Microwave assisted pretreatment for C4 plants in biorefinery

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Abstract

There is a rising global demand for energy and growing concerns about greenhouse gas emissions. Lignocellulosic biomass offers great potential for second generation bioethanol production, based on the biorefinery philosophy. It is composed of a network of interconnected polymers cellulose, hemicellulose and lignin which has evolved to develop recalcitrance against enzyme hydrolysis produced by microorganisms in nature. Therefore, pretreatment is necessary to make the biomass structure more accessible for enzyme to hydrolysis.

The aim of this thesis is to demonstrate the potential of using microwave to assist thermo-chemical pretreatment for lignocellulosic biomass, namely *Miscanthus*, sugarcane bagasse and maize. The pretreatment process was influenced by pretreatment temperature, pretreatment media and holding time. 0.2 M -1 M H_2SO_4 and NaOH were used as preteatment media.

Firstly, temperature optimisation was carried on *Miscanthus* and the results showed that 180 ^oC was the optimal temperature to efficiently release monosaccharides from biomass. In comparison with classic conventional heating pretreatment, microwave assisted pretreatments maximally released 12.5 times more reducing sugars during the pretreatment process.

Secondly, the reducing sugar constitutions were tuned by change holding time or pretreatment media, because hemicellulose was easier to be broken down than cellulose. Xylose and glucose were selectively produced by using NaOH and H_2SO_4 (or $FeCl_3$) respectively as pretreatment media. Chemical compositions and biomass morphological changes were investigated and compared. The significant removal of hemicellulose and lignin, as well as more dismantled fibre structure led to enhanced bioethanol conversion via SSF process (simultaneous saccharification fermentation). Similar study was conducted on sugarcane bagasse and maize. The performance of pretreatment media was similar. However, their optimal conditions for reducing sugar release were different, probably due to different chemical compositions percentages and biomass structure.

Overall, in comparison with conventional heating pretreatment, microwave assisted pretreatment is much energy efficient and effective, showing promising potential in the biorefinery process.

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Author's Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References. Some of the results presented herein were obtained by, or in collaboration with other co-workers. They are fully acknowledged in the list below along with their corresponding institution.

Piece of work	**Chapter**	**Co-worker**	**Institution**
Digestibility analysis	2-6	Rachael Hallam	Senior Research Technician, Centre for Novel Agricultural Products (CNAP), Department of Biology, University of York
SEM analysis	2-6	Meg Stark	Senior Research Technician, Department of Biology, University of York
Ethanol measurement by GC	3	Dr Tony Larson	Department of Biology, University of York

Chapter 1 Introduction

1.1 Project background

Currently, we are facing rising issues of an increasing demand for energy, limited fossil fuel resource and the environment impact of its combustion. Therefore, non-fossil fuel-based energy sources are gaining growing attention. Conversion of abundant lignocellulosic biomass to biofuels presents a viable option for improving energy security and reducing greenhouse emissions.[1] Lignocellulosic materials such as agricultural residue (e.g., wheat straw, sugarcane bagasse, corn stover), forest product (hardwood and softwood) and dedicated crops (switchgrass, salix) are sufficiently abundant and produce very low net greenhouse emissions.[2] Utilising food residues and waste products from other industries means they have little competition with food industry. From a climate-change point of view, biofuels result in lower emissions of greenhouse gases (GHG), due to the fact that agricultural crops, from which biofuels are produced, absorb some or most of the CO_2 released by biofuel-powered vehicles. Biofuels also eliminate sulphur dioxide emissions. It has been reported that on the life-cycle basis, ethanol produced from cellulosic biomass resources is able to cut greenhouse gas emissions by 86% compared with that of gasoline.[3]

Approximately 90% of the dry weight of most plant materials is stored in the form of cellulose, hemicellulose, lignin and pectin, with the remainder being mainly waxes and inorganics such as silicates.[4] Lignocellulosic biomasses are recalcitrant materials, composed of a network of the interconnected polymers cellulose, hemicellulose and lignin, that has evolved to develop recalcitrance against enzyme hydrolysis produced by microorganisms in nature.[5] To release the sugars locked in biomass, various pretreatments have been proposed and trialled at laboratory and pilot scale. Pretreatment is a crucial processing step for biochemical conversion of lignocellulosic biomass into bioethanol, in which the cellulose polymers are made accessible for a more rapid conversion through hydrolysis and fermentation steps. At the same time, hydrolysis of hemicellulose and lignin removal may occur, depending on the process applied. Among these processes are steam explosion,[6] ammonia fibre explosion,[7] hot water,[8] supercritical CO_2, [9] biological [7] and acid or alkaline pretreatments [10, 11] and others. Figure 1 illustrates the effect of pretreatment on lignocellulosic material, showing that the compact biomass structure is opened up and the polysaccharides are more approachable for enzyme to attack.

As Figure 1 indicates, pretreatments may alter the structure of cellulosic biomass to make it more accessible for enzymes and can also decrease the degree of polymerization

and cellulose crystallinity. Additionally, they can selectively remove hemicellulose and lignin from the lignocellulosic matrix, thus improving biomass digestibility. [6, 12]

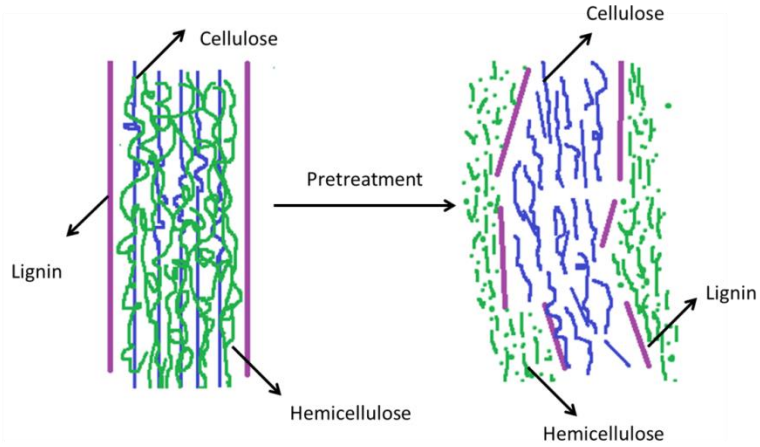


Figure 1 Schematic of the role of pretreatment in the conversion of biomass to fuel[13]

Acid and alkaline pretreatments are considered effective and economic, which explains their extensive use in most cases during biomass pretreatment.[5, 12, 14-21] Acid pretreatment centres around dilute acid pretreatment, using acids such as H_2SO_4 and HCl and has been a crucial technology for hydrolysing lignocellulosic biomass for fermentable sugar production.[22] Acid can partially release monosaccharides, oligosaccharides and lignin monomers by splitting strong chemical bonds under high temperature.[23, 24] Sulfuric acid is most commonly used in pretreatments,[5, 10, 11, 23] although other acid such as phosphoric acid[15, 25] and nitric acid[26] have been assayed, presumably mainly on cost grounds, with nitric acid being potentially oxidising and HCl too corrosive. Alkali can facilitate dissociation of entire wall polymers by breaking hydrogen and other covalent bonds and lignin can be removed without the degradation of cellulose, also hemicellulose is efficiently hydrolysed.[6] It is able to alter cellulose structure and increase amorphous cellulose content, thus improving biomass digestibility.[17] NaOH is widely used for biomass pretreatments.[20, 21, 27, 28] Other alkaline agents have also been studied, such as lime[29] and ammonia.[17]

In recent years, there has been an upsurge of interest in the use of lignocellulosic material, in particular non-food and food waste residues such as corn stover,[29] sugar cane bagasse,[14] *Miscanthus*,[30] rape straw,[31] wheat straw,[32] and so forth, as feedstock for second generation bioethanol. In this work, C4 plants, including *Miscanthus giganteus*, sugarcane and maize are used as feedstock. They are C4 plants and are considered to be promising energy crops. Plants can be divided into C3 and C4 plants, according to their carbon fixing pathways. Compared to C3 plants, C4 plants

have higher CO_2 fixation rate that results in faster photosynthesis. Therefore, C4 plants can grow very fast. Moreover, C4 plants have a very low compensation point (at this light intensity, the rate of photosynthesis of the plant is equal to its rate of respiration), which makes it possible for them to conduct photosynthesis at low light intensity when only low concentration of CO_2 is available.[30]

There is growing interest in using microwaves in various biomass transformation processes. Compared to conventional heating, it is more direct, rapid and uniform.[33, 34] Due to these unique properties, microwaves have wide applications such as food drying and heating, chemical synthesis, sample digestion and extraction.[33] As was mentioned earlier, pretreatment for biomass is a vital process to improve biochemical conversion from biomass to bioethanol which includes enzymatic hydrolysis and fermentation. In this work, microwave is applied to facilitate the thermo-chemical pretreatment of biomass and the purpose is to enhance this biochemical conversion. Different analysis techniques have been used to study both the pretreatment media and biomass residue in order to have a comprehensive understanding of the pretreatment process.

1.2 Project aims

In this work, microwave irradiation is used to facilitate acid or alkaline pretreatment of biomass. Figure 2 demonstrates the conversion from lignocellulosic biomass to bioethanol. After pretreatment, digestible polysaccharides (hemicellulose and cellulose) are more exposed and accessible. With enzyme or acid hydrolysis, polysaccharides will be broken down into their constituent monosaccharides, such as arabinose, galactose, glucose, xylose and mannose, which can be fermented into ethanol or butanol by yeast or bacteria. After distillation, bioethanol or biobutanol is obtained. There are several factors affecting the hydrolysis of cellulose, including the accessible surface area of the biomass material, crystallinity of the cellulose fibres and the content of both lignin and hemicellulose.[35] The hydrolysis process can be significantly improved by removing lignin and hemicellulose, reduction of cellulose crystallinity and the generation of a more open biomass structure through an effective pretreatment process.[2, 35]

In this work, aqueous solutions of acid (H_2SO_4), alkali (NaOH) and $FeCl_3$ are used to pretreat biomass (*Miscanthus*, sugarcane bagasse and maize), different analysis methods are used to investigate their performance on biomass structure, chemical composition and biomass digestibility. In order to compare, water pretreatment is used as control.

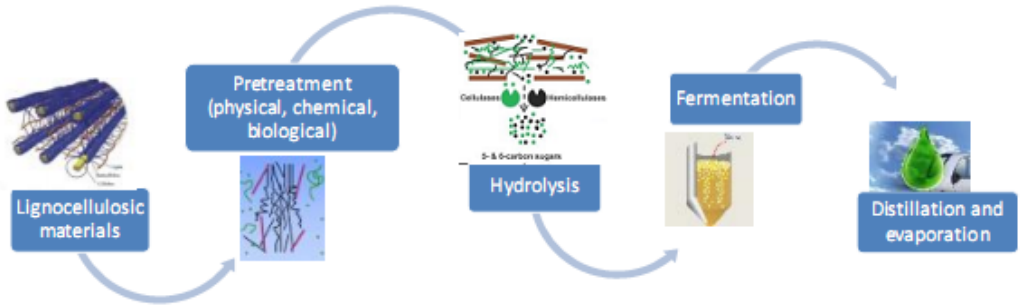


Figure 2 Conversion of lignocellulosic biomass to fuel [13, 36, 37]

1.3 Energy crisis and need for bioethanol

Due to the energy security issue and long term effect of CO_2 on environment, there is an increasing necessity of the developing alternative fuel energy. Over time, petroleum-based resources will be limited and more expensive, due to the combined impact of fossil fuel scarcity and its increasing cost. With the growing interest in bioenergy production, more and more research has been done in the field of biofuels. Oil, natural gas and coal will not only emit climate-threatening greenhouse gases and other pollutants, but also the quantity of undiscovered stocks will be a matter of great concern.[38] According to New Policies Scenario which is a scenario in World Energy Outlook that takes account of broad policy commitments and plans that have been announced by countries, energy-related CO_2 emission rises by 20% to 37.2 Gt by 2015, leaving the world on track for a long-term average temperature increase of 3.6 ^oC. The presumably rising sea levels will do more than just make the beach closer, it diminishes crop yields, increases area affected by drought and causes more frequent and destructive forest fire. It is worth mentioning that 'Carbon neutrality' is a shorthand term that is frequently used assuming that CO_2 emitted during biomass combustion to generate useful energy will be taken in again during the regrowth of an equivalent mass of biomass. At the same time, The International Energy Agency (IEA) standard methodological framework for comparing bioenergy and fossil energy systems in lifecycle analysis presumes stable atmospheric carbon for bioenergy systems and increasing atmospheric carbon for fossil reference systems (Figure 14 shows CO_2 emission reduction by using bioethanol from a variety of biomass on a LCA basis). The stable atmospheric carbon can be assumed for bioenergy system, because the atmosphere and biosphere represent a single carbon pool.[39] Global energy demand increases by one-third from 2011 to 2035.[40] Therefore, it is of great importance to turn to alternative energy resources, such as low-carbon energy sources (renewables and

nuclear), which meet around 40% of the growth in primary energy demand. It is predicted that biofuels use will triple, rising from 1.3 million barrels of oil equivalent per day(mboe/d) in 2011 to 4.1 nboe/d in 2035, by which time it will represent 8% of road-transport fuel demand.[40]

Table 1 Ethanol's Net Energy Value: A summary of major studies on maize

Author	Net Energy Value (Btu/gal)	Reference
Shapouri, et al (1995)	+20,436	[41]
Lorenz and Morris (1995) - Institute for Local Self-Reliance	+30,589	[42]
Agri. and Agri-Food, CAN (1999)	+29,826	[43]
Wang, et. al. (1999) – Argonne National Laboratory	+22,500	[44]
Pimentel (2001)	-33562	[45]
Marland and Turhollow	+18154	[46]
Shapouri, et. al, Update (2002) – USDA	+21,105	[47]

Btu/gal: British thermal unit/gallon of ethanol; 1 Btu= 1.055 KJ; 1 US gallon= 3.78541 Liter

Ethanol derived from biomass has the potential to be a sustainable transportation fuel, as well as a fuel oxygenate that can replace gas line.[48] Table 1 shows several energy balance studies results for ethanol production from maize. The results showed renewable returns on non-renewable energy input for maize ethanol, except the result from Pimentel (2002). The result is expected, due to different conversion approaches being applied in the process. It can be predicted that biomass feedstock will also influence the net energy value. Overall, the net energy output from bioethanol is potentially promising and it is able to reduce domestic consumption of fossil fuels, especially petroleum[49].

The world ethanol production in 2001 was 31 GL. The major ethanol producers are Brazil and the US, which generate 62% of world production. The major feedstock for ethanol is sugarcane from Brazil and maize grain from the US respectively.[50] There is a great potential to use lignocellulosic biomass to produce ethanol, including agricultural waste (e.g. corn stover, crop straw, sugar cane bagasse), herbaceous crop (e.g. alfalfa, switchgrass), forestry wastes, wastepaper and so forth.[51]

1.4 Sustainable development and Green Chemistry

Sustainable development was brought up by United Nations Commission on Environment and Development in 1987 (Bruntland Commission), which defined

sustainable development as: ' …meeting the needs of the present without compromising the ability of future generations to meet their own needs.' Two of the most important aspects of sustainable development from a chemical and energy perspective are: 'how fast should we use up fossil fuels?' and 'how much waste or pollution can we safely release to the environment?' However, rather than have agreed answers to these questions, there is a general agreement to develop more renewable forms of energy and to reduce pollution. Therefore, to develop new products, processes and services that achieve all the benefits of sustainable development is our challenge.[33]

The term 'Green Chemistry' is becoming accepted worldwide as a means of describing the development of more eco-friendly, sustainable chemical products and processes. During the early 1990s the US Environmental Protection Agency (EPA) coined the phrase Green Chemistry as 'The utilisation of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture and application of chemical products'.[52] Over the last twenty years Green Chemistry has gradually become recognized as both a culture and a methodology for achieving sustainability.[52]

The aims of Green Chemistry can be summarized as 12 Principles of Green Chemistry (see Table 2).

Table 2 The 12 Principles of Green Chemistry [52]

1	Waste prevention is better than treatment or clean-up
2	Chemical synthesis should maximise the incorporation of all starting materials
3	Chemical synthesis ideally should use and generate non-hazardous substances
4	Chemical products should be designed to be non-toxic
5	Catalysts are superior to reagents
6	The use of auxiliaries should be minimised
7	Energy demands in chemical syntheses should be minimised
8	Raw materials increasingly should be renewable
9	Derivations should be minimised
10	Chemical products should break down into innocuous products
11	Chemical processes require better control
12	Substances should have minimum potential for accidents

The aims of this project are closely concerned with the concept of renewable raw materials and sustainability. Microwave energy is used as an alternative energy input in this work, fitting with Principle 7.

1.5 Biorefinery concept

The world's primary source of energy and chemical production is oil. Approximately, 84 million barrels a day oil is demanded worldwide and it is predicted to go up to 116 million barrels a day by 2030, with transportation accounting for 60% of such rising demand.[53] It is increasingly acknowledged globally that plant-based raw material (i.e. biomass) has great potential to substitute a large fraction of fossil resources as feedstock for industrial productions, addressing both the energy and non-energy (i.e chemicals and materials) sectors.

Biorefining is defined as the sustainable processing of biomass into a spectrum of marketable products (food, feed, material, chemicals) and energy (fuel, power, heat).[54] An integrated biorefinery maximises the overall added value of one plant system by way of fractionation of the raw material, integration of mass and energy flows and processes, by (ideally) using all components of raw material for a range of different products/intermediates and by working with closed loops (see Figure 3).[55] The biorefinery concept includes a wide range of technologies able to separate biomass (wood, grass, crop etc) into their building blocks (carbohydrate, proteins, triglycerides etc.) which can further be converted to value added products, biofuels and chemicals. A biorefinery is a facility that integrates biomass conversion processes and equipment to generate a combination of transportation biofuel, power and chemicals from biomass. This concept is similar to today's petroleum refinery, which produces multiple fuels and chemical products[56].

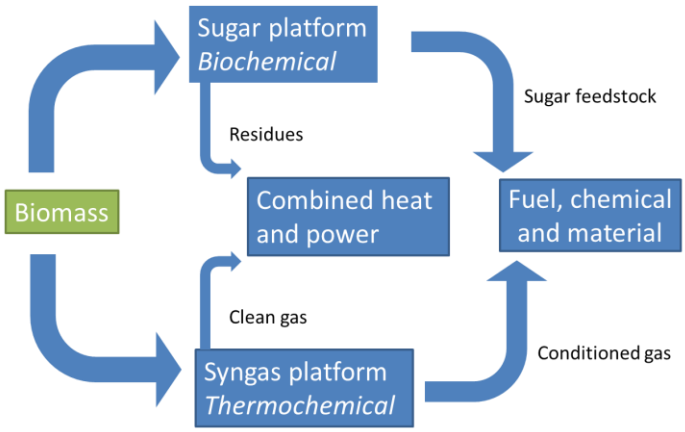


Figure 3 Illustration of biorefinery concept[57]

1.6 Current state of biofuels

Biofuels are made from bio-based materials via thermochemical processes such as pyrolysis, gasification, liquefaction, supercritical fluid extraction, supercritical water liquefaction and biochemical routes. The term biofuels can refer to fuels for direct combustion for electricity production, but is generally used for liquid fuels for transportation sector.[58] As can be seen from Table 3, biofuel offers numerous promising benefits related to energy security, economics and environment. Nevertheless, several challenges must be overcome in order to realize these benefits, such as competition for food and changing land use, as well as the necessary cost of the technology.

Table 3 Benefits and challenges of biofuels [59]

Benefits	**Challenges**
Energy security	**Feedstock**
Domestic energy source	Collection network
Locally distributed	Storage facilities
Well connected supply-demand	Food-fuel competition
chain	**Technology**
Higher reliability	Pretreatment
Economic stability	Enzyme production (mainly for EtOH and BuOH)
Price stability	Efficiency improvement
Employment generation	Technology cost
Rural development	Production of value added co-products

Reduce demand-supply gap	**Policy**
Open new industrial dimensions	Land use change
Control on monopoly of fossil rich states	Fund of research and development
Environmental gains	Pilot scale deployment
Better waste utilization	Policy for biofuels
Reduce local pollution	Procurement of subsidies on biofuels production
Reduce GHGs emission from energy consumption	Tax credits on production and utilization of biofuels
Reduction in landfill sites	

Overall, biofuels can be classified as primary and secondary biofuel (see Figure 4). Primary biofuel refers to the conventional using of bioenergy, such as burning biomass, crops residue, or animal waste etc. Secondary biofuels are concerned with more innovative using of (bio)technology to produce biofuel from substrate. First generation biofuel is produced from raw biomass material in competition with food and feed industries, such as seed, grain or sugar. The most common first generation biofuels are bioethanol, biodiesel and starch-derived biogas, but also unprocessed vegetable oils, bio-methanol and bio-ethers (it can be used as an additive to current fossil fuel to replace petro-ether) may be included as well.[60] Due to this competition, these biofuels give rise to ethical, political and environmental concerns. Therefore, there is an increasing interest in using lignocellulosic agricultural and forest residues and non-food crop feedstocks, which are the sources of 2nd generation biofuel. However, there are several challenges ahead of 2nd generation: 1. Enzymes, pretreatment and fermentation processes need to be more energy and cost efficient; 2. Land competition; 3. The commercialisation of 2nd generation biofuels need to necessitate the new infrastructure for harvesting, transporting, storing and refining biomass.[61] Third generation biofuels specifically derived from microbes and microalgae are considered to be a viable alternative energy resource that is devoid of the major drawbacks associated with first and second-generation biofuels.

