3 of deionised water for 12 h at 170ºC and at standard atmospheric pressure. Samples were filtered using a sintered glass crucible and the solid fraction weighed. The sample was transferred to a 100 cm^3 duran bottle and hydrolysed in triplicate using a commercial enzyme preparation as detailed in Section 2.7.2. Hydrolysates of the solid fraction were subsequently analysed as described in Section 2.5.3.

2.5.3 Sugar Analysis of Biomass Hydrolysates using HPAE-PAD Ion Chromatography

Liquid hydrolysates of pretreated and hydrolysed samples were filtered using a 0.45µm PTFE syringe filter. 1 cm^3 of the filtered sample was transferred to a 1000 cm^3 volumetric flask and diluted using double deionised water. The diluted sample was re-filtered using

a 0.45µm PTFE syringe filter before being injected onto the Ion Chromatograph 5000. Calibration standards (10ppm) for galactose, glucose, mannose, xylose and arabinose were prepared using pure analytical grade sugars purchased from Sigma Aldrich, Ireland. Process conditions for the IC were as follows: SA10 CarboPac column maintained at 45^{o}C, pump pressure 2000 psi, sample flow rate 1.5 $cm^3\ min^{-1}$ and 0.0001 mol L^{-1} KOH mobile phase. Hydrolysate analysis was performed in triplicate on the Dionex ICS 5000 high performance anion exchange chromatographic system with amperometric detection (HPAE-PAD IC, Dionex UK Ltd, Surrey).

2.5.4 Ash Determination

Determination of ash in biomass was conducted following the NREL (National Renewable Energy Laboratory) LAP 005 standard analytical procedure (Sluiter et al, 2005). Porcelain crucibles were labelled and then placed into a muffle furnace at 575^{o}C for 4 h. Once cooled the crucibles were weighed. 0.5 g samples of switchgrass were placed in the crucibles and the total weight recorded. Crucibles and samples were placed back into the muffle furnace at 575^{o}C for 4 h. Percentage ash content was determined once the crucibles had cooled (see Section 2.10).

2.5.5 % Total Solids

Determination of total solids in biomass was conducted following the NREL (National Renewable Energy Laboratory) LAP 008 standard analytical procedure (Sluiter et al, 2008a). Small oven dishes were washed and then dried to a constant weight in a drying oven at approximately 105^{o}C for 4 h. After being cooled the dishes were weighed. 0.5 g samples of switchgrass were weighed and the total weight of sample and dish recorded. Samples were placed into a drying oven where they were dried to a constant weight at 105^{o}C. Samples were cooled and the percentage total solids of the switchgrass was obtained on an as-received basis to that of an oven dry weight basis (see Section 2.10).

2.5.6 Determination of Moisture Content

Stainless steel moisture dishes were weighed and 5 g of biomass sample added (exact weight recorded). The dishes were placed into a drying oven with the lids off at 105^{o}C and dried to a constant weight. The lids were replaced and the dishes transferred to a

desiccator for 1 hour. The dishes were re-weighed and the percentage moisture was determined (Sluiter et al, 2008a) (see Section 2.10).

2.5.7 Acid Soluble / Insoluble Lignin Analysis of Switchgrass

Switchgrass samples (0.3g) were hydrolysed with 3 cm^3 of 72% H_2SO_4 for 2 h at room temperature. Samples were stirred every 10-15 min to ensure equal distribution of the acid. The hydrolysates were diluted to 4% H_2SO_4 with deionised water and the sample was heated in an autoclave to 121^oC for 1 h. After the sample had cooled the hydrolysate was vacuum filtered and the solid fraction washed with 50cm^3 of deionised water. The solid fraction was transferred to an oven dish and dried to a constant weight at 105^oC. Once cooled the liquid fraction was diluted and analysed on a UV-Vis Spectrometer (Shimadzu, Kyoto, Japan) at 205nm for acid soluble lignin (ASL) and a ICS 5000 high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex, England) for monosaccharide analysis (Pronto, 1998). The solid fraction was transferred to a pre-weighed porcelain crucible which was placed in a muffle furnace at 575^oC for 4 h. Upon cooling the crucibles were weighed and the acid insoluble lignin (AISL) calculated (Dence, 1992) as described in Section 2.10.

2.6 Enzyme Assays

Comparisons between commercial cellulase enzyme preparations is complicated by poorly-defined enzyme assays supplied by manufacturers. For this reason, standardised enzyme assays were employed in this study. All experimental analysis conducted using commercial enzyme preparations was performed at a minimum in triplicate.

2.6.1 3, 5-Dinitrosalicylic Acid Method for Reducing Sugars

The DNS reagent was prepared by adding 10 g of 3, 5-dinitrosalicyclic acid and 300 g of potassium sodium tartrate tetrahydrate to 160 cm^3 of a 10% w/v sodium hydroxide solution. The solution was mixed and quantitatively transferred to a 1000 cm^3 volumetric flask. 500 cm^3 of deionised water was added to the volumetric flask which was then wrapped in tin foil to protect the solution from light. The mixture was gently heated in a water bath at 50-60^oC, stirring periodically until all of the solid material had dissolved. Once the solution had cooled, it was made up to 1000 cm^3 with deionised water. The

solution was mixed well before being stored in a cool dry cupboard away from the light (Miller, 1959).

A glucose standard curve was prepared using a stock solution (1 g / 100 cm^3 of 0.1% w/v benzoic acid), from which working standards containing 0-1.4 mg ml^{-1} (0-45 ppm) were prepared. 1 cm^3 of the standard / sample and 1 cm^3 of deionised water was pipetted in triplicate into a series of test tubes. 2 cm^3 of DNS reagent was added to each test tube which was covered and boiled in a water bath for exactly 10 min. The test tubes were cooled on ice, 10 cm^3 of deionised water was added and the samples mixed. Colour development was measured at 540 nm using a Shimadzu UV-Vis 1800 spectrophotometer (Shimadzu, Tokyo, Japan). Reagent blanks were prepared by substituting the 1cm^3 of standard with 1 cm^3 of deionised water. The final reducing sugar concentrations in samples were calculated using the glucose standard curve (Miller, 1959).

2.6.2 Cellulase (EC 3.2.1.4) using Carboxymethyl Cellulose (CMC) (CMCase Activity)

The CMCase assay is based on the principal of estimating a fixed amount of product (glucose) produced from the substrate (CMC) after a defined time. The glucose is measured using the DNS method for estimation of sugars (Miller, 1959). 1 cm^3 of a 1.6% w/v carboxymethyl cellulose (CMC) solution (prepared in 0.1 mol L^{-1} citric acid buffer, pH 4.6) was incubated with 1 cm^3 of enzyme sample (appropriately diluted) at 37^{o}C for 1 h. Following the addition of 2 cm^3 alkali 3,5-dinitrosalicylic acid (DNS) reagent, samples were boiled in a water bath for 10 min. Samples were cooled on ice, 10 cm^3 of deionised water was added and absorbance was measured at 540 nm using a Shimadzu UV-Vis 1800 spectrophotometer.

Sample blanks were prepared by adding enzyme after the addition of DNS reagent. Reagent blanks were prepared by replacing the enzyme sample with deionised water. The corrected absorbance values were converted into µmol of glucose released using a standard glucose curve prepared under corresponding experimental conditions. Enzyme units of activity were calculated from those samples yielding 0.8-1.1 mg of glucose reaction mixture.

2.6.3 Xylanase (EC 3.2.1.8)

Xylanase activity was determined using DNS reagent to monitor the amount of reducing sugar liberated from a solution of xylan (Miller, 1959). The xylan substrate was prepared

by boiling 0.5 g oat spelt xylan in 80 cm^3 deionised water for 2-3 min. Following the submersion of the substrate in an ice-bath, the xylan substrate was added to 10 cm^3 of 1 mol L^{-1} citric acid buffer and the solution was made up to a final volume of 100 cm^3 with deionised water. 1 cm^3 of xylan substrate was pre-equilibrated to 40ºC followed by the addition of 1 cm^3 of enzyme (appropriately diluted) and the reaction allowed to proceed for 10 min before termination by the addition of 2 cm^3 of DNS reagent. The test tubes were placed in a boiling water bath for 5 min. Samples were cooled, 10 cm^3 of deionised water was added and absorbance measured at 540 nm using a Shimadzu UV-Vis 1800 spectrophotometer.

Sample blanks were prepared by adding 2 cm^3 DNS reagent to 1 cm^3 of the xylan substrate before addition of 1 cm^3 of enzyme sample. Reagent blanks were prepared by replacing the sample with 1 cm^3 of deionised water. The corrected absorbance values were converted into µmol of xylose produced using a standard curve prepared under corresponding experimental conditions.

2.6.4 Cellulase Activity using Filter Paper (FPase Activity)

Exo- 1,4 β – glucanase (FPase, EC 3.2.1.91) activity was measured as the amount of reducing sugar liberated from filter paper strips as described by Mandels and Weber (1969). 1 cm^3 of 0.05 mol L^{-1} sodium citrate (pH 4.8), was pre-incubated with 0.05 g of a Whatman No: 1 filter paper strip (6 cm X 1 cm) at 50ºC. After vigorous mixing, 1 cm^3 of enzyme (appropriately diluted) was added to start the reaction which was allowed to proceed for 60 min at 50ºC and was terminated by the addition of 2 cm^3 DNS reagent. Samples were placed in a boiling water bath for 10 min and then cooled. Samples were diluted by the addition of 10 cm^3 deionised water and the paper mash allowed to settle to the bottom of the sample tubes before measuring the absorbance at 540 nm using a Shimadzu UV-Vis 1800 spectrophotometer.

Sample blanks were prepared by the addition of the enzyme after addition of DNS reagent. Reagent blanks were prepared by substituting the 1 cm^3 of enzyme with 1 cm^3 of 0.05 mol L^{-1} sodium citrate (pH 4.8). The corrected absorbance values were converted into µmol of reducing sugar produced using a glucose standard curve prepared under corresponding experimental conditions.

2.6.5 β – Glucosidase (EC 3.2.1.21)

β-glucosidase (EC 3.2.1.21) activity was measured as the release of glucose from cellobiose and is expressed as the amount of enzyme liberating 1 μmol of glucose min^{-1} under standard reaction conditions. 1 cm^3 of 0.015 mol L^{-1} cellobiose (prepared in 0.05 mol L^{-1} sodium citrate buffer pH 4.8) was pre-equilibrated at 50ºC for 2-3 min. 1 cm^3 of the enzyme sample (appropriately diluted using citrate buffer) was added to the substrate and incubated at 50ºC for 30 min. The reaction was terminated by submersion in a boiling water-bath for 5 min.

Sample blanks were prepared by incubating 1 cm^3 of the enzyme at 50ºC for 30 min. Following submersion in a boiling water bath for 5 min, 1 cm^3 of substrate was then added. The amount of glucose liberated (μmol) was measured using a Glucose Assay Kit (GO) –purchased from Sigma Aldrich, Ireland.

2.7 Bioconversion of Willow, Miscanthus and Hemp

2.7.1 Chemical Pretreatment of Feedstocks

Dried lignocellulosic biomass samples were milled through a 1 mm screen using a Retsch cutting mill SM2000 (Retsch, Pennsylvania, USA) to reduce the particle size. 0.5 g feedstock samples were mixed in a 50 cm^3 Duran bottle with 20 cm^3 of the pretreatment chemicals: sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), ammonia solution (NH_3) and methanol (CH_3OH), at various chemical concentrations of 1, 2, 3 and 4 mol L^{-1}. The duran bottles were sealed and placed into an orbital shaker incubator (Gallenkamp, UK) at 40ºC, 100 rpm for 48 h. After this time, the pretreatment mixture was filtered using sintered glass crucibles to separate the solid and liquid fractions (10-15 cm^3 filtrate). The pretreated solid fraction was washed with 100 cm^3 of deionised water (20 cm^3 aliquots) to ensure complete removal of pretreatment chemical and a pH of 7.0-7.6. 10 cm^3 of the liquid fraction was collected (in triplicate) for sugar analysis using the ICS 5000. The resulting solid fraction was either directly hydrolysed and saccharified or subjected to SSF. Recovered chemicals can be reconstituted at this point if required for further use.

2.7.2 Enzymatic Saccharification of Pretreated Biomass Samples

The wet solid fractions of the pretreated biomass was transferred to 100 cm^3 Duran bottles and 15 cm^3 of 0.05 mol L^{-1} citrate buffer was added. The pH was adjusted to 4.6 and 3

cm^3 of the commercial enzyme preparation (appropriately diluted) - 5B06443, C013L or NS22119 – was added at an enzyme loading rate of 0.028 g cm^{-3}. Bottles were sealed and placed in an orbital shaker incubator (Gallenkamp, UK) at 50ºC, 100 rpm for 24 h. Samples were filtered and diluted as described in Section 2.5.3 and sugar quantification conducted in triplicate using the IC 5000.

Process control samples were prepared excluding the sample hydrolysate in order to quantify and correct for any sugars which may have been released from the commercial enzyme preparation during the saccharification process.

2.7.3 Simultaneous Saccharification and Fermentation (SSF) of Willow, Miscanthus and Hemp

Pretreated biomass samples were transferred to 250 cm^3 Duran bottles followed by 25 cm^3 of 0.05 mol L^{-1} citrate buffer, 15 cm^3 mineral solution and 1.5 cm^3 vitamin solution (du Preez and van der Walt, 1983), and samples were gently mixed. The necessary pH adjustments to 5.6 were made to satisfy both the enzyme and the yeast in the fermentation broth. 4 cm^3 of commercial enzyme preparation C013L (Biocatalysts) (appropriately diluted) was added at an enzyme loading rate of 0.017 g cm^{-3} followed by 4 g *S. cerevisiae* (Fermipan Red) yeast. Bottles were sealed with sterile cotton wool and parafilm, and placed in an anaerobic chamber which was then placed in an orbital shaker incubator (Gallenkamp, UK) at 37ºC, 100 rpm for 72 h. After 72 h, the fermentation liquor was immediately decanted to centrifugal tubes, sealed with parafilm and centrifuged at 8000 rpm for 2 min. Ethanol quantification was conducted using gas chromatography.

Both *Saccharomyces cerevisiae* and the commercial enzyme preparations – NS22119, C013L and 5B06443 (a crude microbial cellular extract) may contain varying amounts of carbohydrates. For this reason, control samples were prepared which excluded the pure glucose standard utilised in the trial fermentations, as described in Section 2.9. This is necessary in order to quantify and correct for any sugars which may have been present in the enzyme and / or yeast preparations.

2.7.4 Ethanol Analysis of Biomass Hydrolysates using Gas Chromatography

Hydrolysates generated from the pretreatment and SSF of willow, miscanthus and hemp were filtered using a 0.45 μm PTFE syringe filter, appropriately diluted and an internal standard of methanol and butanol was added. Samples were analysed on the GC – 14A

Gas Chromatograph using the following process conditions: Porapaq Q column maintained at 210^{o}C, air and hydrogen flame and a nitrogen carrier gas. Ethanol hydrolysate analysis was performed on the Shimadzu GC-14A Gas Chromatograph (Shimadzu UK Ltd, U.K.). All sample analysis was conducted in triplicate.

2.8 Bioconversion of Switchgrass

2.8.1 Chemical Pretreatment of Switchgrass

The dried biomass sample was milled through a 1 mm screen using a Retsch cutting mill SM2000 (Retsch, Pennsylvania, USA) to reduce the particle size. Samples (0.5g) were mixed in a 50 cm^3 Duran bottle with 20 cm^3 of 0.5, 1 and 2 mol L^{-1} concentrations of the pretreatment chemicals: sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), ammonia solution (NH_3) and methanol (CH_3OH). Samples were sealed in pressurised duran bottles and placed into a shaker incubator at 40^{o}C, 100 rpm for 48 h (no catalyst was required). After this time the pretreatment mixture was filtered (13-15 cm^3 filtrate) and the solid fraction washed with 100 cm^3 of deionised water (20 cm^3 aliquots) until the final wash had a pH of 7.0-7.6. The filtrate was collected for determination of residual sugars, liberated from the pretreatment biomass. The resulting solid fraction (0.76g approximate wet weight) was either directly hydrolysed / saccharified or subjected to SSF. These four pretreatment chemicals and their concentrations were employed for switchgrass as they have been demonstrated to be very effective on soft crops (Kim et al., 2011; Nlewen and Thrash, 2010; Xu and Cheng, 2011; Zhang et al., 2013), and can be easily recovered and reused to reduce costs.

2.8.2 Enzymatic Saccharification of Pretreated Biomass

The wet solid fraction of the pretreated biomass was transferred to 100cm^3 Duran bottles and 15 cm^3 of 0.05 mol L^{-1} citrate buffer was added. The pH was adjusted to 4.6 and 3 cm^3 of commercial enzyme preparation C013L (appropriately diluted) containing a mixture of cellobiose, β-glucosidase and β-glucanase (Biocatalysts) was added at an enzyme loading rate of 0.028 g cm^{-3}. Bottles were sealed and placed in an orbital shaker incubator (Gallenkamp, UK) at 50^{o}C, 100 rpm for 24 h. Samples were filtered and diluted as described in Section 2.5.3 and the saccharified mixture was analysed in triplicate on the ICS 5000 for quantification of monomeric sugars.

Process control samples were prepared excluding the sample hydrolysate in order to quantify and correct for any sugars which may have been released from the commercial enzyme preparation during the saccharification process.

2.8.3 Simultaneous Saccharification and Fermentation (SSF) of Switchgrass

Pretreatment and saccharification using 0.5 mol L^{-1} produced conversion yields which were significantly lower than the 1 and 2 mol L^{-1} concentrations also employed. Therefore this concentration was eliminated from further investigation with SSF.

Samples pretreated at 1 mol L^{-1} and 2 mol L^{-1} were transferred to 250 cm^3 duran bottles followed by 25 cm^3 of 0.05 mol L^{-1} citrate buffer, 15 cm^3 mineral solution and 1.5 cm^3 vitamin solution (Maurice, 2011), and samples were gently mixed. The necessary pH adjustments to 5.6 were made to satisfy both the enzyme and the yeast in the fermentation broth. 4 cm^3 of commercial enzyme preparation C013L (enzyme loading rate of 0.017 g cm^{-3}) was added, followed by 4g *S. cerevisiae* (Fermipan Red) yeast. Bottles were sealed with sterile cotton wool and parafilm, and placed in an anaerobic reaction chamber which was placed in an orbital shaker incubator (Gallenkamp, UK) at 37ºC, 100 rpm for 72 h. After 72 h, the fermentation liquor was immediately decanted to centrifuge tubes, sealed using parafilm and centrifuged at 8000 rpm for 2 min. Samples were analysed on the GC-14A for ethanol quantification.

Both *Saccharomyces cerevisiae* and the commercial enzyme preparations – NS22119, C013L and 5B06443 (a crude microbial cellular extract) may contain varying amounts of carbohydrates. For this reason, control samples were prepared which excluded the pure glucose standard utilised in the trial fermentations. This is necessary in order to quantify and correct for any sugars which may have been present in the enzyme and / or yeast preparations.

2.9 Determination of Maximum Theoretical Yield using Trial Fermentations

In order to assess the effectiveness and efficiency of the SSF process, it is important to establish how much sugar is potentially available to the fermentation process from the raw lignocellulosic material so that an estimate can be made of the potential for ethanol production.

Conversion of glucose to ethanol via the EMP pathway has a potential maximum theoretical yield of 0.51g of ethanol per g of glucose (Rose and Harrison, 1971; Skoog and Hahn-Hägerdal, 1990). However, it is important to note that this does not take into account the tendency of yeast to utilise glucose for cell growth / biomass production. As a general rule it is assumed that 45 kg of glucose yields between 18 – 23 kg of ethanol or 23 – 28 litres of ethanol (Rose and Harrison, 1971; Skoog and Hahn-Hägerdal, 1990).

The fermentation conditions employed in the present study were assessed for ethanol production using various starting concentrations of pure glucose (Jennings, 1999; Foyle, 2003), and varying reaction times and two different yeast brands (Dried Fast Action Yeast and Fermipan Red Yeast). Stock solutions of glucose were prepared at various concentrations (1, 2, 3, 4, 5% w/v glucose solution). Samples were prepared and analysed as detailed in Section 2.7.3 and 2.7.4. Trial fermentations were conducted at the optimum temperature of 35ºC and at 100 rpm. The reactions were allowed to proceed for 1, 2, 4, 6, 8, 10, 12, 14, 24, 48, and 72 h.

The results confirmed a conversion rate to ethanol of 83.3% (0.42 g of ethanol / g of glucose) at 48 h and 92% (0.46 g of ethanol / g of glucose) at 72 h (Dried Fast Action Yeast and Fermipan Red Yeast) could be achieved with effective pretreatment of the lignocellulosic biomass.

Both *Saccharomyces cerevisiae* and the commercial enzyme preparations – NS22119, C013L and 5B06443 (a crude microbial cellular extract) may contain varying amounts of carbohydrates. For this reason, control samples were prepared which excluded the pure glucose standard utilised in the trial fermentations, and the lignocellulosic hydrolysate in the SSF treated samples (Section 2.7.3 and 2.8.3). This is necessary in order to quantify and correct for any sugars which may have been present in the enzyme and / or yeast preparations.

2.10 Experimental Calculations

Percentage bioconversion yields of cellulose to glucose (1) and cellulose to ethanol (2) of each sample was calculated using the following equations:

- **% Conversion (cellulose to glucose) = ((C / 1000) x V / G x 1.111) x 100% (1)**

  Where:

  C is the concentration of monomeric glucose sugars in ppm (divided by 1000 to convert to g ml^{-1}), **V** is the volume of sugar produced per sample (see section 2.7.1), and **G** refers to the quantity of cellulose in grams per original sample (see section 2.5). **1.111** – converts cellulose to equivalent glucose. Formula adapted from that detailed in Dowe and McMillan (2001).

- **% Conversion (cellulose to ethanol) = ((A/S / 1,000,000 x V) / G) x 100% (2)**

  Where:

  A is the area of the peak, **S** is the slope of the line as calculated using calibration standards, (divided by 1000 to convert to g L^{-1}, divided by 1000 to convert to g ml^{-1}), **V** is the volume of sugar produced per sample and **G** refers to the quantity of cellulose in grams per original sample (Section 2.7.3). % conversion (glucose to ethanol) based on ethanol theoretical yield (0.51 g ethanol / g of glucose) was calculated for some samples and results are presented in Section 4.2.3. Formula adapted from that detailed in Dowe and McMillan (2001).

- **Acid Insoluble Lignin** (Dence, 1992)

  $$AISL = \frac{(Weight\ (Wa) - Weight\ (Wb) - (Weight\ (Wc) - Weight\ (Wb)}{300} \times 100\%$$

  Wa = dry crucible plus sample: Wb = dry crucible: Wc = crucible plus ash

- **Acid Soluble Lignin** (Pronto, 1998)

  $$ASL = \frac{Abs}{110} \times Dilution\ Factor \times \frac{(Final\ volume)}{Initial\ weight} \times 100\%$$

- **Percentage Ash** (Sluiter et al., 2005)

$$\% \text{ Ash} = \frac{(\text{Weight (crucible plus ash)} - \text{Weight (crucible)}}{\text{Oven Dry Weight (sample)}} \times 100$$

- **Percentage Total Solids** (Sluiter et al., 2008a)

$$\text{Total Solids} = \frac{(\text{Weight (dry dish plus sample)} - \text{Weight (dry dish)})}{\text{Weight (sample as received)}} \times 100\%$$

- **Percentage Moisture** (Sluiter et al., 2008a)

$$\text{Moisture} = 100 - \frac{(\text{Weight (dry dish plus sample)} - \text{Weight (dry dish)})}{\text{Weight (sample as received)}} \times 100\%$$

2.10.1 Replication and Calculation of Error

All sample preparation was conducted in duplicate or triplicate and analysed in triplicate. Percentage error was calculated using commercial software (Microsoft Office Excel, 2013).

2.11 Economic Assessment of Pretreatment Chemicals

The economics of ethanol production for bioenergy are influenced not only by feedstock prices, production and distribution costs but also by chemical inputs to the system. Chemical purity, concentration and possibility of recovery can have a substantial impact on the cost of the bioconversion process. The chemical cost per kg of glucose or per litre of ethanol from 1 kg of feedstock was calculated. This cost was influenced by the quantity of product produced from each kg of feedstock as well as the cost of the pretreatment chemicals needed to pretreat 1 kg of feedstock. The initial cost of 1 tonne of chemical was obtained from the suppliers: Tianjin Huaxiang Chemical Co., Ltd, Shijiazhuang Xinlongwei Chemical Co., Ltd, Qingdao HanHaiDa Import and Export Co., Ltd and Weifang Minghan Import and Export Co., Ltd (http://www.alibaba.com/products [accessed December 2015]).

The cost of producing 1 kg of glucose or 1 litre of ethanol was evaluated using conversion yields achieved following pretreatment and saccharification of samples. Yields were extrapolated to demonstrate the cost per kg of glucose at each of the chemical concentrations employed.

2.12 Using SimaPrò LCA Software

Creating an LCA profile of a product or service involves several steps:

- Establishment of the goal and scope of the study,
- Development of the life cycle inventory,
- Assessment of the life cycle impact,
- Interpretation of the data.

Getting started in SimaPrò is assisted by the step by step navigation provided. First the user must open a new project. Following the ISO guidelines, a description of the product / service is defined. Required data libraries are selected and the goal and scope, system boundary and functional unit of the study are described in detail. Secondly, the user must outline the processes and product stages of the LCA profile. Specific materials, processing, energy and transport inputs required for the system are established in the product stages, along with a waste scenario and waste treatment plan.

Life cycle inventory results (LCI) - inventory of emissions to air, soil and water, as well as resource depletion, are calculated following assessment of both the data given by the user and from selected databases employed in the study (see Section 5.2.3). Data is calculated based on information imported from other research studies (Wernet et al., 2016), which then uses CML methods (CML, 2001) to calculate the equivalence values such as CO_2 emissions to air.

The impact of the LCI results are characterised using the environmental parameters: global warming potential, acidification, eutrophication, photochemical oxidation demand, marine toxicity and human toxicity. The classification and characterisation of groups of emissions is mandatory according to ISO 14040 (ISO, 2006a). Normalisation and weighting as described in Chapter 1 are optional. Emissions can be represented in graphic or table format as shown in Section 5.3.

Chapter 3

A Comparative Analysis of Pretreatment Chemicals for the Bioconversion of Willow (*Salix viminalis*), Miscanthus (*x-giganteus*) and Hemp (*Cannabis sativa L*)

Abstract

This study explores the chemical pretreatment of three energy crops – willow (*Salix viminalis*), miscanthus (*x-giganteus*) and hemp (*Cannabis sativa*) – with a view to producing directly comparative information on the performance of four pretreatment chemicals - sodium hydroxide, methanol, sulphuric acid and ammonia employed at various concentrations. The pretreatment efficiency was evaluated in terms of sugar recovery and ethanol production. Bioconversion yields generally increased when the chemical pretreatment concentration was increased (1 mol L^{-1} - 3 mol L^{-1}), declining only once maximum sugar and ethanol yields had been produced. The optimal chemical pretreatment was crop-specific - ammonia (3 mol L^{-1}) was the best pretreatment for willow and miscanthus, whereas sulphuric acid (3 mol L^{-1}) was the optimum pretreatment for hemp. Simultaneous saccharification and fermentation (SSF) significantly increased cellulose conversion yields, up to 42%, for some samples, compared to that of saccharification alone, with cellulose and glucose bioconversion yields as high as 74% and 99% respectively. Three commercial enzyme preparations (Enzyme Complex – NS22119, Biocellulase – 5B06443 and Cellulase 13L – C013L) were evaluated in this study. Choice of enzyme preparation was found to be critical, bioconversion yields were doubled in some instances with different enzyme preparations. Optimising the choice of pretreatment chemical and enzyme preparation can substantially improve the efficiency of energy crop bioconversion.

3.1 Introduction

In recent years, energy crops have been the focus of many research studies (Sørensen et al., 2008; Zhang et al., 2013; Kuglarz et al., 2014; Smullen et al., 2017a; Smullen et al., 2017b), investigating the bioconversion of different feedstocks including corn stover, miscanthus, switchgrass and willow to ethanol and other related bio-products. Bioconversion of the cellulose and hemicellulose fraction of the crops to ethanol is a complex process made difficult by the naturally recalcitrant nature of lignocellulosic biomass. This poses a fundamental challenge in the enzymatic hydrolysis of cellulose to fermentable monomeric sugars and subsequent fermentation to ethanol. Consequently, the objective of this study was the development of an effective and efficient pretreatment technique to increase the susceptibility of cellulose to enzymatic hydrolysis, producing a high cellulose conversion yield with little or no inhibitor formation.

Various physical (milling, chipping and grinding), chemical (acid, alkaline and oxidising agents) and biological (fungi and bacteria) pretreatment techniques have been investigated over the years on disparate lignocellulosic crops and several published reviews provide a general overview of their effects (Behera et al., 2014; Hendriks and Zeeman, 2009; Haghighi-Mood et al., 2013). Although attempts have been made in previous years to define, optimise and improve pretreatment techniques (Mosier et al., 2005; Alvira et al., 2010; Park and Kim, 2012), comparisons between bioconversion studies and other related research studies have been challenged by the use of different research methodologies, insufficient information on the commercial enzyme preparations used and the limited variability in the processing conditions employed.

The current study takes a co-ordinated approach to the investigation and development of pretreatment approaches for three energy crops – willow (*Salix*), miscanthus (*x-giganteus*) and hemp (*Cannabis sativa*) - with a view to producing directly comparative information on the performance of four pretreatment chemicals employed at various chemical concentrations. Importantly, a particular focus has been placed on clearly defining and detailing each process step to facilitate additional future studies on other crop samples and pretreatment approaches. This includes the characterisation of key process components traditionally ill-defined in the literature such as the enzyme complex employed which is central to the saccharification and bioconversion process.

According to our knowledge a comparative analysis of pretreatment chemicals for the bioconversion of energy crops to ethanol on this scale, employing four pretreatment chemicals at four chemical concentrations and three different commercial enzyme preparations has not been reported before. Therefore, the aim of this study was to establish a comparative report on the bioconversion of energy crops to ethanol, assessing the effect of chemical pretreatment and enzyme preparations on bioconversion yields and the necessity for simultaneous saccharification and fermentation (SSF). It is intended that the comparative approaches established in this study will facilitate future development of bioconversion processes, enhancing the commercial viability of ethanol.

3.2 Results and Discussion

3.2.1 Composition of Willow, Miscanthus and Hemp

The composition of lignocellulosic biomass (cellulose, hemicellulose and lignin) can vary significantly depending on several factors including: production inputs, production cycles and environmental conditions. As a result, comparisons between compositional studies can be challenging. To establish the overall efficiency of lignocellulosic bioconversion processes in the current study, and to facilitate comparisons with other bioconversion approaches, it is necessary to determine the cellulose composition of the energy crop(s) under review. Cellulose is the main contributor of monosaccharides in commercial ethanol production and is therefore the sole focus of many bioconversion studies. There are many potential approaches to cellulose quantification including the TFA method (Fengel and Wegner, 1979), the concentrated sulphuric acid method (Grohmann et al., 1984), the NREL method (Sluiter et al., 2008b), the monoethanolamine method (Nelson and Leming, 1957) and the liquid hot water method (Pérez et al., 2007).

The latter two methods were employed in the current study as they are regularly referenced in the literature for such applications. The monoethanolamine approach provides an estimate of the cellulose content of lignocellulose based on the fraction remaining after the solubilisation of lignin and other hemicellulose components. In contrast, the liquid hot water method relies on the enzymatic hydrolysis of the cellulose to glucose after the removal of hemicellulose and lignin. The cellulose composition of willow, miscanthus and hemp in this study was determined to be 35.6%, 49.5% and 43.3%, respectively, using monoethanolamine, while the liquid hot water method produced glucose yields of 25.8%,

43.3% and 25.9%, respectively. However, as will be shown later in this paper the experimental approach which includes enzymatic hydrolysis inherent in the liquid hot water method underestimates the glucose yield.

The cellulose composition of willow, miscanthus and hemp has also been investigated in other studies with reported yields of 43% (Sassner et al., 2008), 38% (de Vrije et al., 2002), and 44% (Sipos et al., 2010) respectively. Table 3.1 presents the conversion yields for willow, miscanthus and hemp pretreated using the four chemicals employed in this study at one concentration (3 mol L^{-1}). Conversion yields are calculated based on cellulose composition as determined by the monoethanolamine method and glucose composition as determined by the liquid hot water method for comparability and relatability to other studies including (Sassner et al., 2008; de Vrije et al., 2002; Sipos et al., 2010). Additional information on conversion yields for 1, 2 and 4 mol L^{-1} pretreated samples is provided in Table 3.3 (a-b).

Table 3.1: Comparison of the percentage bioconversion yields for cellulose (*) and glucose. Samples were pretreated at the same chemical concentration using 3 mol L^{-1} H_2SO_4, NaOH, NH_3 and CH_3OH and samples hydrolysed for glucose analysis using C013L commercial enzyme preparation.