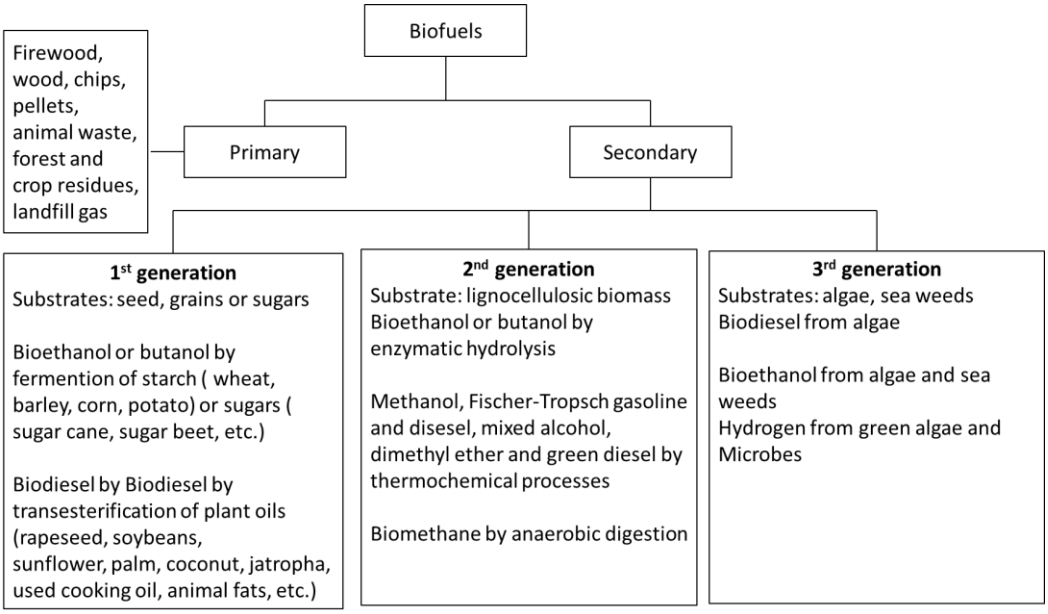


Figure 4 Biofuel classification[62]

Up to now, bioethanol is the most widely used biofuel for transportation globally. It is produced from biomass feedstock such as sugarcane, sugar beet and starch crops (mainly maize and wheat). USA is the largest producer for bioethanol (51.3 billion litres/ year) with maize as main feedstock. The European Union produces 3.44 billion litres of bioethanol per year, with sugar beet and starch crops as the main feedstock[56]. Biodiesel is derived from oil based crops, such as rapeseed, sunflower, soybean but also palm oil and waste edible oils.[56] Biogas is produced from anaerobic digestion of mixtures of corn derived starch, manure, organic waste and grasses. As biofuel can be derived from a wide range of biomass, it can be classified as either first or second generation; when biogas is mainly derived from waste and residue, it can be categorized in second generation energy. The advantages of first generation biofuels is that the raw materials are easy to convert into biofuel, because they are mostly composed of sugar or oil.[56] Most Life Cycle Assessments (LCA) have found a net reduction in global warming emissions and fossil energy consumption when most common transportation biofuels (bioethanol and biodiesel) are used to replace conventional diesel and gasoline.[63-65] However, first generation biofuels currently produced from sugar, starch and vegetable oils have several disadvantages: their production competes with food for their feedstock and fertile land and their potential production is limited by soil fertility and yield. Meanwhile, the CO_2 emission saving effect is limited by the high energy input required for crop cultivation and conversion.[66, 67]

For the purpose of partially overcoming the shortcomings of first generation biofuel, there is a growing interest in the production of second generation biofuels (i.e. from raw

materials based on waste, residue or non-food crop biomass) as a potential alternative to fossil fuels and conventional biofuels[56]. The use of such biomass in biorefinery complexes is expected to ensure additional environmental benefits and improve world energy security, due to the coproduction of both bioenergy and high value chemicals. In contrast to first generation biofuels, where the utilized fraction (grains and seed) correspond to only a small amount of the above-ground biomass, second generation biofuels can rely on the whole plant for bioenergy production. Second generation biofuels (e.g. Fisher Tropsch diesel from biomass and bioethanol from lignocellulosic feedstock) has higher land-use efficiency and environmental performance, according to the LCA studies published before.[68, 69] More importantly, second generation biofuels could be derived from lignocellulosic residue and waste which is already available or non-food crops such as perennial grasses and short-rotation forestry. They allow the coproduction of valuable biofuels, chemical compounds as well as electricity and heat, contributing to a better energy, environmental and economic performance through the development of biorefinery concepts.[70]

Production of the third generation fuel usually relies on the lipid content of microorganisms. Microalgae synthesize and accumulate large quantities of neutral lipids (20–50% dry weight of biomass).[71] They are able to produce 15–300 times more oil for biodiesel production than traditional crops on an area basis. Compared to conventional crop plants which are usually harvested once or twice a year, algae and seaweed (macroalgae) have a very short harvesting cycle (1-10 days depending on the process), allowing multiple or continuous harvests. However, technical challenges such as lipid extraction and dewatering need to be overcome.[72] There is a high content of water in algae, thus dewatering is required, which is carried out either via centrifugation or filtration before extracting lipids. Lipids obtained from algae can be processed through transesterification to give biodiesel.[71] As can be seen from Figure 4, both bioethanol and biodiesel can be derived from algae or seaweed.

1.7 Biomass composition

Biomass is synthesized via the photosynthetic process which converts atmospheric carbon dioxide and water into sugars. Further, plants use sugars to synthesize the complex material that is termed biomass. The primary building block of plant cell wall is lignocellulose. Plant biomass is mainly composed of cellulose, hemicellulose and lignin, along with smaller amounts of pectin, protein, extractives (soluble non-structural material such as non-structural sugars. nitrogenous material, chlorophyll and waxes) and ash.[73] Figure 5 illustrates the structural organization of the plant cell wall. Cellulose is

protected from degradation by hemicellulose and lignin. The compositions of these constituents can vary depending on the plant species (see Table 4 for details of some common lignocellulosic materials). Additionally, the ratios between the constituents within a single plant vary with age, stage of growth and other conditions.[2]

Table 4 Cellulose, hemicellulose and lignin content in common agricultural residues and wastes [5, 7]

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
News paper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	1.4-3.3	
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal bermuda grass	25	35.7	6.4
Switchgrasss	45	31.4	12
Swine waste	6	28	n.a
Sugar cane bagasse	52.45	25.96	12.72

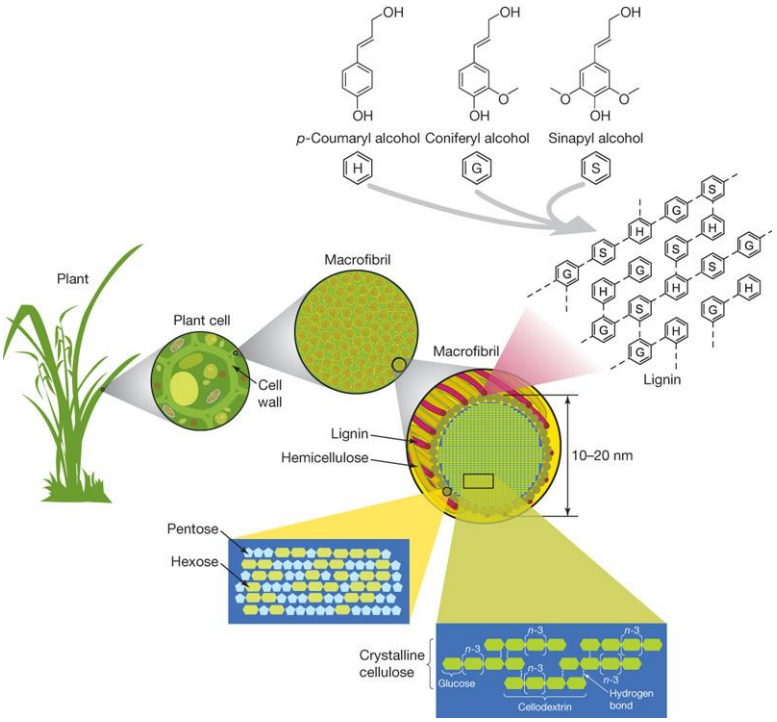


Figure 5 Structure organization of the plant cell wall (taken from ref [74])

1.7.1 Cellulose

Cellulose is the main structural constituent in plant cell walls and is present in an organized fibrous structure. Cellulose is a linear, unbranched homopolysaccharide composed of β-D-glucopyranose units which are linked together by β-1,4-glycosidic bonds (see Figure 6).[5]

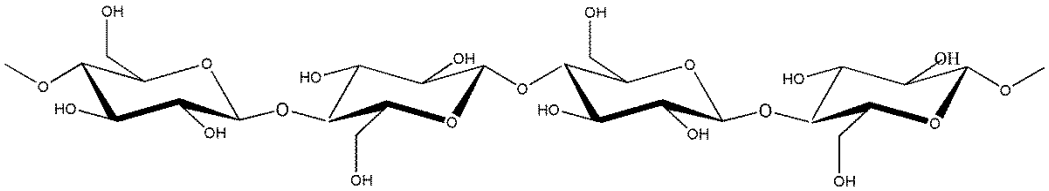


Figure 6 Cellulose structure

Cellulose in biomass presents in two forms, amorphous and crystalline. The long-chain cellulose polymers are linked by hydrogen and Van der Waals bonds, constructing cellulose packed into microfibrils which are about 10-20 nm in diameter (see Figure 5).

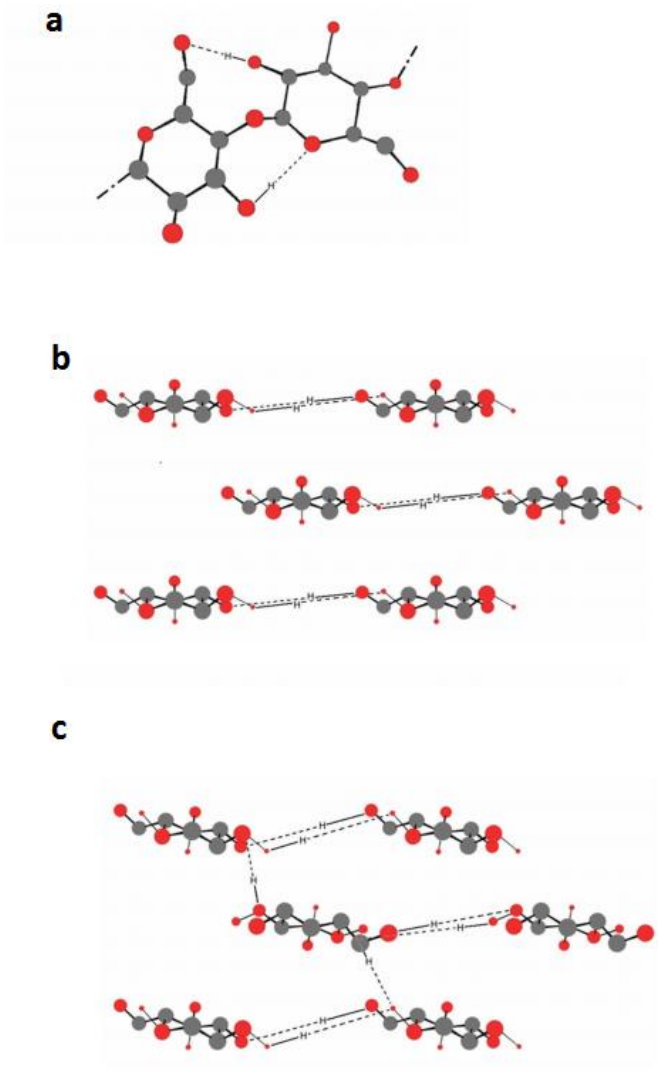


Figure 7 (a) Cellobiose, the repeating unit in crystalline cellulose I, with intramolecular hydrogen bonds shown. Axial cross sections of 3 sheets of (b) cellulose Iand (c) cellulose II, with intermolecular hydrogen bonds shown. Cellulose strands are represented by cellobiose units and hydrogen atoms have been omitted for clarity unless involved in hydrogen-bonds. (Taken from ref. [75])

Three hydrogen bonds occur per glucosyl unit in biosynthetic cellulose: two intramolecular hydrogen bonds and one intermolecular hydrogen bond to a neighbouring cellulose molecule in the same sheet (see Figure 7). Each microfibril is an unbranched polymer with about 15000 anhydrous glucose molecules[76]. The microfibrils are lined up parallel to each other and consist of crystalline regions. Crystalline cellulose is the major component, whereas a small amount of unorganized cellulose chains form amorphous cellulose. Amorphous cellulose is less compact and can be degraded more easily than crystalline cellulose.[2] However, these amorphous regions are staggered, making the overall cellulose structure strong.[76] Although the monomer (glucose) and short oligomers are water-soluble, cellulose is not, because of its high molecular weight

(solubility is usually inversely related to polymer length) and the comparatively low flexibility of cellulose polymer chains.[75]

1.7.2 Hemicellulose

Hemicellulose is a polysaccharide that comprises xyloglucans, xylans, mannans and glucomannans, linked by β-(1-4)-linked backbones with an equatorial configuration and β-(1-3, 1-4)-glucans. It has a lower molecular weight than cellulose. It can be branched and decorated with functionalities such as acetyl and methyl groups, cinnamic, glucuronic and galacturonic acid (see Figure 8). Xyloglucans (XyG) are the most abundant hemicellulose in primary cell walls found in every land plant species that has been analysed. They are branched with α-D-xylose linked to C-6 of the backbone. Xylans are a diverse group of polysaccharides with the common backbone of β-(1,4)-linked xylose residues, with side chains. They usually contain many arabinose residues attached to the backbone which are known as arabinoxylans and glucuronoarabinoxylans.[77] These hemicellulose types are present in all terrestrial plants cell walls, except for β-(1-3, 1-4)-glucans, which are restricted to Poales (They are a large order of flowering plants in the monocotyledons, including families like grasses and bromeliads) and a few other groups. Figure 8 presents chemical structures of xyloglucan and glucurnonarabinoxylan. The structures of hemicelluloses and their abundance vary widely from species to species and cell types. The most significant biological role of hemicellulose is that they strengthen the cell wall by interaction with cellulose and, in some walls, with lignin.[78] It acts as an amorphous matrix material, holding the stiff cellulose fibrils in place (see Figure 5). It has been suggested that the affinity of hemicellulose to lignin is enhanced by the substitution with hydrophobic groups such as acetyl and methyl groups thus improving the cohesion between the three major lignocellulosic polymers.[75]

The main difference between hemicellulose and cellulose is that hemicellulose has branches with short lateral chains composed of different sugars. These monosaccharides include pentose (xylose, rhamnose and arabinose), hexose (glucose, mannose and galactose) and uronic acid (e.g. 4-O-methylglucuronic, D-glucuronic and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1,4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds. Due to its non-crystalline nature, hemicellulose is easier to hydrolyse than cellulose, especially in acidic conditions.[2]

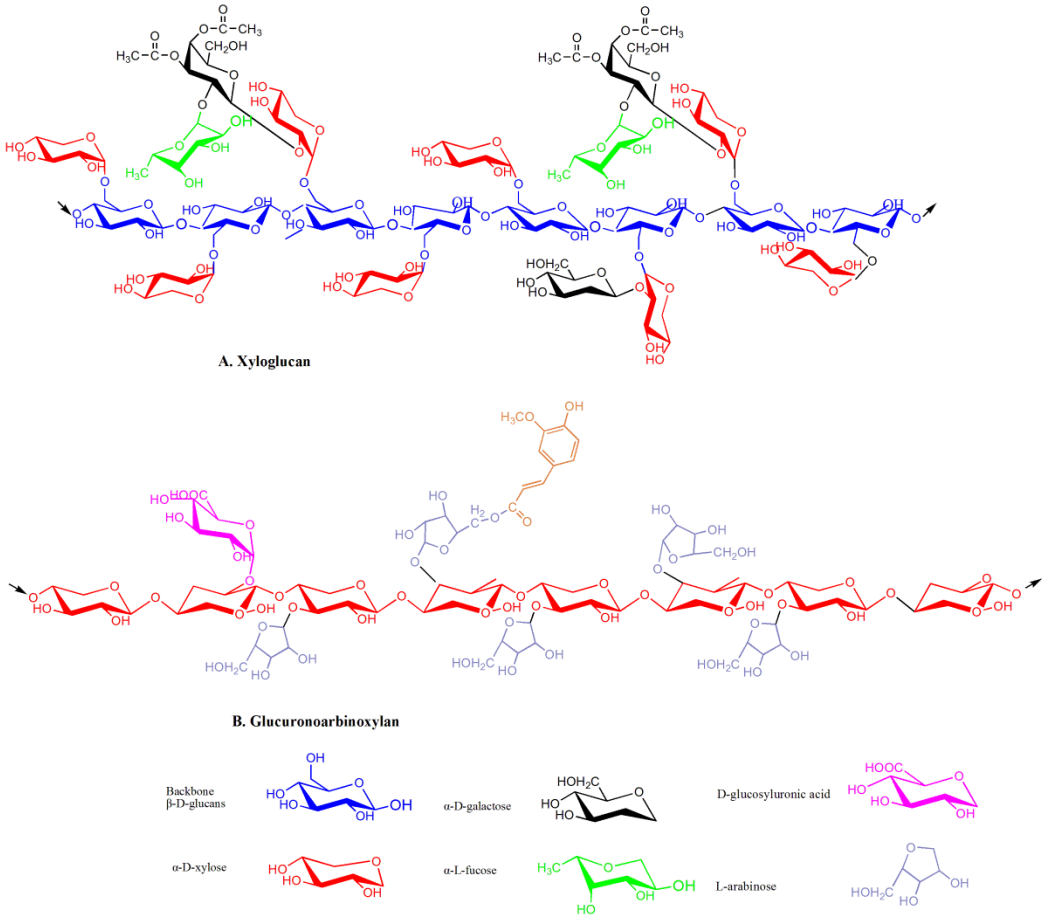


Figure 8 Structure of xyloglucan; the principle component of hemicellulose. The basic heptasaccharide repeating unit structure may bear additional substitutions as indicated. B. A unit structure of the highly substituted glucuronoarabionxylan. Feruloyl groups are esterfied to a few of the arabinosyl units and subsequently from several phenyl-phenyl and ether linkages to other esterified feruloyl units and to lignin.[77, 78]

1.7.3 Lignin

Lignin is a complex and large molecule, composing cross-linked polymers of phenolic monomers. It presents primary cell wall, imparting structural support, impermeability and resistance against microbial attack. The aromatic compounds in lignin form a network with cellulose through ester, phenyl bonds covalent as an ester and others.[2] It acts as nature's glue, forming a protective barrier that limits water and enzyme accessibility to cellulose and gives plants increased resistance to pathogen and insect attack and biomass degradation.[79] Lignin deposition is thought be increased in response to attack by these invaders.[80] Three phenyl propyl alcohols exist as monomers for lignin, coumaryl alcohol (p-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol) and sinapyl alcohol (syringyl alcohol) (see Figure 9). After their biosynthesis, monolignols are transported to the cell wall where they undergo oxidation and radical polymerization to form a complex three dimensional molecular architecture

that contains a variety of bonds with typically around 50% β-O-4 ether linkage. [81] A schematic representation of the general chemical structure of lignin from softwood is depicted in Figure 10. Lignin composition and content are influenced by the species and also by the environment.[82] In general, herbaceous plants such as grasses have the lowest lignin content, whereas softwoods have the highest lignin content (see Table 4).

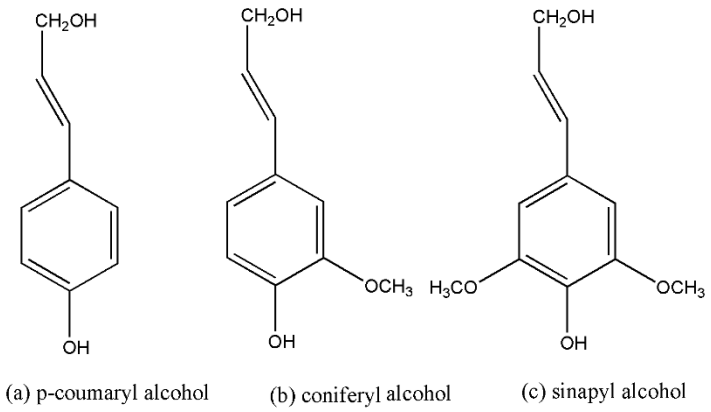


Figure 9 Lignin monomers

Lignin is identified as one of the major obstacles for an energy-efficient biomass destruction process. Not only does lignin prevent efficient hydrolysis of polysaccharides, but also modified lignin after pretreatment causes unproductive binding of hydrolases. Several inhibitors such as syringyl aldehyde and vanillic acid are derived from lignin, which negatively influence hydrolases and fermentative organisms.

In most cases of chemical pretreatments, lignin is modified by hydrolysing its ether bonds. Only some of the lignin is removed from the pulp (e.g. organosolv pulping, some base treatments, sulphite pretreatment and pulping, kraft pulping). The lignin removal is a result of chemical fragmentation and the ability of the liquor to solvate the modified lignin fragments.[75]

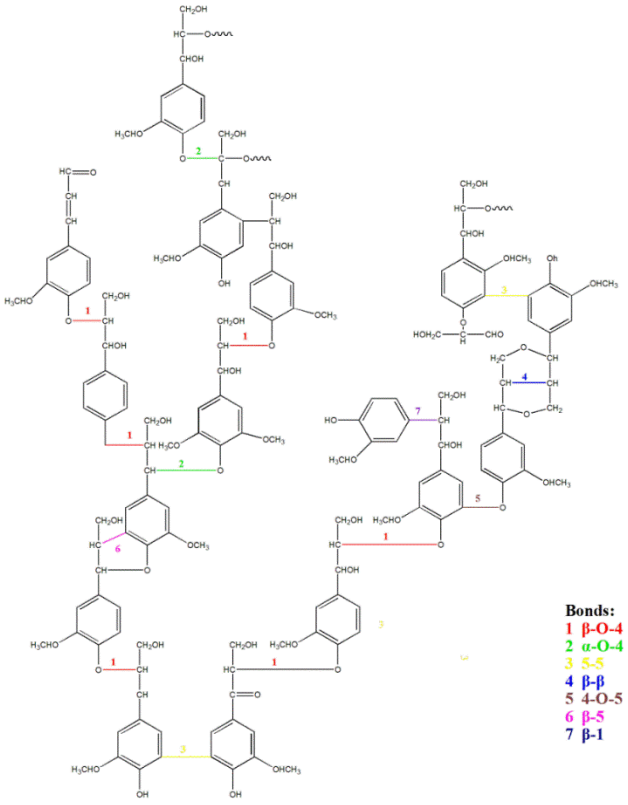


Figure 10 Schematic model of lignin structure[82]

1.7.4 Bonds between lignin and carbohydrates: lignin-carbohydrate complexes

Hemicellulose and lignin are not only entangled, but also covalently cross-linked. In wood, the covalent lignin-carbohydrate linkages include ester and ether linkages through sugar hydroxyl to the α-carbon of phenylpropane subunits in lignin (Figure 11). Ferulic acid can dimerize hemicellulose, see Figure 12. In grasses, these lignin-carbohydrate complexes contain ferulic and p-coumaric acid (Figure 13).[83] Initially, ferulic and p-coumaric acids are bonded to hemicellulose via ester bonds (Figure 13). During lignification, the lignin network grows by participating in the radical polymerisation reaction.[75]

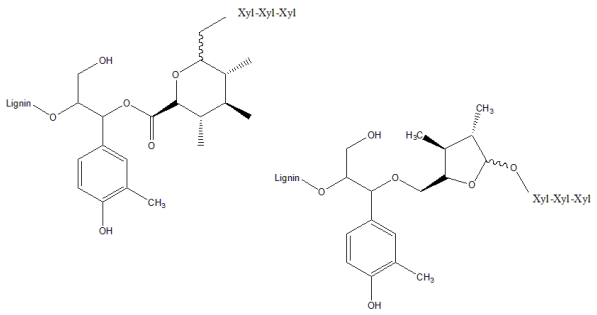


Figure 11 Ester linkage to the α-carbon of phenylpropane subunits in lignin

The cell wall rigidity and resistance to enzymatic digestion is correlated to the extent of cross-linking via lignin-carbohydrate-complex. Therefore, the cross-links must be broken by chemically hydrolysing the ester bonds, so that an effective deconstruction process can be achieved. Direct complexes between lignin and carbohydrates are formed during lignification, when hydroxyl groups of carbohydrates react with electrophilic ketone methide intermediates of the growing lignin polymer chains.[75]

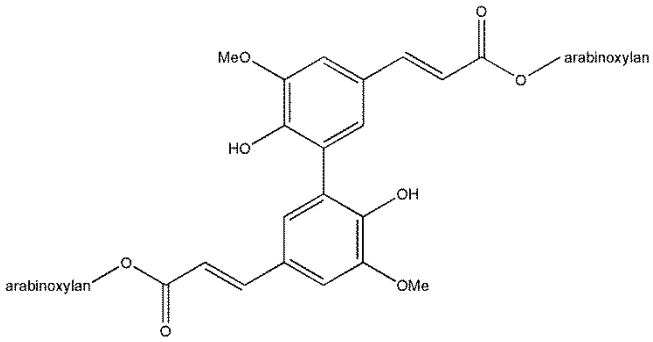


Figure 12 Ferulic acid dimer cross link [75]

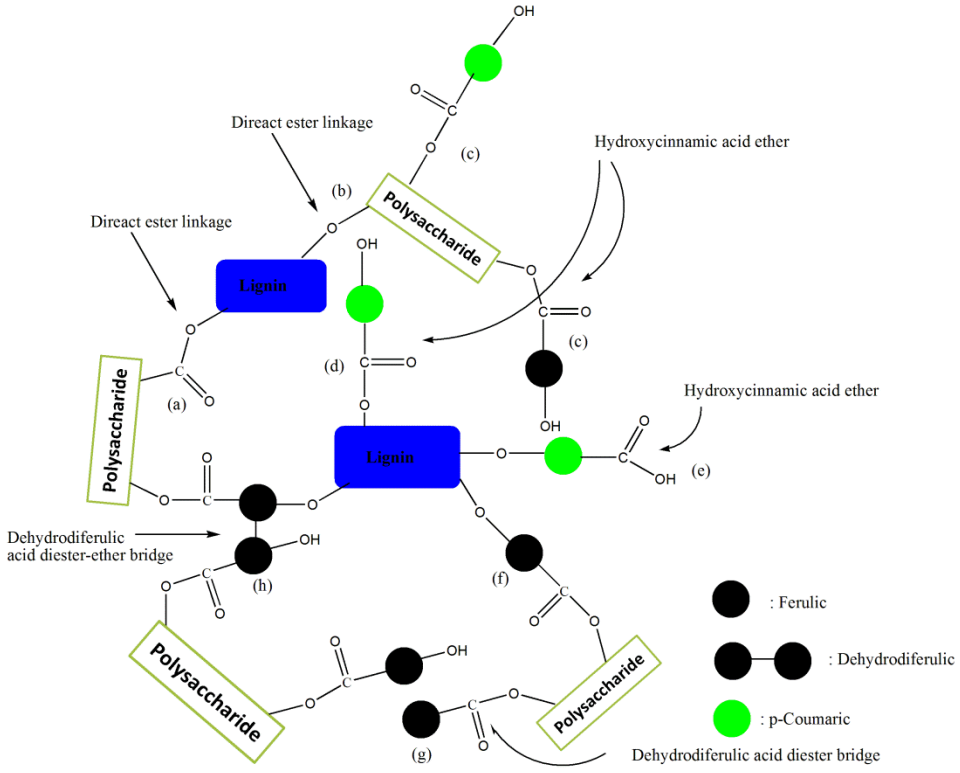


Figure 13 Schematic diagram showing possible covalent cross-links between polysaccharides and lignin in walls. a. Direct ester-linkage; b. direct ether-linkage; c. hydroxycinnamic acid esterified to polysaccharides; d. hydroxycinnamic acid esterified to lignin; e. hydroxycinnamic acid etherified to lignin; f. FA ester-ether bridge; g. dehydrodiferulic acid diester bridge; h. dehydrodiferulic acid diester-ether bridge.[84]

1.8 Promising feedstock

Biomass energy currently contributes 9-13% of the global energy supply- accounting for 45±10 EJ per year. Biomass energy includes both traditional uses (e.g., firing for cooking and heating) and innovative applications (e.g. producing electricity and steam and liquid biofuels). 7 EJ energy a year is generated by using modern technology and the remainder is in traditional uses. Biomass energy is generated from renewable material. Biomass feedstock is considered to be sustainable with proper management and technologies.

Bioethanol is one of the most modern forms of biomass energy and it has great potential to replace gasoline. Potential feedstocks for bioethanol include starch, sugar crops and agricultural residues (e.g. corn, barley, oat, rice, wheat, sorghum and sugar cane). In order to avoid conflicts between food use and industrial use of crops, wasted crops are preferred to produce ethanol. Wasted crops are crops lost during the year at all stages between the farm and the household level during handling, storage and transport. The agricultural residue includes corn stover, crop straws and sugar cane bagasse, generated during sugar cane processing.[50]

There are about 73.9 Tg of dry wasted crops produced every year in the world, which could potentially give rise to 49.1 GL $year^{-1}$ of bioethanol. At the same time, lignocellulosic biomass could produce up to 442 GL $year^{-1}$. Hence, the total potential bioethanol production from crop residue and wasted crops is 491 GL $year^{-1}$, which is able to replace 353 GL of gasoline (32% of the global gasoline consumption). Table 5 and

Table 6 show available wasted crops and lignocellulosic biomass production and their potential bioethanol production every year world widely.[50]

Table 5 Quantities of wasted crop and lignocellulosic biomass potentially available for bioethanol[85]

	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
Wasted crops (Tg)								
Corn	3.12	9.82	1.57	0.3	1.74	0.01	4.13	20.7
Barley	0.17	1.23	2.01	0.01	0.01	0.19	0.04	3.66
Oat	0.004	0.06	0.43	0.01	0.001	0.001	0.05	0.55

Rice	1.08	21.86	0.02	0.96	0.08	0.02	1.41	25.44
Wheat	0.83	10.28	4.09	0.02	0.24	0.82	0.91	17.2
Sorghum	2.27	0.54	0.004	0	0.13	0.001	0.18	3.12
Sugar cane	0.46	1.64	0	0	0.36	0	0.74	3.2
Subtotal	7.94	45.43	8.13	1.3	2.56	1.05	7.45	73.86
Lignocellulosic biomass (Tg)								
Corn stover	0	33.9	28.61	133.66	0	0.24	7.2	203.62
Barley straw	0	1.97	44.24	9.85	0.16	1.93	0.29	58.45
Oat straw	0	0.27	6.83	2.8	0.03	0.47	0.21	10.62
Rice straw	20.93	667.59	3.92	10.95	2.77	1.68	23.51	731.34
Wheat straw	5.34	145.2	132.59	50.05	2.79	8.57	9.8	354.35
Sorghum straw	0	0	0.35	6.97	1.16	0.32	1.52	10.32
Bagasse	11.73	74.88	0.01	4.62	19.23	6.49	63.77	180.73
Subtotal	38	923.82	216.56	218.9	26.14	19.7	106.3	1549.42

Table 6 Potential ethanol production world widely[50]

	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
From wasted crop (GL)								
Corn	2.17	6.82	1.09	0.21	1.21	0.01	2.87	14.4
Barley	0.12	0.83	1.35	0.005	0.01	0.13	0.03	2.46
Oat	0.002	0.04	0.3	0.01	0	0.001	0.03	0.38
Rice	0.71	14.4	0.02	0.63	0.05	0.02	0.93	16.8
Wheat	0.55	6.78	2.7	0.02	0.16	0.54	0.6	11.3
Sorghum	1.55	0.37	0.003	-	0.09	0.0004	0.12	2.14
Sugar cane	0.23	0.82	-	-	0.18	0.0001	0.37	1.59
Subtotal (A)	5.33	30.1	5.45	0.87	1.7	0.7	4.95	49.1
From lignocellulosic biomass (GL)								

Corn stover	-	9.75	8.23	-	0.07	0.07	2.07	58.6
Barley straw	-	0.61	13.7	0.05	0.6	0.6	0.09	18.1
Oat straw	-	0.07	1.79	0.009	0.12	0.12	0.06	2.78
Rice straw	5.86	186.8	1.1	0.77	0.47	0.47	6.58	204.6
Wheat straw	1.57	42.6	38.9	0.82	2.51	2.51	2.87	103.8
Sorghum straw	-	-	0.1	0.31	0.09	0.09	0.41	2.79
Bagasse	3.33	21.3	0.004	5.46	1.84	1.84	18.1	51.3
Subtotal (B)	10.8	261	63.8	7.42	5.7	5.7	30.2	442
Total	16.1	291.1	69.2	9.12	6.39	6.39	35.1	491.1

The availability of feedstock for bioethanol can vary considerably from season to season and depends on geographic locations. Locally available agricultural biomass should be used for bioethanol production.

1.9 Process for bioethanol production

By far, bioethanol is the most widely used biofuel for transportation world widely. Practically, any of the organic molecules of the alcohol family can be utilized as fuel. Several types of alcohol, such as methanol (CH_3OH), bioethanol (C_2H_5OH), propanol (C_3H_7OH), butanol (C_4H_9OH), can be used for motor fuels. However, only methanol and bioethanol fuels are technically and economically suitable for internal combustion engines.[86]

Bioethanol derives from a renewable resource and it represents a closed carbon dioxide cycle because after burning of ethanol, the released carbon dioxide is recycled back into plant material by photosynthesis cycle. The toxicity of the exhaust emission from ethanol is lower than that of petroleum sources. It contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that have health hazard to living beings. It also benefits energy security as it shifts the need for a proportion of foreign-produced oil to domestically-produced energy sources. Therefore, countries which have limited access to crude oil can grow energy crops for energy use and reduce foreign exchange expense.[87]

However, bioethanol has several disadvantages, including its lower energy density than gasoline (but 35% higher than that of methanol), corrosiveness, low flame luminosity, lower vapour pressure (making cold starts difficult) and complete miscibility with water. Therefore, in order to overcome these disadvantages, ethanol is blended with a small fraction of a much more volatile fuel such as gasoline and vapour pressure is increased.[58]

On a life-cycle basis, bioethanol derived from different biomass has various GHGs emission reductions. Figure 14 illustrates that corn ethanol offers rather limited benefits, as it reduces GHGs emission only by 18% compared to gasoline. In contrast, sugarcane and cellulosic ethanol contribute to nearly 90% lower emission. Corn ethanol is inferior to sugarcane and cellulosic ethanol in terms of 'net energy balance'. This is defines as the ratio of renewable energy output over fossil fuel input to product bioethanol on a life-cycle basis. Studies shown that corn ethanol yields 20-30% more energy than fossil fuel energy in making it. Sugarcane and cellulosic ethanol achieve renewable energy 9 times worth the fossil energy used to produce them.[88]

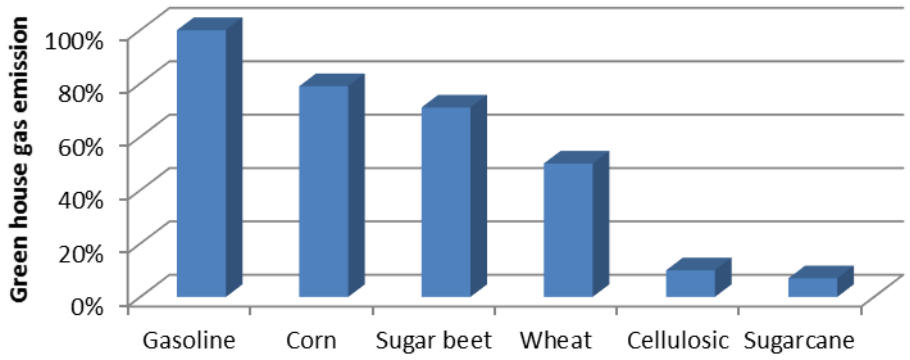


Figure 14 Reduction in greenhouse gas emissions, compared to gasoline, by ethanol produced from a variety of feedstocks (on a life-cycle basis)[88].

Lignocellulosic material can be converted into bioenergy via two different approaches, i.e. thermochemical or biochemical conversions (see Figure 15). The thermochemical route involves processes that require rather extreme temperatures and pressure than that of biochemical conversion system. Thermochemical process includes combustion, gasification and pyrolysis. It is generally more capital-intensive and requires large-scale production for economic benefits. Bioenergy can be produced from lignocellulosic residues by thermochemical or biochemical processing, liquid fuels such as bioethanol or biodiesel, gaseous fuels such as biogas (methane), electricity and heat can be obtained.[62, 89]

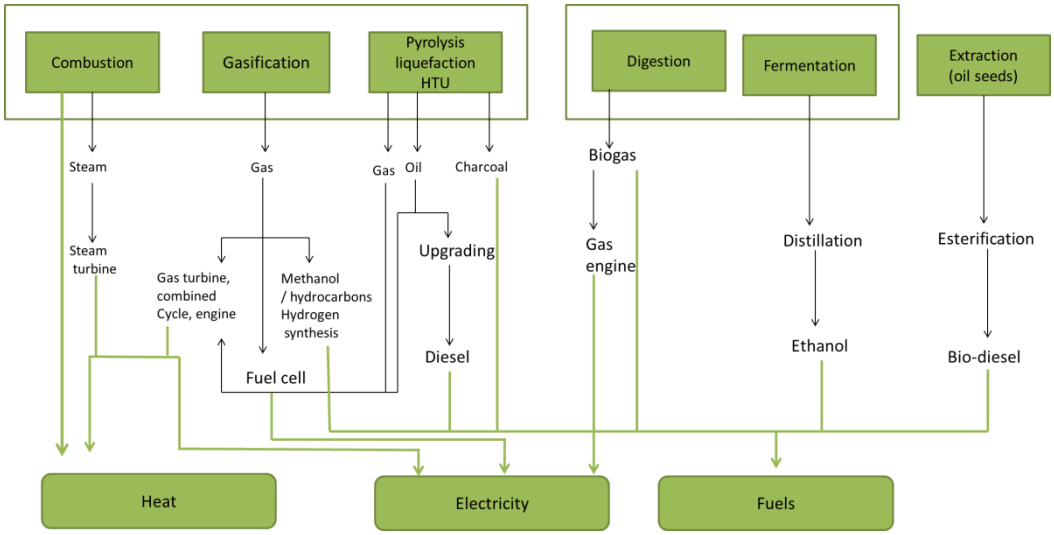


Figure 15 Thermochemical and biochemical conversions of lignocellulosic biomass[89]

The thermochemical process for bioethanol production involves gasification of raw material at a high temperature of 800 ^{o}C followed by a catalytic reaction. Raw material is converted into syngas (hydrogen, carbon monoxide and carbon dioxide). In the presence of catalysts (Rh, Ru, Co, Fe based), the resulting syngas can be synthesized into ethanol, or utilized by the microorganism as biocatalyst to form ethanol and water, which can be further separated by distillation.[90-92]

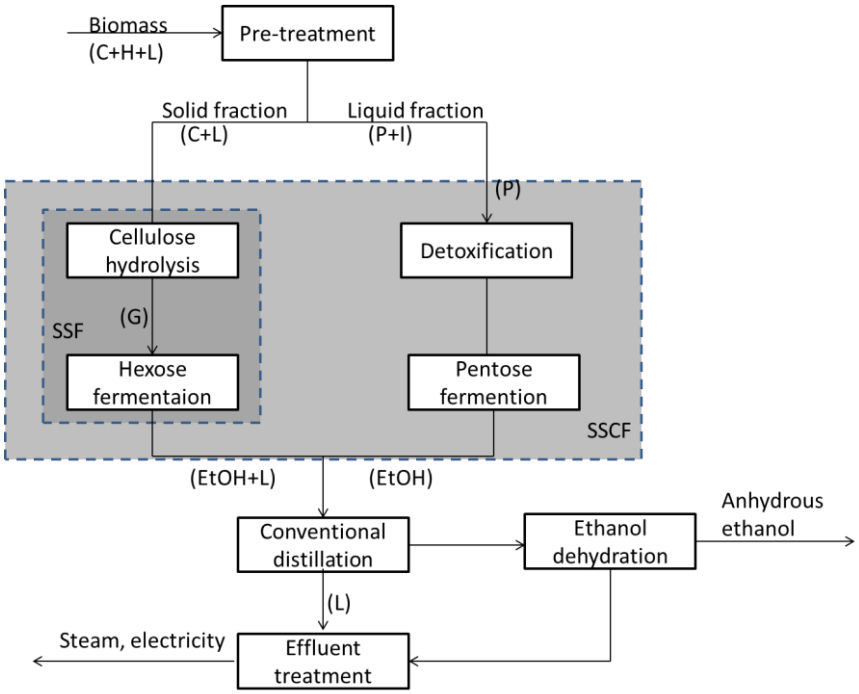


Figure 16 Biochemical pathway to produce bioethanol from lignocellulose biomass. Possibilities for reaction–reaction integration are shown inside the shaded boxes: SSF – simultaneous saccharification and fermentation; SSCF – simultaneous saccharification and co-fermentation. Main stream components are: C – cellulose; H– hemicellulose; L – lignin; G – glucose; P – pentose; I – inhibitors; EtOH – ethanol.[58]

Biochemical route involves physical (i.e. size reduction) or/and thermo-chemical with possible biological pretreatment. Biochemical pretreatment is mainly used to overcome the recalcitrant structure of biomass and increase cellulose accessibility to cellulases. The upstream operation is followed by enzymatic or acidic hydrolysis of cellulosic material and conversion of hemicellulose into monosaccharides (saccharification). Subsequently, the produced sugars are fermented into ethanol and then purified via distillation. Lignin is combusted and converted into electricity and heat. Figure 16 presents the bioethanol production process from lignocellulose biomass via biochemical conversion.