	H_2SO_4		NaOH		NH_3		CH_3OH	
Willow	42.0*	58.0	15.0*	20.0	59.0*	81.0	35.0*	48.0
Mean	41.7	57.6	14.7	20.3	59.0	81.4	34.7	47.9
Std Dev**	±0.2	±0.3	±0.5	±0.7	±0.7	±0.9	±0.6	±0.8
Miscanthus	41.0*	47.0	18.0*	21.0	42.0*	45.0	34.0*	38.0
Mean	41.3	47.2	18.2	20.7	39.2	45.2	33.7	38.4
Std Dev**	± 1.0	±1.1	±0.0	±0.0	±0.3	±0.3	±1.1	±1.3
Hemp	31.0*	51.0	30.0*	49.0	36.0*	60.0	30.0*	50.0
Mean	30.9	50.8	29.5	49.4	35.6	59.7	29.9	50.1
Std Dev**	±0.0	±0.0	±0.4	±0.7	±0.9	±1.6	±0.6	±1.0

Samples were prepared and analysed in triplicate with mean and standard deviation** calculated. Calculation methodology is described in Section 2.10.

Although cellulose the main component of lignocellulosic biomass is the sole contributor of sugar (glucose) for ethanol production, some hemicellulose sugars are referenced as being convertible (with difficulty) to fermentable monomeric sugars (Chandel et al., 2010). Chapter 1 mentioned briefly the major monomers in hemicellulose including hexose (β-D-glucose, α-D-galactose and β-D-mannose), and pentose (β-D- xylose and α-L-arabinose) and while these sugars were not the main focus of our investigation, our compositional analysis of willow, miscanthus and hemp was broadened to include their quantification.

Pretreatment of lignocellulosic biomass produces a "slurry" containing liquid and solid fractions. The solid fraction mostly contains cellulose and lignin as the major components, while the liquid fraction contains xylose as the main sugar, and small concentrations of other sugars including glucose, arabinose, galactose and mannose mainly from the hemicellulose liquid hydrolysate (Njoku et al., 2013).

The liquid hot water method described by Pérez et al. (2007) was employed in the determination of hemicellulose monomeric sugars, similar to that of cellulose. Samples were analysed using the ICS 5000 and results are presented in Table 3.2.

Table 3.2: Quantitative analysis of hemicellulose sugars in willow, miscanthus and hemp using the liquid hot water method. Samples were hydrolysed using C013L commercial enzyme preparation.

Feedstock	Galactose	Mannose	Xylose	Arabinose
Willow	2.02%	2.08%	10.50%	1.37%
Std Dev*	±0.07%	±0.11%	0.00%	±0.06%
Miscanthus	0.51%	0.24%	21.68%	1.27%
Std Dev*	±0.01%	0.00%	±0.01%	0.00%
Hemp	1.94%	2.58%	14.98%	1.17%
Std Dev*	±0.03%	0.00%	±0.01%	0.00%

Samples were prepared and analysed in triplicate with standard deviation* calculated. Calculation methodology is described in Section 2.10.

The hemicellulose composition of willow in this study was found to be similar to that of Sassner et al. (2008) when the monomeric sugars; mannose, xylose and arabinose were analysed and compared. Conversion yields in this study however were lower than those reported by Ali and Tschirner (2010). Our galactose yields of 2.02% were comparable however, with the 2.1% achieved by Ali and Tschirner (2010) and Sassner et al. (2008).

Following analysis of miscanthus, xylose and mannose yields were found to be higher in the current study (21.68% and 0.24%, respectively) than that reported by Scordia et al. (2013) (19.9% and 0.1%, respectively). In contrast, galactose and arabinose yields were lower than the 0.6% and 1.7% (respectively) determined by Scordia et al. (2013).

Analysis of hemp demonstrated a significantly lower galactose and mannose yield when compared to Sebastião et al. (2016). While xylose and arabinose were determined to be higher in the current study than the 14.4% and 0.9% (respectively) reported by Kuglarz et al. (2014).

Further investigation of the composition of willow, miscanthus and hemp in this study determined the following:

- % Total Solids for Willow (96%), Miscanthus (90%) and Hemp (97%).
- % Moisture for Willow (4%), Miscanthus (10%) and Hemp (3%).
- % Ash for Willow (8.5%), Miscanthus (13%) and Hemp (2.5%).

3.2.2 Pretreatment and Saccharification of Willow, Miscanthus and Hemp

Lignocellulose is a complex substrate and as discussed in Chapter 1, pretreatment is essential for delignification, effective and efficient separation of the cellulose / hemicellulose matrix and subsequent conversion of cellulose to fermentable monomeric sugars. The methodology performed in the current study was adapted from established methods utilising crops such as switchgrass, miscanthus, pinewood, beechwood and corn stover (Isci et al., 2008; Chen et al., 2009; Zhao et al., 2009; Li et al., 2010a; Li et al., 2010b; Liu et al., 2013).

Pretreatment utilising NH_3 has been found to be very effective on 'soft crops' such as switchgrass and miscanthus. Isci et al. (2008), reported delignification yields of 40-50% and hemicellulose removal yields of 50% when switchgrass was pretreated with NH_3. While Liu et al. (2013), achieved delignification yields of 76.9% for NH_3 pretreated

miscanthus. Lignin removal yields increased significantly to 81.2% for Chen et al. (2009), who investigated the effects of NaOH pretreatment on corn stover. Dilute acid and organosolvent pretreatment have also been shown to achieve high conversion yields for both hard and softwood crops. Li et al. (2010a), examined the use of dilute acid pretreatment on the delignification and conversion of switchgrass and determined that 50% glucan and 28.5% lignin recovery was possible, compared to ionic liquid pretreatment (13.6% lignin recovery). Highest delignification yields were achieved by Zhao et al. (2009) for the pretreatment of pinewood and beechwood using CH_3OH, with yields of 75% and 90% respectively.

NaOH, CH_3OH, H_2SO_4 and NH_3 were assessed at various concentrations for the pretreatment of willow, miscanthus and hemp in order to establish their effect on the cellulose conversion yields of each crop with results graphically presented in Figure 3.1A (Willow), 3.1B (Miscanthus) and 3.1C (Hemp).

A wide range of chemical concentrations were employed in this study (0.5 – 8 mol L^{-1}) in order to determine the optimum pretreatment concentration range under the conditions utilised in this study. At concentrations less than 1 mol L^{-1} the pretreatment chemicals were deemed to be ineffective with lower conversion yield than those observed at the higher concentration range. When the chemical concentration was increased to 5 mol L^{-1} conversion yields began to decline significantly, as a result of substrate degradation. Therefore the chemical concentration range of 1-4 mol L^{-1} was applied in this study for further investigation.

Samples pretreated at the lowest concentration employing 1 mol L^{-1} NaOH, CH_3OH, H_2SO_4 and NH_3 uniformly produced low conversion yields of < 22% with some as low as 3-4% for each crop (Fig. 3.1). Conversion yields increased only slightly for those samples pretreated using 2 mol L^{-1} concentrations. With similar conversion yields achieved for some samples such as hemp (23.2% (CH_3OH), 24.7% (H_2SO_4), and 24.6% (NH_3). Consequently, the identification of an optimum pretreatment at this stage was very difficult. Conversion yields continued to increase when samples were pretreated at the higher chemical concentration of 3 mol L^{-1} before declining when the highest concentration of 4 mol L^{-1} NaOH, CH_3OH, H_2SO_4 and NH_3 was employed.

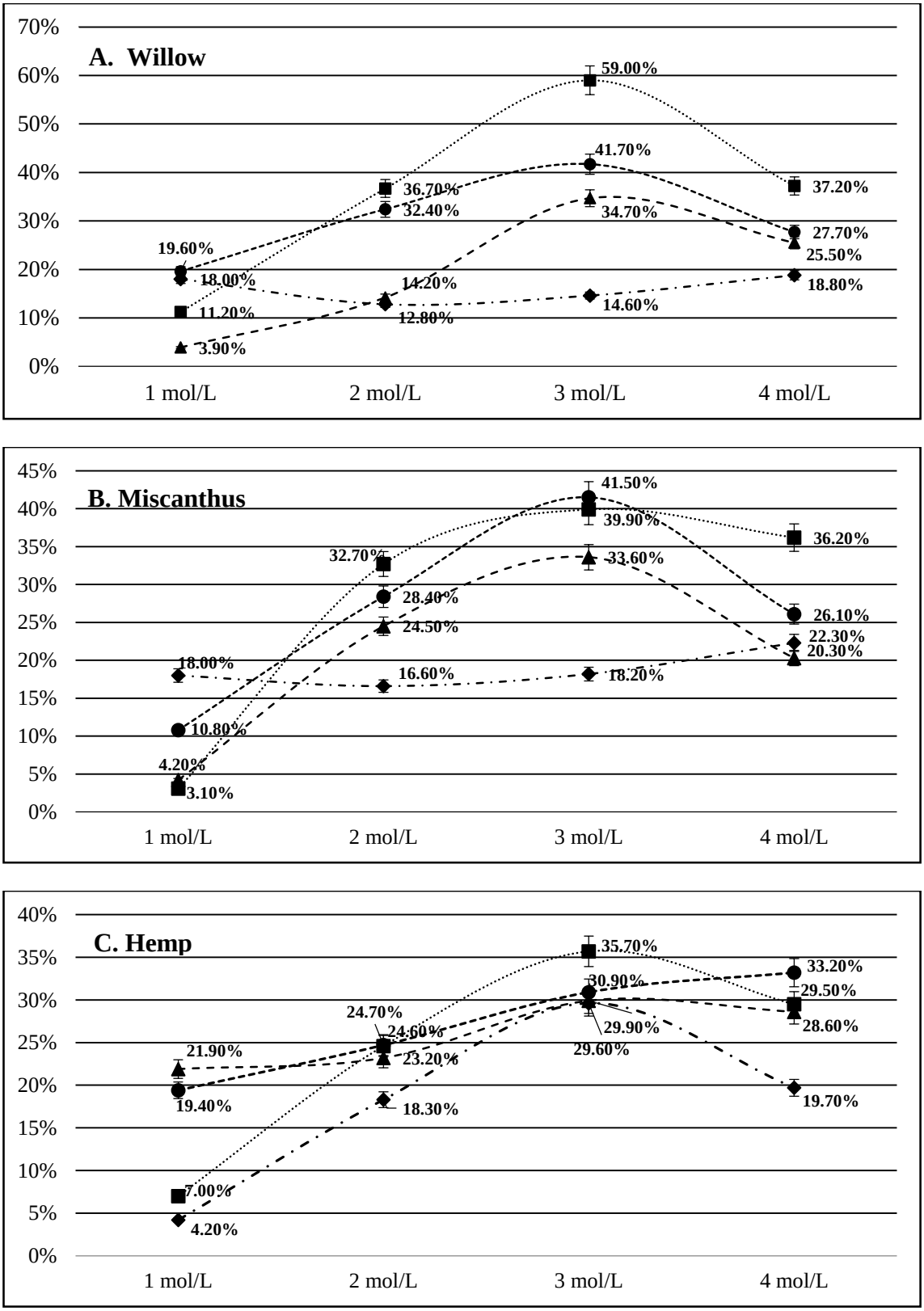


Figure 3.1: Comparative analysis of the % cellulose bioconversion yields for willow (**A**), miscanthus (**B**) and hemp (**C**). Samples were pretreated using: ammonia ■, sulphuric acid ● , methanol ▲ and sodium hydroxide ◆ at various concentrations: 1 mol L^{-1} ◆, ▲, ● and ■, 2, mol L^{-1} ◆, ▲, ● and ■, 3 mol L^{-1} ◆, ▲, ● and ■ and 4 mol L^{-1} ◆, ▲, ● and ■ and saccharified with the enzyme preparation C013L. Samples were performed and analysed in triplicate with percentage error and standard deviation calculated.

Pretreatment employing NH_3 demonstrated the highest cellulose saccharification yields for willow and hemp when 3 mol L^{-1} NH_3 was applied, while 3 mol L^{-1} H_2SO_4 pretreatment was the most effective for miscanthus. NaOH was the least effective chemical for all three crops when employed using different chemical concentrations.

Willow and miscanthus displayed similar conversion yields when pretreated using 3 mol L^{-1} H_2SO_4 (41.7 and 41.5% conversion, respectively), compared to 31% conversion for hemp. Similar conversion yields between willow and miscanthus were also observed when 3 mol L^{-1} CH_3OH was applied, in contrast to the 29.9% achieved by hemp (Fig 3.1). Meanwhile a significant difference was observed in the conversion of willow (59%), miscanthus (39.9%) and hemp (35.7%) when pretreated with 3 mol L^{-1} NH_3.

Conversion yields for pretreated willow varied substantially. Low yields of 14.6% were obtained for 3 mol L^{-1} NaOH pretreated samples compared to 59% when 3 mol L^{-1} ammonia was employed (Fig. 3.1A). The difference in conversion yields was also significant for miscanthus with low yields of 18.2% at 3 mol L^{-1} NaOH compared to the highest yields of 41.5% at 3 mol L^{-1} H_2SO_4 (Fig. 3.1B). Conversion yields for hemp at 3 mol L^{-1} pretreatment chemical were lower at 29.6 to 35.7% (Fig. 3.1C).

Pretreatment of lignocellulosic biomass to monomeric sugars is not a selective process and consequently, the hemicellulose component is also subject to chemical pretreatment. Hemicellulose sugars: galactose, xylose, mannose and arabinose are liberated along with glucose (cellulose) when saccharified using an enyzme preparation. Table 3.3 presents the level of hemicellulose sugars achieved following pretreatment of willow, miscanthus and hemp when 1 mol L^{-1} (Table 3.3a), 2 mol L^{-1} (Table 3.3b), 3 mol L^{-1} (Table 3.3c) and 4 mol L^{-1} (Table 3.3d) NaOH, CH_3OH, H_2SO_4 and NH_3 was applied and samples were hydrolysed with the commercial enzyme preparation C013L.

Hemicellulose compositional analysis using the liquid hot water method confirmed between 10.50% and 21.68% xylose depending on the crop (Table 3.2), while the other pentose sugar, arabinose, was present at much much lower levels of between 1.17% and 1.37% (Table 3.2). In addition to glucose, other pentoses included mannose at between 0.24% to 2.58% and galactose at 0.51% to 2.02% depending upon the crop (Table 3.2).

The recovery of these hemicellulose sugars in hydrolysates under the process conditions investigated in this study can be estimated from the data presented in Table 3.3(a-d). For example, application of the liquid hot water method for willow hemicellulose confirmed

the presence of 2.02% galactose (Table 3.2). Following the pretreatment of willow with 3 mol L^{-1} NaOH, this reduced to 0.97% (Table 3.3c). The conclusion in this example is that there is a 48% recovery of galactose from willow under these specific process conditions.

Table 3.3a: Quantification of hemicellulose sugars (actual yield) for willow, miscanthus and hemp pretreated using 1 mol L^{-1} NaOH, H_2SO_4, CH_3OH, NH_3 as described in Section 2.7.1. and hydrolysed using C013L.

Composition	NaOH	CH_3OH	H_2SO_4	NH_3
		Willow		
Galactose	0.62%	0.43%	0.66%	0.82%
Mannose	1.58%	0.00%	0.10%	0.23%
Xylose	0.68%	3.80%	2.65%	0.79%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Miscanthus		
Galactose	0.69%	0.24%	0.52%	0.79%
Mannose	0.14%	0.00%	0.41%	0.16%
Xylose	0.96%	1.46%	1.83%	5.08%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Hemp		
Galactose	0.67%	0.80%	0.53%	0.85%
Mannose	1.3%	1.71%	0.00%	2.60%
Xylose	0.10%	0.00%	5.50%	0.55%
Arabinose	0.00%	0.00%	0.00%	0.00%

Samples were pretreated and analysed in triplicate.

Table 3.3b: Quantification of hemicellulose sugars (actual yield) for willow, miscanthus and hemp pretreated using 2 mol L^{-1} NaOH, H_2SO_4, CH_3OH, NH_3 as described in Section 2.7.1 and hydrolysed using C013L.

Composition	NaOH	CH_3OH	H_2SO_4	NH_3
		Willow		
Galactose	0.91%	0.50%	0.79%	0.74%
Mannose	1.83%	0.00%	0.31%	1.78%
Xylose	1.97%	5.94%	4.06%	4.00%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Miscanthus		
Galactose	0.66%	0.86%	0.80%	0.80%
Mannose	0.32%	0.00%	0.36%	0.16%
Xylose	2.65%	2.28%	2.24%	1.45%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Hemp		
Galactose	1.32%	0.42%	0.57%	0.92%
Mannose	1.96%	1.81%	1.78%	1.33%
Xylose	0.43%	0.00%	4.60%	1.6%
Arabinose	0.00%	0.00%	0.00%	0.00%

Samples were pretreated and analysed in triplicate

Table 3.3c: Quantification of hemicellulose sugars (actual yield) for willow, miscanthus and hemp pretreated using 3 mol L^{-1} NaOH, H_2SO_4, CH_3OH, NH_3 as described in Section 2.7.1 and hydrolysed using C013L.

Composition	NaOH	CH_3OH	H_2SO_4	NH_3
		Willow		
Galactose	0.97%	0.73%	0.73%	1.00%
Mannose	1.87%	1.02%	1.71%	1.16%
Xylose	2.03%	6.83%	4.09%	2.50%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Miscanthus		
Galactose	0.52%	0.96%	0.77%	0.55%
Mannose	0.36%	0.00%	0.20%	0.23%
Xylose	1.13%	1.88%	2.85%	1.69%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Hemp		
Galactose	1.33%	0.86%	0.96%	1.34%
Mannose	2.40%	2.16%	2.32%	1.48%
Xylose	2.35%	1.96%	2.66%	2.17%
Arabinose	0.00%	0.00%	0.00%	0.00%

Samples were pretreated and analysed in triplicate.

Table 3.3d: Quantification of hemicellulose sugars (actual yield) for willow, miscanthus and hemp pretreated using 4 mol L^{-1} NaOH, H_2SO_4, CH_3OH, NH_3 as described in Section 2.7.1. and hydrolysed using C013L.

Composition	NaOH	CH_3OH	H_2SO_4	NH_3
		Willow		
Galactose	0.23%	0.75%	0.60%	0.80%
Mannose	0.80%	1.73%	0.84%	1.21%
Xylose	0.00%	0.00%	1.38%	2.58%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Miscanthus		
Galactose	0.33%	0.90%	0.72%	0.85%
Mannose	0.50%	0.00%	1.63%	0.00%
Xylose	0.00%	2.00%	1.52%	12.2%
Arabinose	0.00%	0.00%	0.00%	0.00%
		Hemp		
Galactose	0.88%	0.42%	0.55%	0.78%
Mannose	2.26%	1.30%	1.63%	0.90%
Xylose	0.00%	0.00%	1.60%	1.93%
Arabinose	0.00%	0.00%	0.00%	0.00%

Samples were pretreated and analysed in triplicate.

The hemicellulose composition of lignocellulosic biomass and their subsequent conversion methodologies have been investigated and reported in several research studies including that of du Preez (1994), Agbogbo and Wegner (2007) and Njoku et al. (2013). Consistent with these studies, arabinose yields were low and in this study too low to detect or present in trace amounts. Potential explanations for this include early liberation and degradation of arabinose and / or levels below the detection range for the analytical methodology employed (HPAE/PAD ICS 5000). All pretreatment filtrates were analysed on the ICS 5000 in an effort to quantify (if possible) the presence of arabinose sugars. Arabinose sugars present in the filtrate were measured in trace amounts only. Samples were also spiked to ensure the instrument was capable of separating and detecting arabinose sugars (this was confirmed). Finally, a combination of sample alterations was performed such as dilutions, chemical concentration variations and process temperature and time changes. This too was unsuccessful, concluding that arabinose sugars present in the hydrolysate were subjected to early conversion and degradation.

Xylose the second most abundant sugar found in lignocellulosic biomass is present in relatively high concentrations in willow (10.50%), miscanthus (21.68%), and hemp

(14.98%). However, pretreatment of willow, miscanthus and hemp using NaOH, CH_3OH, NH_3 and H_2SO_4 demonstrated significantly lower yields than that expected following the compositional analysis performed in Table 3.3. Xylose yields increased as the concentration of the chemical increased, however, the conversion yields achieved were lower compared to that of the original amount (Table 3.2). It is concluded that partial or complete xylose degradation occurred for some samples under certain process conditions.

The impact of these pentoses, together with other hemicellulose hexoses (mannose and galactose) are discussed further in Section 3.2.5.

3.2.3 Commercial Enzyme Preparations and the Hydrolysis of Pretreated Willow, Miscanthus and Hemp

In an effort to improve saccharification yields a focus was placed on the enzyme complex used in the saccharification process and on applying the SSF approach to eliminate any potential for an underestimation of the pretreatment conditions due to enzyme feedback inhibition.

Accurately estimating the enhanced susceptibility of pretreated biomass cellulose to enzymatic and cellulose hydrolysis is central to optimising the pretreatment processes. However, the majority of reports in the literature generally pay insufficient attention to the critical enzymatic component of the process. While a plethora of commercial cellulase preparations have been employed by various researchers, the value of these studies in informing further process development is hampered by the sporadic availability of these preparations from suppliers and the absence of essential information on the component enzyme activities of the cellulase complex in the commercial products.

Three commercial cellulase products were employed in the current study: 5B06443 Biocellulase W from Kerry Ingredients and Flavours, C013L Cellulase 13L from Biocatalysts and NS22119 Enzyme Complex from Novozymes. The manufacturer's descriptions of their products was very limited and necessary information for the successful conversion of cellulose to glucose and ethanol was challenged by insufficient information on enzyme activities and dosage rates, along with the definition of the enzyme units presented and how these were established (Table 3.4).

As described in Section 1.4.2.2, at least three categories of enzymes are necessary to convert cellulose into soluble sugars (Fig. 3.2). These include endoglucanase (EC.

3.2.1.4) which hydrolyse internal β-1, 4-glucosidic bonds randomly in the cellulose chain; cellobiohydrolase (EC.3.2.1.91) which move progressively along the cellulose chain and cleave off cellobiose units from the ends of the chain and β-glucosidase (EC.3.2.1.21) which converts cellobiose and soluble cellodextrins into glucose. All these enzymes work synergistically to hydrolyse cellulose by creating new unit accessible sites for each other removing obstacles and relieving product inhibition (Glabe and Zacchi, 2002). Individual cellulases have very limited hydrolytic activity, while a mixture of cellulases can exhibit a synergistic effect where the hydrolytic activity of the cellulase mixture is greater than the sum of the hydrolytic activities of individual enzymes (Niddetzky et al., 1994). Optimising this mixture, therefore, becomes important and should be modulated to increase the production of glucose and the lignocellulosic conversion (Berlin et al., 2007; Boisset et al., 2000; Jing et al., 2007).

There are several factors which affect the efficiency of the cellulase enzyme preparation to effectively hydrolyse biomass. These must be taken into consideration when evaluating the performance of a particular enzyme or enzyme preparation.

- *Cellulose Crystallinity* - The degree of polymerisation and cellulose crystallinity have been considered as important factors in determining the hydrolysis rate of the cellulosic substrate (Chang and Holtzapple, 2000).
- *Substrate Available Surface Area* - Accessibility of the substrate to the cellulolytic enzymes is one of the major factors influencing the hydrolysis process. Thus, one of the main objectives of pretreatment is to increase the available surface area (Alvira et al., 2010).
- *Lignin Barrier (Content and Distribution)* - Lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier (Chang and Holtzapple, 2000). Different strategies have been studied to overcome the non-productive adsorption of cellulase to lignin such as alkali extraction and addition of protein or other additives (Börjesson et al., 2007; Pan et al., 2005).
- *Hemicellulose Content* - Removal of hemicellulose increases the mean pore size of the substrate and therefore increases the accessibility and the opportunity for the cellulose to become hydrolysed (Chandra et al., 2007).
- *Porosity:* Pore size of the substrate in relation to the size of the enzyme can be a limiting factor in the hydrolysis of lignocellulosic biomass (Chandra et al., 2007). Cellulases have been known to become trapped in the pores if the internal area is

much larger than the external area, which is the case for many substrates (Zhang and Lynd, 2004). Increasing the pore size either prior to or during the pretreatment process can significantly improve the rate of hydrolysis.

- *Cell Wall Thickness (Coarseness)* - Plant cell walls which are too thick can limit the penetration of liquid by their nature and contributes to the recalcitrant structure of the feedstock (Alvira et al., 2010).

Table 3.4: Manufacturer's description of enzyme preparations used in this study. 5B06443 and C013L enzymes were derived from *Trichoderma* species, NS22119 enzyme is derived from *Aspergillus* species.

Commercial Enzyme Preparation Properties	**5B06443** **Biocellulase W Kerry Ingredients and Flavours**	**C013L** **Cellulase 13L Biocatalysts**	**NS22119** **Enzyme Complex Novozymes**
Activity	1000 Cell[a] T u/ml	1,500 Cell[b] u/g	100 FBG[c] /g
Density (g/ml)	1.10	N/D	1.19
pH	6.0-7.0	3.5-6.0	4.5-6.0
Temperature (^oC)	60	50-50	25-55
Dosage (%w/w)	0.01-0.05%	1.2-5.0%	0.05-0.4%

aAn enzyme preparation derived from *Trichoderma reesei* which contains cellulase, hemicellulase and beta-glucanase activities. Effective in the degradation of the complex carbohydrate found in the plant cell walls. Can be used in a wide variety of applications which involve hydrolysis of non-starch polysaccharides and are effective in the brewing process (Kerry Ingredients and Flavours, 2011).

bAn enzyme preparation for degradation of cellulose and other viscosity forming polysaccharides. The activity performance of this preparation is the result of the synergistic effect of the cellulose and associated side activities. Side activities such as cellobiase, beta-glucosidase and beta-glucanase can result in complete cellulose breakdown. Cellulase 13L results in the liquefaction and maceration of many fruits and vegetables. It is also useful for a range of other applications including the production of low calorie bulking agents for inclusion in slimming foods. (Biocatalysts, 2015, revision 8).

ᶜFBG = Fungal Beta- Glucanase Unit (One FBG is the amount of enzyme that produces reducing carbohydrates equivalent to 1μmol of glucose per minute under the conditions in the method. The activity is determined relative to an enzyme standard). Contains a wide range of carbohydrases, including arabinase, β-glucanase, cellulase, hemicellulase, pectinase and xylanase. Can break down the cell walls for the extraction of useful components from plant tissue (Novozymes, 2011).

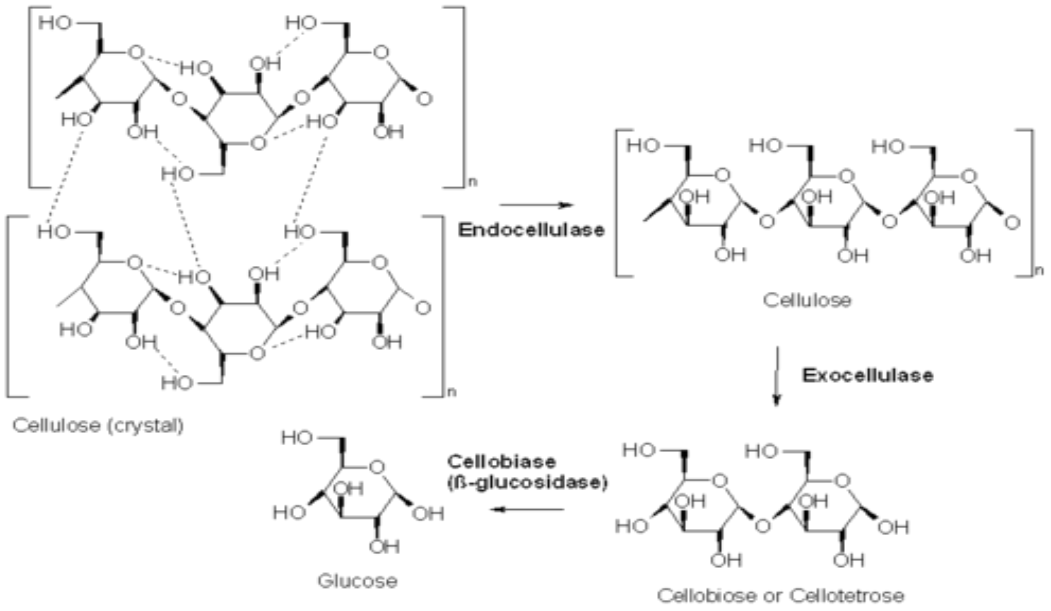


Figure 3.2. Mechanism of cellulolysis. The three types of reaction catalysed by cellulases: 1. Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endocellulase) 2. Hydrolysis of chain ends to break the polymer into smaller sugars (exo-cellulase) 3. Hydrolysis of disaccharides and tetra-saccharides into glucose (beta-glucosidase) (Wood and McCrae, 1979).

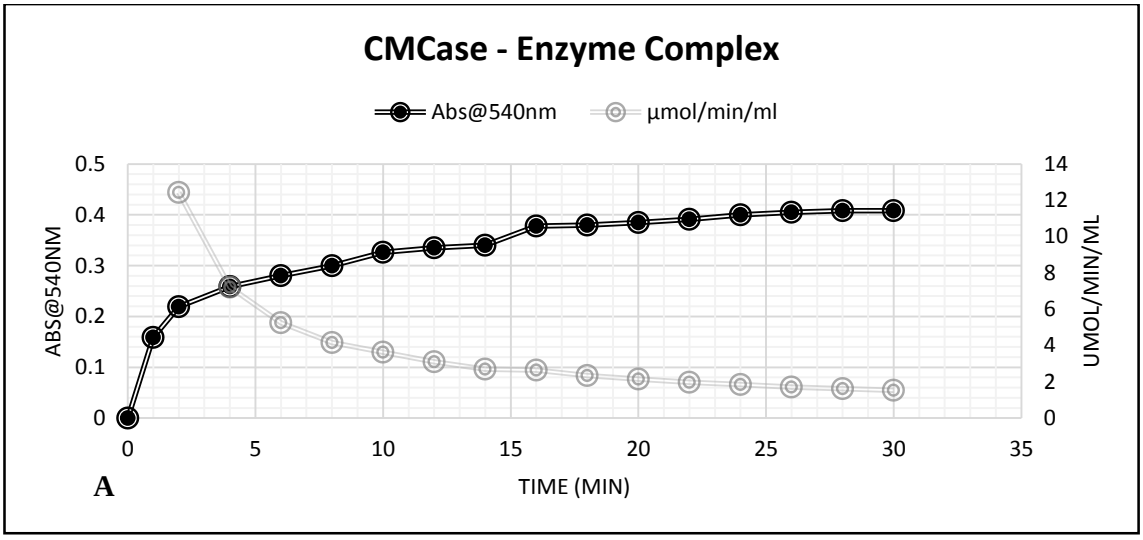
The accurate and reproducible measurements of enzyme levels employed in the biomass bioconversion processes are clearly central to bioconversion studies. It is generally recognised that the more valuable assay methods measure the cellulose enzyme under conditions which reflect the conditions of use. Model substrates include carboxymethyl cellulose, filter paper and cellobiose. Carboxymethyl cellulose (CMC) is a soluble and highly hydrolysable substrate which is routinely used for estimation of endo-β-glucanase activity in cellulose preparations. The CMCase assay relies on a fixed-time assay where a single concentration of reducing sugar produced within a defined reaction time is used

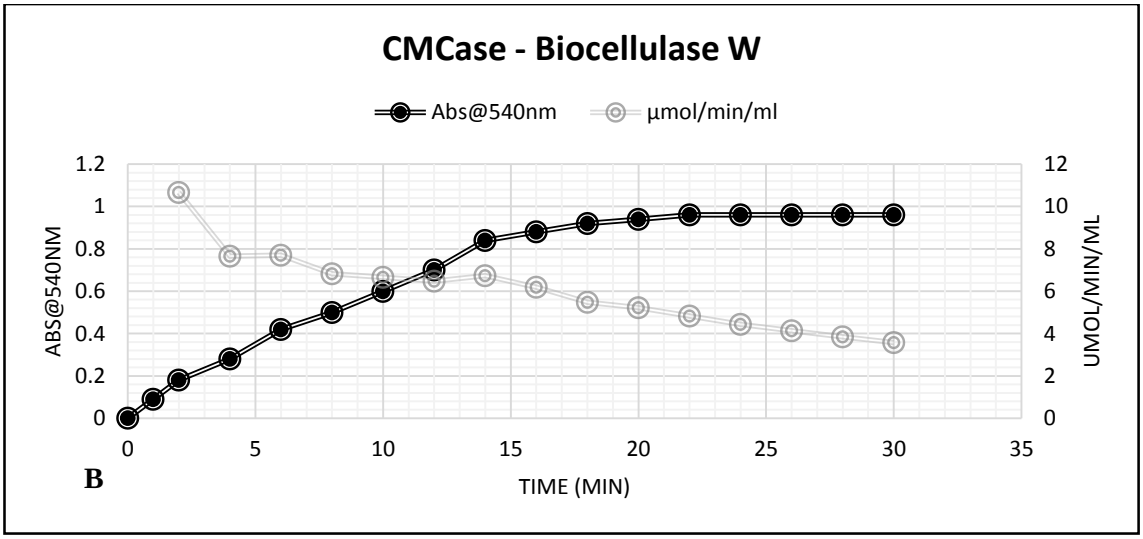
to calculate enzyme activity (Section 2.6). FPase assays based on filter paper as substrate provide an additional practical measurement of saccharifying cellulose activity because this substrate is insoluble and more difficult to hydrolyse (Mandels et al., 1976). β-glucosidase is the only cellulolytic enzyme for which a specific substrate is available - cellobiose (Section 2.6).