1.10 Biomass pretreatment

Lignocellulosic biomass is a recalcitrant structure in which hemicellulose and cellulose are packed with layers of lignin, resulting in resistance towards enzymatic hydrolysis.[5] Therefore, various pretreatments have been studied to improve the yields of fermentable sugars from cellulose and hemicellulose, such as mechanical,[7] steam explosion,[6, 93] ammonia fibre explosion,[7, 94] hot water, supercritical CO_2,[9] ozone pretreatment,[7] biological,[7] ultrasound,[95] acid or alkali[10, 11, 29] and others.

Table 7 An effective pretreatment must meet the following requirements[7]

1	Improve sugar production or ability to subsequently form sugars by hydrolysis;
2	Avoid degradation or carbohydrate loss;
3	Avoid by-product formation, such as inhibitors of subsequent hydrolysis and
4	Be cost effective

Pretreatments may alter the structure of cellulosic biomass to make it more accessible for enzymes, as well as decrease the degree of polymerization and cellulose crystallinity. Additionally, it can selectively remove hemicellulose and lignin from the lignocellulosic matrix.[6, 12] Table 8 presents the characteristics of the pretreatments that currently have been studied. Although most of these treatments can release hemicellulose and cellulose from the cell wall, some of them are economically unfeasible due to technical issues. Furthermore, they are not all able to overcome the recalcitrant material found mainly in wood-based feedstocks. Different from agricultural residues, forest and wood materials are high in lignin content and cellulose content, which renders them more recalcitrant. Agricultural residues such as corn stover, rice and wheat straw are mostly composed of hemicellulose and have a low lignin content conferring on them a less resistant texture. Additionally, they require less energy input than woody biomass to

reach size reduction. Therefore, the ratio of overall energy consumption versus sugar yield with regard to feedstock versatility, as well as toxic inhibitors formed per level of sugar recovery are the primary considerations on the estimation of the pretreatment efficiency and cost effectiveness of the process.[90]

Acid and alkaline pretreatments are considered effective, which explains their extensive use in most cases during biomass pretreatment.[5, 12, 14-21] Acid pretreatment centres around dilute acid pretreatment, which has been a crucial technology for hydrolysing lignocellulosic biomass for fermentable sugars production.[22] Acid can partially release monosaccharides, oligosaccharides and lignin monomers by splitting strong chemical bonds under high temperature.[23, 24] Marasabessy *et al.* reported that, with 30 minutes 0.9% (w/v) H_2SO_4 pretreatment at 178 ^oC before enzymatic hydrolysis, 100% of all pentoses present in *atropha curcas* fruit hull are released (71% yield and 29% degradation to furfural) after 24 hour enzymatic hydrolysis. Meanwhile, 83% of the hexoses (78% yield and 5% degradation to 5-hydroxymethylfurfural) is achieved.[96] Sulfuric acid is most commonly used in pretreatments, [5, 10, 11, 23] although other acid such as phosphoric acid [15, 25] and nitric acid [26] have been assayed .

Alkali can facilitate dissociation of entire wall polymers by breaking hydrogen and other covalent bonds and lignin can be removed without depolymerisation of the other major constituents.[6] Additionally, NaOH and ammonia pretreatment can significantly increase the disordered or amorphous fraction in the cellulose, improving the cellulose digestibility.[17] NaOH is widely used in these pretreatments.[20, 21, 28] Other alkaline agents are also used for pretreatment, such as lime[29] and ammonia.[17] Gomez *et al.* pretreated *Miscanthus*, maize and sugar cane bagasse with dilute NaOH under conventional condition in the temperature range of 20 to 180 ^oC and the results show that hemicellulose and lignin are effective degraded and the total sugar release during pretreatment is between 2-10 mg/g biomass. Zhu *et al.* studied conventional and microwave assisted dilute NaOH (1%) pretreatment of wheat straw and the weight loss of hemicellulose and lignin after pretreatment is 76-84.4% and 81-86% respectively. [21]

However, conventional acidic hydrolyses (usually dilute sulfuric acid with concentration below 4 wt% and temperature higher than 160℃) are always accompanied by the formation of toxic inhibitors such as furfural from xylose and hydroxymethylfurfural (HMF) from glucose in addition to phenolics and acetic acid. Acetic acid resulting from dilute acid pretreatment of lignocellulosic material is pH dependent and presumably mostly comes from hemicellulose acetate and it can reach a high concentration of approximately 10 g /L that is harder to separate and detoxify than HMF and furfural.[97]

Lignocellulosic materials also have been pretreated with hot water at high pressure during a fixed period and this presents elevated recovery rates for pentoses and produces low amounts of inhibitors. The temperature is usually 473-503 K. About 40%-60% of the total mass is dissolved in this process, with 4-22% of the cellulose, 35-60% of the lignin and all of the hemicellulose being removed.[2]

Table 8 Pretreatment methods and key characteristics [90].

Pretreatment	Key characteristics	Reference
Diluted acid	-Practical and simple techniques. Does not require thermal energy. -Effective hydrolysis of hemicelluloses with high sugar yield. - Generates toxic inhibitors - Requires recovery steps	[24, 87, 98-100]
Hot water	- The majority of hemicelluloses can be dissolved. - No chemicals and toxic inhibitors - Average solid load. - Not successful with softwood	[101-104]
Ammonia fibre expansion (AFEX)	- Effective against agricultural residues mainly corn stover without formation of toxic end-products. - Not suitable for high-lignin materials - Ammonia recovery - No wastewater	[7, 94, 105-107]
Ammonia recycle percolation (ARP)	- High redistribution of lignin (85%) - Recycling ammonia - Theoretical yield is attained	[108, 109]
Steam explosion with catalyst	- Effective against agricultural residues and hardwood. - High hemicelluloses fractions removal - Not really effective with softwood	[110]
Organosolv	- High yield is enhanced by acid combination. - Effective against both hardwood and softwood. - Low hemicellulosic sugar concentration - Formation of toxic inhibitors - Organic solvent requires recycling - High capital investment	[111, 112]
Ionic liquid	-Dissolution of cellulose increased amenability to cellulose -Reduce lignin content -Still in initial stages -less energy demanding, easier to operate and more environmentally friendly than current dissolution processes. However, it probably depends on the ionic liquid, as some are very toxic and difficult to prepare -high cost, regeneration requirement, lack of toxicological data and knowledge about basic physico-chemical characteristics	[111, 113]

Ozone	- Effectively remove lignin from a wide range of cellulosic material without generating inhibitors. - Expensive	[7]
Alkaline wet oxidation	- The combination of oxygen, water, high temperature and alkali reduces toxic inhibitors. - High delignification and solubilization of cellulosic material - Low hydrolysis of oligomers	[114, 115]
Alkaline (sodium hydroxide, lime, potassium hydroxide, aqueous ammonia, ammonium hydroxide)	-delignification process -remove significant amount of hemicellulose -remove acetyl and various uronic acid substitutions on hemicellulose -decrease degree of polymerization and crystallinity -disrupt lignin and cellulose structure	[22, 29, 116-118]
Steam explosion without catalyst	-hemicellulose removal -lignin transformation -reduction of particle size -lower environmental impact -cost effective	[22, 58],[119]
Fungal bioconversion	- Environmentally friendly - Low use of energy and chemical - Slow bioconversion	[37, 111]
High energy radiation (using gamma rays, ultrasound, electron beam, pulsed electrical field, UV and microwave heating)	-Increase of specific surface area -decrease the degrees of polymerization and crystallinity of cellulose -hydrolysis of hemicellulose and partial depolymerization of lignin. -some of these methods are energy intensive and prohibitively expensive, but microwave are really quite low energy	[22, 111]
Mechanical comminution(milling, chipping and grinding)	-disrupt cellulose crystallinity -decrease the degree of polymerization -increases the specific surface area of cellulosic biomass by breaking down biomass into smaller particles -renders the substrate more amendable for enzymatic hydrolysis -time-consuming -energy-intensive and expensive -less effective than chemical pretreatment	[22]

$FeCl_3$ pretreatment is also gaining growing attention. It can efficiently remove hemicellulose from the biomass, because it behaves as Lewis acid, which can facilitate decomposition of cellulose.[120] At the same time, Cl^- ions are good hydrogen acceptors and are able to interact with the hydroxyl groups of the sugars, leading to dissolution of cellulose too. López-Linares studied olive tree biomass pretreatment in 0.26 M $FeCl_3$ at 152.6 ^oC for 30 min. The results show that 100% of hemicellulose was removed and enzymatic hydrolysis of pre-treated solids resulted in a yield of 36.6 g glucose /100 g of

glucose in the raw material. Hemicellulosic sugar recovery in the prehydrolysate was 63.2%.[121] Lu *et al.* studied microwave assisted aqueous $FeCl_3$ pretreatment of rice straw and they found the pretreatment damaged the silicified waxy surface of rice straw, disrupted almost all the ether linkages between lignin and carbohydrates and removed lignin.[122]

In this work, *Miscanthus*, sugarcane bagasse and maize were pre-treated with water, H_2SO_4 (0.2 M, 0.4 M or 1 M), NaOH (0.2 M or 0.4 M or 1 M) and 0.2 M $FeCl_3$ with microwave assistance for various holding time (5 minutes to 40 minutes) under various temperature (130 ^oC to 200 ^oC). The sugars released from biomass during pretreatments were evaluated by using HPEAC (High-Performance Anion-Exchange Chromatography). The changes of chemical components, namely lignin, hemicellulose and crystalline cellulose, in biomass were compared, in order to have a further understanding of biomass digestibility variation after pretreatments. Morphological characteristics of biomass were studied by using scanning electron microscopy. The chemical structure of biomass residue was studied by using FT-IR. SSF (Simultaneous Saccharification and Fermentation) is conducted in order to investigate biomass digestibility. The results showed the potential of using microwave in the thermo-chemical pretreatment for biomass.

1.11 Microwave chemistry

1.11.1 Microwave definition

Since the late 1980s, microwave has been drawing growing attention in performing chemistry and has become a widely accepted alternative energy source for conventional energy. Microwaves lie between radio waves and infrared in the electromagnetic spectrum, in the frequency range of 0.3 to 300 GHz (see Figure 17).[123] By international convention it has been agreed that the following frequencies are assigned to industrial and scientific microwave heating and drying: 915 ± 25 MHz; 2450 ± 13 MHz; 5800 ± 75 MHz; and 22125 ± 125 MHz. Hence, not the entire microwave region is available for heating usages. For microwave chemistry, 2450 MHz has been used almost exclusively. [52]

Microwave chemistry is based on the efficient heating of a material by ‘microwave dielectric heating’ effects that are dependent on the ability of a specific material (solvent or reagent) to absorb microwave energy and convert it into heat. There are two main mechanisms through which material interacts with microwave energy, namely dipole rotation and ionic conduction. Dipole rotation means the alignment of molecules that

have permanent or induced dipoles, with the electric field component of the radiation. The heat is generated by sympathetic agitation of the molecules. The efficacy of heat generation through dipole rotation depends on the characteristic dielectric relaxation time of the sample, which in turn is decided by temperature and viscosity. Ionic conduction is the migration of dissolved ions with the oscillating electric field. The kinetic energy is converted into heat. [52]

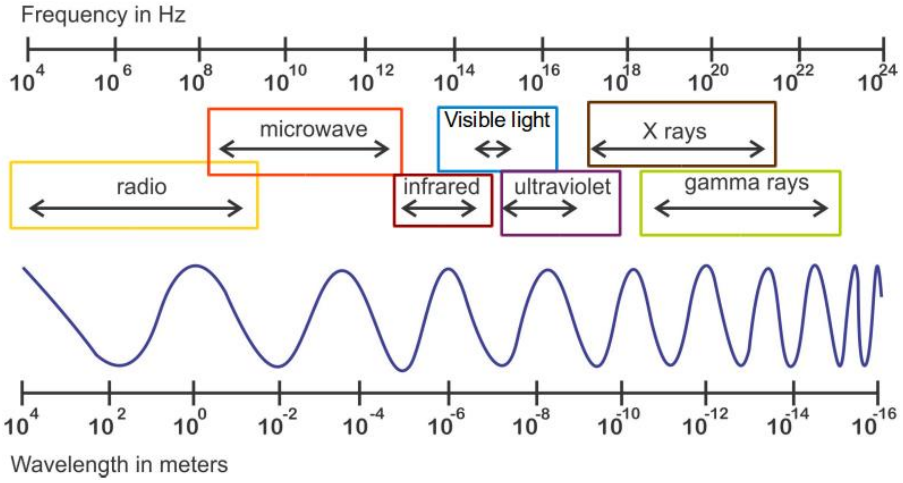


Figure 17 Electromagnetic spectrum

When the applied field oscillates, the dipole or ion field tries to realign itself with the alternating electric field and energy is lost in the form of heat through molecular friction and dielectric loss. The amount of heat generated in this process is decided by the ability of the matrix to align itself with the applied field and is related to the radiation frequency. At high frequencies the change in direction of the field is too rapid to allow rotation to occur, hence there is no heat generated, whereas at low frequencies the rate of rotation is slow, having minimal heating effect. The frequency of 2.45 GHz lies between these two extremes and the molecules will have enough time to align in the field, but not to follow the alternating field precisely.[124]

Conventional heating transfers energy by conduction or convection. From perspective of industrial heating application there are two main techniques that are used in commercial heating systems: hot-air and steam. The utilisation of these techniques is dependant of the application.[125] Microwave energy is transferred primarily by dielectric loss which is a measure of a substance in converting absorbed radiation into heat. The ability of a substance to convert electromagnetic energy into heat at a given frequency and temperature is dependent on loss factor tan δ. It is the quotient tan δ=ε'' /ε', where ε'' is dielectric loss factor, which is a measure of the efficiency with which electromagnetic radiation is converted into heat and the dielectric constant (ε') of a material which

represent the molecule's ability to be polarized by the electric field. High values of the dissipation factor (tan δ) suggest ready susceptibility to microwave energy and consequently rapid heating. In general, solvents can be classified as high (tan δ > 0.5), medium (tan δ > 0.1-0.5) and low microwave absorbing (tan δ < 0.1). Table 9 shows the tan δ value of a variety of the solvents.[124]

Table 9 Loss factors (tan δ) of different solvents [124]

Solvent	Tan value	Solvent	Tan value
Ethylene glycol	1.350	DMF	0.161
Ethanol	0.941	1,2-dichloroethane	0.127
DMSO	0.825	water	0.123
2propanol	0.799	chlorobenzene	0.101
Formic acid	0.722	Chloroform	0.091
Methanol	0.659	Acetonitrile	0.062
Nitrobenzene	0.589	Ethtyl acetate	0.059
1-butanol	0.571	Acetone	0.054
2-butanol	0.447	Tetrahydrofuran	0.047
1,2-dichlorobenzene	0.280	Dichloromethane	0.042
NMP	0.275	Toluene	0.040
Acetic acid	0.174	hexane	0.020

Other common solvents without a permanent dipole moment such as carbon tetrachloride, benzene and dioxane are almost microwave transparent. However, a low tan δ value solvent can still be used in a microwave-heated reaction, because either the substrate or some of the reagents/catalysts are likely to be polar, the overall dielectric properties of the reaction medium will allow sufficient heating by microwave. On the other hand, polar additives such as ionic liquids can be added into low-absorbing reaction mixture to increase the absorbance level of the medium. [124]

By using microwave, energy can be introduced remotely, without contact between the source and the chemicals. It is also easy to control by turning on or off the power. Heating rates are higher than can be achieved conventionally as long as one of the components can interact strongly with microwaves.[52] Conventional heating is carried

out by conductive heating with and external heat source. It is relatively slow and inefficient method for transferring energy into the system, because it depends on the thermal conductivity of different materials that need to be penetrated, resulting in the temperature of the reaction vessel being higher than that of the reaction mixture. However, microwave irradiation gives efficient internal heating (in-core volumetric heating) by direct interaction of microwave energy with the molecules (solvents, reagents, catalysts) that are present in the reaction mixture.

The reaction vessel employed are typically made out of (nearly) microwave-transparent material, such as borosilicate glass, quartz or Teflon, leading to an inverted temperature gradient compared to conventional thermal heating, see Figure 18. The wall effects (no hot vessel surface) is minimized by very efficient internal heat transfer.[124]

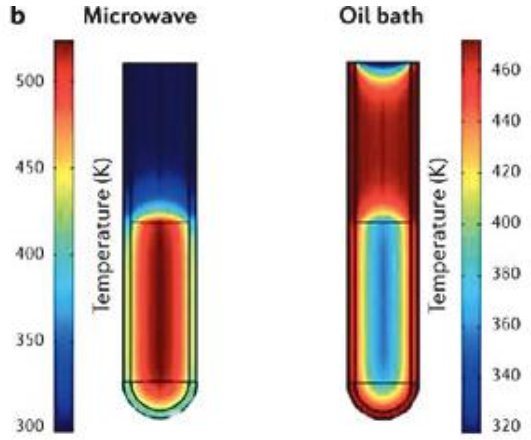


Figure 18 Inverted temperature gradients in microwave versus oil-bath heating, Difference in the temperature profiles (finite element modeling) after 1 Min of microwave irradiation (left) and treatment in an oil-bath (right)(taken from ref. [124])

1.11.2 Microwave effect

The energy of microwave photon is too low to directly cleave molecular bonds and therefore microwaves cannot directly induce molecules to react.[126] Accelerated rates and altered reaction pathways under microwave lead to a debate of whether the observation can be rationalized by purely thermal/kinetic effects arising from the rapid heating and bulk reaction temperature obtained by microwave dielectric heating, or whether some effects are concerned with so-called specific microwave effects, for example,

1. The superheating effect of solvent at atmospheric pressure.
2. The selectively heating of, for instance, good microwave-absorbing heterogeneous catalysts or reagents in a less polar reaction medium

3. The formation of ‘molecular radiators’ by direct interaction of microwave energy to specific reagents in homogeneous solution (microscopic hotspots) and
4. The elimination of wall effects caused by inverted temperature gradients.[126]

However, more research need is required to understand these phenomena.[126]

1.11.3 Microwave effect on biomass

Because the dielectric loss data for biomass is limited, a most important rule is that polar and mobile components will absorb microwaves effectively, whilst components which are either non-polar (waxes) are far less effective.

As was discussed previously, pretreatment is essential in order to make lignocellulosic material more accessible for acids or enzyme to digest and hence results in more efficient hydrolysis of biomass. Due to its unique qualities, such as rapid and uniform heating, penetration and selectivity of affected materials, microwave technology has numerous applications in food processing, wood drying, plastic and rubber treating, as well as curing and preheating of ceramics and so forth. [127] Based on these existing applications, microwave-assisted pretreatment of lignocelluloses material is gaining growing attention. It was initially reported by Ooshima *et al.*[128] and Azuma *et al.*[129]. Up to now, numerous feedstocks have been used, such as sugarcane bagasse [5], rape straw [31], switchgrass [19] and wheat straw[21]. Nikolic *et al.* studied microwave pretreatment for corn and the results showed that the glucose concentration in pretreatment liquor was increased by 8.48% compared to untreated control sample and the percentage of theoretical ethanol yield was 92.27% after 44 hours of the simultaneous saccharification and fermentation (SSF).[130] Lu *et al.* reported that the glucose yield of rape straw from enzymatic hydrolysis was enhanced by 56.2% (11.5% for raw rape straw) after microwave pretreatment.[31] Chen *et al.* studied the microwave assisted sulfuric acid pretreatment for sugarcane bagasse and revealed that, when the temperature is 190 ^{o}C, the fragmentation of particles became very pronounced, almost all hemicellulose was removed and the crystalline structure of cellulose disappeared.[5] When microwave is used to treat lignocelluloses, it can selectively heat the more polar part of biomass and create a ‘hot spot’ with the inhomogeneous materials. In this case, an ‘explosion’ effect may occur among the particles, which enhances the disruption of the recalcitrant structures of lignocellulose.[127]

In the sense of the bio-refinery, apart from using microwave as a pretreatment method for biomass hydrolysis, it can also be used to facilitate biomass thermal decomposition, such as gasification and pyrolysis, to decompose feedstock into smaller molecules that

can be used as energy source or input of other synthesis process. For example, hydrocarbons are broken down into syngas (H_2+O_2) which can further be used as a fuel directly, or converted into liquid fuel through the Fischer-Tropsch process. Pyrolysis is a thermal process that decomposes a substance in an oxygen excluded environment. Lignocelluloses can be converted into bio-oil, gases and char. By altering parameters such as temperature or reactant residence time, any of these constituents yields can be maximised, e.g. a high temperature and high residence time result in increased yield of gases; a high temperature and low residence time promote bio-oil yield; and a low temperature and heating rate lead to increased char production or to no chemical reactions at all. Various feedstocks have been studied in microwave assisted pyrolysis, such as rice straw[131], corn cobs[132], wheat straw[133], coffee hulls[134], Pine wood sawdust[135], Corn stover[136], etc.

1.11.4 Limitations of microwave technology

As mentioned before, microwave technology has many attractive advantages, such as uniform heating, instantaneous control, selective heating, clean energy transfer and chemical reactions driven. However, it also has limitations. Firstly, it's not easy to accurately predict the exact nature of electromagnetic field interaction with materials. Secondly, compared to conventional heating, it requires a higher initial capital cost. Thirdly, the largest single microwave source for industrial application is 100 kW. Multiple sources would be needed if larger amounts of energy were required. [137]

1.12 Introduction to work in this thesis

In recent years, there has been an upsurge of interest in the use of lignocellulosic material, as feedstock for second energy generation bioethanol. *Miscanthus*, sugarcane bagasse and maize are the most promising energy feedstocks available for the process of realising second generation biofuels. Due to the recalcitrant nature of biomass, a number of strategies have been put forward to achieve a more efficient bioethanol production, in which pretreatment is playing an essential role. Conventional hydrothermal pretreatments of biomass have been widely studied, whereas little research has been done in the area of microwave assisted chemical pretreatments. In this work, microwave was used to assist chemical pretreatments of C4 plants, namely *Miscanthus*, sugarcane bagasse and maize, in order to investigate the microwave performance on pretreatment process. Pretreatment media, holding time and temperature condition were assayed for each biomass, Biomass morphological characteristics were studied by scanning electron microscope. The type and quantity of sugar released during the pretreatment process

were evaluated and chemical components were compared. Furthermore, the fermentation ability of pre-treated biomass was studied by SSF process. The results showed that a good yield of sugar release during pretreatment process, which was contributed by a selective removal of lignin and hemicellulose. Compared to conventional hearing method, microwave assisted pretreatment released better yields of reducing sugars during pretreatment, due to its unique heating mechanism in which crystalline cellulose plays an important role as microwave absorber under the right conditions. The results showed promising potential of using microwave to assist thermo-chemical pretreatment for lignocellulosic material.

Chapter 2 and 3 discussed the results of *Miscanthus* under various temperature and holidng time. Chapter 4 and 5 discussed the results obtained from sugarcane bagasse and maize respectively. Chapter 6 investigated the results from ferric chloride pretreatments for these three types of biomass.

Chapter 2: Temperature optimization on microwave assisted pretreatment of *Miscanthus* biomass in biorefineries

Aspect of work describle in this chapter has been published in:

Microwave assisted chemical pretreatment of *Miscanthus* under different temperature regimes

Zongyuan Zhu, Duncan J. Macquarrie, Rachel Simister, Leonardo D. Gomez, Simon J. McQueen-Mason

Sustainable Chemical Process, October, 2015

2.1 Introduction

There is a rising global demand for energy and growing concerns about greenhouse gas emissions. Lignocellulosic biomass offers great potential for biofuel production, based on the biorefinery philosophy. As a crucial biomass energy crop with relatively low maintenance and high yield/energy content, *Miscanthus* plays an important role in the sustainable production of renewable fuels and chemicals.

Figure 19 *Micanthus* × giganteus

The genus *Miscanthus* includes about 17 species of perennial non-wood rhizomatous tall grasses native to subtropical and tropical regions of Asia. Among them *Miscanthus tinctorius*, *Miscanthus sinensis* and *Micanthus sacchariflorus* are of primary interest for biomass production.[138, 139] The sterile hybrid genotype *Miscanthus* × *giganteus* from *Miscanthus sacchariflorus* and *Miscanthus sinensis* is widely used in Europe and, more recently, in North America. Much research is devoted at present to broaden the genetic base of *Miscanthus*, maximise the productivity and the adaptive range of the crop.[140, 141]

Miscanthus was first introduced from Japan and cultivated in Europe in the 1930s. Since 1980s, field trials have been carried out in order to investigate the biomass potential of *Miscanthus*. In the US, for instance, the Freedom Giant genotype of *Miscanthus* was commercialized by REPREVE Renewables LLC.

Table 10 shows information of its yield reported by Europe and North America.[142] As can be seen, the the harvestable *Miscanthus* yield (dry matter) was estimated around 27 to 44 t ha^{-1} in small scale trials at spring harvest in Montreal Canada.[143-146] Nevertheless, there is very limited data of its production from other continents.

Table 10 *Miscanthus* productions in Europe and North America[142]

Location	Genotype	harvest period	Age of stand (Years)	Yield(t ha^{-1}) dry matter
Sweden	Sin -H	Autumn	3	11.0–24.7
		Winter	3	11.2–14.7
	Sin	Autumn	3	9.7–17.3
		Winter	3	7.1–10.3
Denmark	Sac	Autumn	3	1.4
		Winter	3	0.4
England	Sin-H	Autumn	3	18.2
		Winter	3	10.9
	Sin	Autumn	3	6.8–15.0
		Winter	3	4.9–8.6
	Gig	Autumn	3	13.8–18.7
		Winter	3	9.2–12.7
	Sac	Autumn	3	11.1
		Winter	3	6.3
Germany	Sin-H	Autumn	3	6.5–17.7
		Winter	3	5.4–12.8
	Sin	Autumn	3	4.6–10.9
		Winter	3	3.1–7.3
	Gig	Autumn	3	22.8–29.1
		Winter	3	17.5–20.7
	Sac	Autumn	3	12.6
		Winter	3	12.7
Portugal	Sin-H	Autumn	3	10.3–20.0
		Winter	3	5.9–14.3
	Sin	Autumn	3	9.1–12.8
		Winter	3	6.8–11.1
	Gig	Autumn	3	34.7–37.8
		Winter	3	19.6–26.4
	Sac	Autumn	3	35.2
		Winter	3	22.4
	Sin-H	Autumn	3	20.3–40.9
		Winter	3	12.2–31.9
	Sin	Autumn	3	16.1–22.4
		Winter	3	11.6–17.6
Northwestern Spain	Gig		4	14–34
Northern Greece	Gig	September	2	44
Central Greece	Gig	End of growing season	2 to 3	26
Western Turkey	Gig	Spring	3	28
Southern Italy	Gig		2 to 3	30–32
USA Illnois	Gig	Spring	2 to4	24–44
Canada Monteral	Gig		1	10–11

Gig: *Miscanthus* × giganteus; Sac: *Micanthus* sacchriflorus; Sin- H: *Miscanthus* sinensis hybrids; Sin: naturally occurring diploid *Miscanthus* sinensis

Miscanthus yield is greatly influenced by genotype, location and harvest time. *Micanthus × giganteus* has a larger potential for biomass yield compared to others. Better yield can be obtained in southern Europe than in northern Europe due to its higher average temperature and abundant global radiation. The best *Miscanthus* yield in UK is demonstrated by *Micanthus × giganteus* and its yield is between 13.8 to 18.7 t ha^{-1}. It is one of the most promising candidates for future European based bio-refinery.

2.2 Previous pretreatment methods studied on *Miscanthus*

As it has been mentioned previously in Chapter 1, like any lignocellulosic feedstock, *Miscanthus* is recalcitrant to chemical and enzyme hydrolysis. Major information about *Miscanthus* activation is summarised in Table 2. According to their pretreatment steps, there are three major categories of pretreatment of the *Miscanthus*. As can been seen, they need high temperature (up to 190 ^{o}C), substantial amount of additives and long holding time (up to 40 hours).

Table 11 Previous pretreatment methods studied on *Miscanthus*

	Methods	Conditions	References
1. One step pretreatment	Soda	145°C, 30 Min, 1.5M NaOH	[30, 147]
	AFEX(ammonia fibre expansion)	160°C, 5 Min, 2:1 (w/w) ammonia to biomass	[148]
	Wet explosion	170°C, 5 Min, 18 bars, O_2, H_2O_2	[149]
	Organosolv	Formic acid/acetic acid/water for 3 h at 107°C	[150]
		EtOH-H_2O 170-190°C, 60 Min, H_2SO_4 0.5-1.2%	[151]
		EtOH-H_2O 180°C, 90 Min	
		AcOH, HCl, 60-180 Min	[152, 153]
		Milox : formic acid–hydrogen peroxide–water	[154, 155]
	Ammonia	Aqueous ammonia (25% w/w) for 6 h at 60°C.	[150]
	Dilute acid	130°C, 15 Min, 1-4% H_2SO_4	[156, 157]
	Photocatalytic	TiO_2, UV-irradiation	[158]
2. Two steps pretreatment	Dilute acid and wet explosion	1. 80-100°C, 3-25 h, 0.5-1.5% H_2SO_4 2. 170°C, air was added, 200 bar, 5 Min	[149]
	Dilute acid and ethanol organosolv	1. 100°C, 17 h, H_2SO_4 2.170-180°C, 60 Min, H2SO4, 0.5-0.9%	[151]
	Enzyme and ethanol organosolv	1. Cellulyve® 2. 2. 150-170°C, 30-60 Min, H_2SO_4, 0.5-1%	[159]
	Autohydrolysis and ethanol organosolv	1. 130-150°C, 1-40 h, H_2O 2. 170-180°C, 60 Min, H_2SO_4, 0.5-0.9%	[160]
3. Ionic liquids pretreatment		[C2mim][OAc], 140°C, 3 h	[161]
		[C2mim][OAc], H_2O, K_3PO_4 70 -140°C for 1– 44 h	[162]
		[C_4C_1im][$MeSO_4$], [C_4C_1im][HSO_4], 120°C, with H_2O	[163]
		[Emim]Cl + H_2SO_4 6–10 h at 343 K	[164]
		[C2mim][OAc] + H_2O	[165]

Therefore, new technology has to be developed to solve these problems. Microwave is a promising candidate. As is well known, microwave has unique heating mechanism and it is a very efficient way of heating. Microwave heating can effectively disrupt the recalcitrant structures of lignocellulosic biomass, because cellulose, hemicellulose and other low molecular compounds are dielectrics.[5] Limited studies of microwave assisted pretreatment for *Miscanthus* have been previously reported.[25] As was mentioned in the Introduction, acid and base play a key role in biomass pretreatment. In this chapter, the microwave technology will be studied in the presence of acid and alkali on *Miscanthus × giganteus*, with an aim of obtaining a more efficient sugar release and more digestible biomass residue.

2.3 MW pretreatment of *Miscanthus*

MW pretreatment of lignocellulosic material could enhance their saccharification. Temperature plays a significant role during the pretreatment,[166] a higher temperature typically achieves higher biomass solubility, shortens the pretreatment time, reducing the biomass recalcitrance.[167] However, high temperatures also lead to the formation of compounds such as furfural, hydroxymethylfurfural (HMF) and phenolics, that are inhibitors of subsequent hydrolysis and fermentation.[166] Hence, different temperatures ranging from 130 ^{o}C to 200 ^{o}C are assayed here, in order to investigate temperature influence on biomass under microwave irradiation. Figure 20 shows the biomass material used in this study. Figure 21a shows the microwave equipment and auto-sampler. The reaction is conducted in a closed system with a 35 ml glass tube (Figure 21b) and a magnetic stir bar is used to make sure the pretreatment media is homogeneous. The temperature is measured by a vertically focused IR temperature sensor.

Figure 20 Untreated *Miscanthus* material

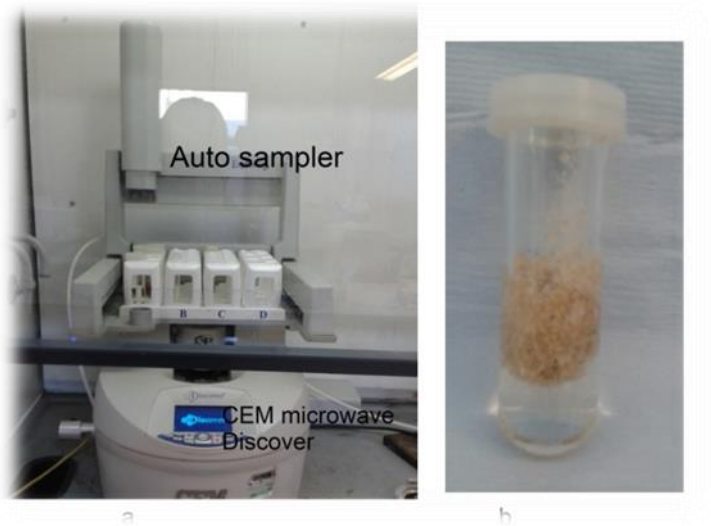


Figure 21 CEM microwave machine; b. Microwave reaction vessel

Figure 22 shows the experimental diagram and analysis techniques which have been used in this study. The pretreatment was performed in the CEM microwave machine and each condition was performed in triplicate in order to make sure the data is repeatable. After pretreatment, the biomass solid residue and liquid media was separated by centrifuge to assay the biomass solid fraction properties and liquor sugar components.

2.4 Development of carbohydrate analysis procedures and biomass morphological characterization methods

In the current study, the types and quantities of sugars release during the pretreatment process is presented and crystalline cellulose, hemicellulose and lignin are quantified and compared. Hence, it is important to know the methods used in this study to analyse the chemical compositions in the lignocellulosic material before the results and discussion.

The reducing sugars released in pretreatment media and hemicellulose content in the solid fraction are quantified by High Performance Anion Exchange Chromatography (HPEAC). As we know, High-performance-liquid-chromatography (HPLC) is a separation technique appropriate for heat labile non-volatile molecules. Separation of is based on the different affinity of a given analyte for the stationary and mobile phase. The polarity of mobile phase is used to elute the analytes one by one. HPLC offers several advantages of analysis carbohydrates, such as high resolution, fast analysis, direct injection of sample without or minimal pretreatment and easy of automation. Quaternary ammonium polymer-based stationary phases and high pH are frequently used for HPLC to analysis carbohydrates, thus the method is named as (HPEAC). This part of analysis was conducted in CNAP (Centre of Novel Agricultural Products,

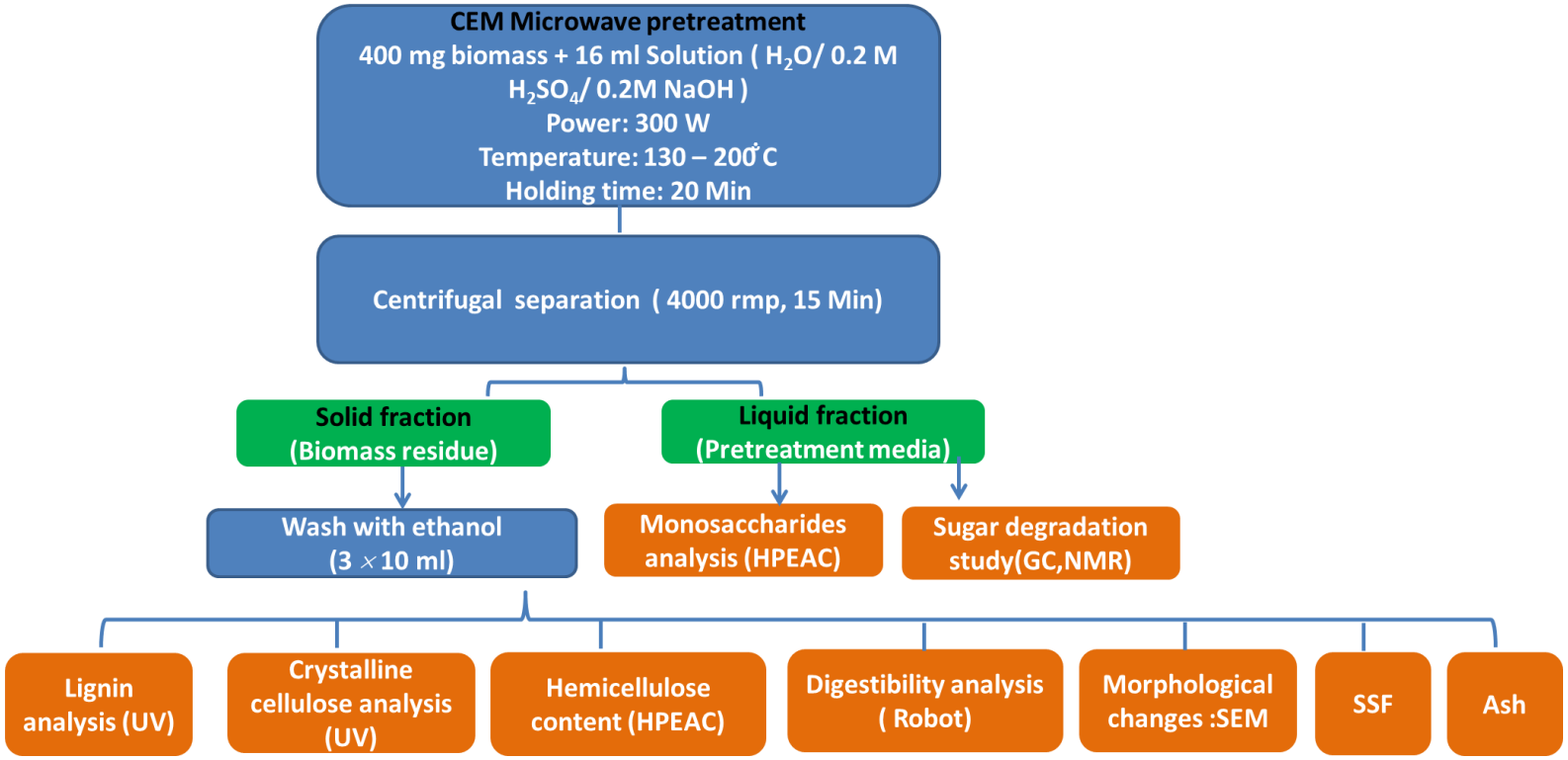


Figure 22 Experimental diagram and analysis process

University of York) by using HPEAC (Dionex IC 3000) on a Dionex Carbopac PA-20 column with integrated amperometry detection. The separated monosaccharides can be quantified by using external calibration with an equimolar mixture of nine monosaccharides standards, which were subjected to same experimental procedures in parallel with the samples. Common monosaccharides follow the molecular formula $C_nH_{2n}O_n$ (e.g $C_6H_{12}O_6$, $C_5H_{10}O_5$). Figure 5 presented a typical chromatogram of a standard sugar mixture obtained by Dionex. As can be seen, fructose, arabinose, rhamnose, galactose, glucose, xylose, mannose and galacturonic acid, are eluted. Figure 24 shows their chemical structures.

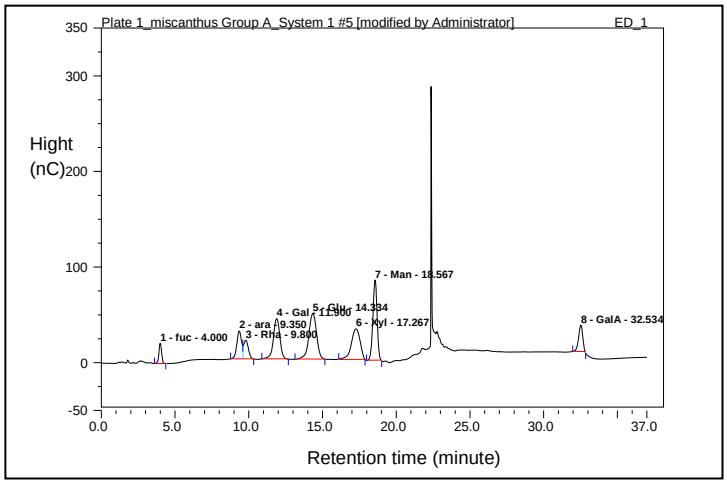


Figure 23 HPEAC of standard monosaccharides mixture

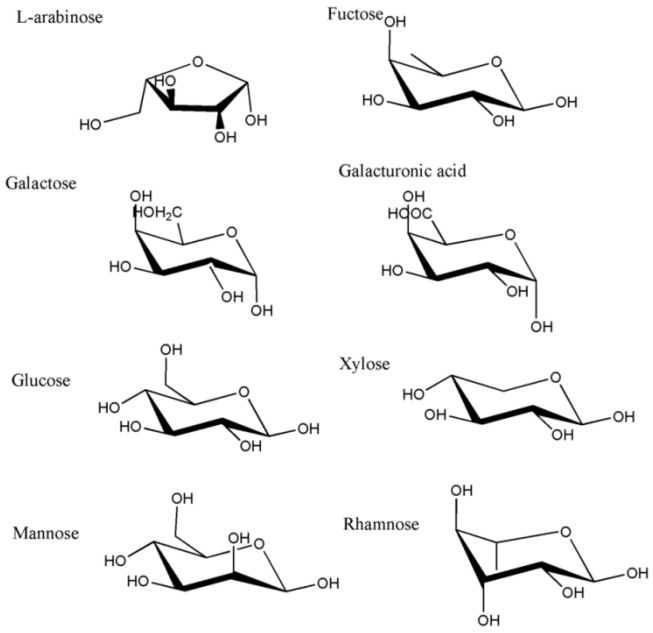


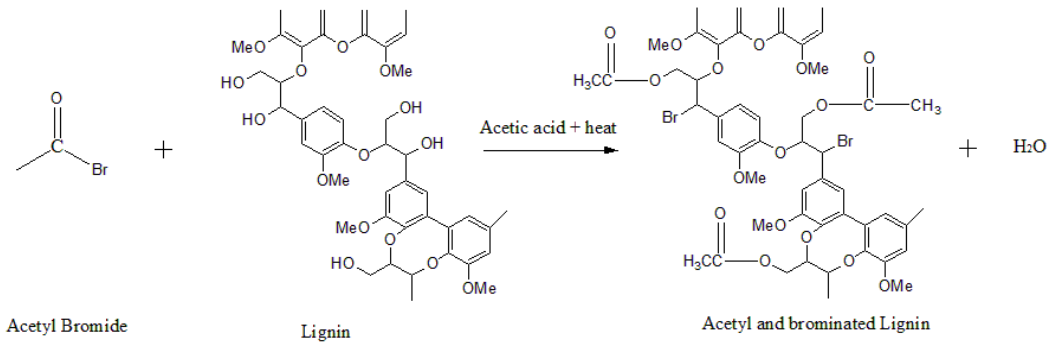
Figure 24 Chemical structure of standard monosaccharides

2.4.1 Hemicellulose analysis

Hemicellulose is one of the major components of lignocellulosic material. It is measured by using a protocol described by Foster *et al.*, which in short is to hydrolyse hemicellulose into its monomers by using TFA (Trifluroacetic acid) treatment. [168] Then the monosaccharides are measured by using Dionex and their sum represent the quantity of hemicellulose presented in the biomass sample. The monosaccharides in the pretreatment media and hemicellulose content in the solid fraction (biomass residue) and results are expressed as their average value with standard deviation. Arabinose, galactose, glucose, xylose and mannose are identified in the pretreatment media.