Many commercial cellulase preparations can also include hemicellulases, a group of enzymes that are defined and classified according to their substrate hemicellulose (Section 1.3.6 and Section 1.4.2.2). The synergistic action of a multitude of different enzymes is required to hydrolyse a particular hemicellulose and the substrate used to measure this activity in the current study was oak spelt xylan (Section 2.6.3).

The matter of defining commercial cellulase preparations is further complicated by the fact that the progress curve of the cellulase reaction is not linear. The estimation of units of enzyme activity generally relies upon measurement of the initial rate (Vo) of the enzyme catalysed reaction (when the rate of product formation is directly proportional to assay time) under standard experimental conditions. All of the standard enzyme assays used in the present study are fixed-time assays (Section 2.6) and it is well recognised that with such assays, initial reaction rates should not be determined solely on the basis of a single measurement since this presumes that the reaction is proceeding effectively at a constant rate, i.e. from time zero up to at least the time at which the reaction is terminated and the product estimated. For this reason, the general practice has been to take measurements at multiple time points so as to ensure that the units of enzyme activity are calculated from measurements in the initial velocity range. However, the situation with cellulase and xylanase enzyme assays is even more complex due to the non-linear progress curves obtained for CMCase, Xylanase, FPase and β-glucosidase activity, even in the initial stages of the reaction (representative samples are presented in Figures 3.3a-d). The practical consequence of these non-linear curves is that estimates of enzyme activity vary significantly depending upon the point of the progress curve where the reaction is terminated and the product concentration estimated. It is therefore vital that a clear list of criteria be laid down for cellulase and xylanase assays so that accurate and reproducible results can be obtained. This has been achieved in the current study (Section 2.6) for the three commercial cellulase products employed in the bioconversion processes: 5B06443 Biocellulase W (Kerry Ingredients and Flavours), C013L Cellulase 13L (Biocatalysts) and NS22119 Enzyme Complex (Novozymes).

The results are presented in Table 3.5 where IU cm^{-3} of FPase (F), CMCase (C), β-Glucosidase (G) and Xylanase (X) activities are detailed for the three commercial preparations. The relative levels of the four activities are also presented in Table 3.5 as the ratio F/C/G/X.





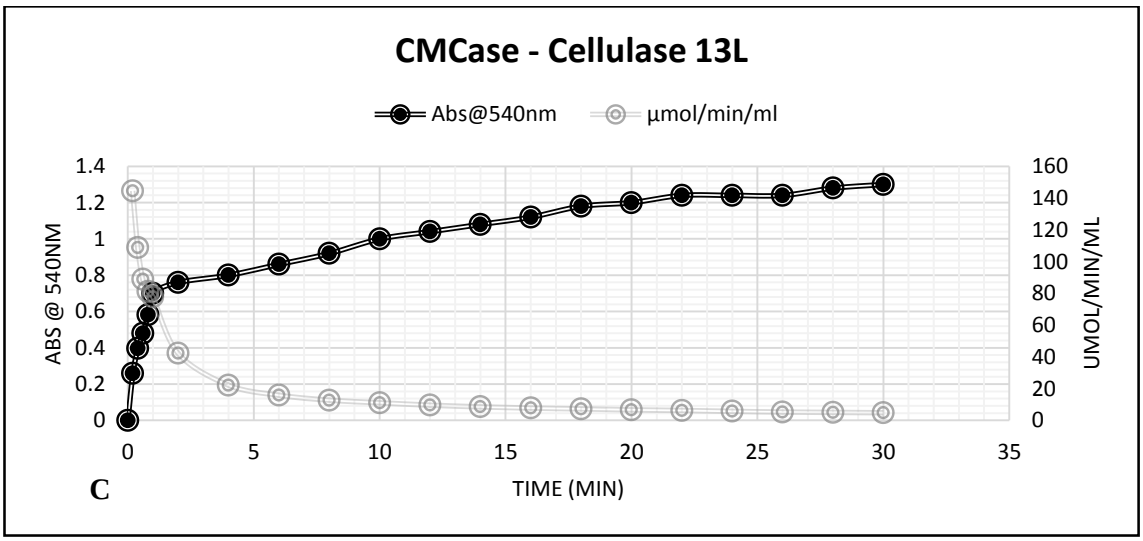


Figure 3.3a: Analysis of CMCase activity for the commercial enzyme preparations Enzyme Complex (A), Biocellulase (B) and Cellulase 13L (C). Samples were prepared and investigated in triplicate as described in Section 2.6.

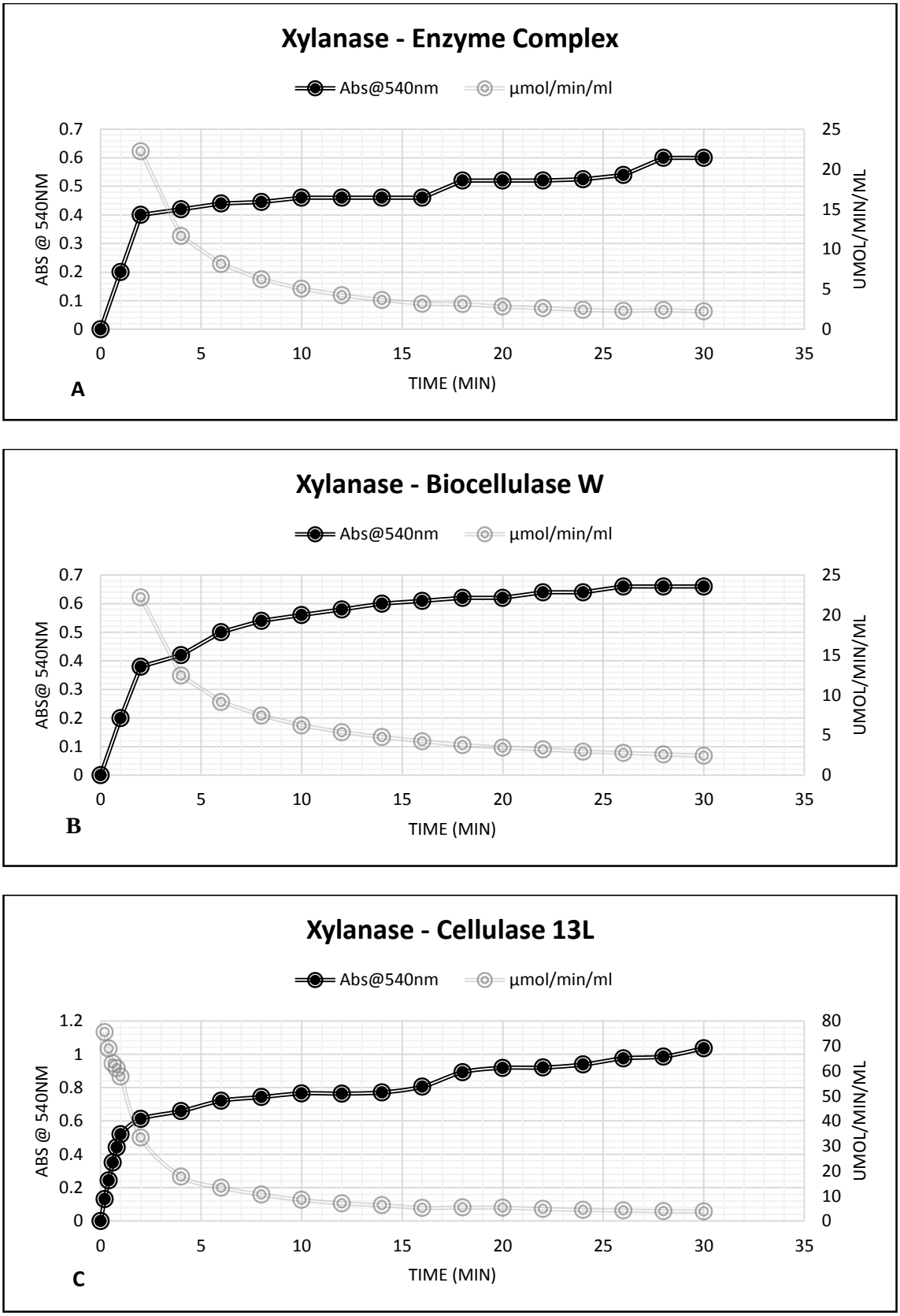
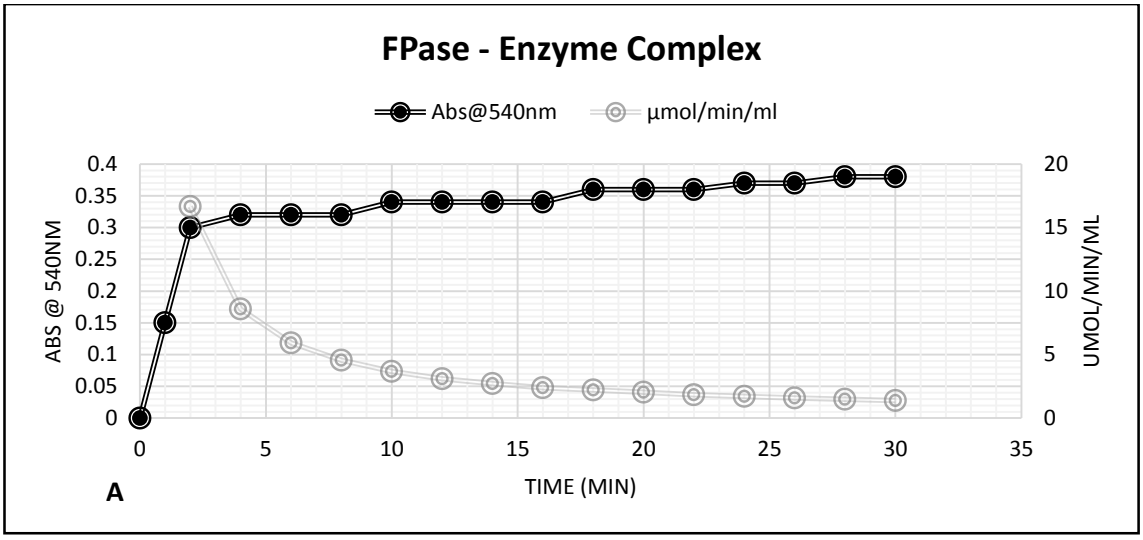
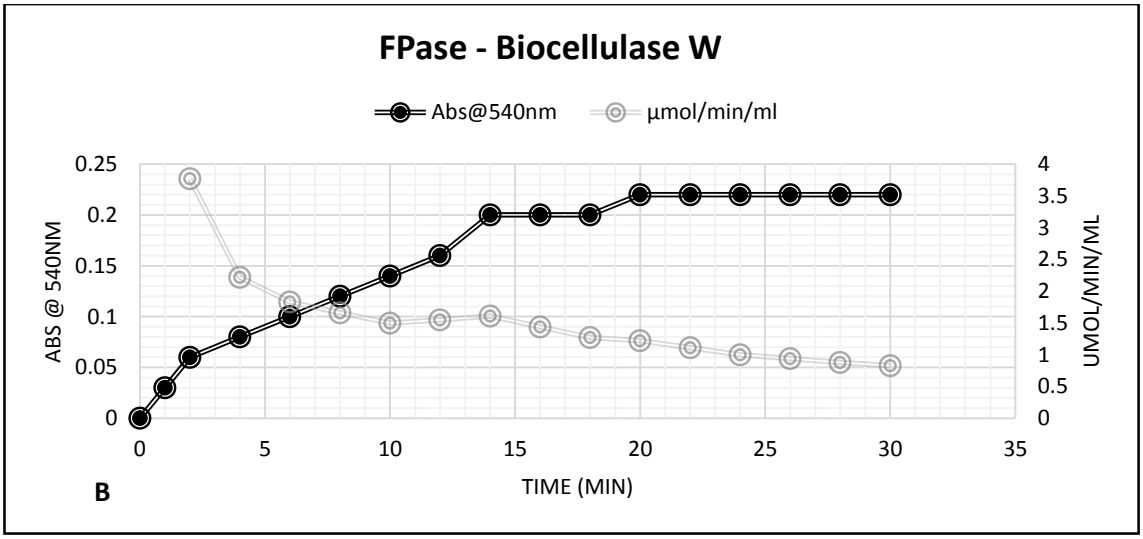


Figure 3.3b: Analysis of Xylanase activity for the commercial enzyme preparations Enzyme Complex (A), Biocellulase (B) and Cellulase 13L (C). Samples were prepared and investigated in triplicate as described in Section 2.6.





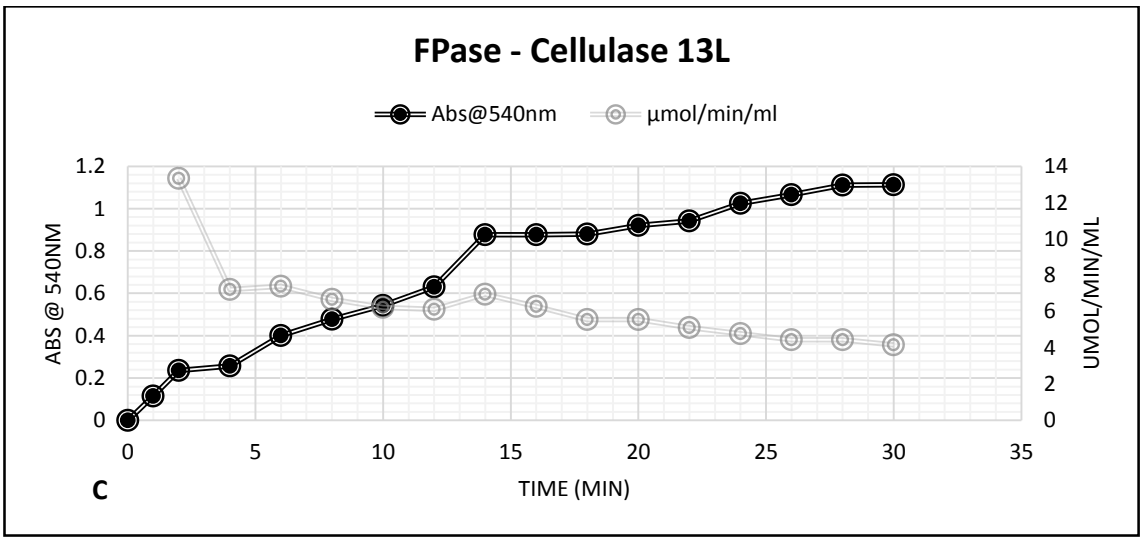


Figure 3.3c: Analysis of FPase activity for the commercial enzyme preparations Enzyme Complex (A), Biocellulase (B) and Cellulase 13L (C). Samples were prepared and investigated in triplicate as described in Section 2.6.

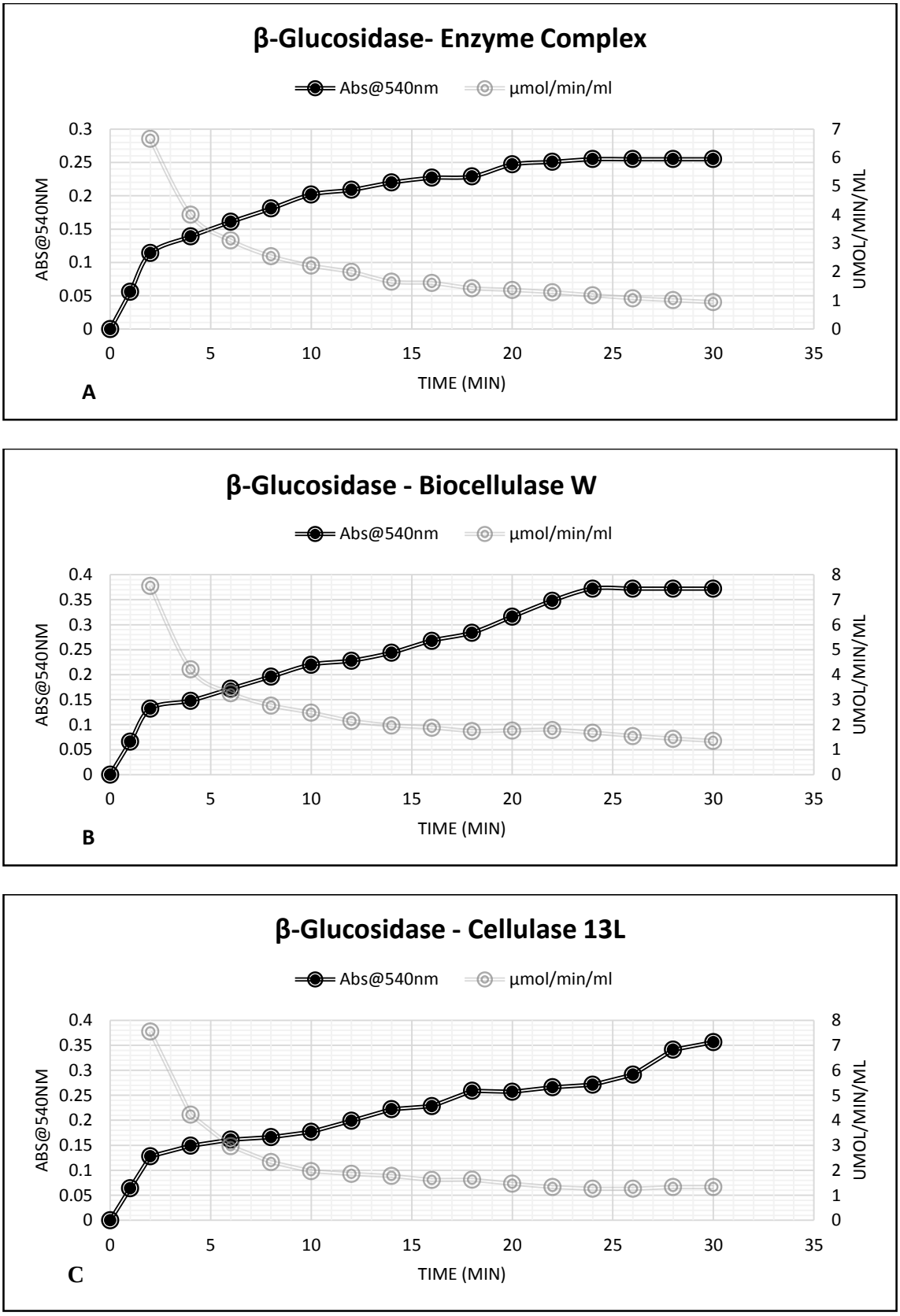


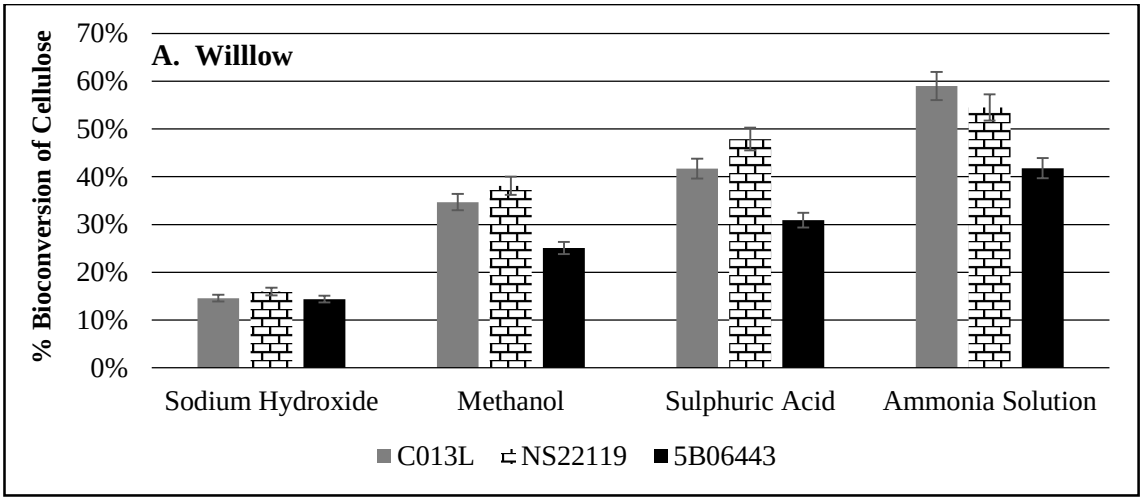
Figure 3.3d: Analysis of β-glucosidase activity for the commercial enzyme preparations Enzyme Complex (A), Biocellulase (B) and Cellulase 13L (C). Samples were prepared and investigated in triplicate as described in Section 2.6.

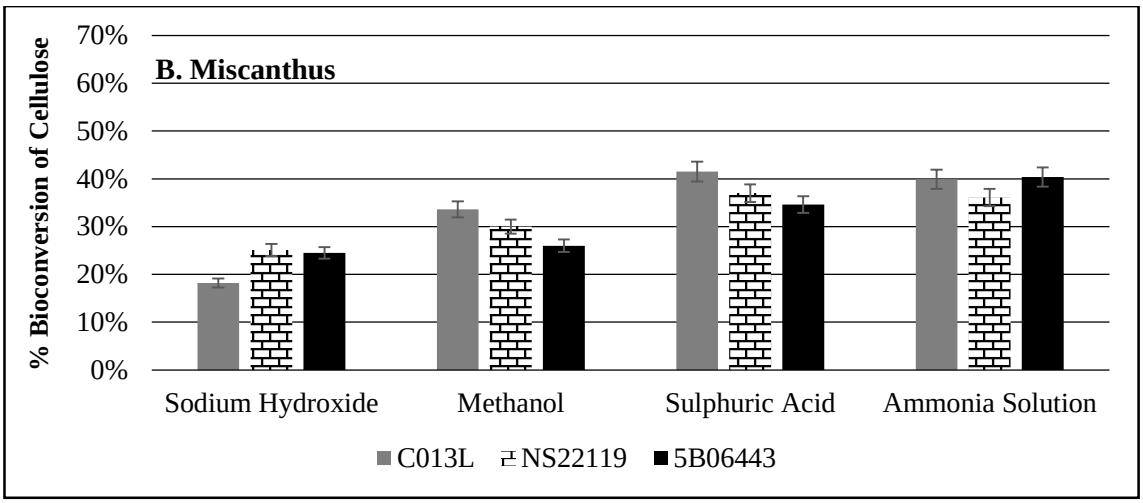
Table 3.5: Enzyme analysis of commercial preparations used in this study. Units of enzyme activity are in international units cm^{-3} (IU cm^{-3}) which equates to µmol min cm^{-3} of reducing sugar.

Enzyme Activity	Substrate	5B06443 Biocellulase W Kerry Ingredients and Flavours	C013L Cellulase 13L Biocatalysts	NS22119 Enzyme Complex Novozymes
		IU cm^{-3}	IU cm^{-3}	IU cm^{-3}
FPase (F)	Insoluble filter paper	2.09	6.59	2.97
CMCase (C)	Soluble CMC	7.06	9.26	5.87
β-Glucosidase (G)	Soluble Cellobiose	3.18	1.71	1.87
Xylanase (X)	Soluble Xylan	6.36	7.27	4.04
Dosage		11%	11%	11%
Ratio	F/C/G/X	1/3.4/ 1.5/ 3.0	1/1.4/ 0.3/ 1.1	1/2.0/ 0.6/ 1.4

Following the comparative analysis performed on willow, miscanthus and hemp in Section 3.2.2, highest conversion yields were observed following the application of the pretreatment chemicals at a concentration of 3 mol L^{-1}. It was therefore decided that this concentration (3 mol L^{-1}) and its expected yields would be the basis for our comparative assessment of the commercial enzyme preparations and their effectiveness in the conversion of cellulose to glucose. Results are presented in Figure 3.4.

Analysis of samples pretreated at a concentration of 3 mol L^{-1} showed that similar cellulose bioconversion yields were obtained for NaOH pretreated willow with all three enzymes preparations (Fig. 3.4A). However, the very low conversion yields of 14-16% would suggest that the cellulose in this pretreated sample was largely inaccessible to any enzyme complex. The 5B06443 preparation underperformed relative to C013L and NS22119 with CH_3OH, H_2SO_4 or NH_3 pretreated willow (Fig. 3.4A). For example, just 25% of CH_3OH pretreated willow was hydrolysed by 5B06443 compared to 38% with NS22119; 31% of H_2SO_4 pretreated willow was hydrolysed by 5B06443 compared to 48% with NS22119; 42% of NH_3 pretreated willow was hydrolysed by 5B06443 compared to 59% with CO13L. Low conversion yields and low FPase activity suggests that the cellobiohydrolase is a rate limiting step for the 5B06443 preparation.





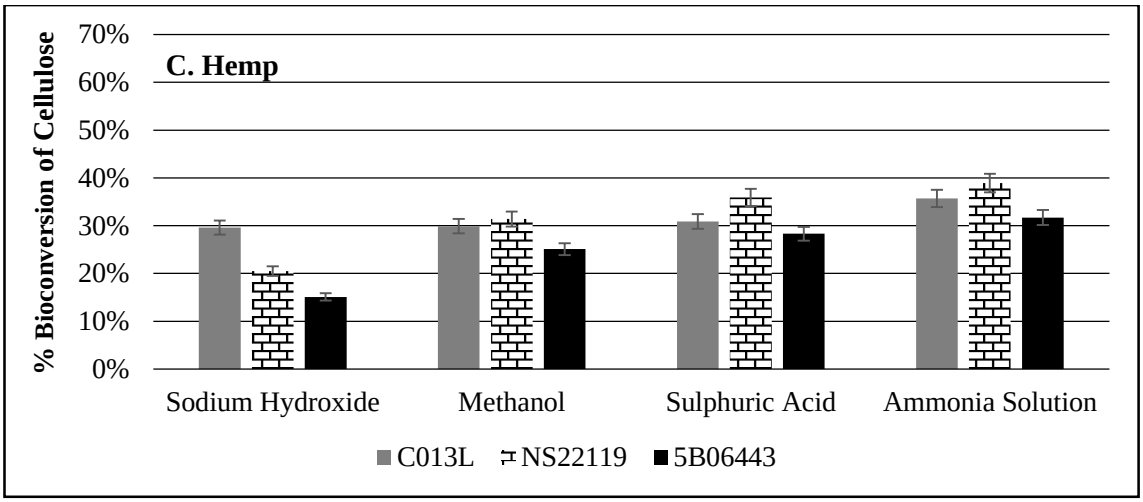


Figure 3.4: Effect of various commercial enzyme preparations on the bioconversion of willow (**A**), miscanthus (**B**) and hemp (**C**) (cellulose to glucose). Samples were chemically pretreated using 3 mol L^{-1} NaOH, CH_3OH, H_2SO_4 and NH_3 and hydrolysed using C013L, NS22119 or 5B0644 (Table 3.5). Samples were performed and analysed in triplicate with error bars representative of percentage error.

In the case of pretreated miscanthus (Fig. 3.4B) and hemp (Fig. 3.4C) the differences in enzyme preparations were less apparent. NaOH pretreated miscanthus hydrolysed by C013L yielded a lower conversion rate compared to NS22119 and 5B06443, while miscanthus hydrolysed using C013L performed better following pretreatment with CH_3OH or H_2SO_4 (Fig. 3.4B). A similar increase in conversion yield was observed for NS22119 and 5B06443 when H_2SO_4 and NH_3 pretreated miscanthus were hydrolysed. NaOH pretreated hemp demonstrated the greatest difference in bioconversion yields among the three enzyme preparations (15-30% conversion, Fig. 3.4C). NS22119 generated the highest yield of glucose monomers for CH_3OH, H_2SO_4 and NH_3 pretreated hemp (31-38% conversion, Fig. 3.4C), while 5B06443 was the least effective in the bioconversion of hemp, irrespective of the pretreatment used with conversion yields ranging from 15-32%.

Enzyme preparation C013L was generally the most effective in the bioconversion of miscanthus, while NS22119 was the most effective for willow and hemp. 5B06443 was the least effective for all three crops and was therefore eliminated from the study. Supply issues with NS22119 necessitated its elimination from the study and therefore all further process developments were conducted using C013L.

The relative effectiveness of the three commercial cellulase preparations in hydrolysing pretreated willow, miscanthus and hemp samples at 2, 3 and 4 mol L^{-1} is summarised in more detail in Table 3.6. A particular focus going forward was placed on the application of 2 and 3 mol L^{-1} chemical concentrations in the pretreatment of willow, miscanthus and hemp. Yields achieved at these concentration demonstrated enhanced conversion yields compared to that of 1, and 4 mol L^{-1} concentrations.

Table 3.6: Comparative analysis of the bioconversion of cellulose to glucose for willow, miscanthus and hemp using four chemical pretreatments employing 2 (A), 3 (B) and 4 (C) mol L^{-1} H_2SO_4, NaOH, NH_3 and CH_3OH and three commercial enzymes C013L, NS22119 and 5B06443. Samples were pretreated and hydrolysed as described in Section 2.7. Analysis was carried out using HPAE IC.

Feedstock **A**	*Commercial Enzyme Preparation*	*Acid Pretreatment (H_2SO_4)*	*Standard Deviation*	*Alkaline Pretreatment (NaOH)*	*Standard Deviation*	*Ammonia Fibre Explosion (NH_3)*	*Standard Deviation*	*Organosolvent Pretreatment (CH_3OH)*	*Standard Deviation*
Willow	C013L	32.4%	± 0.24%	12.8%	± 0.15%	36.7%	± 0.30%	14.2%	± 0.02%
	NS22119	28.9%	± 0.04%	15.4%	± 0.80%	39.5%	± 0.07%	28.3%	± 0.00%
	5B06443	27.8%	± 1.17%	13.1%	± 0.89%	31.6%	± 0.42%	18.8%	± 0.61%
Miscanthus	C013L	28.4%	± 0.01%	16.6%	± 0.12%	32.7%	± 0.14%	24.5%	± 0.01%
	NS22119	43.8%	± 0.73%	18.8%	± 0.24%	31.2%	± 0.08%	26.0%	± 0.19%
	5B06443	32.6%	± 0.03%	14.4%	± 0.03%	38.5%	± 0.13%	26.8%	± 2.27%
Hemp	C013L	24.7%	± 0.13%	18.3%	± 0.85%	24.6%	± 0.66%	23.2%	± 0.03%
	NS22119	36.2%	± 0.36%	13.5%	± 0.04%	23.7%	± 0.39%	28.0%	± 0.79%
	5B06443	20.2%	± 1.83%	11.2%	± 0.53%	21.9%	± 0.28%	19.7%	± 0.30%

Table 3.6 continued overleaf

Feedstock **B**	*Commercial Enzyme Preparation*	*Acid Pretreatment (H_2SO_4)*	*Standard Deviation*	*Alkaline Pretreatment (NaOH)*	*Standard Deviation*	*Ammonia Fibre Explosion (NH_3)*	*Standard Deviation*	*Organosolvent Pretreatment (CH_3OH)*	*Standard Deviation*
Willow	C013L	41.7%	± 0.24%	14.6%	± 0.66%	59%	± 0.95%	34.7%	± 0.85%
	NS22119	47.9%	± 0.21%	16.0%	± 0.76%	54.5%	± 0.31%	38.1%	± 1.61%
	5B06443	30.9%	± 0.55%	14.4%	± 0.40%	41.8%	± 0.09%	25.1%	± 1.16%
Miscanthus	C013L	41.5%	± 1.40%	18.2%	± 1.11%	39.9%	± 1.21%	33.6%	± 1.53%
	NS22119	37.0%	± 0.08%	25.1%	± 0.08%	36.1%	± 1.87%	30.0%	± 0.35%
	5B06443	34.6%	± 0.13%	24.5%	± 0.09%	40.4%	± 0.04%	26.0%	± 2.55%
Hemp	C013L	30.9%	± 0.01%	29.6%	± 0.40%	35.7%	± 1.34%	29.9%	± 0.81%
	NS22119	35.9%	± 2.29%	20.5%	± 3.23%	38.9%	± 0.25%	31.4%	± 2.96%
	5B06443	34.6%	± 1.76%	15.1%	± 0.87%	31.7%	± 0.49%	25.1%	± 0.79%

Table 3.6 continued overleaf

Feedstock **C**	*Commercial Enzyme Preparation*	*Acid Pretreatment (H_2SO_4)*	*Standard Deviation*	*Alkaline Pretreatment (NaOH)*	*Standard Deviation*	*Ammonia Fibre Explosion (NH_3)*	*Standard Deviation*	*Organosolvent Pretreatment (CH_3OH)*	*Standard Deviation*
Willow	C013L	27.7%	± 1.96%	18.8%	± 0.45%	37.2%	± 0.93%	25.5%	± 0.84%
	NS22119	33.5%	± 0.46%	19.8%	± 0.41%	45.9%	± 0.19%	33.1%	± 0.00%
	5B06443	20.4%	± 2.38%	15.5%	± 1.34%	37.9%	± 1.82%	25.4%	± 0.56%
Miscanthus	C013L	26.1%	± 0.29%	22.3%	± 0.77%	36.2%	± 0.60%	20.3%	± 1.94%
	NS22119	32.0%	± 0.45%	23.0%	± 0.52%	40.6%	± 0.04%	32.9%	± 0.24%
	5B06443	25.3%	± 0.76%	23.1%	± 0.40%	32.8%	± 0.20%	25.8%	± 0.60%
Hemp	C013L	33.2%	± 0.05%	19.7%	± 0.98%	29.5%	± 0.23%	28.6%	± 0.15%
	NS22119	29.3%	± 0.26%	21.2%	± 0.49%	45.3%	± 0.03%	34.8%	± 1.07%
	5B06443	29.0%	± 0.59%	17.8%	± 0.59%	35.1%	± 1.02%	24.7%	± 1.26%

3.2.4 Inhibitors and Mitigation Strategies

The sub-optimal cellulose conversion yields following the pretreatment and enzymatic hydrolysis of lignocellulosic biomass (Section 3.2.2) may be a consequence of cellulase feedback inhibition as a result of increased concentrations of monosaccharides generated and / or inhibitor formation in biomass hydrolysates.