2.4.2 Lignin analysis method

Additionally, lignin content was measured. There are a number of lignin measurement methods which have been studied, for example, 1. indirect methods which quantify lignin present in sample by quantifying the amount of oxidant (e.g. chlorine or potassium permanganate) consumed during reaction; 2. methods involving the dissolution of lignin in certain solvents (thioglycolate or acetyl bromide) and quantification their sufficient derivatizations is measured by UV spectrometer; 3. direct method by using mineral acids to solubilize and hydrolyze carbohydrate in samples leaving the lignin residue to be determined by gravimetric measurement.[169] In this work, the lignin amount of biomass residue after pretreatment is determined by using acetyl bromide method. [170] Scheme 1 display the reaction mechanism of lignin and acetyl bromide. Compared to other methods, it is rapid and simple, especially appropriate for small sample size (3-6 mg), providing precise absorbance value for determining total lignin content and having less interference from non-lignin products.[171] However, there is a need for a well defined lignin standard with which to calibrate the method to obtain the correct absorbance values for quantifying lignin in an unknown sample.

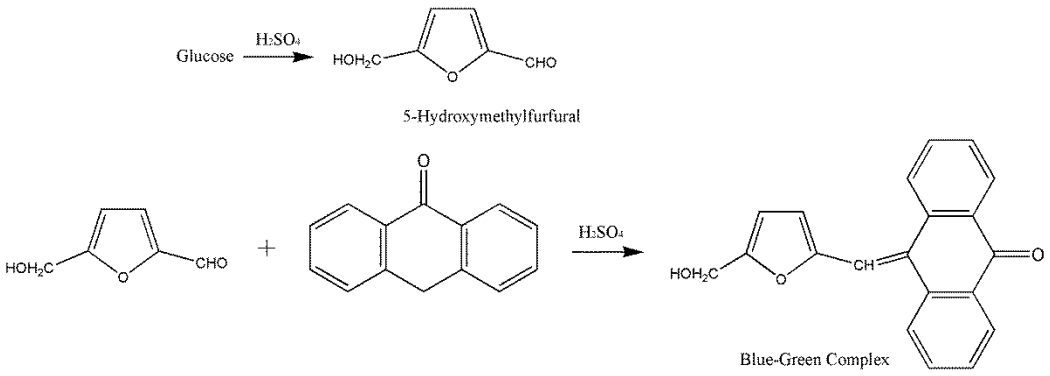


Scheme 1 Reaction mechanism for acetyl derivatization of lignin by acetyl bromide reagent under acidic conditions.

2.4.3 Crystalline cellulose analysis

The importance of measuring crystalline cellulose can be addressed from two aspects: 1. In this study, due to the low amount of amorphous cellulose, the total amount of cellulose is defined as the amount of crystalline cellulose; 2. Crystalline cellulose percentage is an significant factor influencing the following digestion process.

The crystalline cellulose percentage in biomass sample is measured by using a protocol described by Foster *et al*.[168] The biomass is firstly hydrolysed by TFA and Undegraf reagent (Acetic acid: nitric acid: water, 8:1:2 v / v) to remove hemicellulose and possible oligosaccharides and then hydrolysed with 72% H_2SO_4 to degrade crystalline cellulose into glucose.[168] The glucose is measured by using the Anthrone test (Scheme 2) and the formed complex has an absorbance at 625nm which can be quantified by UV. The Anthrone test is run with a rang concentration of glucose to produce a standard curve, which is used to quantify the crystalline cellulose content (see Figure 25 for standard glucose and biomass samples). Hence, the crystalline cellulose content in the biomass sample is analysed by measuring the glucose from its decomposition.



Scheme 2 Anthrone test pathway

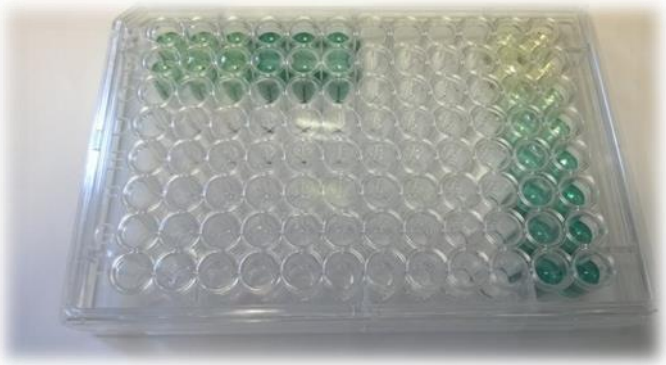


Figure 25 Anthrone test with commercial glucose and biomass samples

The saccharification of biomass was investigated by using a high throughput saccharification assay which is based on a robotic platform that can carry out the enzymatic digestion and quantification of the released sugars in a 96-well plate format (see Figure 26). The hydrolysis time is 8 hours in total. The pretreated biomass will be undergoing enzymatic hydrolysis and quantification of the released glucose.

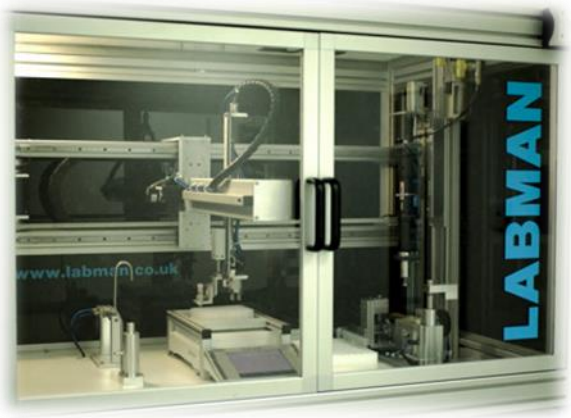


Figure 26 Robotic platform for measuring digestibility of biomass samples

2.4.4 Morphological study of biomass

Scanning electron microscopy (SEM) has been extensively used to study the cell walls after biomass pretreatment (see Figure 27). It is able to describe anatomical features and degradation at cellular and nano-resolution of biomass surface. When the specimen is irradiated with a fine electron beam (electron probe), secondary electrons are emitted from the specimen surface. Topography of the surface can be observed by two-dimensional scanning of the electron probe over the surface and acquisition of an image from the detected secondary electrons. The objective lens is used for focusing and this lens is to determine the final diameter of the electron probe. The specimen is observed at a high magnification in an electron microscope. A specimen stage stably supports the specimen and moves smoothly. Secondary electron detector is used for detecting the secondary electrons emitted from the specimen. The output signals from secondary electron are amplified and then transferred to the display unit. Inside the electron optical system and the specimen chamber should be kept at a high vacuum of 10^{-3} to 10^{-4} Pa. Therefore, these components are evacuated generally by a diffusion pump. Figure 27 shows the basic construction of a SEM. The electron gun produces an electron beam. The electron beam can be adjusted by using condenser lens.

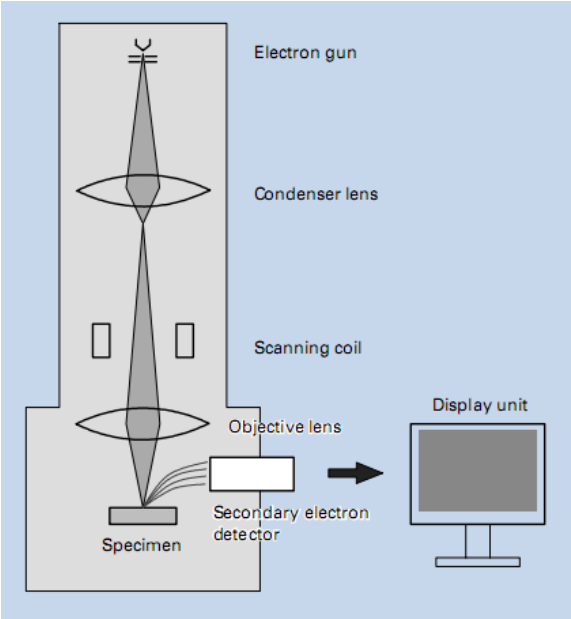


Figure 27 Basic construction of a SEM(taken from [172])

2.5 Results and discussion

As mentioned in the Introduction, both hemicellulose and cellulose are composed of digestible monosaccharides and they can be depolymerised into their monomers under certain conditions. However, due to its non-crystalline nature, hemicellulose is easier to break down than cellulose, especially in acidic conditions. The biomass material is composed of 34 ± 2.5% cellulose, 42 ± 2.8% hemicellulose, 28 ± 2% lignin and 0.83 ± 0.03 ash.

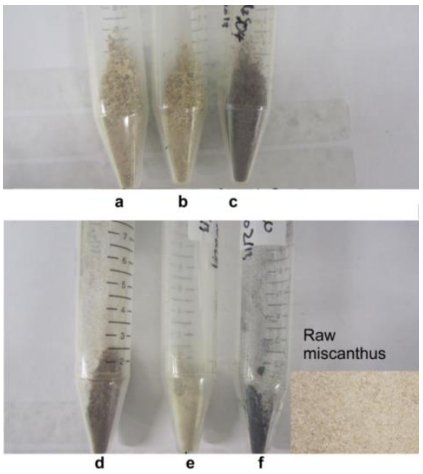


Figure 28 Biomass appearance after 130 ºC and 200 ºC pretreatment. 130 ºC: a. H_2O; b. NaOH; c. H_2SO_4. 200 ºC: a. H_2O; b. NaOH; c. H_2SO_4

Previous studies have found that the optimum temperature for hot water pretreatment for lignocellulosic materials is in the range between 160-200 ºC. In order to study the

temperature influence on pretreatment process, a range of temperatures is assayed here (130 ^{o}C to 200 ^{o}C). Figure 28 shows biomass appearance after 130 ^{o}C and 200 ^{o}C pretreatments. In comparison with 130 ^{o}C, 200 ^{o}C gives rise to darker biomass residue when H_2O and H_2SO_4 are used as pretreatments media, whereas little difference is observed in the case of NaOH pretreatment. It is important to note that acidity of water increasing with temperature and at 200 ^{o}C, the pH of pure water is close to 5.0. [101] Therefore, real acidity of experimental solutions at high temperature range could be higher than at room temperature. This may be partly the reason why the biomass appearance of water pretreated sample at 200 ^{o}C is similar to that of H_2SO_4 pretreated sample.

In the following section, the yields of reducing sugar released during pretreatment process are presented. The biomass composition results and morphological features are compared.

2.5.1 Monosaccharides analysis in the pretreatment media

As was mentioned, the monosaccharides released in the pretreatment media are analysed by using Dionex. Figure 29 shows the total amount of reducing sugar released from *Miscanthus* during pretreatment by using water, NaOH and H_2SO_4 as pretreatment media under various temperatures. In this study, it is not clear in every case whether two results are different or not. However, the statistical significance of the result has not been calculated due to the limited amount of samples. More data needs to be obtained in order to determine this.

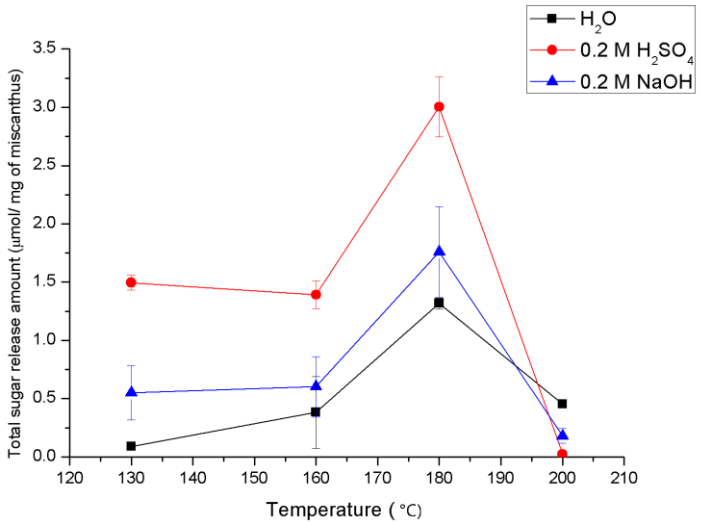


Figure 29 Total sugar amount at different temperature (130 °C, 160 °C, 180 °C and 200 °C; Holding time: 20 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

With the increasing temperature, similar patterns can be observed that the sugar production has a clear maximum at temperature around 180 °C. In this temperature range, the better sugar yields (up to 3.0 µmol/mg, yield from carbohydrate: 75.3%) were achieved in the presence of H_2SO_4 in comparison to neutral and basic solutions. However, the reducing sugar amount released from *Miscanthus* in the neutral and basic conditions is also remarkably high, (1.31 µmol/mg and 1.76 µmol/mg biomass respectively). Further increase of the pretreatment temperature to 200 °C leads to a significant drop in sugar yield for all investigated solutions. According to previous research, the reducing sugars yield could be explained by their degradation at high temperature.[173] Both the sugar production and decomposition rates increase significantly with the rising temperature. Nevertheless, from the results here, it can be predicted that decomposition rate is more sensitive to the temperature. At temperatures ca. 200 °C, the lowest sugar amounts were measured. During acid pretreatment/hydrolysis, sugar degradation is a prominently observed.[174, 175] Previous research suggested that 5-Hydroxymethylfurfural(HMF) is derived from the dehydration of hexoses and furfural is formed from dehydration of pentoses.[176] These by-products (inhibitors), have an inhibiting effect on the reaction rate during fermentation process by damaging the yeast and other microorganisms and slow down yeast metabolism and enzymatic activity. [174] In our study, levulinic acid was identified as the major degradation product (detail will be discussed in section 2.6). In the case of alkaline conditions, degradation of sugars is also observed.[177-179] Yan *et*

al. reported lactic acid yields from carbohydrate biomass by using NaOH/$Ca(OH)_2$, amounting to 27% and 20% respectively.[177]

Particular features of microwave activation of cellulose at temperature 180 ^oC have been discussed by Fan *et al*. It has been found that when the temperature is below 180 ^oC the CH_2OH groups on cellulose are hindered from interacting with microwaves when they are strongly involved in hydrogen bonding within both the amorphous and crystalline regions. When temperature is above 180 ^oC, these CH_2OH groups could be involved in a localized rotation in the microwave radiation, allowing for the transfer of microwave energy to the surrounding environment.[180] Figure 30 shows the interaction between microwave and CH_2OH groups under different temperature conditions. Microwave energy is efficiently absorbed by biomass and maximum sugar yield is achieved at 180 ^oC, which further leads to optimum sugar production.

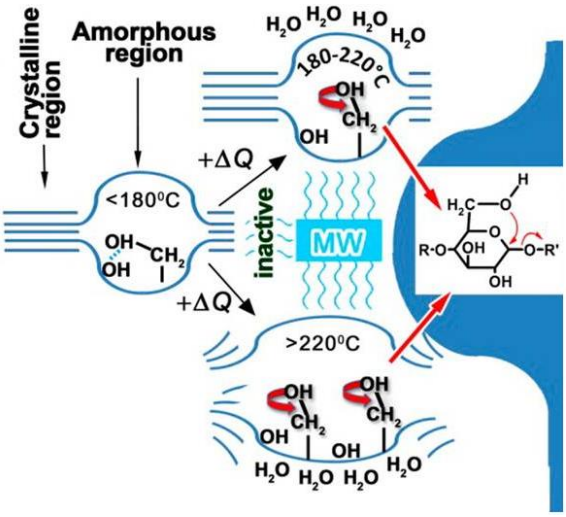


Figure 30 Cellulose and microwave interaction as a function of temperature[180]

Figure 31 shows the monosaccharide composition in the pretreatment media when temperature is 130 ^oC. The monosaccharide compositions of the liquor after acid and alkaline MW pretreatment suggest a breakdown of hemicelluloses from *Miscanthus*, where xylose is the major component. The second major constituent is arabinose, with small amounts of glucose and galactose. A minor amount of mannose is also detected. Therefore, water, alkali and acid pretreatment extracts soluble hemicellulose fractions which were composed mainly of glucuronoarabinoxylan or 1-arabino-D-xylans.[27] NaOH leads to a sugar removal of 0.55 μmol/mg biomass with xylose as major

constituent. By using 0.2 M H_2SO_4 pretreatment, high xylose yield from available carbohydrate (22%) is achieved, which is 1.5 µmol/mg biomass.

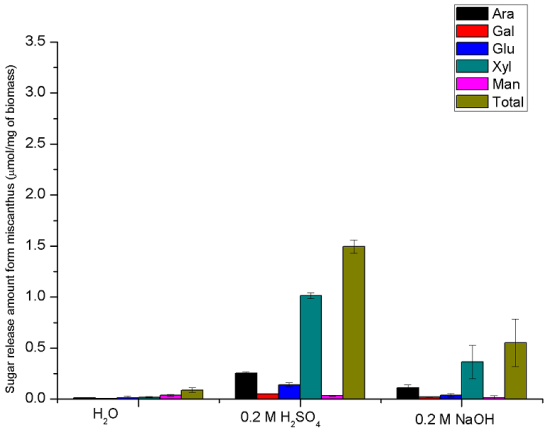


Figure 31 Monosaccharides released to pretreatment media at 130 ºC (Holding time: 20 Min; each condition was repeated triplicates and average value was reported here; error bar was reported as standard deviation)

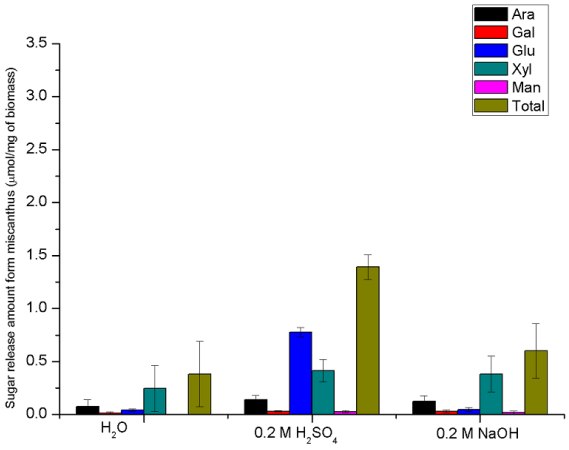


Figure 32 Monosaccharides released to pretreatment media at 160 ºC (Holding time: 20 Min; each condition was repeated triplicates and average value was reported here; error bar was reported as standard deviation)

Figure 32 shows monosaccharides released when the temperature is 160 ºC. The xylose production from 0.2 M H_2SO_4 is decreased from 1 to 0.4 µmol/mg biomass when temperature increases from 130 ºC to 160 ºC, because it degrade into other chemicals under high temperature acid condition such as furfural and levulinic acid. As can be seen, xylose is the major component in the monosaccharides mixture for water and NaOH pretreatments, suggesting hemicellulose is degraded under these conditions. In contrast in the presence of acid, a remarkable glucose yield (20%) was achieved. This glucose could be a result of both glucan and cellulose degradation.

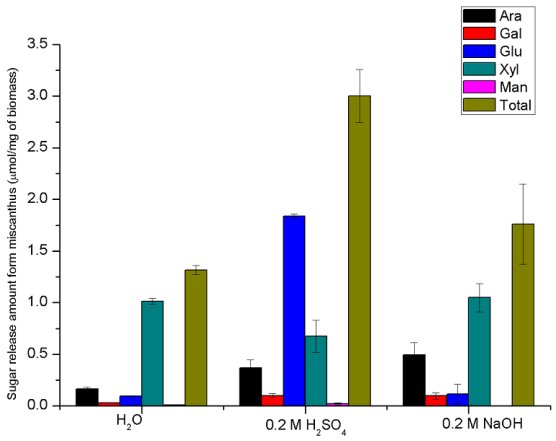


Figure 33 Monosaccharides released to pretreatment media at 180 ºC (Holding time: 20 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Similar results are observed when temperature is 180 ºC (see Figure 33). Xylose yields from available carbohydrate are enhanced up to 1 μmol / mg biomass when water and NaOH are used as pretreatment media, suggesting higher temperature can facilitate hemicellulose breakdown. H_2SO_4 is able to give maximum glucose yield from available carbohydrate (1.8 μmol / mg biomass), due to the efficient decomposition of cellulose under high temperature. This result highlighted the distinctive performance of H_2SO_4 and NaOH on biomass, demonstrating that sugar can be selectively produced by using H_2SO_4 or NaOH at a controlled condition. Figure 34 shows very low amount of reducing sugar present in the pretreatment media when temperature is 200 ºC, due to the further degradation of sugar.

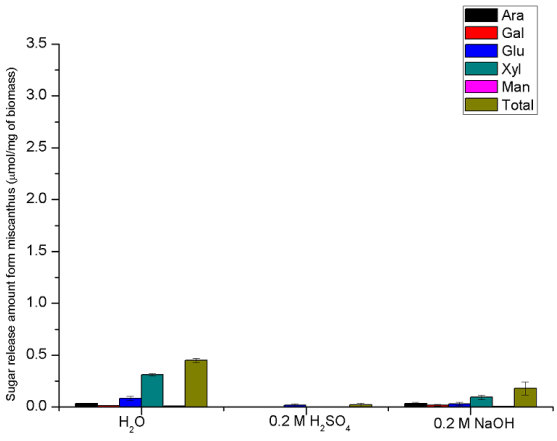


Figure 34 Monosaccharides released to pretreatment media at 200 ºC (Holding time: 20 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation))

Conventional heating pretreatment method has been studied before. As can be seen from Table 12, various materials have been used under various conditions. Gia *et al*., Guo *et al*. and Funazukuri obtained promising sugar production. However, the conditions they used involved either very long holding times or high temperatures up to 543 K. *Hu et al*. used relatively mild conditions and the reducing sugar yield is very low. In comparison with these studies, the microwave assisted acid/alkali pretreatment presented here leads to much better sugar removal with lower concentration of pretreatment media and shorter reaction time.

Table 12 Conventional heating pretreatment has been reported

Author	Pretreatment conditions	Main results	Reference
Gia *et al.*	*Miscanthus* 1-3% H_2SO_4 Temperature: 121 ^oC Time: 10-180 Min	With the increasing severity factor xylose yield was firstly improved then gradually declined. Maximum xylose yield from available xylan was 70-75%.	[156]
Guo *et al.*	Bagasse 1%, 2%, 4% (w/w) H_2SO_4 Temperature: 130 ^oC Time:15 Min	0.9 g xylose/g xylan and above was achieved when the H_2SO_4 concentration was increased from 2% to 4% during pretreatment. Glucose release also increased with the increasing concentration of H_2SO_4	[157]
Hu *et al.*	Switchgrass NaOH solustion: 0.125-0.75 M Temperature: 190 ^oC Time: 30 Min	Total sugar release was about 8 g/ 100 g biomass. The major sugar component is xylose.	[181]
Funazukuri	Cotton cellulose 1% Formic acid Temperature: 503-543 K Time: 0-60 Min	Yields of glucose form the three components increased with increasing time and temperature. The maximum yield of 88 % for total sugar was obtained after 20 min at the highest temperature (543 K).	[182]
This study	*Miscanthus* 0.2 M H_2SO_4 0.2 M NaOH Temperature: 180 ^oC Time 5-30 Min	Maximal sugar yield is 3 μmol/mg biomass, by using 0.2 M H_2SO_4 for 20 Min (yield from carbohydrate in biomass: 75.3%). Selective produce glucose (1.8 μmol/mg biomass; maximal yield: 47.6%) or xylose (1 μmol/mg maximal yield: 21%) by using H_2SO_4 or NaOH as pretreatment media.	

Figure 35 Conventional pretreatment acid digestion vessel (Parr Instruments, Moline, IL)

In order to compare, conventional heating pretreatment were performed in a high pressure vessel and heated in the oven (see Figure 35). As highlighted before, 180 ^{o}C is the optimum temperature condition when microwave is applied. Hence, temperature here was controlled at 180 ^{o}C and hold time was 40 Min. Visually, biomass samples change colour differently after pretreatment and H_2SO_4 lead to a darker sample than others, which is similar to that of MW pretreatment when temperature is 130 ^{o}C (compare Figure 36 with Figure 28). Figure 37 shows the monosaccharides released in pretreatment media during conventional heating pretreatment process. The reducing sugar production from water and NaOH pretreatment is 0.21 and 0.16 μmol / mg biomass respectively, with xylose and glucose as major sugar constitutions. H_2SO_4 contributes to better reducing sugar production (0.24 μmol / mg biomass), in which xylose is the major constituent. Compare Figure 37 to Figure 31, it is noticed that the sugar constituent percentage here is similar to that of MW pretreatment when pretreatment temperature is 130 ^{o}C. It suggests during both microwave heating and conventional heating pretreatments, hemicellulose is degraded in preference to cellulose and give xylose as major sugar constituent. Less reducing sugars are released into pretreatment media by using conventional heating method. The reducing sugar release from MW pretreatment is 12.5 times more than that of conventional heating pretreatment within half time.

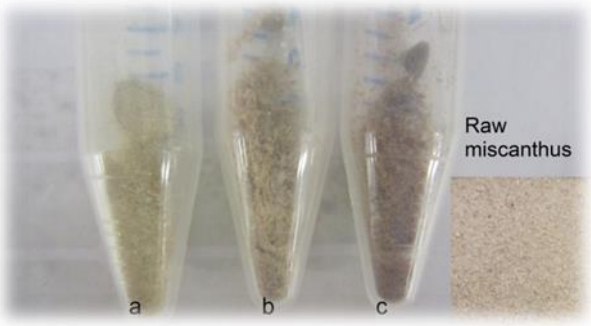


Figure 36 Biomass samples pretreated with different media. a. H_2O; b. NaOH; C. H_2SO_4

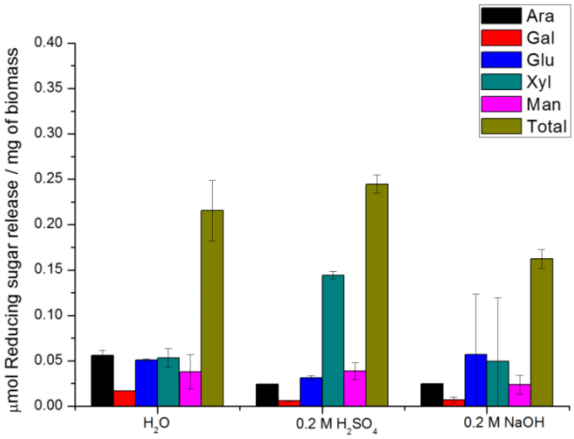
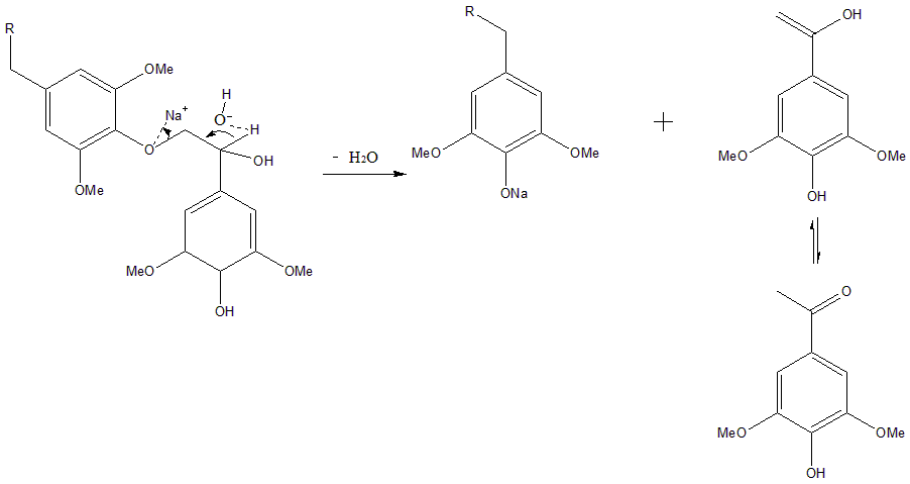


Figure 37 Reducing sugar release during conventional pretreatment at 180 ºC for 40 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

More sugar released into pretreatment media by using MW pretreatment, because the polar part of biomass is significantly involved in the alignment with oscillating microwave field, resulting in more efficient biomass degradation. Moreover, with microwave assistance, 180 ºC is the optimal temperature to efficiently remove sugars from biomass during pretreatment process. The possible explanation is that cellulose phase transition occurs, leading to a sofer cellulose (less crystalline cellulose). Below 180 ºC the polar groups in cellulose have less freedom to rotate easily, resulting in a less effective interaction. Above 180 ºC, the number of groups capable of rotating increases particularly, leading to a more effective interaction between cellulose and microwave[183]. Hence, they can act as 'molecular radiators' allowing for the energy transfer of microwave energy to their surrounding environment.[180]

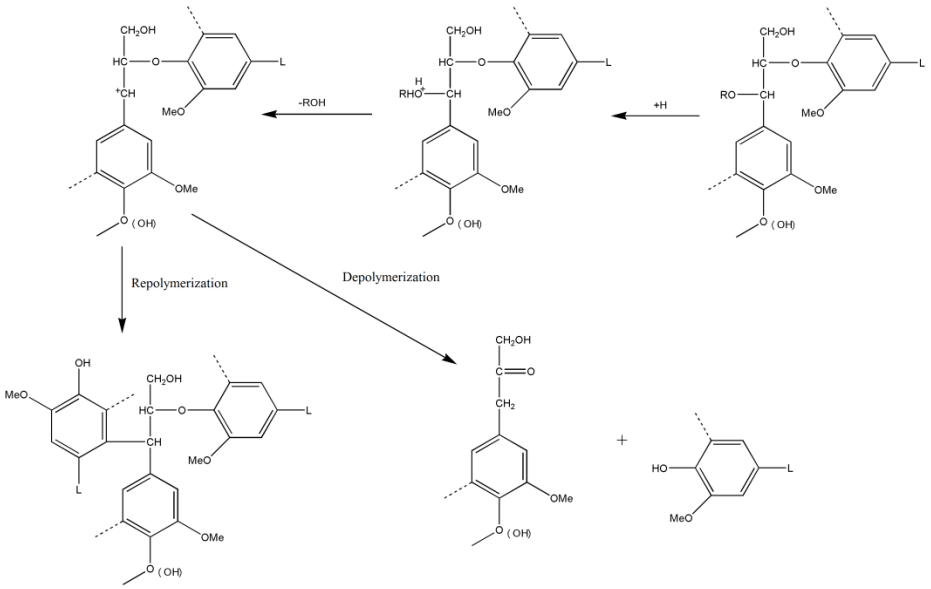
2.5.2 Lignin amount

Lignin is a complex and large molecule, composing cross-linked polymers of phenolic monomers. After their biosynthesis, monolignols are transported to the cell wall to form a complex three dimensional molecular architecture that contains a variety of bonds with typically around 50% β-O-4 ether linkage (as it can be seen from Chapter 1, Figure 10). Although it has multiple potential to use as a product feedstock or as a fuel on their own, it is also generally considered as a barrier for efficient enzyme hydrolysis of biomass.[184] Hence, the presence of lignin is considered one of the most important factors limiting the hydrolysis of lignocellulose.[12] Alkaline and oxidation treatments, such as alkaline peroxide and lime and oxygen, have been utilized to remove lignin.[18, 80, 116]



Scheme 3 Cleavage of the β-O-4 bond and formation of syringyl derivatives

It was suggested that under alkaline condition, cleavage of the β-O-4 ether bond takes place heterolytically through a six-membered transition state, in which the sodium cation and hydroxide ion participate (see Scheme 3). The sodium cations catalyse the reaction by forming cation adducts with lignin and polarizing the ether bond, resulting in an increased negative partial charge on the oxygen atom and reduced energy required for heterolytic bond cleavage[185]. The acid-catalyzed depolymerization also focused on the cleavage of β-O-4 bond of the lignin. The lignin model compounds show α-ether elimination reactions resulting in benzylic carbonium intermediate products, which quickly rearrange into other chemicals or undergo repolymerization (see Scheme 4).[186]



Scheme 4 Cleavage of the β-O-4 bond and formation of benzylic carbonium intermediate for depolymerisation and repolymerization. [187]

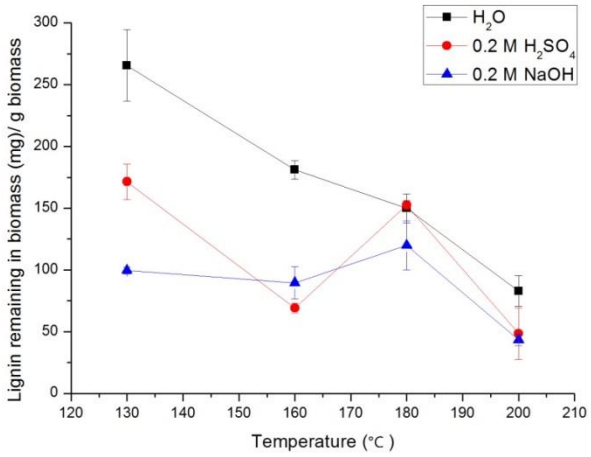


Figure 38 Lignin amount after pretreatment under different temperature (Holding time: 20 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

In untreated *Miscanthus*, lignin represents 304 mg / g biomass (Figure 38). NaOH removed lignin more efficiently than water at lower pretreatment temperatures. All pretreatments remove the similar amount of lignin at 200 ºC. The lignin removal is up to 221 mg/g of biomass when the temperature was 200 ºC in all pretreatments. At 180 ºC, the lignin content of biomass pretreated with H_2SO_4 was considerably higher. This could be explained by lignin extraction from the inner regions of the cell wall and subsequent condensations and re-deposition on the surface as reported for wood samples. [187] Scheme 4 shows that under H_2SO_4 conditon, carbonium ions was produced and can lead

to repolymerisation. This would explain the higher lignin content after H_2SO_4 pretreatment. At the same time, it probably changed in to other components. Li *et al.* reported that depolymerisation and subsequent re-polymerization of lignin occurs, with increasing severity of steam pretreatment of aspen wood. [187] Acetic acid assisted pretreatment of aspen wood also lead to similar results.[187]

Xu *et al.* studied conventional NaOH pretreatment for Switchgrass and the results showed that with the increasing temperature of pretreatment (50-121 ^oC) or NaOH cocentration, increasing amount of lignin was removed from biomass. Optimally, 85.5% lignin was reduced by 2% NaOH when temperature was 121 ^oC within 1 hour. In contrast, the results in the current study are more efficient with lower concentration of NaOH and faster. [188] At higher temperature (160-220 ^oC), increasing severity of NaOH pretreatment also leads to better lignin removal, which is in good agreement with the results in this study. [189] Gomez *et al.* studied conventional thermo-chemical pretreatment for *Miscanthus* (180 ^oC, 40 Min) and similar amount of lignin is removed by 0.2 M NaOH pretreatment, whereas more lignin (210-240 mg/ g biomass) is presented in the biomass after water and 0.2 M H_2SO_4 pretreatments[190].

Table 13 shows the lignin amount of pretreated biomass by using conventional heating pretreatment. In terms of water pretreatment, conventional heating method remove similar amount of lignin, compared with that of microwave heating method when its temperature is 130℃. With higher temperature, microwave assisted pretreatment removed more lignin. With NaOH and H_2SO_4 pretreatments, microwave heating method is more efficient than conventional heating method (lower temperature and shorter reaction time). Due to its chemical structure, lignin is much less polar than polysaccharides and significant poorer microwave absorber than cellulose and hemicellulose.[34] However, in our study, microwave assistance promotes lignin removal. The explanation could be the ester linkages between polysaccharides and lignin are influenced by microwave effect, which leads to its cleavage and removal of lignin.

Table 13 Lignin mount present in 1g biomass residue after conventional heating pretreatment

	H_2O	NaOH	H_2SO_4
Lignin amount (mg)	119.48 ± 6.76	50.61 ± 4.756	117.8 ± 9.06

2.5.3 Hemicellulose analysis

Untreated *Miscanthus* has 42% of hemicellulose, comprising arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid, with xylose and glucose as major component. Figure 39 shows the hemicellulose percentage in the biomass residue after various microwave assisted pretreatments. The results show that it suffers different degrees of removal depending on the pretreatment conditions. When the temperature is 130 ^{o}C, hemicellulose percentages slightly decrease after water and NaOH pretreatment, whereas H_2SO_4 reduces hemicellulose percentage in the biomass residue to 21% (see Figure 39a). When temperature increases to 160 ^{o}C, the hemicellulose percentages are 32% and 31.5% respectively by using water and sodium hydroxide pretreatment (see Figure 39b). It is significantly reduced to 14.7% by 0.2 M using H_2SO_4.

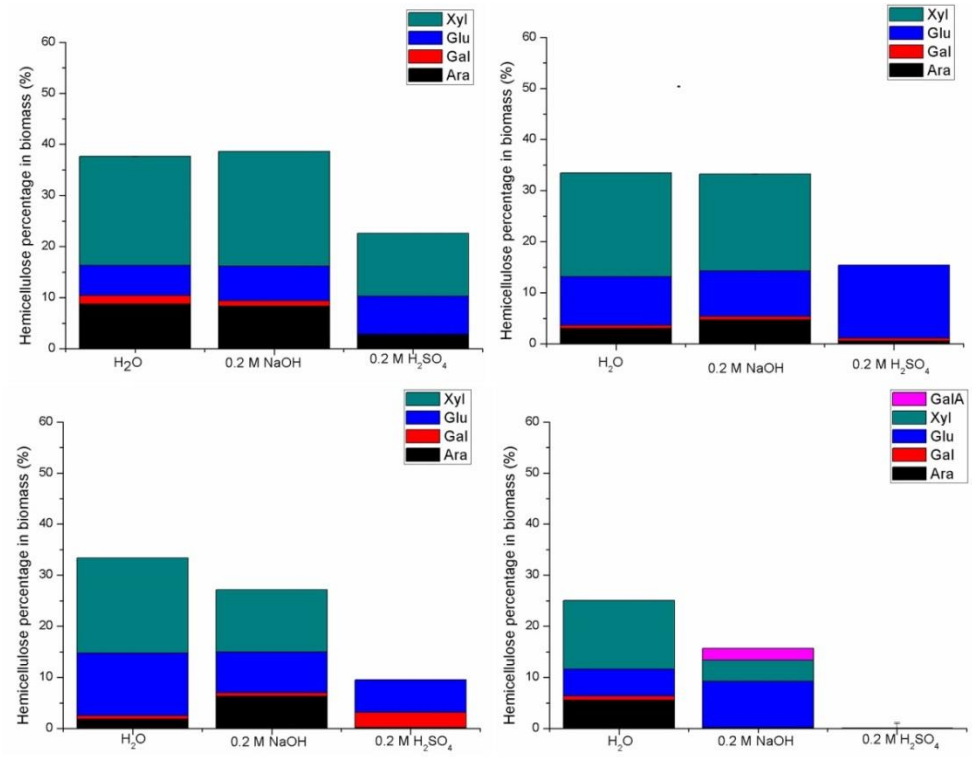


Figure 39 Hemicellulose percentages after different temperature pretreatments. a.130 ^{o}C; b. 160 ^{o}C; c. 180 ^{o}C; d. 200 ^{o}C; Holding time: 20 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

When temperature is further increased to 180 ^{o}C, more hemicellulose is removed from biomass and the hemicellulose percentage is only 8.8% by using H_2SO_4 (see Figure 39c). When temperature is further increased up to 200 ^{o}C, all the hemicellulose is removed completely from biomass by H_2SO_4 (see Figure 39d). Therefore, the increasing temperature promotes hemicellulose removal process. Water and NaOH pretreatments

remove similar amount of hemicellulose from biomass, which is in agreement with the previous results of monosaccharides analysis in pretreatment media (see Figure 31 to Figure 34). H_2SO_4 more efficiently removed hemicellulose into pretreatment media. Figure 40 presents the hemicellulose percentages after conventional heating pretreatment (180 ^oC, 40 Min). As can be seen, hemicellulose is effectively removed from biomass. Under the H_2SO_4 conditions, almost all the hemicellulose is removed from biomass. Compared to conventional pretreatment, less hemicellulose is removed by MW pretreatment at 180 ^oC. When MW pretreatment temperature is 200 ^oC, the hemicellulose percentage presented in biomass is similar to that of conventional heating pretreatment (temperature: 180 ^oC). The reason could be that holding time has a more important influence on hemicellulose removal than temperature and heating methods. However, it would be interesting to pretreat *Miscanthus* under conventional heating method for shorter holding time (e.g. 5-10 Min).