The latter can occur during the chemical pretreatment process. Most lignocellulose-derived inhibitors such as furfurals, hydroxymethylfurfurals (HMF), formic acid, lactic acid and glycolic acid (Sjöström, 1991; Taherzadeh et al., 1997) are formed when hemicellulose and / or lignin are solubilised and degraded (Jönsson and Martín, 2016). Under acidic pretreatment conditions employing acids such as sulphuric acid, the pentoses and uronic acids resulting from hydrolysis of the hemicelluloses undergo dehydration with the formation of furfurals, while the hexoses are dehydrated to HMF (Jönsson and Martín, 2016). In addition, the splitting of β-O-4 ether and other acid labile linkages in lignin macromolecules during acidic treatments results in the formation of a high number of phenolic products (Du et al., 2010; Mitchell et al., 2014) including; 4-hydroxybenzoic acid, vanillin, conferyl alcohol and conferyl aldehydes (Mitchell et al., 2014). Under alkaline pretreatment conditions the carbohydrates are better preserved, but some degradation can occur leading to the formation of carboxylic acids. Acetic acid formed by saponification of the acetyl groups, is another typical product of alkaline treatments (Klinke et al., 2002).

A further consideration is the inhibition of cellulase, xylanase and β-glucosidase activities by lignin or lignin-carbohydrate complexes. For example, Berlin et al. (2006) reported on cellulase inhibition by various softwood lignin preparations concluding that different cellulase preparations can differ significantly in their sensitivity to lignin.

Cellulase feedback inhibition as a result of increased concentrations of monosaccharides generated during the saccharification process must also be considered. Accumulation of glucose and cellobiose, end products of hydrolysis, can inhibit cellulases and decrease glucose yields. Other monosaccharides such as mannose and galactose have also been shown to decrease glucose yields, but the mechanism behind the cellulase inhibition is not fully understood (Hsieh et al., 2014). It is generally accepted that cellulases are inhibited by cellobiose (Gruno et al., 2003), while β-glucosidase is inhibited by glucose (Andric et al., 2010). Indeed, Andric et al. (2010) suggested increasing the β-glucosidase

concentration in cellulase preparations to alleviate inhibition. However, as indicated in Table 3.5 for the present study, the best performing commercial enzyme preparation (NS22119) for willow and hemp had the lower ratio of β-glucosidase activity.

Efforts to counteract inhibition problems with hydrolysates have been investigated with limited success. These include:

- *Feedstock selection and engineering* – selecting feedstocks such as wheat straw (Larsen et al., 2012) and miscanthus (Chiaramonti et al., 2012) with low recalcitrance, thus, requiring milder conversion conditions.
- *Detoxification / conditioning* – using chemical additives and separation techniques to remove the inhibitory compound (Alriksson et al., 2006; Cavka and Jönsson, 2013).
- *Genetic and metabolic engineering* (Jönsson and Martín, 2016).
- *Bioabatement* – microbial treatment (Cao et al., 2015).

Based on the premise that the low cellulose conversion yields reported in Section 3.2.2 were most likely to be a consequence of feedback inhibition as a result of increased concentrations of monosaccharides generated during the saccharification process, SSF was investigated as a means of improving yields. This is detailed in Section 3.2.5 and this approach proved to be highly effective in achieving significant increases in bioconversion yields. While these results suggest that other types of inhibitors were not significant contributors to the sub-optimal enzymatic saccharification yields with the Irish-grown crops and the chemical pretreatment used in this study, further investigation on the identity of specific inhibitors in the pretreatment and fermentation broth could form a basis for future study.

3.2.5 Simultaneous Saccharification and Fermentation (SSF) of Pretreated Biomass

SSF (Section 1.4.3) was employed in this study to relieve any potential inhibition which may have been produced and to establish if the C013L cellulase enzyme complex employed in these studies (Table 3.5) was performing optimally.

Many studies including Sørensen et al. (2008) focus solely on the saccharification of the pretreated biomass sample to yield monomeric sugars for ethanol production. While some researchers including Kuglarz et al. (2014) have employed SSF where the glucose is subsequently converted to ethanol. When both approaches were assessed in the present

study, significantly higher bioconversion yields were recorded for all three crops and results are summarised in Figure 3.5.

SSF was performed at two different pretreatment concentrations (following review of the data in Figure 3.1) employing 2 mol L^{-1} NaOH, CH_3OH, H_2SO_4 and NH_3 (Fig. 3.5A) and 3 mol L^{-1} NaOH, CH_3OH, H_2SO_4 and NH_3 (Fig. 3.5B). Samples pretreated at the lower chemical concentrations (Fig. 3.5A) achieved lower bioconversion yields compared to those pretreated at the higher concentration employed (Fig. 3.5B). A similar trend to that observed in Table 3.6, in which a decrease in concentration from 3 mol L^{-1} to 2 mol L^{-1} coincided with a decrease in conversion yield.

NaOH was the least effective pretreatment for all three crops with bioconversion yields as low as 23% (2 mol L^{-1}) and 35% (3 mol L^{-1}) slightly higher than that achieved following pretreatment and saccharification alone. NH_3 was the most effective pretreatment technique when applied to willow, miscanthus and hemp at 2 mol L^{-1} (Fig 3.5A). In contrast, 3 mol L^{-1} NH_3 pretreatment was most effective for willow and miscanthus, while sulphuric acid was the most effective on hemp. Meanwhile, the conversion yield for methanol pretreated samples increased by 100% when the concentration of the pretreatment chemical was increased to 3 mol L^{-1} (Fig. 3.5B).

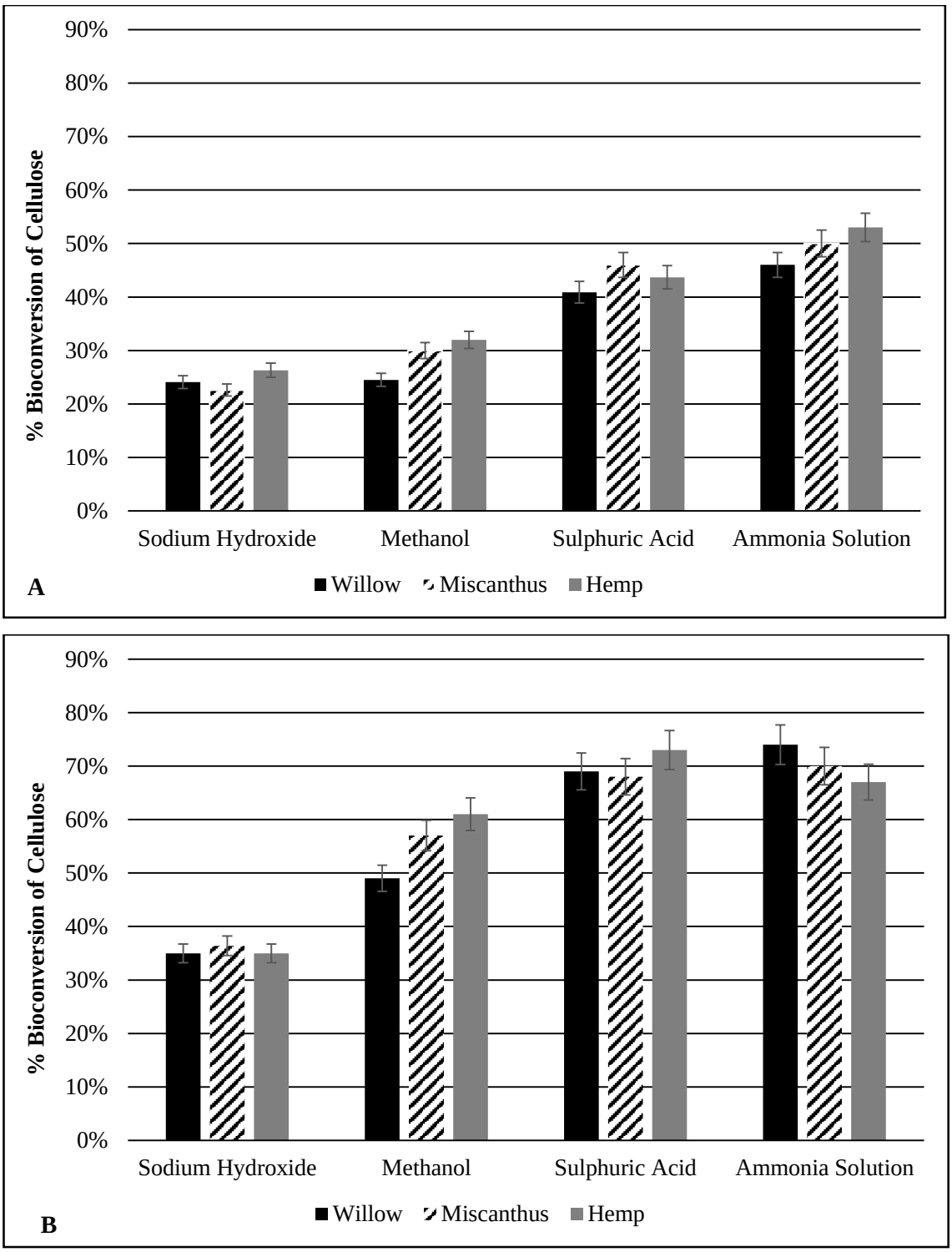


Figure 3.5: Percentage bioconversion of cellulose to ethanol using pretreated willow, miscanthus and hemp employing pretreatment chemicals 2 mol L^{-1} **(A)** and 3 mol L^{-1} **(B)**, NaOH, CH_3OH, H_2SO_4 and NH_3. Samples were pretreated, followed by SSF using the enzyme C013L as described in Section 2.7. Samples were performed and analysed in triplicate. Error bars are representative of percentage error.

Table 3.7: Comparison of ethanol conversion yields for cellulose (*) and glucose. Samples were pretreated at the same chemical concentration using 3 mol L^{-1} H_2SO_4, NaOH, NH_3 and CH_3OH and hydrolysed using C013L commercial enzyme preparation.

	H_2SO_4		NaOH		NH_3		CH_3OH	
Willow	69.0%*	96.0%	35.0%*	48.0%	74.0%*	99.0%	49.0%*	67.0%
Mean	69.3%	95.9%	34.8%	48.0%	73.7%	99.1%	49.0%	66.6%
Std Dev**	± 0.4%	± 0.6%	± 1.0%	± 0.9%	±1.0%	± 1.4%	±0.1%	± 0.7%
Miscanthus	68.0%*	77.0%	36.0%*	41.0%	70.0%*	80.0%	57.0%*	64.0%
Mean	68.4%	76.7%	36.4%	40.9%	70.1%	79.5%	56.7%	63.5%
Std Dev**	±0.7%	±0.8%	±0.8%	±0.6%	±0.6%	±0.9%	±0.9%	± 0.3%
Hemp	73.0%*	98.0%	35.0%*	58.0%	67.0%*	96.0%	61.0%*	97.0%
Mean	73.0%	98.3%	35.5%	58.2%	67.2%	96.3%	60.9%	97.3%
Std Dev**	±0.7%	±1.1%	±0.6%	±1.1%	±0.6%	±0.2%	±0.2%	± 0.7%

Samples were prepared and analysed in triplicate. Calculation methodology is described in Section 2.10**. Glucose conversion yields are representative of the actual yields achieved and not the theoretical yields.

A comparative analysis of the bioconversion yields achieved from the saccharification of pretreated crops and that of SSF is presented in Figure 3.6. A significant increase in the bioconversion yields of cellulose was observed in samples which have been subjected to the SSF process. NaOH identified as the least effective pretreatment chemical liberated bioconversion yields of 35% for all crops subjected to SSF compared to 14-30% conversion achieved following saccharification alone (Fig. 3.6B). Samples pretreated with CH_3OH increased to 49%, 61% and 57% for willow, miscanthus and hemp, respectively, compared to 35%, 30% and 34% determined following saccharification. Meanwhile, H_2SO_4 pretreated samples experienced an average increase of 32%, while NH_3 pretreated samples were calculated to have an average increased conversion yield of 25% (Fig. 3.6B). A summary of results calculated based on cellulose and glucose composition is presented in Table 3.7.

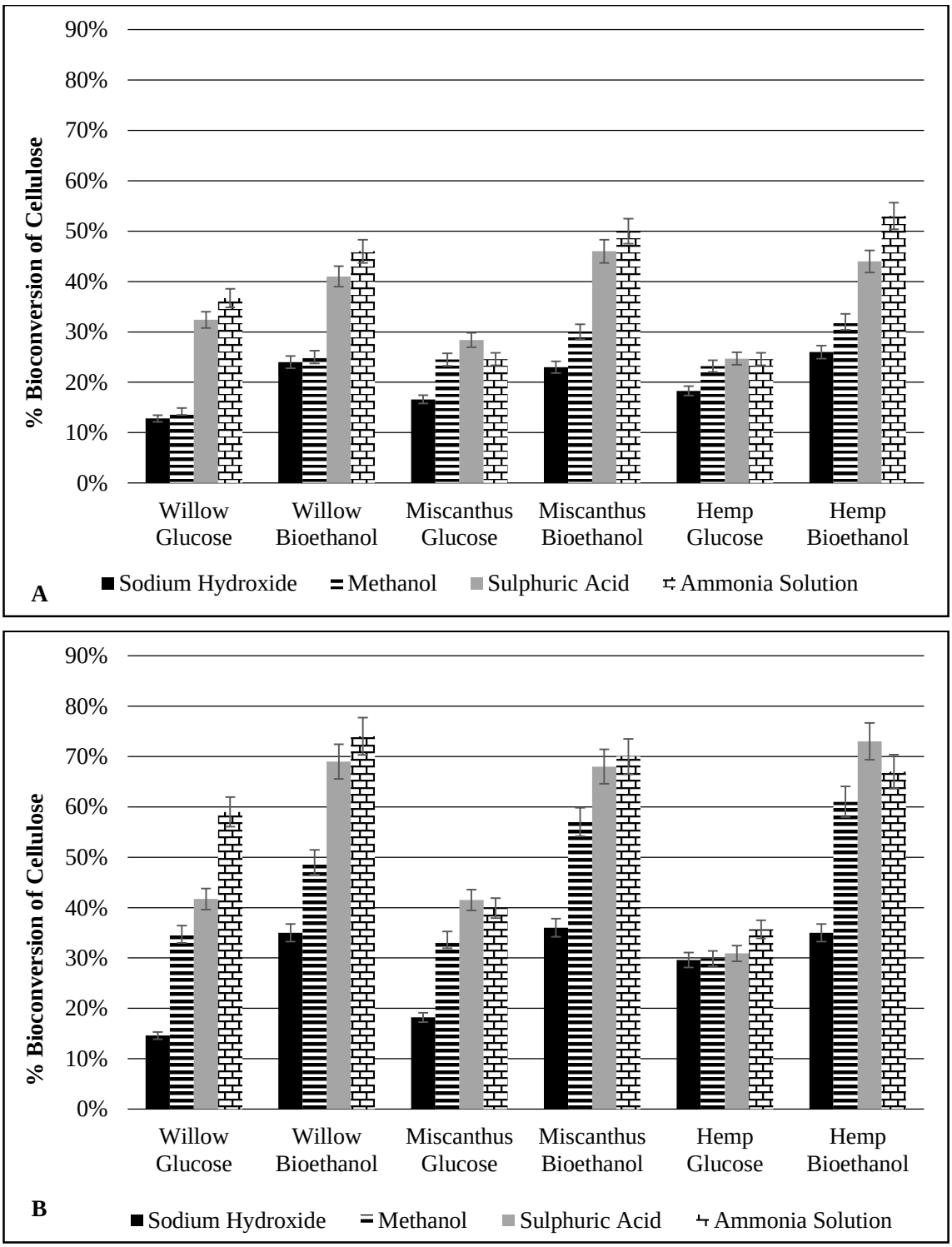


Figure 3.6: Comparative analysis of glucose yields (cellulose to glucose) produced from pretreated willow, miscanthus and hemp at 2 mol L^{-1} (**A**) and 3 mol L^{-1} (**B**), NaOH, CH_3OH, H_2SO_4 and NH_3 and ethanol yields (cellulose to ethanol) resulting from the SSF of pretreated willow, miscanthus and hemp as described in Section 2.7. Samples were performed and analysed in triplicate. Error bars are representative of percentage error.

As discussed in Section 3.2.3, hydrolysates contained four other sugars in addition to glucose and therefore consideration had to be given to the possibility that some or all of these other sugars contributed to ethanol production. This is not the case for xylose, arabinose and galactose, which is clear from the following:

- The pentoses xylose and arabinose are not fermented by *S.cerevisiae* (Yoon et al., 2003).
- Under the anaerobic conditions employed in this study galactose too is not fermented by *S.cerevisiae* (Quarterman et al., 2016).
- *S.cerevisiae,* however, is capable of fermenting mannose using the EMP pathway (Wendland et al., 2009).

A summary of the levels of the hexoses galactose and mannose in the pretreated lignocellulosic samples is presented in Table 3.8.

Table 3.8. Comparative analysis of galactose and mannose sugars for willow, miscanthus, hemp and switchgrass using the optimised pretreatment and hydrolysis conditions detailed in Section 2.7 and 2.8 and discussed in Section 3.2.2 and 4.2.2.

Crop	**Pretreatment**	**% Bioconversion following SSF**	**Galactose as a Percentage of Total Hexoses in Hydrolysates**	**Mannose as a Percentage of Total Hexoses in Hydrolysates**
Willow	3 mol L^{-1} NH_3	99%	2.5%	2.9%
Miscanthus	3 mol L^{-1} NH_3	80%	1.1%	0.47%
Hemp	3 mol L^{-1} H_2SO_4	98%	2.0%	4.8%
Switchgrass	1 mol L^{-1} CH_3OH	97%	2.0%	0.5%

% Bioconversion yields for Switchgrass presented in Table 3.8 are reported in detail in Chapter 4.

The high bioconversion yields (80% - 99%) achieved following SSF cannot be attributed to galactose as it is not fermented by *S.cerevisiae* under the strict anaerobic conditions employed in this study. However, as mannose can be fermented to ethanol it must be considered that some of the sugar (0.47% - 4.8%) potentially contributed to these high bioconversion yields.

3.2.6 A Comparative Analysis of Pretreatment Chemicals for the Bioconversion of Willow, Miscanthus and Hemp.

Over the years, many research papers have been written on the effects of different pretreatment techniques on lignocellulosic biomass. A review of the literature shows that very few studies have taken a comparative approach to evaluating these pretreatment techniques and identifying the most effective research methodology (feedstock, pretreatment chemical, commercial enzyme and processing conditions) necessary for the production of 2nd generation ethanol. As a result, comparison and reproducibility among these studies has been difficult due to varying or insufficient process information. In addition, a leading biomass pretreatment technique for energy crops has yet to be established.

A comparative analysis conducted in the current study determined that willow, miscanthus and hemp can produce varying degrees of monomeric sugars depending on the pretreatment chemical and concentration employed, which is often crop specific. When the concentration of the chemical employed was increased the conversion yields increased significantly, peaking and then declining once the highest concentration had been applied. Pretreatment utilising NH_3 was determined to be the most effective chemical for willow and hemp following saccharification, while H_2SO_4 was the most effective for miscanthus. NaOH was the least effective pretreatment chemical for all three crops employed in the study.

The selection of an appropriate commercial enzyme for effective and efficient bioconversion is also critical to the success of ethanol production. Three enzymes were evaluated in this study and were found to vary significantly depending on the feedstock and pretreatment chemical employed. The commercial enzyme NS22119 was the most effective for willow and hemp, while C013L was the most effective for miscanthus. Due

to supply issues however, the enzyme C013L was selected for use on all three crops in the second part of the study, evaluating the necessity of SSF.

Employed to improve the efficiency of the bioconversion process, SSF has been shown to minimise any potential feedback inhibition which may occur during or after the pretreatment process (Smullen et al., 2017a). When pretreated samples were subjected to SSF, conversion yields increased significantly. Consequently, cellulose and glucose bioconversion yields as high as 74% and 99%, respectively were achieved, demonstrating the necessity for SSF.

Other research studies have also examined the effects of these pretreatment chemicals on energy crops, including willow, miscanthus and hemp. Sassner et al. (2008) investigated the effect of H_2SO_4 impregnated steam on willow, using various chemical concentrations, temperatures and residence times and achieved high glucose and ethanol yields of 92% and 79% respectively. Kuglarz et al. (2014) reported significantly different yields for hemp using the same pretreatment with glucose and ethanol yields maximised at 74% and 92% respectively. However, when miscanthus was evaluated by Sørensen et al. (2008) these yields declined with just 61.3% (glucose conversion) being achieved. Glucose conversion yields achieved in the current study were slightly lower than that reported by Sassner et al. (2008), Kuglarz et al. (2014), and Sørensen et al. (2008), when H_2SO_4 was applied, with glucose conversion yields of 58%, 47% and 51% respectively. However, conversion yields increased significantly to 96%, 77% and 98% respectively, when SSF was employed.

Although pretreatment employing H_2SO_4 appears to be favoured among researchers for the bioconversion of lignocellulosic biomass, other acidic pretreatment approaches have also been explored. Haverty et al. (2012) investigated the effect of formic acid / hydrogen peroxide solution on miscanthus and found a significant difference in yield between treated and untreated biomass samples. Those samples that were treated at the lowest peroxide concentration of 2.5 % (w/w) achieved a glucose yield of 52.4%, compared to untreated samples achieving just 40.3%. Similar to our own study, the yields (79.16%) increased proportionally when the chemical concentration was increased (7.5% w/w) (Haverty et al., 2012). Cellulose recovery yields were also high (99.7%, 99.65% and

95.63%) for Haverty et al. (2012). However, these yields declined as the chemical concentration was increased (2.5%, 5.0% and 7.5% w/w, respectively).

Pretreatment employing NH_3 has also been shown to be very effective on lignocellulosic feedstocks. Little research has been conducted to-date on aqueous ammonia pretreated willow, miscanthus and hemp and so comparison between this study and other related studies is very difficult. However in 2013, a study conducted by Liu et al. (2013) showed that ammonia pretreated miscanthus liberated a glucose conversion yield of 53.4%. This yield increased significantly when sugar bagasse was investigated using a combined pretreatment technique employing liquid hot water and aqueous ammonia, with glucose conversion yields of 87% (Yu et al., 2013). In the current study, NH_3 was determined to be the most effective pretreatment for willow and miscanthus with conversion yields (based on actual yield) of 99% and 80% respectively, following SSF of the pretreated biomass. Similar yields to that of "soft crops" barley straw (Park and Kim, 2012) and wheat straw (Zhang et al., 2013).

Sodium hydroxide the least efficient pretreatment chemical employed in this study has been proven to be effective in other studies utilising crops such as miscanthus. A recent conversion study performed by Soares Rodrigues et al. (2016) concluded that an increase in pretreatment temperature can significantly increase conversion efficiency with cellulose conversion yields of approximately 59-85%. However, the process requires the biomass to be steam treated prior to pretreatment, increasing the demand for both energy and water. Not an option in the current study, which aims in Chapter 4 and 5 to identify the most environmentally and economically friendly approach.

Pretreatment engaging an organic solvent (methanol or ethanol) can be applied with or without an additional treatment, specifically a catalyst (H_2SO_4). Shimizu and Usami (1978) achieved delignification yields of 90% with 90% methanol when the pretreatment of pinewood was analysed. While a combined pretreatment method of a 1% H_2SO_4 catalyst and 80% ethanol enhanced the glucose conversion yield of miscanthus, achieving conversion yields of 75% for Obama et al. (2012). An option warranting further investigation in future works.

With comparable high yields achieved for the bioconversion of energy crops to that of pretreatment methods used currently for other forms of biomass, it is now intended that

the comparative approaches established in this study will facilitate the future development of the bioconversion process for energy crops.

3.3 Conclusion

Various pretreatment technologies for lignocellulosic biomass have been described to improve ethanol production (Maurya, 2015). A major bottleneck in this technology is the recalcitrant nature of the biomass and the inaccessibility of enzymes to cellulose due to the presence of lignin (Zhang et al., 2013). Chemical and thermochemical are currently the most promising technologies for industrial application (Maurya, 2015). However, it must also be noted that no treatment technology is 100% effective in the conversion of biomass to fermentable monomeric sugars (Maurya, 2015).

Chapter 3 investigated the effects of four chemical pretreatments at various concentrations on the conversion of three energy crops, using three commercial enzyme preparations. This study showed that pretreatments were crop specific and no one pretreatment was equally effective on all three crops. In fact, pretreatment employing ammonia was identified as the most effective and efficient for willow and miscanthus at a chemical concentration of 3 mol L^{-1}, while sulphuric acid was determined to be the best pretreatment technique for hemp. In general, sodium hydroxide was demonstrated to be the least effective pretreatment chemical for all four chemicals under investigation.

The commercial enzyme preparation employed was also found to be crop specific. The enzyme preparation NS22119 was most effective in the conversion of willow and hemp, whereas C013L was more effective for miscanthus in the conversion of cellulose to monomeric sugars. Inhibitor formation and/or feedback inhibition was identified as a possible issue during the conversion of cellulose to ethanol. Cellulose to glucose conversion yields were unexpectedly low and it was assumed that the sugars being produced were inhibited or were indeed the inhibitor themselves. Subsequently, the use of SSF was investigated as a means of releviating the issue. The efficiency of energy crop bioconversion can be substantially improved by optimising the choice of pretreatment maximising the potential for fossil fuel replacement and greenhouse gas mitigation.

Chapter 4

Bioconversion of Switchgrass: Identification of a Leading Pretreatment Option Based on Yield, Cost and Environmental Impact

Abstract

Switchgrass *(Panicum virgatum L*) is considered to be one of the best feedstocks for second generation ethanol production. However, its use as a biofuel resource for the ethanol market is challenged by high investment costs and inconsistent production methodologies. This study explores the use of switchgrass as a potential feedstock for ethanol production, investigating the effects of different pretreatment chemicals – sodium hydroxide, methanol, sulphuric acid and ammonia – employed at various concentrations of 0.5, 1 and 2 mol L^{-1} on conversion yields, while also minimising cost and assessing potential environmental impacts.

Glucose and ethanol yields showed that methanol was the most effective pretreatment chemical at 1mol L^{-1}, producing 230 g of glucose and 340 cm^3 of ethanol kg^{-1} of feedstock with a 97% conversion yield. Pretreatment employing sodium hydroxide was found to be the least effective, with cellulose to glucose conversion yields of 38% and 62% following simultaneous saccharification and fermentation (SSF). In general, SSF significantly increased cellulose conversion yields, up to 32% for some samples. At €0.55 kg^{-1} glucose and €0.50 L^{-1} ethanol methanol was also found to be the most cost effective pretreatment technique compared to sodium hydroxide at €1.96 kg^{-1} glucose and €7.94 L^{-1} ethanol.

4.1. Introduction

In recent years, new policies and incentives have been implemented across the world to encourage an increase in the production and consumption of renewable energies, including, renewable fuels such as biofuels. The EU biofuel directive (Hamelinck et al., 2005) and the American Policy Energy Act (PEA, 2009) both aim to increase the consumption of ethanol by 2022. In the last decade the use of biofuels across the world has steadily been increasing. In 2000, approximately 18.2 billion litres of ethanol were consumed in North America compared to 83 billion litres in 2012, accounting for approximately 83% of the total ethanol produced globally (Sainz, 2009). Europe remains the largest producer and consumer of biodiesel, accounting for 42% of total biodiesel production (REN, 2014). Despite an increase in global production of biofuels, several market challenges still remain. These include sustainability concerns, environmental impact and the economic value of the fuel compared to other transportation fuels such as biomethane (REN, 2014). As a result, financial incentives such as tax reliefs, subsides and feedstock establishment grants have been implemented to help stimulate production and consumption of liquid biofuels in Europe (Hamelinck et al., 2004).

Current production of biofuels relies on starches and sugars. However, there has been considerable debate surrounding the sustainability and the use of food crops for fuel, which are alleged to have driven up food prices and raised concerns of a food crisis. In addition, some studies have demonstrated that first generation biofuel can have a negative environmental impact, increasing GHG emissions (Zhang et al., 2013). In contrast, second generation biofuels produced from lignocellulosic biomass (municipal wastes, grasses, waste paper and energy crops) have been identified as an alternative as food crops are not used as feedstocks, are sustainable and have a positive environmental impact as the feedstocks grown are carbon neutral (Zhang et al., 2013).

Second-generation biofuels are not yet produced commercially, but a considerable number of pilot and demonstration plants have been announced or set up in recent years, with research activities taking place mainly in North America, Europe and a few emerging countries (e.g. Brazil, China, India and Thailand) (IEA, 2010). The main obstacle for second-generation biofuels is high initial investment costs as well as higher costs for the end-product compared to fossil fuels or many first generation biofuels. Some companies have reported they will start commercial production of second-generation biofuels within

the coming years, with the help of government subsidies and new blending mandates (IEA, 2010).

Research and development efforts have been undertaken for different conversion processes with the aim of reducing processing costs to be comparable with current 1st generation applications (IEA, 2010). Consequently, the pretreatment process has become the main focus of many conversion studies (Agbor et al., 2011; Chiaramonti et al., 2012; Haghighi-Mood et al., 2013) including our previous study investigating the bioconversion of energy crops, detailed in Chapter 3.

Switchgrass (*Panicum virgatum L*) is a native North American perennial grass which is considered to be one of the best feedstocks for ethanol production due to its high annual biomass yield and high carbohydrate content (Xu and Cheng, 2011). Its potential as an energy crop has been recognised as a major cellulosic ethanol source that could potentially displace 30% of current petroleum consumption (Perlack et al., 2005). In Europe, research on switchgrass as a biomass crop for energy began in 1998 (Alexopoulou et al., 2008) and switchgrass has since been demonstrated as an inexpensive, low input crop estimated to produce > 700% more output than input energy (Farrell et al., 2006).

Switchgrass can be converted into liquid biofuels through a biochemical pathway, employing pretreatment, enzymatic hydrolysis and fermentation. Among all the investigated pretreatment methods, chemical pretreatment has been found to be the most widely studied (Kim et al., 2011; Nlewen and Thrash, 2010; Xu and Cheng, 2011). Research studies to-date have focused on the bioconversion of switchgrass to monomeric sugars in order to examine selected pretreatment techniques. Typically, these studies have been limited to one or two selected pretreatment techniques, which has made the identification of an effective and/or leading chemical pretreatment process for ethanol production from switchgrass difficult and often misleading.

The current study takes a co-ordinated approach to the investigation and development of chemical pretreatment processes which can be applied to switchgrass – with a view to producing directly comparative information on the performance of four pretreatment chemicals (sodium hydroxide, sulphuric acid, ammonia and methanol), while also investigating the necessity for simultaneous saccharification and fermentation (SSF) to improve conversion and ethanol yield. Our objective is to identify a leading pretreatment

chemical for the bioconversion of switchgrass based not only on conversion yield but also environmental impact and economic value.

4.2. Results and Discussion

4.2.1 Compositional Analysis of Switchgrass

The composition of lignocellulosic biomass is instrumental to the performance and efficiency of both pretreatment and biodegradation processes and is an essential component of any bioconversion study. The chemical composition of switchgrass used in the current study is shown in Table 4.1.

Cellulose, the main constituent of switchgrass, is a polysaccharide that consists of a linear chain of D-glucose molecules linked by β-(1, 4)-glycosidic bonds (Agbor et al., 2011). Typical cellulose content can vary between 35-50%, depending on environmental and growth conditions. The cellulose content of switchgrass in this study was determined to be 39.6%, with structural glucan contributing 33.3%. Other switchgrass studies (Li et al., 2010a; Xu and Cheng, 2011) reported cellulose contents of between 32% and 59.4% respectively. Cellulose is of particular importance in this study as it is converted to ethanol during fermentation with *S. cerevisiae* yeast.

Hemicellulose, the second main constituent of switchgrass, differs from cellulose in that it is not chemically homogeneous. Hemicellulose is composed of branched heterogeneous polymers of pentose (xylose, arabinose), hexoses (mannose, glucose and galactose) and acetylated sugars (Haghighi-Mood et al., 2013). Hemicellulose is more easily solubilised then cellulose or lignin, its concentration in switchgrass was determined to be 22%. Typical hemicellulose content is expected between 20-35% and again can vary substantially (Haghighi-Mood et al., 2013).

Lignin is the third most abundant polymer in lignocellulosic biomass comprising of 15-20%. Lignin can be extremely challenging to degrade and is resistant to microbial attack. Total lignin content in switchgrass was 17.7%, consisting of 3.0% acid soluble lignin and 17.4% acid insoluble lignin compared to the 18.8% reported in another study (Kim et al., 2011). As lignocellulosic biomass is naturally recalcitrant, the higher the lignin content the more difficult the feedstock to breakdown. This must be taken into account when selecting a pretreatment chemical to use in the bioconversion process i.e. ammonia is ineffective if lignin content is too high (Gupta and Lee, 2010).

Switchgrass has a higher ash content than other similar crops such as miscanthus and was found to contain 8.7% ash. Other studies (Xu and Cheng, 2011) reported much lower ash contents of 3.7% for switchgrass. The moisture content of the dried switchgrass used in the current study was 11%.

Table 4.1. Compositional analysis of switchgrass harvested at Teagasc Crop Research Centre Carlow.

Parameter	% of dry weight
Cellulose	39.6%
Structural Glucan	33.3%
Hemicellulose	22.0%*
Xylan	17.1%
Arabinan	3.8%
Galactan	0.8%
Mannan	0.2%
Total Lignin	17.7%
Acid Soluble Lignin	3.0%
Acid Insoluble Lignin	17.4%
% Total Solids	89.0%
Moisture	11.0%
Ash	8.7%

*Hemicellulose content was calculated on the basis of total pentose sugars.

4.2.2 Pretreatment and Saccharification of Switchgrass

In theory, an ideal pretreatment process produces a disrupted, hydrated substrate that is easily hydrolysed but avoids the formation of sugar degradation products. The methodology used in this study was adapted from similar established methods utilising crops such as switchgrass (Nlewen and Thrash, 2010), corn stover (Chen et al., 2009) willow, miscanthus and hemp (Smullen et al., 2017b).

Switchgrass samples were pretreated using different chemical treatments – NaOH, H_2SO_4, NH_3 and CH_3OH – at different concentrations of 0.5 mol L^{-1}, 1 mol L^{-1} and 2 mol L^{-1}. These chemicals are representative of the different pretreatment chemicals used in other studies and results are graphically presented in Figure 4.1.

In general, low conversion yields were obtained at pretreatment concentrations of 0.5 mol L^{-1}. NH_3 was the most effective pretreatment at 0.5 mol L^{-1} with a 47% glucose

conversion yield, compared to CH_3OH pretreatment which had the lowest yields of 26%. Pretreatment concentrations of 1 mol L^{-1} produced higher conversion yields than pretreatment concentrations of 0.5 mol L^{-1} and 2 mol L^{-1}. CH_3OH was the most effective pretreatment chemical when a concentration of 1 mol L^{-1} (69% glucose conversion) was employed, while NaOH was the least effective (38% glucose conversion yield). The most effective pretreatment at 2 mol L^{-1} was NH_3 (55% conversion), compared to NaOH (34% conversion yield).

Pretreatment employing NaOH was the least effective pretreatment chemical in this study. However, it has been shown to be very effective in other switchgrass studies. Gupta and Lee (2010) achieved a glucose conversion yield of 54% when the NaOH concentration was increased to 5% (1.25 mol L^{-1}), while, Xu and Cheng (2011) demonstrated that a decrease in residence time achieved a higher glucose yield of 70% with NaOH.

Conversion yields for H_2SO_4 pretreated switchgrass exceeded those reported in other studies. At a lower chemical concentration and a higher reaction temperature than employed in the current study, Jensen et al. (2010) achieved a glucose conversion yield of 26% using H_2SO_4. Similarly, pretreatment employing $NH_{3(aq)}$ was more effective in the present study compared to previous studies, with highest glucose conversion yields of 63% compared to 53% achieved by Gupta and Lee (2010) when the reaction was performed at a higher temperature.

Little data is available on methanol pretreated conversions. Research studies however, do suggest that organosolvent pretreatment in general is very effective in the conversion of cellulose to glucose with yields of 90% being achieved when applied to other crops (Zhao et al., 2009).

Other pretreatment techniques not investigated in this study have also been shown to be effective in the pretreatment of switchgrass for ethanol production. Li et al. (2010a) employed ionic liquids in the bioconversion of switchgrass, utilising high temperatures and short residence times, a glucan yield of 67.7% was achieved. Some researchers have taken a combined pretreatment approach to the bioconversion of switchgrass. Capecchi et al. (2016) examined the effects of lime soaking prior to steam pretreatment and demonstrated glucan yields of 1.5-1.9 g L^{-1}.

In the current study methanol was the most effective pretreatment chemical at 1 mol L^{-1} with glucose conversion yields approaching 70%. Conversion yields declined however when the chemical concentration increased from 1 mol L^{-1} to 2 mol L^{-1}, which might suggest product inhibition of the enzyme complex and/or the generation of inhibitors of the saccharification process. Simultaneous saccharification and fermentation (SSF) was investigated in the study in an effort to mitigate this issue and increase conversion yields.

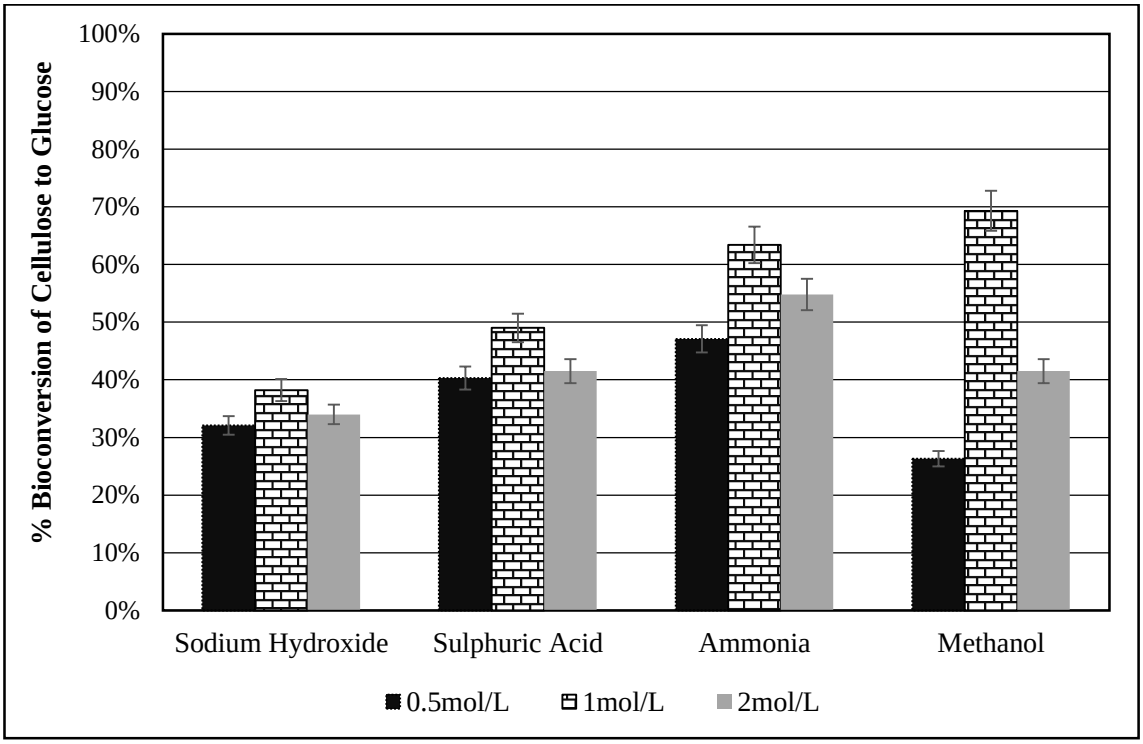


Figure 4.1. Comparative analysis of the bioconversion yields for switchgrass (cellulose to glucose). Samples were pretreated using: sodium hydroxide, sulphuric acid, ammonia and methanol at 0.5 mol L^{-1}, 1 mol L^{-1} and 2 mol L^{-1} concentrations and the commercial enzyme preparation CO13L Error bars are representative of percentage error.

4.2.3 Simultaneous Saccharification and Fermentation (SSF) of Pretreated Switchgrass

SSF (Section 1.4.3) was employed in this study to relieve any potential feedback inhibition or inhibitor formation which may have been produced during the pretreatment of switchgrass, similar to that of willow, miscanthus and hemp in Chapter 3. The theoretical yield for conversion of glucose to ethanol using pure glucose was also established in our previous study. It can therefore be assumed that using the Fermipan

Red yeast employed in this study, that a maximum theoretical yield of 0.46 g of ethanol / g of glucose (92%) could be achieved, with actual glucose to ethanol conversion yields being slightly higher.

Similar to yields achieved following saccharification (Fig. 4.1) samples pretreated at a concentration of 1 mol L^{-1} produced higher conversion yields of 62-97% than those at 2 mol L^{-1} (30-72% conversion) following SSF (Fig. 4.2). CH_3OH was the most effective pretreatment chemical when employed at 1 mol L^{-1} with highest conversion yields of 97%, while H_2SO_4 was the most effective pretreatment chemical at 2 mol L^{-1} (72% conversion). NaOH was the least effective pretreatment chemical with lowest yields of 62% and 30% for 1 mol L^{-1} and 2 mol L^{-1} respectively. Pretreatment utilising NH_3 demonstrated a conversion yield of 90% when employed at 1mol L^{-1} compared to 53% when the concentration was increased to 2 mol L^{-1}. In general, a significant decline in conversion yield was observed for samples pretreated at 2 mol L^{-1} compared to those at 1mol L^{-1}.

A comparative analysis of bioconversion yields achieved from the saccharification of pretreated switchgrass (Fig. 4.1) to that of SSF (Fig. 4.2) demonstrated a significant increase in the bioconversion yields for both 1 and 2 mol L^{-1} concentrations following SSF. Table 4.2 shows the actual yields of glucose (g of glucose / kg of feedstock) produced from switchgrass following pretreatment at various concentrations and ethanol (cm^3 of ethanol / kg of feedstock and g ethanol / g of glucose (theoretical yield, see Section 3.2.5)).

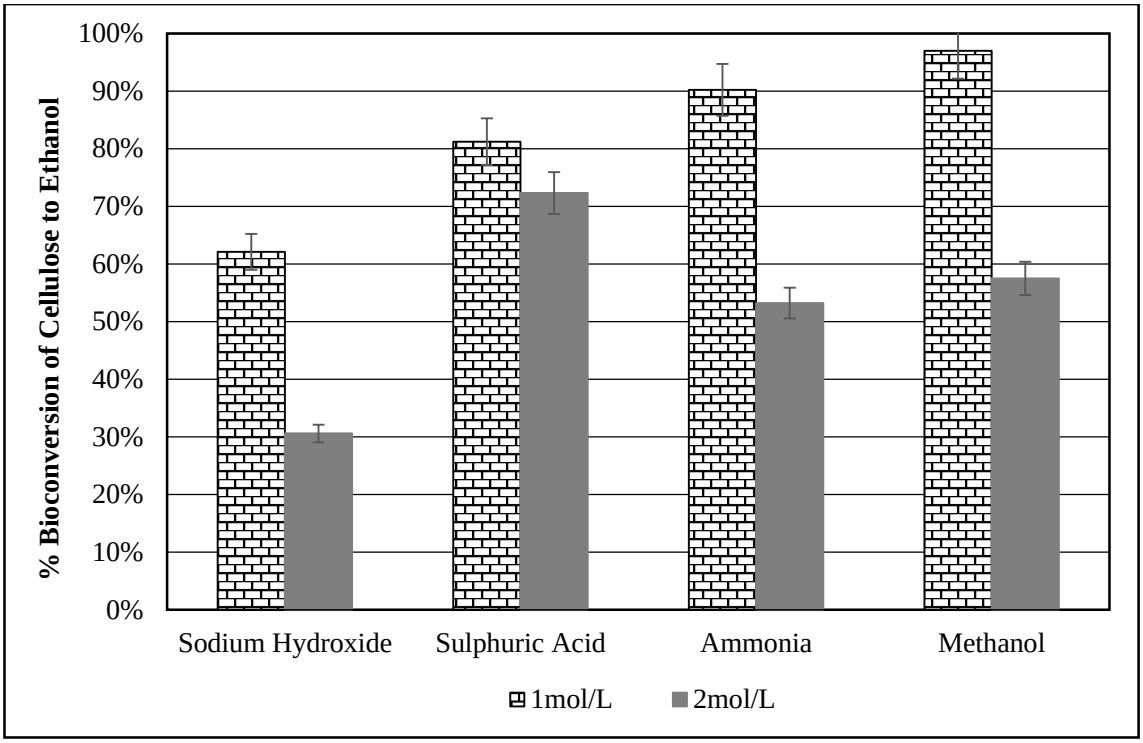


Figure 4.2. Comparative analysis of the percentage bioconversion yields for switchgrass (cellulose to ethanol). Samples were pretreated using: sodium hydroxide, sulphuric acid, ammonia and methanol at 1 mol L^{-1} and 2 mol L^{-1} concentrations. Samples were pretreated, followed by SSF using the enzyme C013L and *S.cerevisiae* yeast. Error bars are representative of percentage error.

NaOH pretreated samples produced 127 g of glucose when saccharified at 1 mol L^{-1} and 218 cm^3 of ethanol when simultaneously saccharified and fermented. H_2SO_4 pretreated samples produced significantly higher yields of 163 g of glucose at 1 mol L^{-1} and 285 cm^3 of ethanol following SSF. This increase in yield was consistently observed for both CH_3OH pretreated samples (230 g of glucose and 340 cm^3 of ethanol) and NH_3 pretreated samples (211 g of glucose and 316 cm^3 of ethanol). The increase in conversion yield following SSF indicates that saccharification alone is not effective in the conversion of pretreated lignocellulosic biomass. This suggests inhibitor formation or feed-back inhibition during the pretreatment stage when samples are saccharified only. Potential inhibitors can include weak acids, furan derivatives, and phenolic compounds which can be removed by various physical (extraction), biological (fungi) and chemical (change in pH) processes (Palmqvist and Hahn-Hägerdal, 2000). Various products of the cellulose hydrolysis process can also inhibit the action of the cellulose complex via feedback

inhibition of individual enzyme activities. For example, cellobiose inhibits the action of endoglucanase and cellobiohydrolase, while β-glucosidase is inhibited by the presence of D-glucose. Where this occurs, the use of glucose or cellobiose fermenting organisms can reduce or eliminate the feedback inhibition because of it immediate conversion to ethanol (Wyman et al., 1986).

Table 4.2. Comparative analysis of conversion yields (cellulose to glucose) achieved for pretreated switchgrass subjected to saccharification and those samples subjected to simultaneous saccharification and fermentation (SSF) (cellulose to ethanol).

		NaOH	H_2SO_4	CH_3OH	NH_3
Non-SSF		**g Glucose kg^{-1} Feedstock**			
	0.5mol L^{-1}	107.0	134.0	87.6	131.5
	1mol L^{-1}	127.0	163.0	230.0	211.0
	2mol L^{-1}	113.0	138.0	138.0	182.0
SSF		**g Ethanol kg^{-1} Feedstock**			
	1mol L^{-1}	218.0	285.0	340.0	316.0
	2mol L^{-1}	107.0	253.0	206.0	187.0
		g Ethanol g^{-1} Glucose			
	1 mol L^{-1}	0.21	0.27	0.32	0.30
	2 mol L^{-1}	0.10	0.24	0.19	0.18

4.2.4 Environmental Impact of Pretreatment Chemicals

Biofuels offer many advantages over petroleum-based fuels including the mitigation of greenhouse (GHG) emissions (Demirbas, 2008). The use of lignocellulosic feedstocks such as switchgrass for biofuels have been widely encouraged as they mitigate GHG emissions not only through fossil fuel substitution but also through carbon sequestration (Demirbas, 2008). A major environmental concern, however, is the production and usage of large quantities of potentially environmentally harmful chemicals during the pretreatment process. This study examines the potential environmental effects associated with the use of pretreatment chemicals (NaOH, CH_3OH, H_2SO_4 and NH_3) using different impact categories including: eutrophication, acidification, aquatic toxicity and global warming potential (GWP).

Excessive soil acidity can often be very damaging to certain types of terrestrial ecosystems and as a source of nitrogen, ammonia (NH_3) can raise the nitrogen levels in soil and water accelerating the rate of eutrophication (Lynch and Kercher, 2005). Sulphuric acid (H_2SO_4), a highly corrosive liquid which dissociates readily in water to sulphate ions and hydrated protons, can have a significant effect on both eutrophication and acidification (Amdur, 1971). H_2SO_4 emissions released into the atmosphere during the pretreatment process can dissolve in clouds forming what is commonly known as acid rain damaging plant, animal and marine life (Amdur, 1971). Subsequently, the most conspicuous effect of this type of eutrophication is the creation of dense blooms of noxious foul-smelling phytoplankton that reduces water clarity and quality (Lehtiniemi et al., 2005). Sodium hydroxide (NaOH) and methanol (CH_3OH) in low concentrations have been shown to have very little effect on the rate of eutrophication and acidification, rapidly diluting in water and air reducing their effects on the surrounding environment.

Aquatic toxicity, is one of the most common environmental impacts associated with accidental spillages and environmental pollution. Most microorganisms have the ability to break down chemicals when released in small quantities, with little adverse effect on the surrounding area. Sulphuric acid (H_2SO_4) released into the environment can have a negative effect on aquatic life if the pH of the water is altered. Trent et al. (1978) demonstrated that a decrease in water pH to 5 would result in severe mortality, while a pH of 3 would cause all organisms to be killed within a 24 h period. Similarly, ammonia (NH_3) released into the environment can be highly toxic to aquatic life, particularly fish (Constable et al., 2003) while also inhibiting the nitrification process, the severity of which depends on several factors including pH, temperature, dissolved oxygen and salinity (Russo, 1985). The hazards associated with sodium hydroxide (NaOH) are as equally harmful, caused by the hydroxyl ion, its effect is highly dependent on its buffer capacity in the aquatic environment (Cooper, 1979). Methanol (CH_3OH), however, if released into the environment is oxidised in a chemical reaction to formic acid, which in turn converts to carbon dioxide in the presence of folic acid. Subsequently, CH_3OH emissions in the aquatic environment will biodegrade rapidly, while large spillages will remain localised (Fiddler, 2005).

The mostly widely debated and regulated impact category is the global warming potential (GWP) of a chemical. The concept was developed to allow comparisons between greenhouse gas emissions (GHG) in the atmosphere. The intergovernmental panel on

climate change (IPCC, 2007) provides the GWP values for all chemical emissions and their CO_2 equivalence $_{(eq)}$. Ammonia (NH_3) is reported to have a $CO_{2\ (eq)}$ of 2.11 kg per kg of chemical. Significantly higher than that of NaOH (0.63 kg $CO_{2\ (eq)}$), CH_3OH (0.30 kg $CO_{2\ (eq)}$) and H_2SO_4 (0.14 kg $CO_{2\ (eq)}$). As climate change and global warming are arguably the most important impact categories of any environmental study, they became the main focus of our LCA study (Chapter 5), examining the emissions output of the chemical pretreatment process on the environment and its recipient's air, soil and water (Smullen et al., 2017c).

The use of chemicals for ethanol production has both its advantages and disadvantages. One major disadvantage is the environmental concern associated with the use and disposal of different chemicals. In a pilot plant or a small scale operation the quantities of chemicals utilised would be minimal compared to industrial production. In the event of an accidental release of these chemicals it would be assumed that the effects would remain localised and could be remediated in a timely manner. However, with increased production, the risks and potential for a larger exposure area would be significantly greater. Consequently, researchers are examining the long term environmental effects of ethanol production in conjunction with developing methodologies, with the view to producing both a cost effective and environmentally friendly fuel.

4.2.5 Economic Analysis

Commercial-scale production of cellulosic ethanol is challenged by a number of technical and economic issues that have restricted the production of 2nd generation ethanol worldwide. In recent years, many companies with government assistance have attempted to overcome the high investment and conversion costs associated with 2nd generation technologies. Novozymes, a Danish biotech company, recently claimed that the first commercial plants will be able to produce cellulosic ethanol at a production price lower than €7.56 L^{-1}, with enzyme costs as low as €1.89 L^{-1} (Bryant, 2010). Perkins (2012) reported that an Iowa plant claims to produce cellulosic ethanol for €11.34 L^{-1} including capital and depreciation costs. However, both feedstock production and feedstock pretreatment still contribute to 50% and 30% respectively, of the overall total cost of ethanol production from lignocellulosic biomass (Service, 2010). As the cost of the feedstocks can vary significantly depending on factors including species type, chemical inputs and harvest methodology, the economics of the pretreatment process exclusively

were evaluated in this study, to establish the most cost effective chemical, minimising potential costs of producing ethanol on an industrial scale.

Table 4.3 presents the chemical cost of producing 1 kg of glucose in the current study (excluding capital costs which can vary from plant to plant), and provides the initial cost of each chemical (ton^{-1}). The cost of NH_3 was highest at €280 ton^{-1}, while H_2SO_4 and CH_3OH were the lowest at €100 ton^{-1}. The cost per kg of glucose increased with increasing concentration of NaOH, H_2SO_4 and NH_3 (progressing from 0.5 – 2 mol L^{-1}). However, in the case of CH_3OH, cost was minimised at a concentration of 1 mol L^{-1} as a result of the high yields at this concentration compared to 0.5 mol L^{-1} and 2 mol L^{-1}. The cost of each pretreatment employed was calculated based on initial chemical cost, molecular weight and quantity utilised.

At a concentration of 0.5 mol L^{-1}, both NH_3 and CH_3OH were the least costly at €0.72 kg^{-1} of glucose, compared to NaOH which was the most expensive at €1.50 kg^{-1}. The cost of each chemical at a concentration of 1mol L^{-1} increased slightly except when CH_3OH was used, the cost reduced to €0.55 kg^{-1} of glucose. Pretreatment at 2 mol L^{-1} was generally the most expensive with chemical costs as high as €5.67 kg^{-1} for H_2SO_4.

The cost of the pretreatment chemicals varied significantly when evaluated in terms of ethanol production. Samples pretreated at 1 mol L^{-1} concentrations were substantially more cost effective compared to those at 2 mol L^{-1}. CH_3OH employed at 1 mol L^{-1} was found to be the least expensive pretreatment chemical (€0.50 L^{-1}) compared to 2 mol L^{-1} CH_3OH where the cost of producing 1 L of ethanol significantly increased to €2.42. Ethanol production utilising NaOH was the most expensive at both 1 and 2mol L^{-1} (€1.96 and €7.94 L^{-1} respectively).

High conversion yields and low purchase price ensured that the lowest costs per unit of output were achieved when methanol was used as the pretreatment chemical. Additionally, the use of pretreatments chemicals with high conversion yields will also maximise output and sales from the second generation ethanol plant. Capital costs for each pretreatment are difficult to estimate and will differ from plant to plant depending on resources and methodology employed. The recovery, reuse and disposal of waste products and chemicals should reduce the cost of the process when applied on an industrial scale.

Table 4.3. Economic analysis of pretreatment chemicals used in the current study. Assessment based on individual quantity and concentration employed.

	Initial cost of chemical*	NaOH	H_2SO_4	CH_3OH	NH_3
		€200 ton^{-1}	€100 ton^{-1}	€100 ton^{-1}	€280 ton^{-1}
Pretreated Switchgrass Samples		**€ kg^{-1} Glucose**			
	0.5 mol L^{-1}	1.50	1.46	0.72	0.72
	1 mol L^{-1}	2.52	2.41	0.55	0.92
	2 mol L^{-1}	5.66	5.67	1.85	2.76
		€ L^{-1} Ethanol			
	1 mol L^{-1}	1.96	1.83	0.50	0.80
	2 mol L^{-1}	7.94	4.03	2.42	2.73

* The cost of the NaOH, H_2SO_4, CH_3OH and $NH_{3(aq)}$ chemicals were obtained from industrial manufacturers; Tianjin Huaxiang Chemical Co., Ltd, Shijiazhuang Xinlongwei Chemical Co., Ltd, Qingdao HanHaiDa Import and Export Co., Ltd, Weifang Minghan Import and Export Co., Ltd. All prices were correct at time of study.

4.3. Conclusion

Over the last 50 years, the concentration of CO_2 in the atmosphere has increased by 30% and other GHG's have also increased alarmingly (Herbert and Krishnan, 2016). The production of biofuels utilising energy crops is expected to bring environmental, social and economic benefits to the bio-economy (Fazio and Monti, 2011) with the cost of producing biomass for use as fuels and as an energy source lower than that of finding and extracting fossil fuels (Rahman et al., 2013). A comparison of the overall emissions released from the combustion of gasoline with those from biofuels showed a 94% lower emissions quantity from biofuels, resulting in a lower environmental impact (Schmer et al., 2008). However, cost, sustainability and environmental impact still remain the key obstacles to the commercial viability of 2nd generation biofuels.

Evaluation of the pretreatment process showed that methanol was the most effective and efficient pretreatment chemical in conversion of switchgrass to ethanol. A theoretical yield of 0.32 g of ethanol / g of glucose was achieved at a concentration of 1 mol L^{-1}, and a cellulose to ethanol yield of 97%. Pretreatment employing sodium hydroxide was the

least effective pretreatment liberating a theoretical ethanol yield of just 0.21 g of ethanol / g of glucose, and a cellulose to ethanol yield of 62.1%.

A similar trend was also observed when the environmental impact of the chemical was reviewed. In general, ammonia and methanol appeared to have the lowest environmental impact on air, soil and water. While accidental exposure and spillages involving sulphuric acid and sodium hydroxide appeared to have a greater impact for many reasons, including significant changes in soil and water pH. An assessment of the process economics indicated that methanol was the most cost effective chemical for the conversion of switchgrass to ethanol and other related by-products. The cost of employing methanol (1 mol L^{-1}) in the pretreatment of switchgrass was estimated to be €0.55 kg^{-1} of glucose and €0.50 L^{-1} of ethanol.

When evaluating the environmental impact of a chemical, the severity of the chemical (pH, concentration, quantity, etc.) must also be taken into account. Consequently, the identification of a leading pretreatment chemical based on environmental impact is specific to the parameter under investigation and subsequently, no one pretreatment chemical can be established as an environmentally friendly technique. However, reuse, reduce and recycling techniques utilised during the process could demonstrate a substantial reduction in energy, cost and environmental impact.

Chapter 5

A Life Cycle Assessment of Pretreatment Technologies for the Bioconversion of Lignocellulosic Biomass to Ethanol

Abstract

Second generation biofuels have been proven to have a lower environmental impact than 1st generation biofuels, and more significantly, fossil based fuels. The present study, examines the processes (pretreatment and simultaneous saccharification and fermentation) in which lignocellulosic biomass is converted to ethanol, with a particular focus on the chemicals employed during the pretreatment process.

In recent years, questions have been raised regarding the environmental impact of the process compared to the environmental benefits. This study quantifies the impact of four pretreatment chemical processes employing sodium hydroxide, ammonia, methanol and sulphuric acid on five environmental receptors, in order to identify the pretreatment process with the lowest environmental impact. Using SimaPrò LCA software, the emissions output to air, soil and water contributing to the environmental parameters: global warming potential (GWP) / climate change, eutrophication, acidification, photochemical oxidation potential and marine and human ecotoxicity were assessed.

On evaluation, impacts on the two most widely reported environmental receptors (GWP and Human ecotoxicity) differed significantly. Methanol exhibited the lowest GWP (0.0019 kg $CO_{2\,(eq)}$ 100 kg^{-1} of ethanol) and was the second lowest (0.015 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol) contributor to human ecotoxicity. In contrast sodium hydroxide had the highest impact on GWP of 14.71 kg $CO_{2\,(eq)}$ 100 kg^{-1} of ethanol and on human ecotoxicity (0.612 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol).

In general, emissions output varied significantly among all four pretreatment chemicals when compared using selected environmental receptors. Methanol was identified as having the lowest environmental impact overall.

5.1. Introduction

Since the energy crisis of the 1970's interest in the production of biomass for energy purposes has increased considerably in Europe, particularly in the area of biofuel production (Fazio and Monti, 2011). Over the past decade, biofuels have moved from being a niche energy source in the European transport sector to being a significant source of road transportation fuel, with liquid biofuels (biodiesel and bioethanol) being at the forefront of renewable energy in EU transport policies i.e. the renewable energy directive 2009/28/EC (EC, 2009). Consequently, Europe is expected to see a further increase in the consumption of biofuels with Denmark, the UK and Ireland expecting to be 100%, 87.7% and 70% (respectively) dependant on biofuels by 2020 (Shine et al, 2010). As a result of newly introduced policies in the U.S and Europe, ethanol production is predicted to double in the coming decade (OECD-FAO, 2015).

Lignocellulosic ethanol has been identified as the best alternative to 1st generation biofuels because of the wide diversity of feedstock sources worldwide, low agricultural input production opportunities and beneficial greenhouse gas (GHG) balances, compared with current fuel sources (González-García et al., 2012b). Subsequently, in order to meet global ethanol demand and renewable energy targets a complete transition from 1st generation to 2nd generation ethanol is imperative.

The use of large quantities of chemicals during the pretreatment stage however, still remains an environmental concern which has raised questions as to the environmental benefit of a transition to this non-fossil fuel alternative. As a result, conversion studies utilising lignocellulosic biomass for ethanol production are being assessed for their sustainability and environmental performance to evaluate the overall environmental impact of 2nd generation ethanol.

SimaPrò, life cycle assessment software was employed in this study in order to evaluate and establish the environmental impact of each of the four pretreatment chemicals – sodium hydroxide, methanol, sulphuric acid and ammonia utilised in Chapters 3 and 4. Life cycle assessment (LCA) methodology has been proven to be a valuable tool in conversion studies, analysing the environmental performance of a process and the potential impacts of a product. LCA assesses and interprets data over a product / processes life cycle (production, use and end-of-life) evaluating both the entire life cycle, often referred to as a cradle-to-grave study or part of the life cycle, referred to as a cradle-to-

gate or gate-to-gate study. Chapter 1 briefly described the working principals of an LCA study, used to identify specific areas of the process, establish and compare energy and resource consumption rates, and environmental emissions to air, soil and water (ISO, 14040; Borrion et al., 2012). Several studies have been conducted over the years, using LCA methodology to examine the environmental performance of ethanol production from different lignocellulosic feedstocks such as corn stover (Spatari et al., 2010), wheat straw (Borrion et al., 2012), miscanthus (Parajuli et al., 2015) and switchgrass (Spatari et al., 2005; Bai et al., 2010). In general, these studies can conclude that lignocellulosic derived ethanol would provide environmental advantages over fossil fuels by reducing non-renewable energy consumption and GHG emissions (González-García et al., 2012b). Unfortunately, methodological differences among these reports, have created uncertainty and prevented comparisons between the feedstocks and technologies employed. This has made the selection of pretreatment chemicals, cellulase enzyme preparations and the choice of yeast strain more difficult, in turn challenging the commercialisation of 2nd generation ethanol production.

The objective of the present study was to evaluate the environmental performance of ethanol production from switchgrass (*Panicum virgatum L*), with a particular focus on the pretreatment process, specifically the chemicals employed. A comparative approach was taken in this study, investigating the effects of different chemical pretreatments on emissions output, with the aim of identifying the pretreatment technique with the lowest environmental impact, while also demonstrating the highest cellulose to ethanol yield. To facilitate a direct comparison of fuel ethanol from switchgrass using different pretreatment chemicals, the same methodology was used to convert the biomass to ethanol (described in Section 2.7) and the same system boundary was defined.

The switchgrass feedstock employed in this study was chemically pretreated at a single concentration of 1 mol L^{-1}, in order to accurately and uniformly compare the pretreatment chemicals employed, and establish the most effective pretreatment technique. Information obtained from the conversion process was assessed for its environmental performance using SimaPrò LCA software version 7.3.3.

5.2 Methodology Analysis

5.2.1 Goal and Scope

Prè Consultants SimaPrò LCA software (version 7.3.3) (Prè Consultants, 2016) was employed in this gate-to-gate study. The goal of the study was to quantify and compare the inputs and outputs of the process including energy, chemicals and emissions produced. The scope included the emissions released during the pretreatment process and use of these chemicals and conversion processes alone. All other inputs and outputs were excluded.

5.2.2 System Boundary and Functional Unit

The system boundary employed in this LCA was specific to the goal outlined for the study. An overview of the industrial ethanol production process is outlined in Figure 5.1. As the goal of the study was to evaluate the environmental performance of the pretreatment process, the system boundary is representative of this and excludes all other external elements outside the scope of this study. The system boundary for the study is inclusive of the pretreatment process. Production of inputs (feedstocks, chemicals, enzymes, electricity, heat and transport) are not included in this study. No allocation criteria was applied in this study since feedstock cultivation only yielded one product, lignocellulosic biomass and the ethanol production process, produces only one main product, ethanol. The functional unit for the study was the production of 100 kg of ethanol.

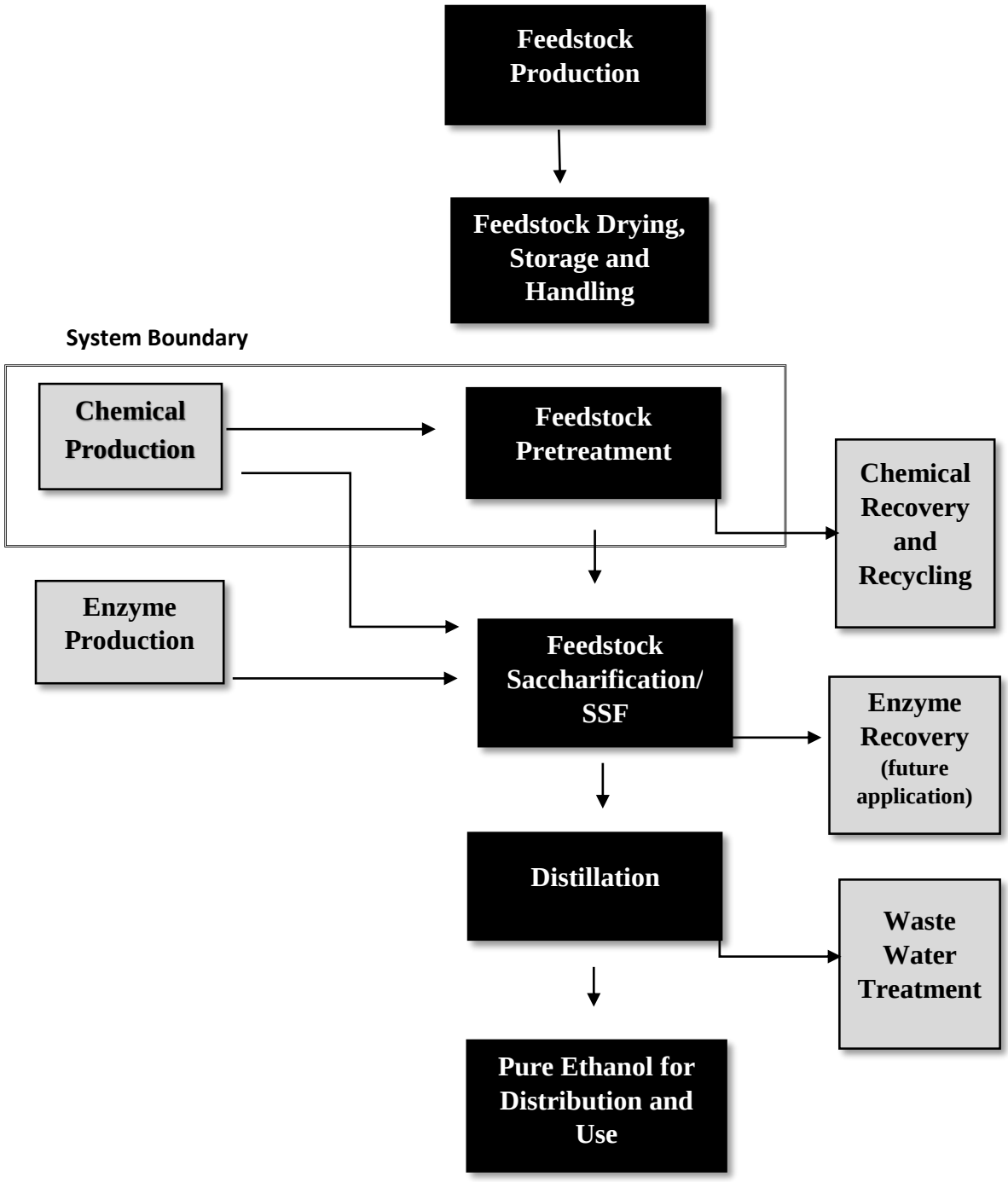


Figure 5.1: Cradle-to-grave LCA profile of ethanol production with system boundaries. Production of inputs are not included in the present study (Gate-to-gate).The current study investigates the feedstock pretreatment processes (chemical and energy use). Subsequent emissions produced in the feedstock saccharification process are discussed following a literature review.

5.2.3 Life Cycle Inventory

Life cycle inventory, the data collection portion of the LCA, is a critical step which identifies and accounts for every input and output to the system of interest (Baumann and Tillman, 2004). Usually consisting of detailed tracking of all flows in and out of the product system, it is an extremely complex process which can often consist of many different data sources. Inventory data for the ethanol production system employed in the current study is summarised in Table 5.1.

Table 5.1: Data sources for the life cycle inventory of ethanol production from switchgrass employed in the current study.

Subsystem	Data Required	Data Source
S1 Switchgrass Pretreatment Process	Production Scale	Laboratory Results: (1)
	Chemical Use	Laboratory Results: (1)
	Electricity and Heat Use	Laboratory Results: (1)
S2 Cellulose Bioconversion Process	Production Scale	Laboratory Results: (1)
	Enzyme Production/Use	Laboratory Results: (1)
	Saccharomyces cerevisiae Use	Research Reports: (3)
	Nutrient Use	Laboratory Results: (1)
	Water Requirements/Treatment	Research Reports: (2,3,4)

(1) Smullen et al., 2017a, (2) Borrion et al., 2012, (3) González-García et al., 2012a and 2012b, (4) Agostinho et al., 2015

To effectively build and analyse LCA models, transparent, high quality and widely accepted inventory data is required for the most commonly used materials and processes. SimaPrò extracts large amounts of background data from different processing studies including chemical and food production, and imports the data from a wide selection of available databases. The following databases were employed in this study:

- Ecoinvent v3 LCI Database
- EU and Danish input and output library.
- European Life Cycle Database

Of these the ecoinvent v3 LCI Database is the most regularly employed and referenced (Wernet et al., 2016). LCA databases collect data from scientific studies on the environmental impact of products and processes so that the environmental consequences of the manufacture and use on a range of impact categories can be quantified.

Impact assessment was carried out using the CML method (Centre of Environmental Science at Leiden University) which uses a set of characterisation methods to quantify the effect of the process on pre-defined environmental receptors. This method calculates the equivalent carbon dioxide (CO_2), phosphate (PO_4^{-3}), sulphur dioxide (SO_2), ethylene (C_2H_4) and 1, 4-dichlorobenzene ($C_6H_4Cl_2$) emissions for the different chemicals and inputs used in this study in order to provide a basis for comparison of the effect of the different pre-treatments on each environmental receptor (eg climate change, eutrophication etc).

5.2.4 Ethanol Production

Switchgrass variety Shawnee analysed in the present study was produced at the Crop Research Centre, Carlow, Ireland (52.86^{o}N, 6.90^{o}W) sown in April 2008 and harvested in September 2011. Compositional analysis as detailed by Smullen et al. (2017a) was performed on the switchgrass prior to being subjected to chemical pretreatment.

Switchgrass samples were pretreated and saccharified as described in Section 2.8. Various concentrations of 0.5, 1 and 2 mol L^{-1} were investigated for their effects on the conversion yields of switchgrass and it was determined (Chapter 4) that 1 mol L^{-1} chemical concentration was the most effective and efficient for the bioconversion of switchgrass. Subsequently, the chemical concentration of 1 mol L^{-1} was the main focus of this particular study, although for comparative purposes the chemical concentrations 0.5 mol L^{-1} and 2 mol L^{-1} were also examined and results are presented in Table 5.4 (0.5 mol L^{-1}), Table 5.5 (1 mol L^{-1}), and Table 5.6 (2 mol L^{-1}).

For the purposes of the environmental assessment the process inputs (pretreatment chemicals, electricity and heat) used in the conversion process have been extrapolated, increasing the quantities of those used in the laboratory to those employed in full scale industrial ethanol production. In order to prepare the chemical concentration of 1 mol L^{-1} employed in this study the following quantities of each chemical were used: 0.34 g NH_3,

0.8 g NaOH, 0.32 g CH_3OH and 1.96 g H_2SO_4 per 20 cm^3 of deionised water. These were then extrapolated (340 kg NH_3, 800 kg NaOH, 320 kg CH_3OH and 1960 kg H_2SO_4 per 20 L of deionised water). The electricity and heat required during the pretreatment process (24 KWh) was calculated according to the manufacturers specifications for the Gallenkamp, Environmental Shaker Incubator 10x 400 (Cheshire, UK) used, operating at 1000 Wh. Based on these figures, an assumption was made as to the amount of electricity and heat required for small scale industrial production. There is a certain amount of uncertainty regarding the amount of electricity and heat that would be required and the value stated in Table 5.2 is not necessarily required. Table 5.2 presents a summary of the key inputs into the system of interest.

Table 5.2: Industrial inputs for the bioconversion of switchgrass to 100 kg of ethanol*.

Pretreatment	Chemical	Electricity / Heat	Water	Enzyme	Yeast
Ammonia	5.0 kg	24.0 MWh	161.5 L	4.0 L	4.0 kg
Sodium Hydroxide	17.0 kg	24.0 MWh	161.5 L	4.0 L	4.0 kg
Sulphuric Acid	31.8 kg	24.0 MWh	161.5 L	4.0 L	4.0 kg
Methanol	8.7 kg	24.0 MWh	161.5 L	4.0 L	4.0 kg

* Industrial inputs are inclusive of the entire bioconversion process as performed in our laboratory analysis and extrapolated accordingly.

5.3. Results and Discussion

5.3.1. Environmental Performance of the Pretreatment Process

The software package SimaPrò 7.3.3 was used in this study to assess the emissions to air, soil and water of the pretreatment process using the following impact categories: global warming potential (GWP), eutrophication (EP), acidification (AP), photochemical oxidation demand (POD) and marine and human ecotoxicity. CML methodology (CML, 2002) was then applied for the evaluation / comparison of the associated impact categories. The CML method categorises the emissions in terms of equivalent values according to the potential environmental impact. Global warming potential is measured

in kg of $CO_{2\,(eq)}$, eutrophication is measured in kg of $PO_4^{-3}{}_{(eq)}$, acidification is measured in kg of $SO_{2\,(eq)}$, photochemical oxidation demand is measured in kg of $C_2H_{4\,(eq)}$, while marine and human ecotoxicity is measured in kg of 1, 4 $C_6H_4Cl_{2\,(eq)}$.

5.3.1.1. Global Warming Potential (GWP)

The global warming potential (GWP) of a product or process and its subsequent impact on climate change, are the two most widely debated and regulated consequences in the assessment of environmental performance. At the forefront of global environmental policies (PEA, 2005; IEA, 2015) GWP and climate change have been identified among many, as the most significant environmental parameters and so were the main focus of this particular study. However, due to insufficient information on the pretreatment conditions employed and the use of different functional units, the value of comparison between this study and existing environmental studies has been limited.

Figure 5.2 shows the total emissions in CO_2 equivalence (eq) associated with the chemical conversion of switchgrass pretreated using ammonia, sulphuric acid, methanol and sodium hydroxide at a concentration of 1 mol L^{-1}. Pretreatment employing sodium hydroxide exhibited the highest quantity of GHG emissions measuring 14.71 kg $CO_{2\,(eq)}$ 100 kg^{-1} ethanol, compared to methanol pretreatment which produced the lowest quantity of GHG emissions, measuring just 0.0019 kg $CO_{2\,(eq)}$. Ammonia pretreatment produced the second highest quantity of GHG emissions measuring 12.03 kg $CO_{2\,(eq)}$, while pretreatment utilising sulphuric acid produced 7.77 kg $CO_{2\,(eq)}$ 100 kg^{-1} ethanol.

The identification of methanol as the lowest contributor of emissions to air is significant to this study. As demonstrated in Chapter 4, methanol was the most effective and efficient pretreatment chemical, exhibiting the highest conversion yields, in addition to being the most economic chemical. Climate change is arguably the most important impact category as the purpose of biomass utilisation for energy purposes is to reduce GHG emissions. Therefore, as methanol has the lowest, by far, carbon footprint (GHG emissions) compared to other pretreatments, it has huge potential to become a leading pretreatment chemical.

The emissions output of each pretreatment chemical per kg of chemical employed, was also evaluated in the current study. It was determined that methanol and sulphuric acid had the lowest (0.24 kg $CO_{2\,(eq)}$ kg^{-1} chemical) emissions per kg of chemical employed. Significantly lower (10 times) than that of ammonia (2.4 kg $CO_{2\,(eq)}$ kg^{-1} chemical).

However, when evaluated in terms of the functional unit outlined, results varied substantially.

Emissions output in the present study varied significantly from the emissions outputs provided in other research studies, where total emissions was calculated based on the functional unit of the study. Borrion et al. (2012) determined that wheat straw pretreated using sulphuric acid could potentially contribute 150 kg $CO_{2\ (eq)}$ 100 kg^{-1} of ethanol, significantly higher than that calculated in the current study.

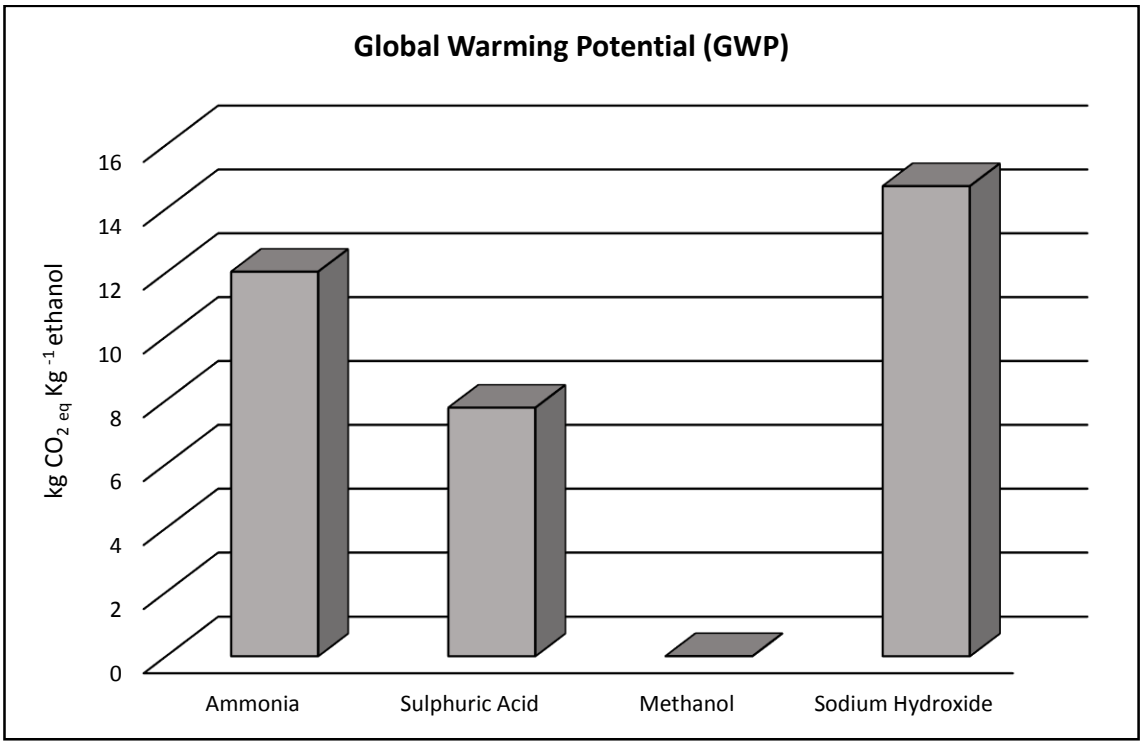


Figure 5.2. Global Warming Potential of pretreatment processes employed in this current study, quantified as kg $CO_{2\ (eq)}$ 100 kg^{-1} ethanol.

5.3.1.2. Eutrophication (EP)

Eutrophication, characterised by excessive plant and algal growth (Schindler, 2006) is the second most investigated impact category. Not confined to water alone, Galloway et al. (1995) reported that eutrophication can also have a detrimental effect on both the natural rate of nitrogen fixation in soil and nitrous gases in the atmosphere. Described as a syndrome of ecosystem responses to human activities, eutrophication can fertilise water bodies with nitrogen (N) and phosphorus (P), often leading to changes in animal and plant populations, while also degrading water and habitat quality (Cloern, 2013).

Emissions to soil (N and P) were generally very low and only detectable in trace amounts when all four pretreatments were evaluated.

In contrast, the emissions to air (NH_3 and N) were considerably greater than those released into the soil. Pretreatment employing sulphuric acid had the greatest of eutrophication emissions to air measuring 0.216 kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} of ethanol, while methanol pretreatment had the second lowest quantity of emissions at 0.042 kg $PO_4^{-3}{}_{(eq)}$. The emissions output for ammonia was even lower measuring 0.025 kg $PO_4^{-3}{}_{(eq)}$, compared to sodium hydroxide which demonstrated a total emissions output of 0.099 kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} of ethanol.

Total emissions output to water differed completely to those released into the air and soil. Sodium hydroxide and sulphuric acid were the lowest contributors, when their emissions and effects were evaluated. Sulphuric acid pretreatment had the lowest quantity of emissions of 0.017 kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} of ethanol, which increased to 0.051 kg $PO_4^{-3}{}_{(eq)}$ for sodium hydroxide pretreated switchgrass. The total emissions for ammonia pretreatment increased significantly to 0.084 kg $PO_4^{-3}{}_{(eq)}$, while methanol pretreated switchgrass exhibited the highest quantity of emissions (0.36 kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} of ethanol) (Fig. 5.3).

González-García et al (2012b) investigated the effect of ethanol production from poplar, on eutrophication and found that total emission released for the bioconversion process measured 1.07 kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} of ethanol following sulphuric acid and steam pretreatment. In contrast, González-García et al (2012a) calculated an emissions output of 92.6 kg $PO_4^{-3}{}_{(eq)}$ kg^{-1} ha^{-1} following the dilute acid pretreatment of willow.

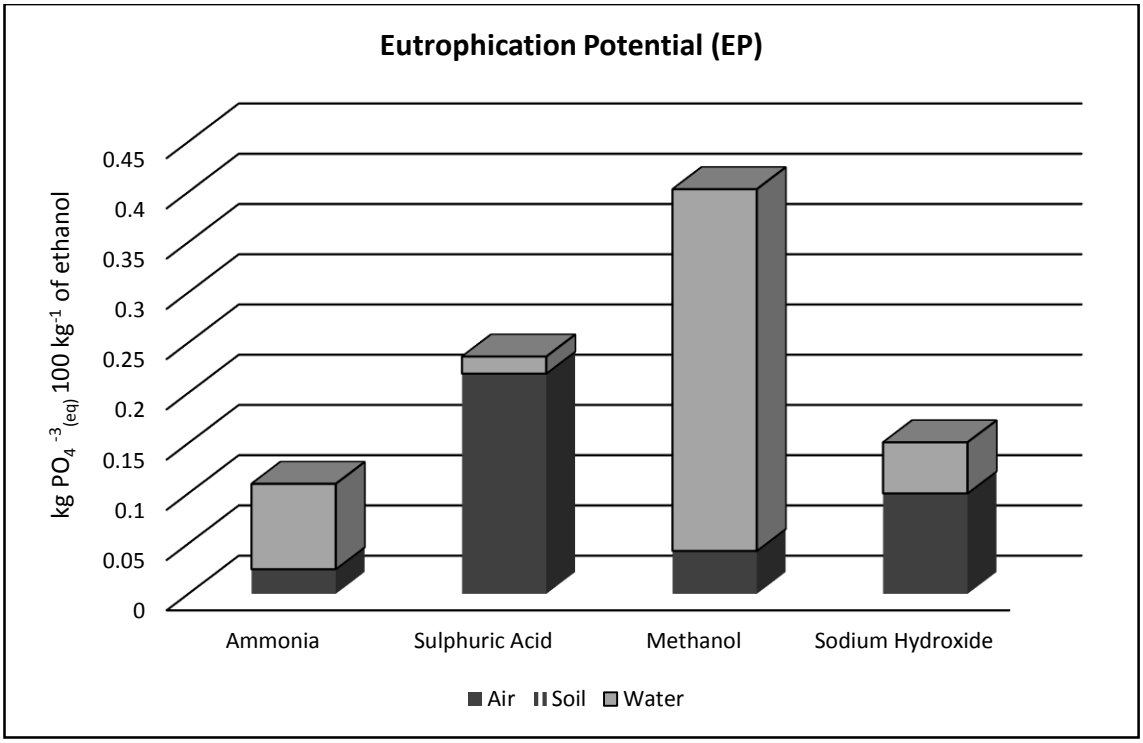


Figure 5.3. Eutrophication Potential of the pretreatment processes employed in the current study, quantified as kg $PO_4^{-3}{}_{(eq)}$ 100 kg^{-1} ethanol.

5.3.1.3. Acidification (AP)

Acidification originates primarily from anthropogenic emissions of sulphur dioxide (SO_2), nitrogen oxides (NO_x) and ammonia (NH_3). Most of the SO_2 and NO_x is emitted to the atmosphere by the combustion of fossil fuels, while NH_3 emissions are more commonly associated with agricultural activities. Sulphuric acid employed during the pretreatment process has the potential to make a significant contribution to acidification, generating both SO_2 and NO_x emissions which are easily emitted to the atmosphere. The acidifying potential of ammonia, sulphuric acid, methanol and sodium hydroxide employed in this study were all assessed for their emissions of SO_2 and NO_x and in particular NH_3. Emissions are expressed as kg of $SO_{2\,(eq)}$ 100 kg^{-1} of ethanol and results are shown in Figure 5.4.

Pretreatment employing ammonia liberated the highest quantity of emissions to air when its acidification potential was measured (0.113 kg $SO_{2\,(eq)}$ 100 kg^{-1} of ethanol). Five times higher than that of sulphuric acid pretreatment (0.023 kg $SO_{2\,(eq)}$) and three and a half times higher than pretreatment employing methanol (0.033 kg $SO_{2\,(eq)}$). The total

emissions for sodium hydroxide pretreated switchgrass were determined to be 0.077 kg $SO_{2\ (eq)}$ 100 kg^{-1} of ethanol.

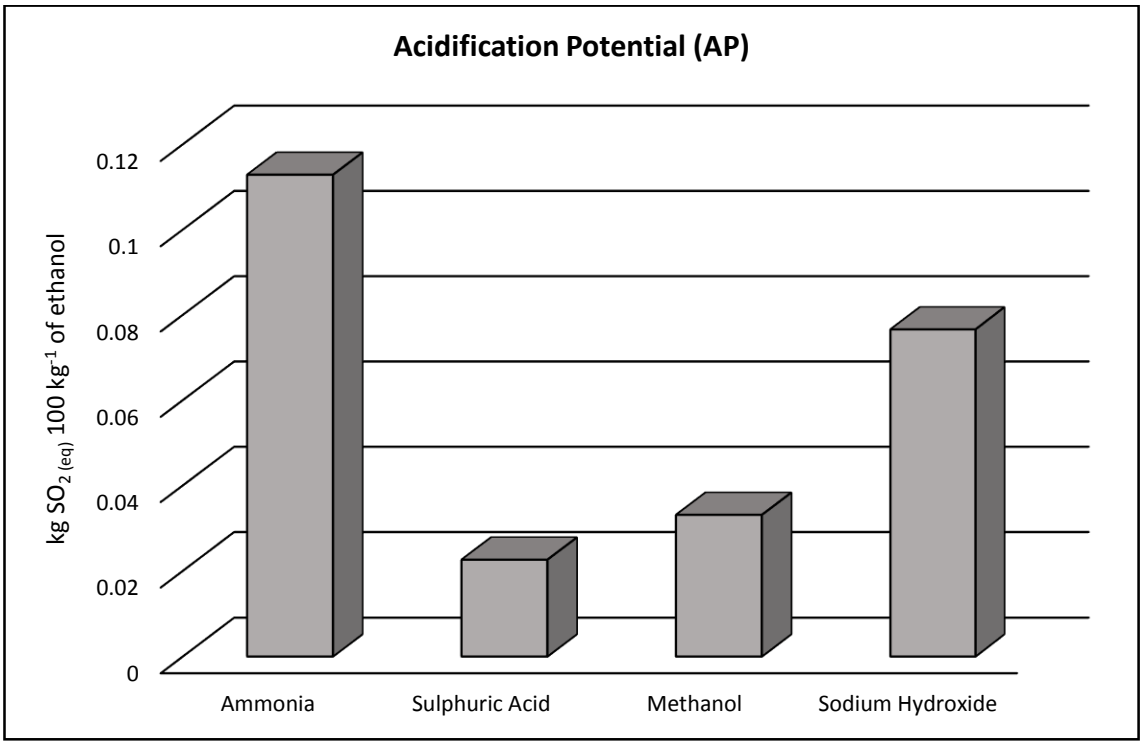


Figure 5.4. Acidification Potential of the pretreatment processes employed in the current study, quantified as kg $SO_{2\ (eq)}$ 100 kg^{-1} of ethanol.

5.3.1.4. Photochemical Oxidation Demand

Photochemical oxidation often referred to as summer smog is the result of reactions between nitrogen oxides (NO_x) and volatile organic compounds (VOC) exposed to UV radiation. Emissions to air, carbon monoxide (CO), hydrocarbons - benzene (C_6H_6) and Toluene (C_7H_8) and carbonyls – acetone (C_3H_6O) and acetic acid (CH_3COOH) produced during the chemical pretreatment of switchgrass were evaluated for their contribution to photochemical oxidation, resulting in urban and rural air pollution. Emissions are reported in ethylene equivalence ($C_2H_{4\ eq}$) and are presented in Figure 5.5.

Highest emissions output was measured following sodium hydroxide pretreatment with 0.195 kg $C_2H_{4\ (eq)}$ 100 kg^{-1} of ethanol. This reduced minimally when sulphuric acid was used as a pretreatment chemical (0.190 kg $C_2H_{4\ (eq)}$ 100 kg^{-1} of ethanol). Pretreatment employing ammonia demonstrated a further reduction in emissions with 0.108 kg $C_2H_{4\ (eq)}$ 100 kg^{-1} of ethanol. Furthermore, total emissions output continued to decrease for

methanol which produced the lowest quantity of emissions to air (0.001 kg $C_2H_{2\,(eq)}$ 100 kg^{-1} of ethanol).

The effects of photochemical oxidation demand (POD) as an environmental consequence of ethanol production are similar among most research studies, however, the source from which they originate can often differ, with comparisons made difficult by changing functional units. Borrion et al. (2012) suggested that pretreatment and SSF process contributes approximately 5.44g and 3.00g of NMVOC kg^{-1} of ethanol respectively, to POD, while González-García et al. (2012b) believes that feedstock cultivation in addition to the ethanol conversion process is the biggest contributor. In comparison to emissions resulting from the pretreatment of poplar and willow, González-García et al. (2012a; 2012b) quantified emissions of 0.161 kg $C_2H_{4\,(eq)}$ $100kg^{-1}$ of ethanol and 50.6 kg $C_2H_{4(eq)}$ kg^{-1} ha^{-1} respectively.

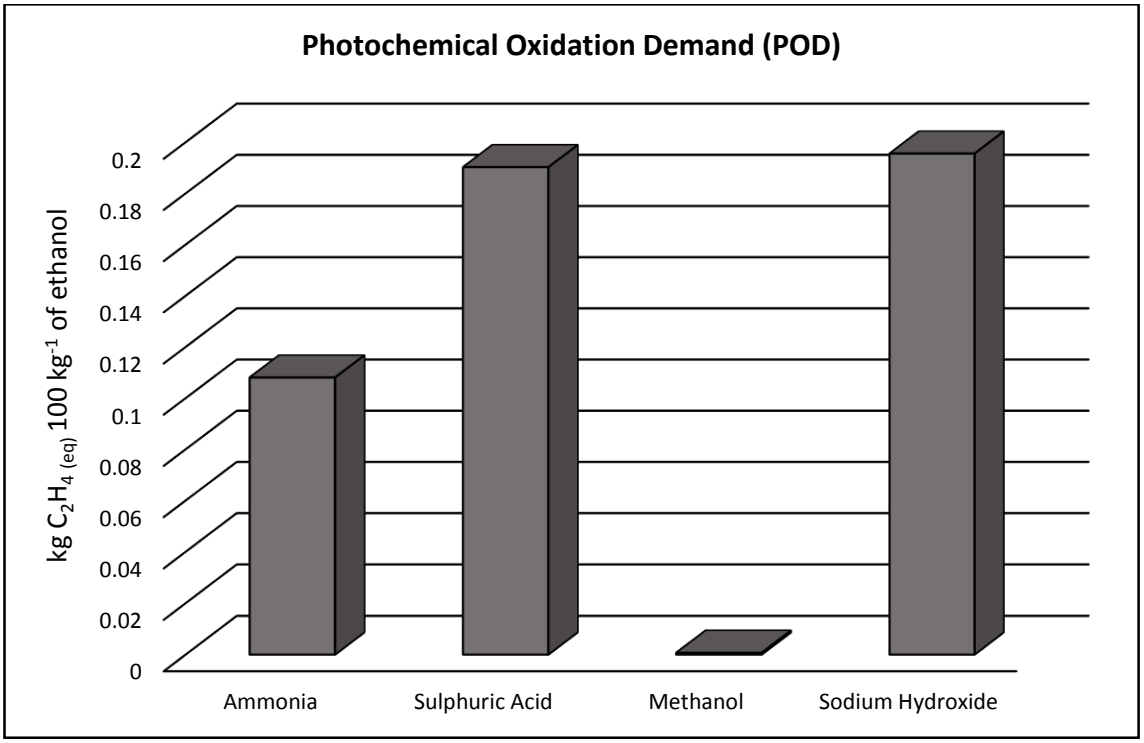


Figure 5.5. Photochemical Oxidation Demand of the pretreatment processes employed in the present study, quantified as kg $C_2H_{4\,(eq)}$ 100 kg^{-1} of ethanol.

5.3.1.5. Marine and Human Ecotoxicity Potential

Marine and human ecotoxicity is an environmental consequence of heavy metal (copper, zinc, lead, cadmium, nickel, mercury and arsenic) and hydrocarbon (benzene and toluene)

release to air, soil and water. Quantitative analysis of the emissions released to the two main recipients air and water during and as a result of chemical pretreatment were examined in this study. Table 6.3 presents a comparative view of the emissions to both air and water contributing to marine and human toxicity, measured in kg of 1, 4-dichlorobenzene $_{(eq)}$ ($C_6H_4Cl_2$).

Sulphuric acid produced the highest emissions output to water (8.823 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol) when marine ecotoxicity was assessed. This reduced substantially to 0.566 kg $C_6H_4Cl_{2\,(eq)}$ when methanol pretreatment was employed. Sodium hydroxide was shown to have the second highest quantity of emissions to water, approximately 3.056 kg $C_6H_4Cl_{2\,(eq)}$. A significant decrease in emissions output compared to that of sulphuric acid. Pretreatment employing ammonia produced a similarly high quantity of emissions measuring 2.484 kg $C_6H_4Cl_{2\,(eq)}$.

The emissions output for some pretreatment chemicals decreased when their effects to air were evaluated. Ammonia had the lowest impact on marine toxicity (0.003 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol) - significantly lower than that observed for water. Pretreatment employing methanol also produced a lower emissions output than that to water, measuring 0.363 kg $C_6H_4Cl_{2\,(eq)}$. As previously observed the quantity of $C_6H_4Cl_{2\,(eq)}$ released following the utilisation of sulphuric acid and sodium hydroxide pretreatment processes was extremely high relative to that of ammonia and methanol. However, when compared to the emissions output to water, sulphuric acid (2.712 kg $C_6H_4Cl_{2\,(eq)}$) liberated a substantially lower emissions output, while sodium hydroxide (7.463 kg $C_6H_4Cl_{2\,(eq)}$) had a significantly higher output. In general, the chemical pretreatment process has a lower environmental impact to air when its effects on marine toxicity are compared (Table 5.3).

Human ecotoxicity is the most widely reported parameter of any environmental hazard or process. Table 5.3 presents a summary of the effect of the chemical pretreatment processes on human toxicity, with the release of 1, 4-dichlorobezene to air, water and soil. On evaluation of these results, sodium hydroxide was demonstrated to have the highest emissions output to water measuring 0.373 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol, more than 3 times higher than that of sulphuric acid (0.101 kg $C_6H_4Cl_{2\,(eq)}$). Emissions output continued to decrease for both methanol and ammonia pretreated switchgrass with quantities of 0.001 kg and 0.001 kg $C_6H_4Cl_{2\,(eq)}$ 100 kg^{-1} of ethanol, respectively, recorded.

A similar trend in emissions output to air was observed for human toxicity and marine toxicity. Pretreatment employing ammonia was found to have the lowest environmental impact on human toxicity when release of 1, 4-dichlorobenzene was evaluated, which increased slightly for the methanol pretreatment processes (0.015 kg $C_6H_4Cl_{2\ (eq)}$). There was a further increase in emissions for the sulphuric acid pretreatment processes (0.361 kg $C_6H_4Cl_{2\ (eq)}$, while the largest contributor of emissions to air was demonstrated to be sodium hydroxide measuring 0.612 kg $C_6H_4Cl_{2\ (eq)}$ 100 kg^{-1} of ethanol.

Emissions to soil were low. Pretreatment employing ammonia demonstrated the lowest emissions output, while sulphuric acid (0.005 kg), methanol (0.002 kg) and sodium hydroxide (0.013 kg) produced minimal amounts of $C_6H_4Cl_{2\ (eq)}$ 100 kg^{-1} of ethanol.

Table 5.3. Environmental performance of chemical pretreatments and their effect on marine and human ecotoxicity. Total emissions is measured as kg $C_6H_4Cl_{2\ eq}$ (1, 4-dichlorobenzene) 100 kg^{-1} of ethanol.

Environmental Parameter	Recipient	Ammonia	Sulphuric Acid	Methanol	Sodium Hydroxide
Marine	Air	0.003	2.712	0.363	7.463
	Water	2.484	8.823	0.566	3.056
Human	Air	0.000	0.361	0.015	0.612
	Water	0.001	0.101	0.000	0.373
	Soil	0.000	0.005	0.002	0.013

Table 5.4. Comparative analysis of emissions to air, soil and water following the chemical pretreatment of switchgrass employing ammonia, sulphuric acid, methanol and sodium hydroxide at a concentration of 0.5 mol L^{-1}. CML methodology (CML, 2002) was applied for the evaluation and comparison of associated impact categories. Emissions were calculated in kg per kg of ethanol produced.

Environmental Parameter	Recipient	Unit	Ammonia	Sulphuric acid	Methanol	Sodium Hydroxide
GWP	Air	kg	0.0408	0.024	7.90×10^{-6}	0.035
	Air	kg	8.39×10^{-10}	6.74×10^{-9}	2.67×10^{-10}	2.33×10^{-9}
Eutrophication	Water	kg	2.88×10^{-9}	5.22×10^{-10}	1.30×10^{-8}	1.19×10^{-9}
Acidification	Air	kg	3.83×10^{-9}	6.98×10^{-10}	1.22×10^{-9}	1.79×10^{-9}
Photochemical Oxidation Demand	Air	kg	3.67×10^{-9}	5.87×10^{-9}	2.5×10^{-11}	4.60×10^{-9}
	Air	kg	0.000	0.0111	1.0 x 10-4	0.0242
Human Ecotoxicity	Water	kg	4.0×10^{-9}	3.5×10^{-6}	2.0×10^{-9}	9.0×10^{-6}
Marine Ecotoxicity	Air	kg	1.1×10^{-7}	0.001	0.000	2.1×10^{-3}
	Water	kg	1.0×10^{-4}	2.7×10^{-3}	0.000	6.0×10^{-3}

Table 5.5. Comparative analysis of emissions to air, soil and water following the chemical pretreatment of switchgrass employing ammonia, sulphuric acid, methanol and sodium hydroxide at a concentration of 1 mol L^{-1}. CML methodology (CML, 2002) was applied for the evaluation and comparison of associated impact categories. Emissions were calculated in kg per kg of ethanol produced.

Environmental Parameter	Recipient	Unit	Ammonia	Sulphuric acid	Methanol	Sodium Hydroxide
GWP	Air	kg	0.120	0.077	1.9×10^{-5}	0.147
Eutrophication	Air	kg	2.5×10^{-4}	2.16×10^{-3}	4.2×10^{-4}	9.9×10^{-4}
	Water	kg	8.4×10^{-4}	1.7×10^{-4}	3.6×10^{-3}	5.1×10^{-4}
Acidification	Air	kg	1.13×10^{-3}	2.3×10^{-4}	3.3×10^{-4}	7.7×10^{-4}
Photochemical Oxidation Demand	Air	kg	1.08×10^{-3}	1.9×10^{-3}	1.0×10^{-5}	1.95×10^{-3}
	Air	kg	0.000	3.61×10^{-3}	1.5×10^{-4}	6.12×10^{-3}
Human Ecotoxicity	Soil	kg	0.000	5.0×10^{-5}	2.0×10^{-5}	1.3×10^{-4}
	Water	kg	1.0×10^{-5}	1.01×10^{-3}	0.000	3.73×10^{-3}
Marine Ecotoxicity	Air	kg	3.0×10^{-5}	0.027	3.63×10^{-3}	0.074
	Water	kg	0.024	0.088	5.66×10^{-3}	0.030

Table 5.6. Comparative analysis of emissions to air, soil and water following the chemical pretreatment of switchgrass employing ammonia, sulphuric acid, methanol and sodium hydroxide at a concentration of 2 mol L^{-1}. CML methodology (CML, 2002) was applied for the evaluation and comparison of associated impact categories. Emissions were calculated in kg per kg of ethanol produced.

Environmental Parameter	Recipient	Unit	Ammonia	Sulphuric Acid	Methanol	Sodium Hydroxide
GWP	Air	kg	1.64	0.59	3.2×10^{-4}	1.38
Eutrophication	Air	kg	3.35×10^{-3}	2.7×10^{-2}	1.07×10^{-3}	9.30×10^{-3}
	Water	kg	1.2×10^{-3}	2.08×10^{-3}	5.3×10^{-2}	4.77×10^{-3}
Acidification	Air	kg	1.5×10^{-2}	2.78×10^{-3}	4.88×10^{-3}	7.2×10^{-3}
Potential Oxidation Demand	Air	kg	1.5×10^{-2}	2.3×10^{-2}	1.02×10^{-4}	1.8×10^{-2}
Human Ecotoxicity	Air	kg	8.0×10^{-3}	0.045	0.59	0.01
	Water	kg	1.8×10^{-4}	1.4×10^{-2}	1×10^{-5}	3.6×10^{-2}
Marine Ecotoxicity	Air	kg	0.04	0.37	0.017	0.82
	Water	kg	0.5	0.55	9.32×10^{-2}	0.146

5.3.2 Environmental Performance of the Saccharification / Fermentation Process

Enzymes and yeast have been found to be essential components for SSF (second stage of the bioconversion process). Although less expensive alternatives are being investigated (Dhutta et al., 2010) they still remain the most attractive option commercially. The cellulase enzymes utilised in the present study were purchased from the manufacturer Biocatalysts and it can be assumed that the enzymes used in other studies under review were also purchased from a manufacturer. In our own previous studies (Smullen et al., 2017a; Smullen et al., 2017b) it has been demonstrated that SSF is a necessary requirement for ethanol production, in order to achieve high conversion yields and reduce the possibility of inhibitor formation.

Dunn et al. (2012), and Mac Lean and Spatari. (2009) have shown that the production of enzymes and yeast themselves, can result in the release of chemical emissions significantly contributing to the overall environmental impact of the ethanol produced. This has made reasonable estimates on the environmental performance of enzymes and yeast to be included in life cycle models difficult due to a lack of crucial information, as well as the limited availability of software inventory databases. Enzyme specific data and activities vary from manufacturer to manufacturer with some manufacturers restricting access to the necessary information for environmental studies such as the current one.

Dunn et al. (2012) carried out an investigation into the energy consumption and GHG emission release of enzyme and yeast manufacturing for ethanol production and determined that cellulosic ethanol produced from switchgrass contributed 11 kg $CO_{2\ (eq)}$ 100 L^{-1} of ethanol, approximately 27% to the GWP of 2nd generation ethanol production. A second study conducted by Mac Lean and Spatari. (2009), reported an enzyme contribution of 8 kg $CO_{2\ (eq)}$ 100 L^{-1} of ethanol, a slight reduction in the CO_2 released (4.84 to 2.26 kg $CO_{2\ (eq)}$ kg^{-1} enzyme) for corn and switchgrass ethanol (respectively), with zero emissions being released following the production of yeast. These values have since been disputed by Agostinho and Ortega (2013), who have reported an estimated 21.93 kg $CO_{2\ (eq)}$ kg^{-1} enzyme, which is equivalent to 0.02% of the GWP for cellulase production. Meanwhile an investigation of the acidification potential, obtained a value of 0.007 kg $SO_{2\ (eq)}$ kg^{-1} enzyme representing 0.01% of the total emissions released (Agostinho and Ortega, 2013).

In a recent study, Jegannathan and Nielsen (2013) summarized an environmental assessment of enzyme use in industrial processes and reported that the implications of enzymatic processes in place of conventional processes particularly, lead to a reduction in global warming, acidification, eutrophication, photochemical ozone formation and energy consumption. Concluding that enzymes are a promising process technology which provide cleaner industrial production. Although a certain amount of GHG emissions can be attributed to enzyme production, Felix and Tilley (2009) believe that on evaluation of the entire bioconversion process, these emissions are negligible compared to the other input resources employed. The cost of enzyme production can also impact on their use in the bioconversion process. According to Zhuang et al. (2007), the estimated cost of cellulase enzyme production ranges from 25% to 50% of the total lignocellulosic ethanol production cost. Dias et al. (2012) estimates the influence of enzyme costs ($0.11 L^{-1} of ethanol) as approximately 30% of the total lignocellulosic ethanol production cost.

Enzymes are effective catalysts and using them often results in significant reductions in water and energy demand, and an increase in economic and environmental performance of the production process (Agostinho et al., 2015). Water consumption during enzyme manufacturing and the bioconversion process has been reported as potentially the highest input requirement for 2nd generation ethanol (Agostinho et al., 2015). A number of attempts have been made to estimate water consumption in fuel production since the early 90's. The amount of water required for the manufacturing and the bioconversion process depends on the production process itself and the degree of water reuse and recycling. The biochemical process employed in the current study and other research studies requires additional water for pretreatment to breakdown the lignocellulosic feedstock. With current technologies, producing 1L of cellulosic ethanol per 9.8 L of water consumed. As the ethanol yield is increased, it is estimated that water consumption could be reduced to as low as 5.9 L (Aden et al., 2002). In the case of enzyme manufacturing, approximately 9870 kg H_2O kg^{-1} enzyme is required, corresponding to 98% of the total materials consumed. Suggesting that a reduction in water consumption and waste water treatment could significantly reduce external impacts caused by enzyme and ethanol production (Agostinho et al., 2015).

5.4 Conclusion

The environmental performance of chemicals used in the pretreatment of switchgrass for ethanol production yielded varying results. Pretreatment employing sulphuric acid and sodium hydroxide demonstrated the highest quantity of emissions to the GWP, EP, POD, MEP and HEP environmental receptors. The lowest chemical contributor of emissions varied depending on the impact category in question. In general, ammonia and methanol appeared to have the lowest environmental impact to air, soil and water, with methanol exhibiting the lowest environmental impact on the climate change receptor (GWP) (0.0019 kg CO_2 100 kg of ethanol). This result is significant as it shows that the use of methanol in the pretreatment step will minimise GHG emissions from the overall process of biomass conversion to fuel. One of the principal drivers for the use of second generation biofuels is GHG mitigation and the use of liquid biofuels needs to have significant savings over the use of fossil fuels. In this context, the climate change environmental receptor is particularly important and the identification of methanol as the pretreatment with the lowest impact on climate change is particularly significant.

However, the severity of the chemical's environmental impact must also be taken into account. Chapter 4 describes the effect of each chemical on the local environment and this too must be considered. Consequently, the identification of a leading pretreatment chemical based on environmental impact is specific to the parameter under investigation and subsequently, no one pretreatment chemical can be established as an environmentally friendly technique. However, reuse, reduce and recycling techniques utilised during the process could demonstrate a substantial reduction in energy, cost and environmental impact.

Chapter 6. General Discussion and Conclusion

It is often said that change is the only constant in life. Yet, humans are evolutionarily predisposed to resist change because of the risks associated with it. In today's society the pace of change is immensely fast, and it will continue to accelerate.

The transition from non-renewable fossil based fuels to clean and sustainable renewable sourced fuels, is one such change. For many years, the European Union have encouraged, promoted and incentivised change, introducing support policies, renewable energy (biofuel) targets and tax subsidies. These policies are driven by various objectives including:

- Reducing oil prices,
- Strengthening energy security,
- Sustaining the agricultural sector and rural economy,
- Decarbonising the transport sector (Bourguignon, 2015).

In 2009, the European Union (EU) enacted its Climate and Energy Package which outlined three key targets for the year 2020:

- A 20% reduction in GHG emissions compared to 1990 levels,
- A 20% share of renewable energy sources in the final energy consumption,
- A 20% reduction of the final energy consumption through improved energy efficiency (IEA, 2014).

For the 27 EU member countries involved, various approaches for distributing the burden over different sectors have been investigated (IEA, 2014).

Biotechnology offers technological solutions to many of the problems facing the world. The emergence of the bioeconomy / green economy is one such solution (OECD, 2017). Worldwide, energy crops are representing the new green economy, in which sustainably sourced fuels produced from lignocellulosic materials are attempting to dominate the biofuel industry; with a particular focus on producing a low carbon, resource efficient and socially inclusive economy. In which the primary objective is to reduce polluting emissions, prevent loss of biodiversity and valuing ecosystem services. As detailed in Chapter 1 this transition is challenged by insufficient capital funding, agricultural development and the limited availability of process data.

A study published in 2014, by environmental Non-Government Organisations (NGO's) and advanced biofuel companies suggested that production of 2nd generation biofuels could potentially contribute an additional €15 billion in revenue to rural economies and create an estimated 300,000 jobs, significantly revitalising and regenerating smaller communities (ECF, 2014) such as rural Ireland. With this in mind, the research project "Coordinated development of leading biomass pretreatment technologies for the generation of bioethanol from Irish crops" was developed.

Irish energy crops have the potential to contribute to the evolving national green economy for the production of second generation biofuels. Selected crops – willow, miscanthus, hemp and switchgrass – do not compete directly with food and fibre crops and can be grown on marginal and degraded land, with virtually no additional inputs such as fertilisers and water (Sims et al., 2010; Finnan and Styles, 2012). Furthermore potential benefits to agricultural food crops are increased, as seen in Australia, where the growing of eucalyptus mallee crops in strips on the millions of hectares of soils with increasing salinity levels could potentially drive down the water table and reduce surface salt concentrations that prohibit cereal crop production (Wu et al., 2005).

Conversion of lignocellulosic biomass is a complex process made difficult by the recalcitrant nature of the biomass structure (Zhang et al., 2013). The employment of an appropriate methodology (pretreatment) to overcome this issue has been investigated for many years and developments are being continually improved through research studies (Agbor et al., 2011; Smullen et al., 2017a). Although many pretreatment options have been developed so far, there is not a single "optimum" process or pathway available for all types of lignocellulosic feedstocks and end-products (Demirbas, 2009; Hayes, 2009; Smullen et al., 2017a). In addition, access to a fully comprehensive and descriptive review of available pretreatment techniques is difficult, due to insufficient or limited process information. Therefore the primary aim of the study was to explore, define, develop and compare biomass pretreatment approaches for willow, miscanthus, hemp and switchgrass and to gain an insight into their potential for the production of second generation biofuels.

The first objective of the study was to conduct a comprehensive review of leading biomass pretreatment technologies and to select prospective approaches for the bioconversion of the Irish energy crops. Chapter 1 provides a comprehensive review of the biological, physical, chemical and physicochemical pretreatment technologies which have been developed. The detailed review was established to inform bioconversion

studies and allow researchers to access the necessary process information, enabling comparisons between pretreatment techniques and their relative attributes. Using this comprehensive review, four chemical pretreatment approaches were selected for further study.

The second objective of the study was to perform a comparative analysis of various chemical and enzymatic pretreatment approaches for lignocellulosic hydrolysis and bioethanol production using the four crops. The effect to which these pretreatment chemicals have on the conversion of the energy crops was investigated in Chapter 3 and 4.

Dedicated energy crops - willow (Salix), miscanthus (*x-giganteus*) and hemp (*Cannabis sativa L*) were selected in Chapter 3, with a view to producing directly comparative information on the performance of four pretreatment chemicals (sodium hydroxide, methanol, sulphuric acid and ammonia) employed at various chemical concentrations (1, 2, 3 and 4 mol L^{-1}). A particular focus has been placed on clearly defining and detailing each process step to facilitate additional future studies on other crop samples and pretreatment approaches. This includes the characterisation of key process components traditionally ill-defined in the literature such as the enzyme complex employed which is central to the saccharification and bioconversion process.

Three commercial enzyme preparations were employed in this study: Biocellulase – 5B06443 from Kerry Foods and Ingredients, Cellulase 13L – C013L from Biocatalysts and Enzyme Complex – NS22119 from Novozymes. On receiving the commercial enzyme preparations, it was noted that the information provided by the manufacturer regarding the enzyme activities and other related information was limited and it was therefore necessary to explore and characterise the enzymatic activities within the crude enzyme preparations (Section 3.2.3).

Pretreatment and enzymatic hydrolysis was demonstrated to be crop specific. Pretreatment employing ammonia was proven to be most effective on willow and hemp, while sulphuric acid was most effective for miscanthus.

Low conversion yields (35.7% - 69%) were achieved following the pretreatment and saccharification of the samples, suggesting that feedback inhibition was present (Section 3.2.2).

In an effort to relieve this issue and increase bioconversion yields, simultaneous saccharification and fermentation (SSF) was employed in the second stage of the study. Through a series of process refinements and improvements, a significant increase in conversion yield was achieved for willow (99 %), miscanthus (80%) and hemp (98%) utilising ammonia and sulphuric acid pretreatments respectively.

Results achieved in Chapter 3 indicate that a leading biomass pretreatment technique for all three crops willow, miscanthus and hemp is difficult to achieve, demonstrating that pretreatment is crop specific. Importantly, critical process details were described including: the optimum chemical type, commercial enzyme preparation and necessary processing parameters required specifically for willow, miscanthus and hemp. In addition, this study shows that a significant increase in bioconversion yield can be achieved with the application of SSF.

The third objective of the study was to select one crop for a more detailed investigation and analysis, focussed on bioconversion yield, cost and environmental impact. Chapter 4 used an exploratory approach to investigate and develop chemical pretreatment techniques which could be applied to switchgrass. The aim of the study was similar to that outlined in Chapter 3, advanced by a detailed assessment of the cost and environmental impact of the pretreatment and enzymatic hydrolysis process.

Four pretreatment chemicals: sodium hydroxide, sulphuric acid, methanol and aqueous ammonia were employed at various concentrations of 0.5, 1 and 2 mol L^{-1} on the energy crop switchgrass (*Panicum virgatum L).* Pretreatment employing methanol at a concentration of 1 mol L^{-1} was demonstrated to be most effective, producing a cellulose to ethanol conversion yield of 97% following SSF. Similarly to analysis performed in Chapter 3, sodium hydroxide proved to be the least effective and efficient pretreatment chemical yielding a cellulose to ethanol conversion yield of just 62% at 1 mol L^{-1}. It is of no surprise that a lower chemical concentration than that highlighted in Chapter 3 was identified as the most effective in this study, as switchgrass is less recalcitrant than willow, miscanthus and hemp and so is easier to breakdown.

When conversion yields were evaluated in relation to the feedstock utilised, pretreatment employing methanol produced approximately 230 g of glucose kg^{-1} of feedstock, which increased significantly to 340 g when expressed as grams of ethanol kg^{-1} of feedstock (cellulose to ethanol). The cost of producing the relative amounts of glucose and ethanol

generated in the study were also assessed. As highlighted in Chapter 1, cost is one of the biggest impediments to the commercialisation of 2nd generation ethanol production. Therefore, any potential reductions in production costs would be advantageous.

The initial cost of each chemical was first assessed and it was determined that methanol and sulphuric acid cost approximately €100 ton^{-1} to purchase from selected suppliers detailed in Section 4.2.5. Substantially lower than that of sodium hydroxide and ammonia (€200 and €280 ton^{-1}, respectively). Pretreatment employing methanol at 1 mol L^{-1} was found to be the most cost effective at €0.55 kg^{-1} of glucose and €0.50 L^{-1} of ethanol. Sodium hydroxide was the most expensive, accounting for approximately €2.52 kg^{-1} of glucose and €1.96 L^{-1} of ethanol. Costs significantly increased when the concentration of the chemical was increased. For example, at a concentration of 2 mol L^{-1}, the cost of employing sodium hydroxide was estimated to be €5.66 kg^{-1} of glucose and €7.94 L^{-1} of ethanol. Consequently, the use of sodium hydroxide for commercial application is non-viable both financially and based on product yield.

Environmental performance is an equally important consideration of ethanol production. Biofuels have been shown to have a lower environmental impact than conventional fuels, producing less GHG emissions (Demirbas, 2008). The cultivation of feedstocks such as willow, miscanthus, hemp and switchgrass is a carbon neutral process or as some studies have demonstrated carbon negative (Pimental and Patzek, 2005; Caslin et al., 2010; Chandel et al, 2011). It is in fact, the large quantities of chemicals required for the bioconversion process that has created the biggest environmental concern. For this study (Chapter 4) a literature review of the effects of the pretreatment chemicals was conducted with a view to outlining the environmental impact of each pretreatment chemical. An assessment of the four pretreatment chemicals on the environmental parameters eutrophication, acidification, aquatic toxicity and global warming potential was conducted.

An interesting conclusion was made in Chapter 4:

- The environmental performance of ethanol production from switchgrass is variable depending on the parameter being assessed and the pretreatment chemical employed. Therefore, no one pretreatment chemical can be generalised as environmentally friendly. Instead, a pretreatment chemical can be classified as

low, moderate and severe relative to its environmental impact on the parameter under evaluation.

Evaluating the performance of a pretreatment chemical based on a literature review can be quite challenging. Many review articles have been published assessing the environmental impact of 2nd generation ethanol production and their relative attributes. However, reports and opinions within these studies differ and it can often be difficult to obtain an accurate description of the process being examined and the potential hazard entailed.

The fourth objective was to create a Life Cycle Assessment (LCA) profile of the pretreatment technologies. Chapter 5 quantified the environmental impact of the pretreatment processes utilised in Chapter 4. Using SimaPrò life cycle assessment (LCA) software, the composition and concentration of each pretreatment chemical was examined and its effect on the environmental recipient's air, soil and water were identified and calculated. Total emission release was characterised and represented using the impact categories: global warming potential (GWP), eutrophication (EP), acidification (AP), photochemical oxidation demand (POD) and marine and human toxicity. CML methodology was applied for the evaluation / comparison of the associated impact categories. The direct environmental impact of each pretreatment technique was subsequently determined.

The LCA profile for switchgrass pretreatment processes demonstrated that the environmental receptors are pretreatment – specific. Pretreatment employing sodium hydroxide was found to have the highest impact on global warming potential compared to methanol which generated the lowest quantity of emissions and thus the lowest global warming potential. This is significant, as methanol was also the most effective pretreatment chemical in the conversion of cellulose to glucose and cellulose to ethanol, in addition to being the most cost efficient pretreatment chemical (Chapter 4). Climate change as discussed in Chapter 5, is arguably the most important impact category, and for methanol to be the lowest contributor of GHG emissions means methanol has the potential to become a leading pretreatment chemical.

Eutrophication described as excessive plant and algal growth was the second impact category evaluated in the study. Total emissions were found to vary significantly when the two recipient's air and water were compared. Pretreatment utilising sulphuric acid

was shown to have the greatest impact on eutrophication when emissions were released to air and the lowest when released to water. While methanol demonstrated the lowest quantity of emissions to air and the highest quantity to water.

The third impact category assessed was acidification. Acidification originates primarily from anthropogenic emissions of sulphur dioxide, nitrogen oxides and ammonia. When evaluated in the current study ammonia was found to have the highest emissions release to air contributing to acidification and sulphuric acid the lowest. Ammonia being the highest contributor to acidification is probably due to the fact it is directly related and easily converted to NO_x. In contrast, emissions released to air contributing to photochemical oxidation demand differed considerably. Pretreatment employing sodium hydroxide measured the highest quantity of emissions, sizably higher than that of methanol.

The final impact categories assessed in Chapter 5 were marine and human ecotoxicity. Marine and human ecotoxicity are both environmental consequences of heavy metal and hydrocarbon release to air, soil and water. Pretreatment employing sodium hydroxide was shown to exhibit the highest contribution of emissions to air for both marine and human ecotoxicity, while ammonia measured the lowest quantity of emissions. When emissions to water were evaluated, methanol was demonstrated to contribute the lowest quantity of emissions for both marine and human parameters. Sulphuric acid and sodium hydroxide liberated the highest quantity of total emissions to water for marine and human ecotoxicity respectively, while ammonia was found to have relatively lower environmental impact.

As outlined in Chapter 5 and evident in the summary above, the identification of a single environmentally friendly chemical is difficult. However, the comparative analysis performed in Chapter 5, can identify the biggest contributors to selected impact categories (as suggested in Chapter 4) and therefore provide important and relevant information for both future industrial and laboratory operations.

The development and improvement of 2nd generation biofuels is on-going and will remain so until associated challenges (capital funding, agricultural development etc.) are resolved. Chapter 1, 3, 4 and 5 established that energy crops can and will play a significant role in the future of 2nd generation biofuel production. However, as a consequence to the utilisation of large quantities of chemicals for the conversion of lignocellulosic biomass

to monomeric sugars, harmful amounts of emissions could potentially be generated and have an adverse effect on both human and marine life.

As a result of aquatic toxicity, a considerable amount of plant and algal bloom could be produced, effecting local lakes and rivers. As a means of releviating this environmental issue and potentially bioremediating affected areas, plant and algal bloom could be removed and used as a feedstock in combination with existing feedstocks for 2nd generation ethanol production.

It is understood following the literature review in Chapter 1 that 3rd generation biofuel production utilising algae alone is not feasible due to its inability to produce bioethanol and the cost of cultivation. However, remediated algae combined with existing feedstocks employed in 2nd generation biofuel production, could potentially:

- Boost biodiesel manufacturing,
- Be cost effective as it would not have to be specifically grown,
- Aid in the remediation of polluted rivers and lakes,
- Assist in the "race" to meet government targets and renewable energy directives.

In addition to these proposed developments it would be intended that any future work would include a more detailed and comprehensive LCA analysis of the bioconversion process for 2nd generation ethanol production. This would include the cultivation of the chosen feedstock, which was outside the scope of the current study and the distribution and use of the fuel; providing a complete representation of the entire 2nd generation biofuel production system.

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Appendices

Appendix 1. IC Method Development for Carbohydrate Analysis of Lignocellulosic Hydrolysis and Quantification of Monomeric Sugars.

Analysis of biomass hydrolysates was conducted using high-performance anion exchange (HPAE) ion chromatography (IC), coupled with pulsed amperometric detection (PAD). The use of IC for this type of analysis is a relatively new idea with previous methods employing gas chromatography (GC) or high-performance liquid chromatography (HPLC) in the quantification of carbohydrates (Lamb et al., 1993; Foyle, 2003).

Both HPLC and GC present challenges in the analysis of hydrolysates as most HPLC methods offer poor separation of samples containing multiple sugars, while GC is highly accurate, it is a lengthy process. IC has been demonstrated as a highly accurate method for carbohydrate analysis (Hayes, 2010). HPAE chromatography takes advantage of the weakly acidic nature of carbohydrates to give highly sensitive separations at high pH using a strong anion-exchange stationary phase (Dionex, 2004). HPAE-PAD is extremely selective and specific for carbohydrate because:

- Pulsed amperometry detects only those compounds that contain functional groups that are oxidisable at the detection voltage employed (in this case, sensitivity for carbohydrates is orders of magnitude greater than for other classes of analytes).
- Neutral or cationic sample components in the matrix elute in, or close to, the void volume of the column. Therefore, even if such species are oxidisable, they do not usually interfere with the analysis of the carbohydrate components of interest (Dionex, 2004).

<u>Chromatographic Conditions:</u>

- SA 10 CarboPac guard and column maintained at 45^{o}C
- Eluent flow rate 1.5 cm^3 min^{-1}
- 0.0001 mol L^{-1} KOH mobile phase followed by a 0.0060 mol L^{-1} wash, prepared using deionised water by eluent generator.
- Pump pressure 2000 psi

Prior to commencing sample analysis, the detection limits of the IC were established using a range of sugar standards at various concentrations (4, 6, 8, 10, 12, 16, 20 mg ml^{-1}). Once an optimum concentration (10 mg ml^{-1}) was determined, calibration standards using pure analytical grade monomeric sugars (glucose, galactose, xylose, mannose and arabinose) were prepared. The instrument was set up as per the processing conditions outlined above and allowed to equilibrate for 30 min prior to sample analysis.

Raw lignocellulosic biomass was pretreated and hydrolysed as detailed in Section 2.7. Samples were filtered and diluted as discussed in Section 2.5.3, and 20 µl samples were injected into the detector (maintained at 45^{o}C) via the auto-sampler. Samples were mixed with 0.0001 mol L^{-1} KOH (mobile phase) and pumped through the stationary phase (Analytical SA 10 column). The eluent loaded onto the column displaces any anions bonded to the resin and saturates the resin surface with the eluent anion. The samples then pass through the column to the electro-chemical cell, containing the pH and gold (Ag/AgCl) electrodes. Sugars are identified and concentrations determined using the process (standards concentration and retention times) and instrument (start-up and shut-down details) methods detailed in the IC software system "Chromeleon".

Method Validation

To ensure the IC was performing optimally, the process method employed was validated for:

- Selectivity and Sensitivity
- Accuracy and Precision
- Range and Linearity
- Detection Limits
- Robustness / System Suitability

Ion chromatographic analysis for anions / cations offers good separation but there can often be interference in early eluting peaks from organic compounds. To help minimise this issue and to validate the instrument processing method employed, internal standards (reference solutions) (10 mg ml^{-1} of Fructose) were randomly applied among the samples for analysis. In addition fresh calibration standards were prepared and analysed before and after each sample run.

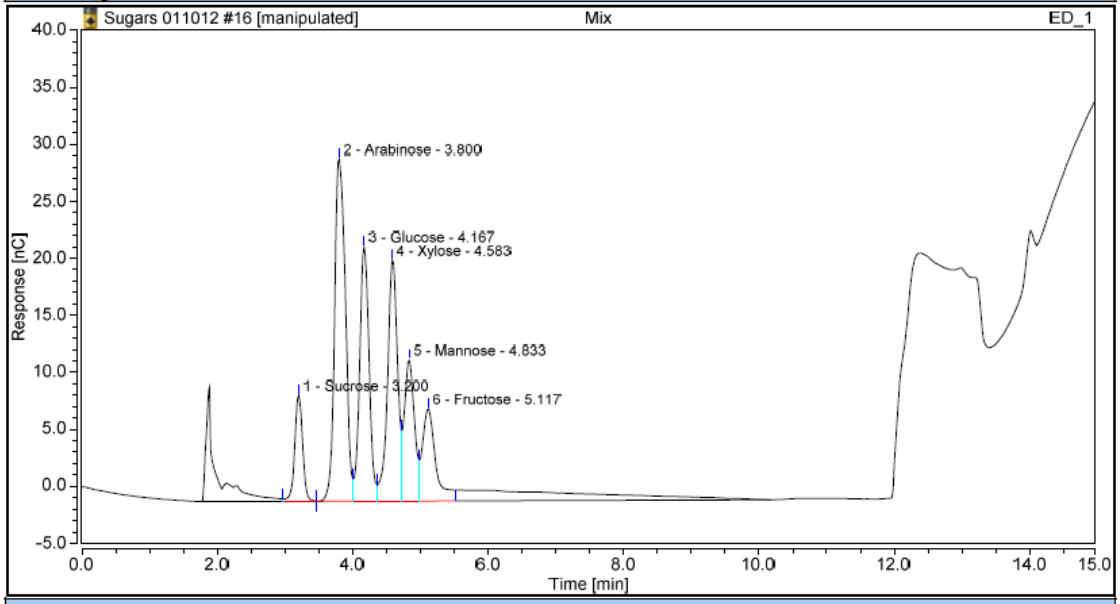
Appendix 2. ICS Chromatogram of Sugar Mix Calibration Standard.

Chromatogram and Results

Injection Details

Injection Name:	**Mix**	*Run Time (min):*	**14.97**
Vial Number:	**RA8**	*Injection Volume:*	**10.00**
Injection Type:	**Calibration Standard**	*Channel:*	**ED_1**
Calibration Level:	**01**	*Wavelength:*	**225.0**
Instrument Method:	**Carbohydrate SA20 26.01.2011**	*Bandwidth:*	**n.a.**
Processing Method:	**Citrate Method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**01/Oct/12 19:10**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area nC*min	Height nC	Relative Area %	Relative Height %	Amount ppm
1	Sucrose	3.200	1.266	9.310	7.00	9.02	60.3626
2	Arabinose	3.800	5.528	29.993	30.56	29.07	64.9564
n.a.	Galactose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	Glucose	4.167	3.632	22.315	20.08	21.63	58.1903
4	Xylose	4.583	3.618	21.122	20.00	20.47	40.5051
5	Mannose	4.833	2.214	12.365	12.24	11.98	42.3199
6	Fructose	5.117	1.832	8.069	10.13	7.82	32.0543
Total:			**18.090**	**103.174**	**100.00**	**100.00**	

Figure 1: ICS chromatogram of sugar standards employed in the calibration of the ICS 5000 for the analysis of lignocellulosic sample are described in Section 2.7. Randomised sample concentration; Flow rate 1.5 ml min^{-1}; Column temperature 45ºC; 0.001 mol L^{-1} KOH mobile phase.

Appendix 3. ICS Chromatograms of Commercial Enzyme Preparations.

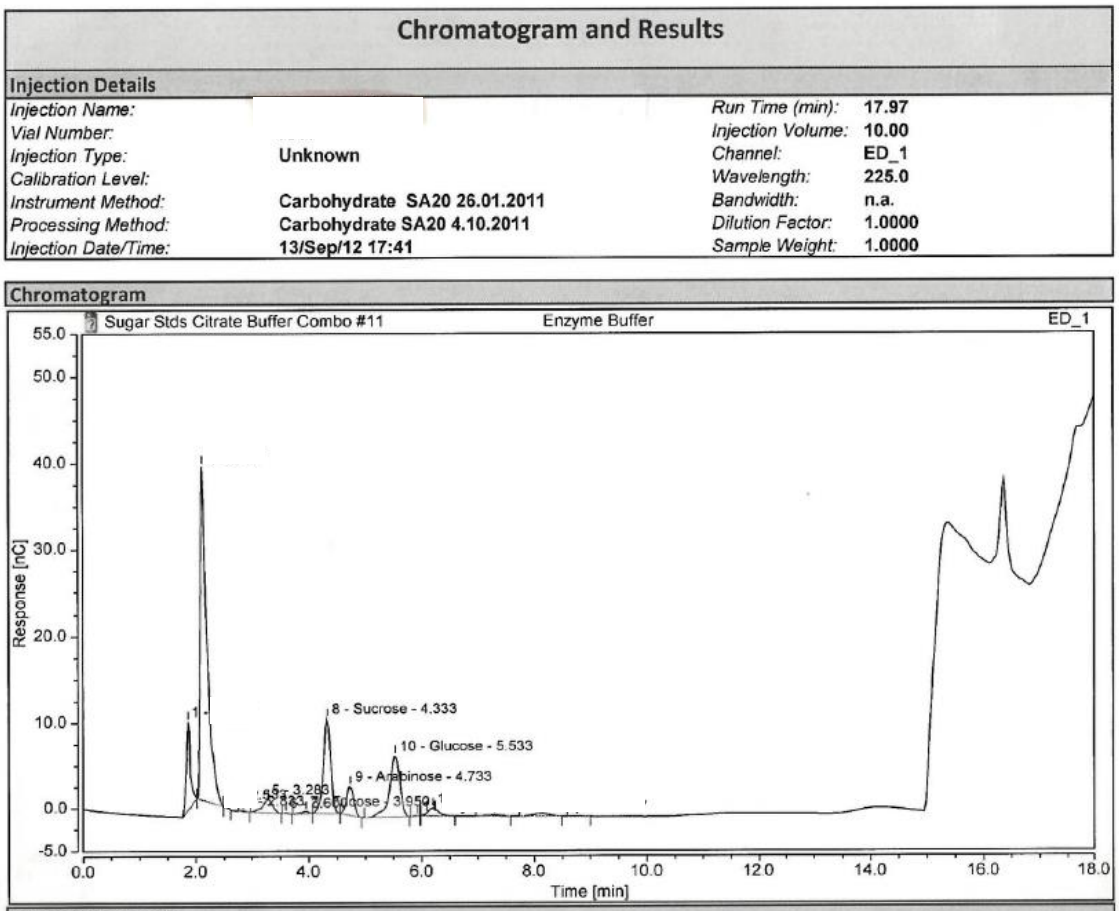


Figure 2: ICS chromatogram of commercial enzyme preparation – Biocellulase (5B06443) employed in this study. Undiluted sample; Flow rate 1.5 ml min^{-1}; Column temperature 45^{o}C; 0.001 mol L^{-1} KOH mobile phase.

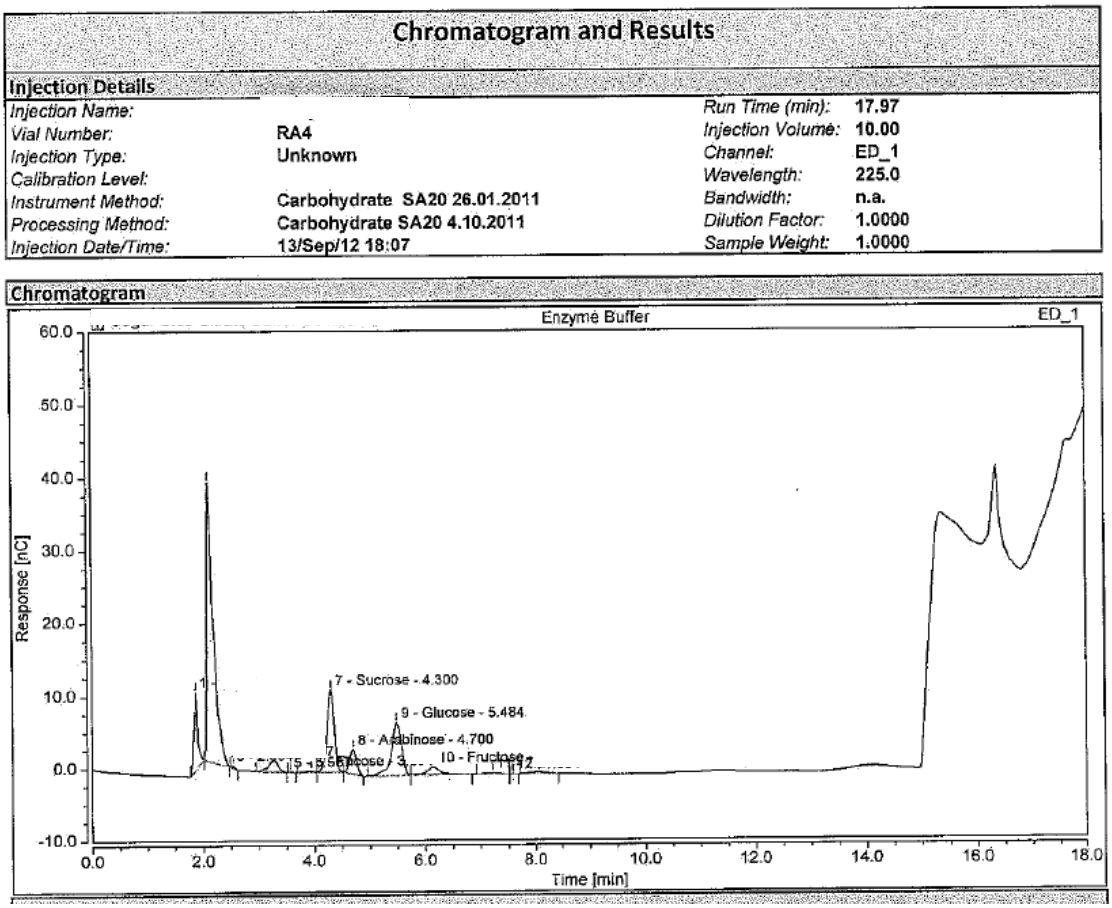


Figure 3: ICS chromatogram of the commercial enzyme preparation - Enzyme Complex (NS22119). Undiluted sample; Flow rate 1.5 ml min^{-1}; Column temperature 45ºC; 0.001 mol L^{-1} KOH mobile phase.

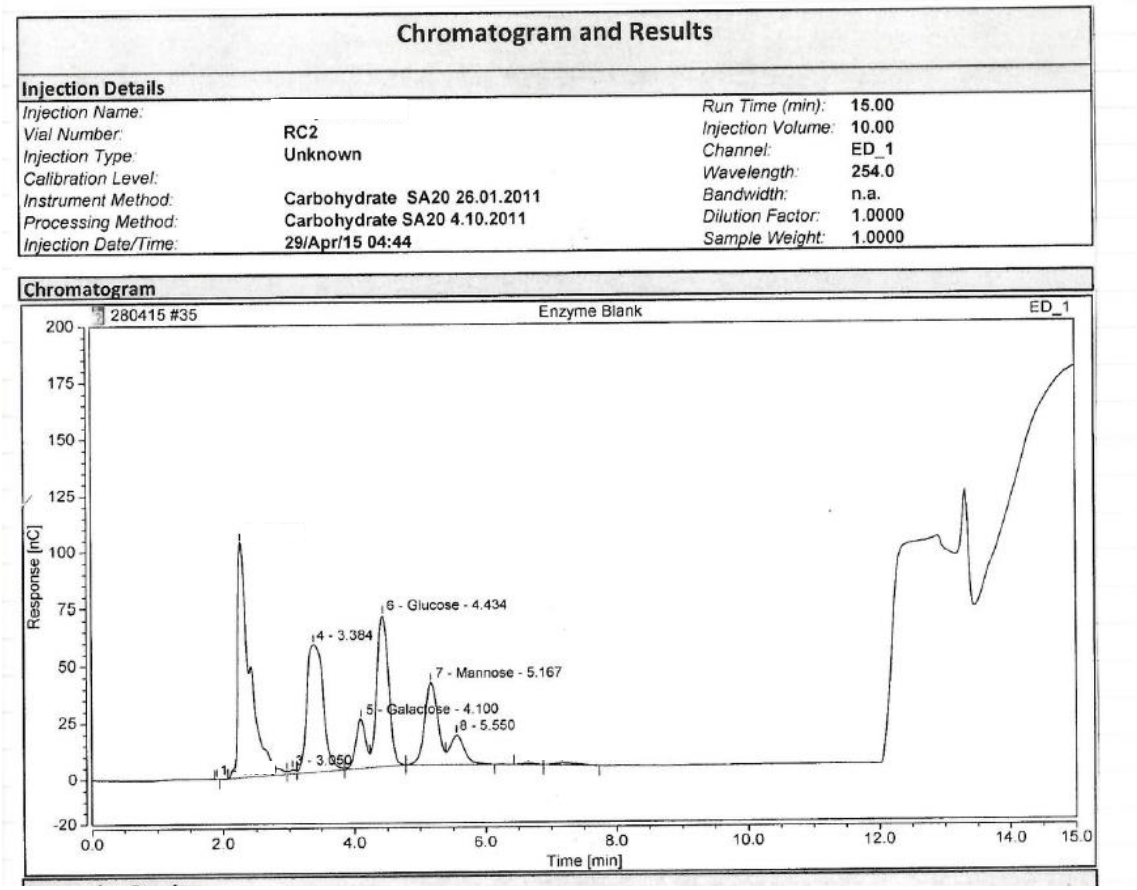


Figure 4: ICS chromatogram of commercial enzyme preparation – Cellulase 13L (C013L) employed in this study. Undiluted sample; Flow rate 1.5 ml min^{-1}; Column temperature 45^{o}C; 0.001 mol L^{-1} KOH mobile phase.

Appendix 4. Representative Sample of ICS Chromatograms for the Quantification of Monomeric Sugars in Pretreated Lignocellulosic Biomass.

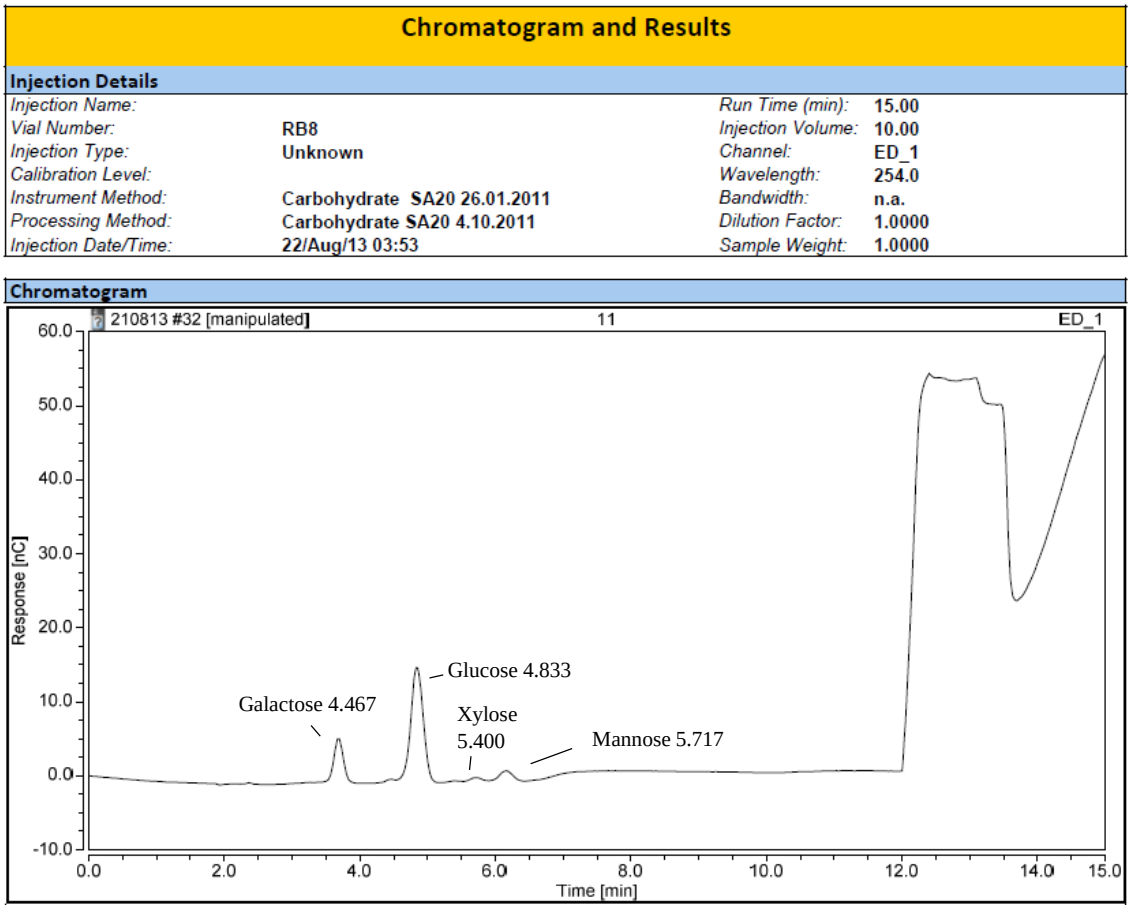


Figure 5: ICS chromatogram of Willow pretreated with 3 mol L^{-1} CH_3OH and hydrolysed using the commercial enzyme preparation - Cellulase 13L. Willow samples were pretreated and hydrolysed as described in Section 2.7 and analysed as detailed in Section 2.5.3.

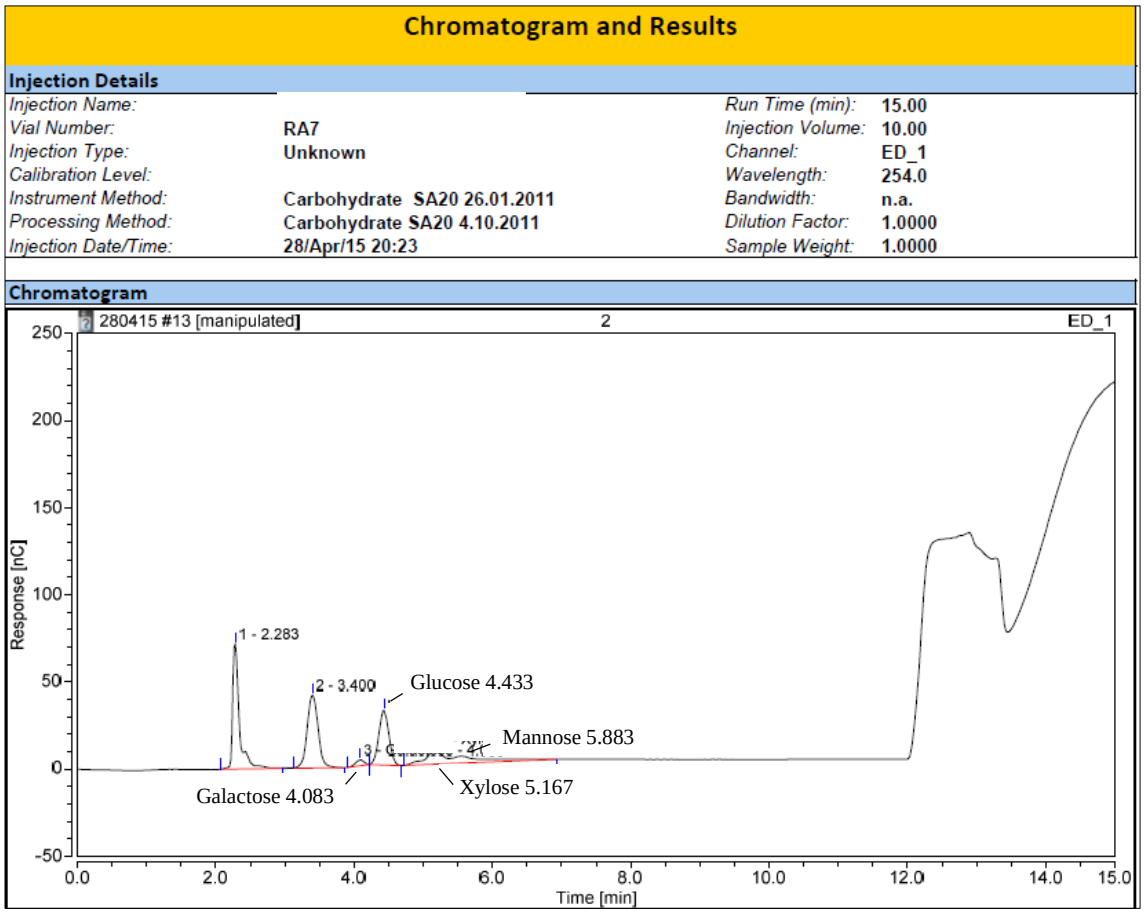


Figure 6: IC Chromatogram of Switchgrass pretreated with 1 mol L^{-1} H_2SO_4 and hydrolysed with the commercial enzyme preparation - Cellulase 13L. Switchgrass samples were pretreated and hydrolysed as described in Section 2.8 and analysed as detailed in Section 2.5.3.

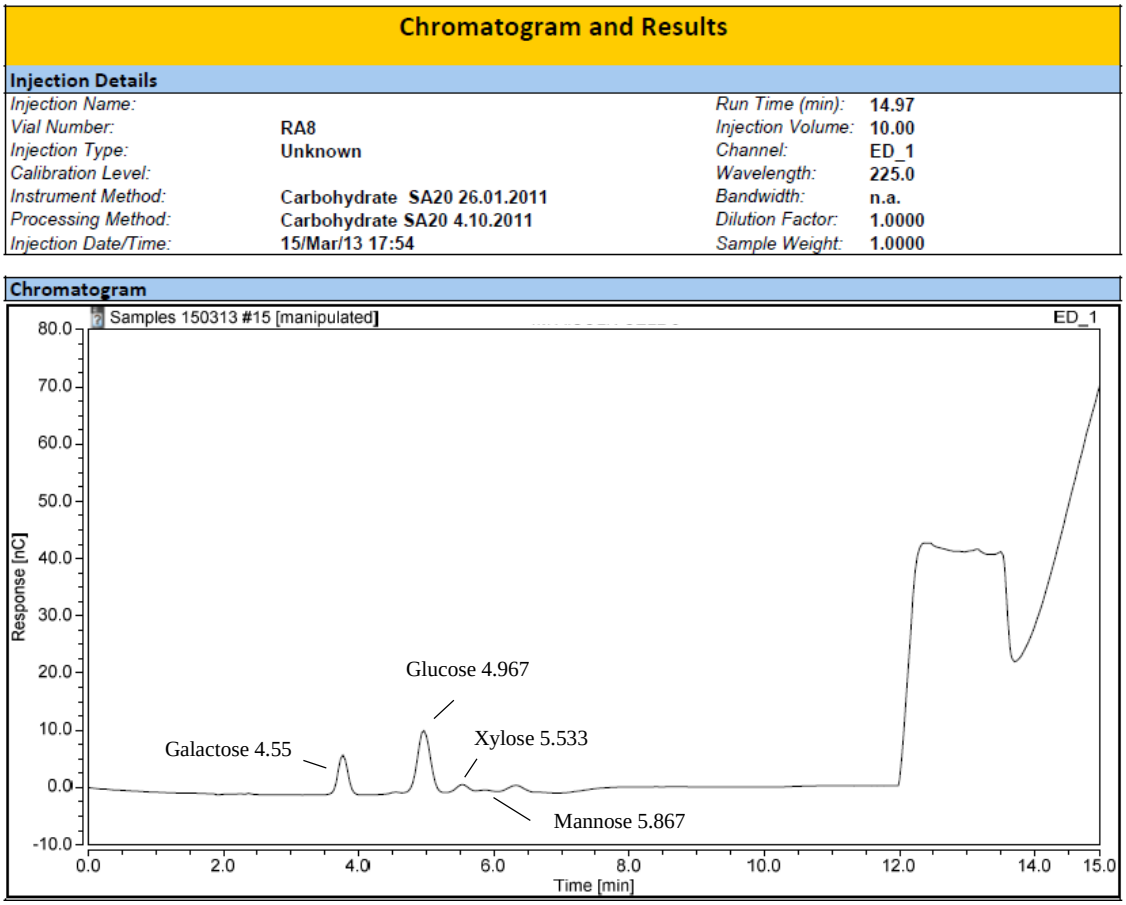


Figure 7: IC Chromatogram of Miscanthus pretreated with 4 mol L^{-1} NH_3 and hydrolysed with the commercial enzyme preparation Cellulase 13L. Miscanthus samples were pretreated and hydrolysed as described in Section 2.7 and analysed as detailed in Section 2.5.3.

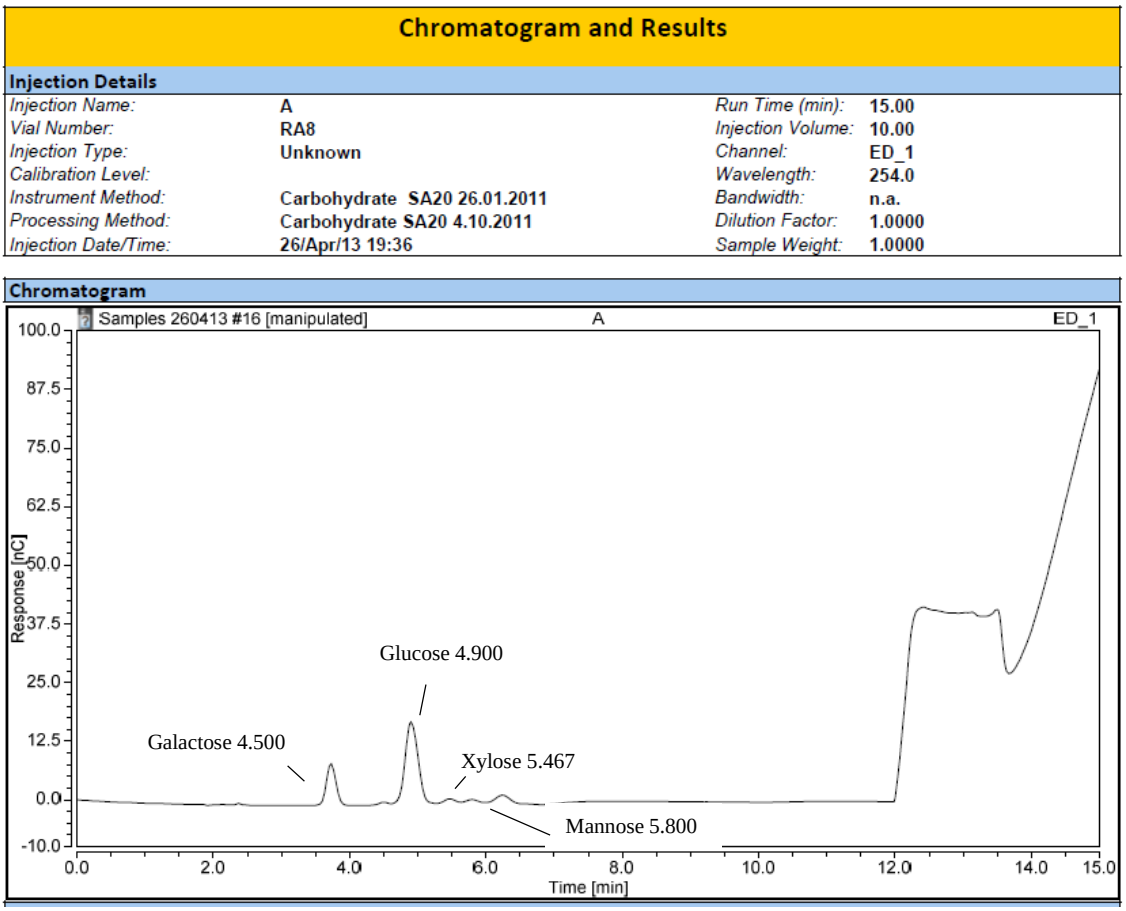


Figure 8: IC Chromatogram of Hemp pretreated with 2 mol L^{-1} NaOH and hydrolysed with the commercial enzyme preparation Cellulase 13L. Hemp samples were pretreated and hydrolysed as described in Section 2.7 and analysed as detailed in Section 2.5.3.

Appendix 5. GC Method Development for Carbohydrate Analysis of Lignocellulosic Hydrolysates and Quantification of Ethanol Product.

Gas chromatography (GC) is an analytical technique for volatile and semi-volatile compounds. As ethanol and its impurities are volatile, GC is an excellent technique for the quantification of ethanol from lignocellulosic biomass (Campo et al., 2007; Rodrigues et al., 2008). The use of HPLC in the analysis of ethanol is also well known, however, GC provides higher resolution and sensitivity. In addition, GC can be coupled to mass spectrometry (MS) capable of establishing unequivocal identification of compounds (Jham et al., 2007). Analysis of fermentation hydrolysates in this study was conducted using the Shimadzu GC-14A.

Chromatograhic Conditions

- Porapak Q column maintained at a temperature of 210^{o}C
- Eluent flow rate 1.4 ml min^{-1}
- Air / Hydrogen flame
- Nitrogen carrier gas
- Flame Ionisation Detector (FID) maintained at 230^{o}C

Prior to commencing sample analysis, the detection limits of the GC were established using a range of alcohol standards at various concentrations (5, 7, 8, 9, 10, 11 and 12 g 100 ml^{-1}). Once an optimum concentration (5 g ml^{-1}) was determined, calibration standards using pure analytical grade ethanol were prepared. The instrument was set up as per the processing conditions outlined above and allowed to equilibrate for 30 min prior to sample analysis.

Raw lignocellulosic biomass was pretreated, hydrolysed and fermentated as detailed in Section 2.7. Samples were filtered and diluted as discussed in Section 2.7.4, and samples were injected into the detector (maintained at 230^{o}C) via a split /splitless injector. The sample was vapourised at the injection port by heat and sent to the Porapak Q column packed with adsorbent or absorbent material. Inside the column, each component in the sample is separated depending on its physical and chemical properties and the concentration of ethanol measured by the FID detector.

Method Validation

To ensure the GC was performing optimally, the process method employed was validated for:

- *System Suitability and Precision* - Trial fermentations were conducted using pure glucose to ensure the yeast was capable of conversion to ethanol. System suitability and precison was then assessed using multiple replicate samples / injections.
- *Linearity and Range* - Various concentrations of ethanol standards (5-12 g 100 ml^{-1}) were prepared and analysed to determine the limit of detection and optimum concentration.
- Ruggedness of the Method
- *Stability of Analytical Solution* - It is important to know if the solutions used in the analytical of both the standard solutions and the sample solutions are stable over time.

To aid the validation of the instrument and processing method employed, internal standards (Methanol and Butanol) were randomly applied to the samples before analysis.

Criteria for Selction of Internal Standard

- Volatility: A similar volatility to that of the analyte (ethanol)
- Peak Separation: Peaks generated from the analysis of the internal standard must be easily separated from the analyte (ethanol) at the optimised chromatography conditions.
- Internal standard must be contained in the sample and thus must be stable and non-reactive.
- Overlapping Peaks: Peaks generated from the analysis of the internal standard must not overlap with the impurities of the sample (Quan et al., 2012).

Appendix 6. GC Chromatogram of Calibration Standard Mix (Methanol, Ethanol and Butanol).

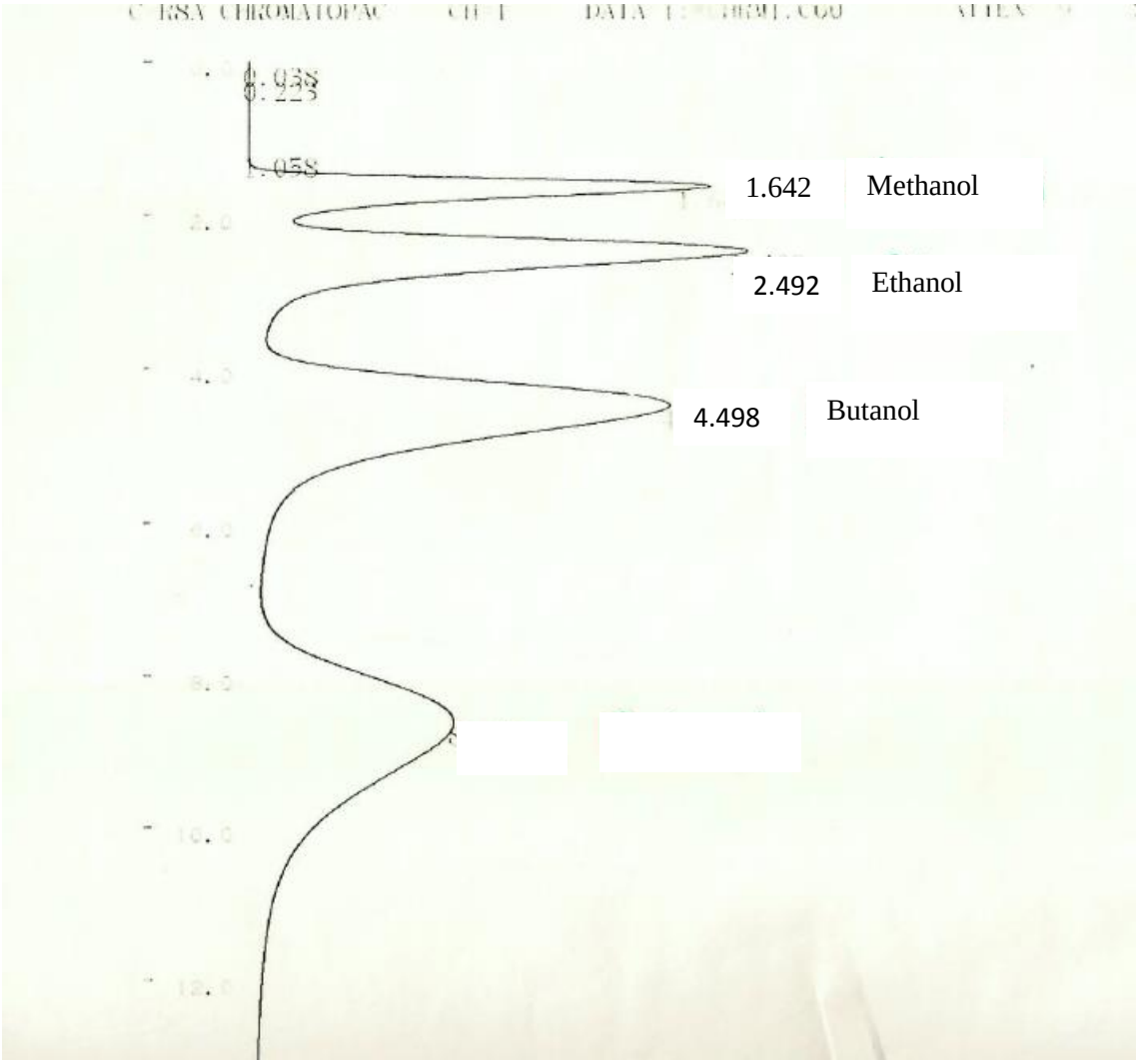


Figure 9: Elution of various alcohols from GC Shimadzu GC – 14A. Randomised sample concentration; Flow rate 20 ml min^{-1}; Column temperature 210^{o}C; Porapak Q column.

References to Appendices

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