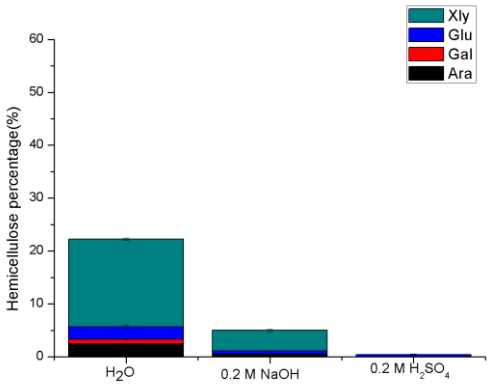


Figure 40 Hemicellulose percentages after conventional heating pretreatment (180 ^oC, 40 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

2.5.4 Crystalline cellulose percentage

Crystalline cellulose percentage is a vital property for biomass and provides mechanical rigidity and toughness for composite material. It also influences wall extensibility, which is an important determinant of plant growth and differentiation.[191] Pretreatment for biomass is an important step to make cellulose more amenable and accessible to cellulose enzymes, thereby enhancing glucose production in the following digestion process.[17] Completely disordered or amorphous cellulose is able to be hydrolysed at a much faster rate than partially crystalline cellulose.[17]

Crystalline cellulose percentage in untreated *Miscanthus* is 36%. Figure 41 presents the crystalline cellulose percentages in microwave pretreated biomass residue. As can be seen, water pretreatment has little effect on crystalline cellulose percentage when the pretreatment temperature is 130 ºC. It increases to 44% and 45% when the pretreatment temperature is 160 ºC and 180 ºC respectively, because lignin and hemicellulose are slightly removed. When pretreatment temperature is 200 ºC, the crystalline cellulose percentage is further brought up to 53%. By using H_2SO_4 pretreatment, crystalline cellulose percentages in solid fraction are similarly enhanced when temperature is between 130 ºC to 180 ºC, but it remarkably drops to 9% when pretreatment temperature is 200 ºC. When biomass is pretreated under 130 ºC to 180 ºC, similar amount of lignin and hemicellulose are removed. With the more severe acid condition (200 ºC), crystalline cellulose is significantly degraded and carbonized (biomass morphological characteristics can be found in Figure 47 and Figure 48). In the case of NaOH pretreatment, the crystalline cellulose percentage in solid fraction is greatly enhanced to 67% when the pretreatment temperature is 180 ºC, which is in good agreement with the earlier discussion that hemicellulose is effectively removed at this condition. When the pretreatment temperature is 200℃, it is 39%, because NaOH can alter cellulose structure and increase amorphous cellulose content in the biomass, making it more disordered.[17]

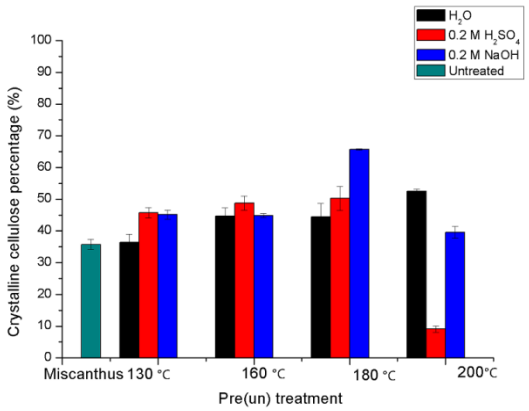


Figure 41 Crystalline cellulose percentage after various pretreatment(each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Table 14 shows crystalline cellulose percentage of biomass residue by using conventional heating pretreatment. Compared with microwave assisted pretreatment, conventional pretreatment leads to lower crystalline percentage of pretreated biomass residue. The reason could be they have different heating mechanisms. Under conventional heating, biomass structure is disrupted by heat penetration from outside to inside and under this condition cellulose probably changes from crystalline to

amorphous structure. However, under microwave condition the heat is generated by the interaction between polar part of biomass and oscillating microwave field. The cellulose fibres could be represented as ionic conducting (crystalline) and non-conducting (amorphous).[183] A very ordered hydrogen bonded network is contained in the crystalline cellulose which could lead to a proton transport network under an electromagnetic field.[183] Therefore, the crystalline cellulose kept the structure at the beginning and is able to act as an active microwave absorber, promoting the biomass decomposition. Therefore, along with the process of lignin/hemicellulose removal, crystalline cellulose percentage goes up, enhancing the microwave absorbing effect, leading to better reducing sugar release than conventional heating pretreatment.

Table 14 Crystalline cellulose percentage after conventional heating pretreatment

	H_2O	H_2SO_4	NaOH
Crystalline cellulose percentage (%)	24.72 ± 0.32	19.12 ± 1.2	24.58 ± 0.86

2.5.5 Digestibility analysis

As we know, lignocellulosic biomass is widely considered as a promising feedstock to reduce the world's reliance on petroleum for liquid transportation fuels and other chemicals, due to its cheap price, abundance and energy rich polysaccharides that make up approximately 75% of its mass. Theoretically, these polysaccharides can be broken down to produce sugars (saccharification) from which a range of useful products, such as biofuels, bioplastics, fine and bulk chemicals, food and feed ingredients. However, one of the greatest barriers to realize the potential of lignocellulose is its digestibility. Hence in this section, the saccharification of biomass was investigated by using a high throughput saccharification assay which is based on a robotic platform that can carry out the enzymatic digestion and quantification of the released sugars in a 96-well plate format. The total hydrolysis time used is 4 hours and the values showing in Figure 64 is based on the amount of glucose released from per gram of un / pretreated biomass during one hour of enzymatic digestion.

After the pretreatment and removal of the soluble fractions, the insoluble biomass residue were subjected to digestibility analysis using a protocol reported by Gomez *et al.* [192]. Figure 42 shows that *Miscanthus* digestibility is increased after all the microwave assisted pretreatments, albeit to widely differing extents. For untreated *Miscanthus*, the digestibility of *Miscanthus* is 10.25 nmol/ mg biomass.hour, meaning 10.25 nmol glucose is produced from 1 mg biomass during each hour of enzymatic hydrolysis (the

total enzymatic hydrolysis is 4 hours). For water pretreatment, the digestibility is slightly increased when temperature is 130 ^{o}C. It is further enhanced to 40-50 nmol/ mg biomass.hour when temperature increases from 160 ^{o}C to 200 ^{o}C. In the case of H_2SO_4, the digestibility is marginally increased when hold temperature is 130 ^{o}C and thereafter it declines. NaOH pretreatment remarkably improve *Miscanthus* digestibility, due to hemicellulose and lignin being efficiently removed from biomass. Acid and alkali pretreatments lead to rather different biomass digestibility. As we know, lignin plays a synergistic and negative role for sugar production by the enzymic hydrolysis.[11] Due to the delignification effect of NaOH, alkaline pretreated *Miscanthus* with low lignin percentage and higher cellulose percentage could produce more sugar in the hydrolysis process.

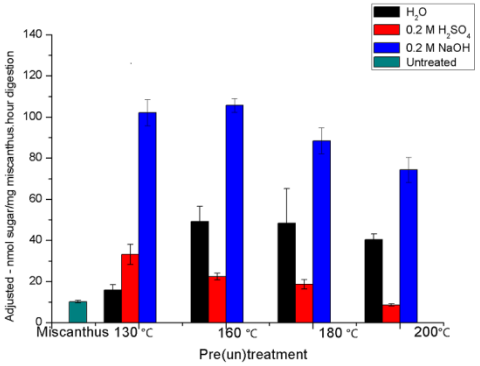


Figure 42 Digestibility of biomass after various pretreatments (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

As has been mentioned previously, there is a significant soluble sugar yield during H_2SO_4 assisted pretreatment process. The release of sugar during the acid pretreatment limits the availability of substrates for subsequent enzyme hydrolysis. Otherwise, hot acid treatment can contribute to the formation of inhibitors of enzyme hydrolysis and therefore reduce sugar release.[100] In this work, the substrates are rinsed after pretreatment prior to enzyme saccharification. Hence, the later explanation seems unlikely. Therefore, less sugar is produced from H_2SO_4 pretreated *Miscanthus* during sacccharification process, because it has relatively high lignin percentage and lower cellulose percentage. Several factors affecting the absolute enzymatic hydrolysis rate were outside the scope of the present study and were not optimised. These include: (1) solids loading; (2) enzyme loading; (3) the effect of various additives; (4) the enzyme cocktail used; and (5) the temperature and duration of digestion.

Table 15 shows digestibility of *Miscanthus* pretreated with conventional heating method. When water is used as pretreatment media, better digestibility is obtained by using microwave assisted pretreatment in comparison with conventional heating pretreatment. However, when NaOH is used as pretreatment media, conventional heating gives rise to better digestibility. The result is expected, due to the fact that more digestible sugar are released during the microwave assisted pretreatment process, meaning less reducing sugar remained in the biomass. However, when H_2SO_4 is used as pretreatment medium, the biomass digestibility is similarly low. As discussed before, with conventional heating pretreatment, a small amount of reducing sugars is released into the pretreatment media and there is a significant drop of crystalline cellulose percentage when H_2SO_4 is used as pretreatment media. Theoretically, in this case, the digestibility of biomass pretreated by conventional heating method should be higher than that of microwave heating method. However, the fact is both heating methods lead to similarly low digestibility by using H_2SO_4. As mentioned before, the inhibitor effect on enzyme is unlikely, due to the washing-up step. Hence, the only explanation is that hemicellulose and cellulose are still decomposed by using conventional heating method, but they are absent from monosaccharides analysis due to their being decomposed during the long holding time pretreatment. Therefore, the analysis of decomposition production from conventional heating pretreatment and inhibitors in the biomass residue would be worth to study in the future.

Table 15 Digestibility of biomass pretreated by using conventional heating

	H_2O	NaOH	H_2SO_4
Digestibility (nmol/ mg biomass.hour)	20.17 ± 1.89	150.37 ± 18.5	32.9 0± 1.6

2.5.6 SEM analysis

Scanning electron microscope is applied to study the morphological characteristics of un / pretreated *Miscanthus*. Figure 43 shows micrographs of the surface of raw *Miscanthus* particles, which present a flat and smooth surface under both magnifications.

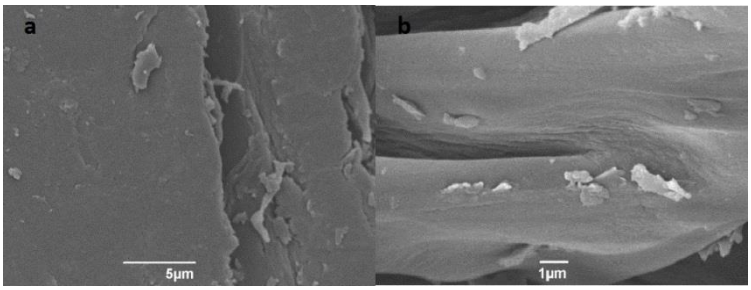


Figure 43 Surface images of the untreated *Miscanthus* obtained by SEM. Flat surface of a fibre showing, a. bar scale: 5 µm; b. bar scale: 1 µm.

Images from *Miscanthus* samples pre-treated with 0.2 M NaOH under various temperatures are presented in Figure 44. Compare Figure 44a and Figure 43a, it can be found that when pretreatment temperature is 130 ^{o}C, parallel strips and small particles appear on the biomass surface. These particles could be 'lignin deposit' which has been reported in previous studies on hydrothermal pretreatment of ligniocellulosic material, both under acid or alkaline conditions. [28, 193-195] These lignin aggregates, formed by lignin extraction from the inner regions of the cell wall, followed by condensation due to pH conditions and re-deposition on the surface. Under different NaOH concentration conditions, different amounts and appearances of lignin droplets are observed. At 130 ^{o}C, there is small amount of 'lignin deposits' on the biomass surface, indicating that this temperature has a mild influence on the biomass structure (see Figure 45 for the image under enhanced magnification). When the temperature is increased to 160 ^{o}C, the other type of lignin deposits matrix appeared on the biomass surface and they tend to combine together, indicating NaOH has a stronger performance on biomass structure at 160 ^{o}C than that of 130 ^{o}C. In contrast, 180 ^{o}C has a rather distinctive performance on biomass. Compare to raw *Miscanthus*, the biomass surface becomes rough, with more exposed cellulose fibres, due to the removal of hemicellulose and lignin (compare Figure 39c to Figure 43a). When pretreatment temperature is enhanced to 200 ^{o}C, a different type of 'lignin deposits' matrix is observed on the biomass surface, which shows a large amount of lignin droplet compared to others (see Figure 44d). Under higher magnification (see Figure 45d), lignin deposits are tend to combined together to form a network when temperature is 160 ^{o}C, whereas they are more separated from each other when temperature is 200 ^{o}C.

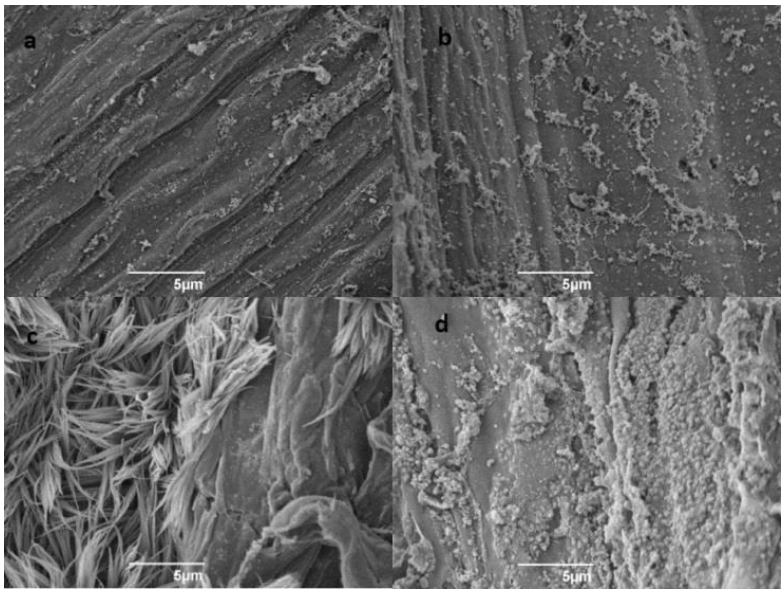


Figure 44 Surface images obtained by SEM on *Miscanthus* treated with 0.2M NaOH pretreatment under various temperature; microwave power: 300 W; magnification scale bar: 5µm. a. 130 ºC; b. 160 ºC; c. 180 ºC; d. 200 ºC.

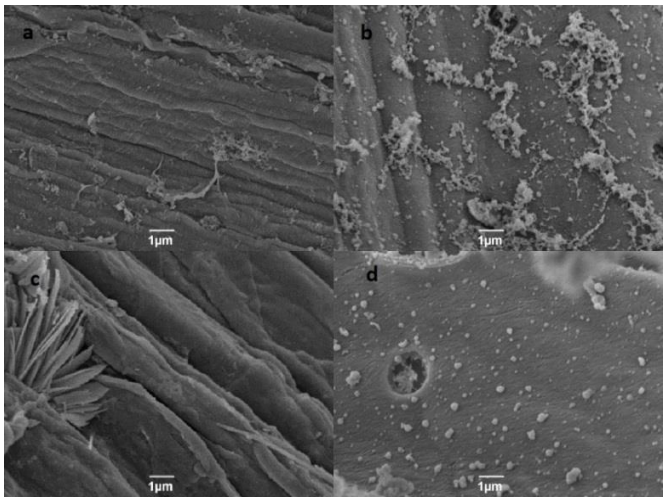


Figure 45 Surface images obtained by SEM on *Miscanthus* treated with 0.2M NaOH pretreatment under various temperature; microwave power: 300 W; magnification scale bar: 1µm. a. 130 ºC; b. 160 ºC; c. 180 ºC; d. 200 ºC.

Therefore, despite the fact that the lignin content in the solid fraction of *Miscanthus* are similarly low after various 0.2 M NaOH pretreatments under different temperature (Figure 38), alkali performances on biomass surface are remarkably distinctive under different temperature. This may influence the sugar removal process during the pretreatment, as the reducing sugar release firstly increases then declines when the temperature increases from 130℃ to 200℃ and maximum reducing sugars are released from biomass during pretreatment when the temperature is 180 ºC(see Figure 29). Here in SEM study, when temperature is 180 ºC, the lignin deposit is absent from biomass

surface, which could be one of the reasons contributing to a good sugar removal during pretreatment.

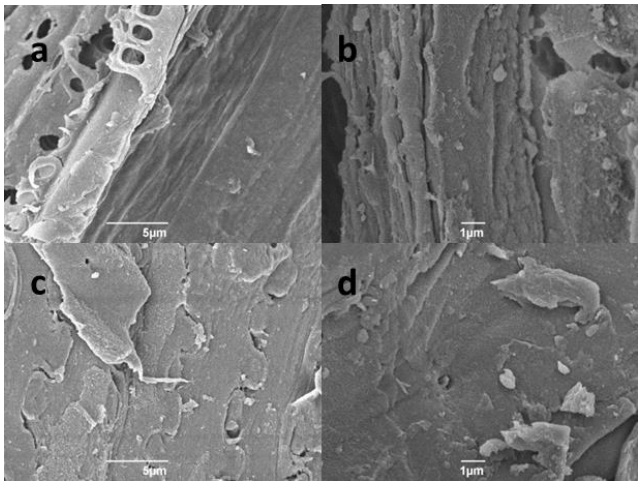


Figure 46 Surface images obtained by SEM on *Miscanthus* treated with H_2O and 0.2M H_2SO_4 pretreatments under 180℃; a. H_2O, bar scale: 5 µm; b. H_2O, bar scale: 1 µm;c. 0.2M H_2SO_4 bar scale: 5 µm; d. 0.2M H_2SO_4 bar scale: 1 µm.

Figure 46 presents the biomass surface features when H_2O and 0.2 M H_2SO_4 are used as pretreatment media when temperature is 180 ºC. Both water and 0.2 M H_2SO_4 has little influence on biomass structure, as the surface keeps smooth and flat as untreated *Miscanthus*, without appearance of cracks or strips which appeared in NaOH pre-treated samples. Hence, these conditions are too mild to bring any surface change of biomass. However, in lignin discussion, we preclude that the lignin probably redeposits back on the biomass surface after H_2SO_4 pretreatment at 180 ºC. There was no obvious 'lignin deposits' appearing on the biomass surface. Selig *et al.* pretreated maze stem with 0.7% H_2SO_4 under 170 ºC for 12.5 Min and they observed the presence of spherical formations on the surface of the residual biomass. They proposed that upon melting, lignin in biomass becomes fluid, coalesces and potentially can move throughout the cell wall matrix.[196] Donohoe *et al.* observed similar 'lignin droplets' under similar conditions (0.8% H_2SO_4 under 150 ºC for 20 Min) and SEM, TEM, NMR and FTIR were used to support the hypothesis.[184] Under 180 ºC and H_2SO_4 pretreatment condition, the lignin droplets were not observed, but a higher lignin content was measued in lignin quantification (see Figure 38). Therefore, the other lignin quantification methods (such as thioglycolate lignin method or Klason lignin measurement [169]) should be try in the future to check the lignin content in the biomass, as acetyl bromide lignin method overestimates lignin content due to the presence of furfural during pretreatment. [197]

As can be seen from Figure 47, biomass presents different colour after pretreatments, especially in the case of H_2SO_4 pretreatment which leads to a carbonized degradation of biomass sample. Figure 48 presents the biomass surface features of *Miscanthus* undergoing water pretreatment and 0.2 M H_2SO_4 pretreatment, when the pretreatment temperature is 200℃. Compare Figure 48a to Figure 43a, it can be noticed that parallel strips appear on the biomass surface, in addition to the appearance of the other form of droplets . When the magnification bar scale is 1 μm (see Figure 48c), it can be observed that the size of these deposits are larger than that of 0.2 M NaOH pretreatment under 200 ^oC (see Figure 45d) and they also tend to separate from each other. These could be the lignin deposits mentioned by Selig and Donohoe. Overall, it can be observed that multiple classes of 'lignin droplets' can be distinguished by morphological criteria, such as size, shape and surface texture. This variability is not surprising, due to the complex structure of lignin molecules and plant cell wall matrix.

However, sample treated with 0.2 M H_2SO_4 is completely carbonized. Macroscopically, the sample becomes a black powder like coal (Figure 47) and under microscope, it presents a degraded aspect, with spherical particles typical of burned sample. Catalysed degradation of sugars to furans to hydrothermal carbon explains the very low amount of sugar released from the biomass samples treated with higher concentration acid (Figure 29). [198]

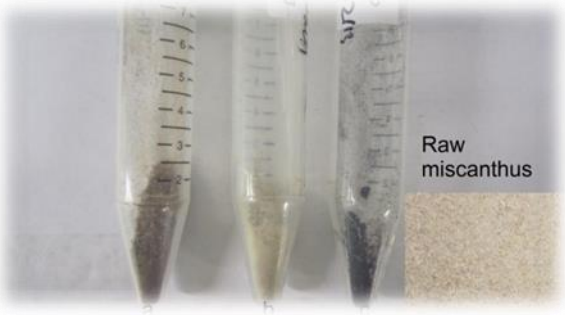


Figure 47 Biomass appearance after microwave assisted pretreatment. a. H_2O; b. NaOH; C. H_2SO_4 (temperature: 200 ^oC; hold time: 20 Min)

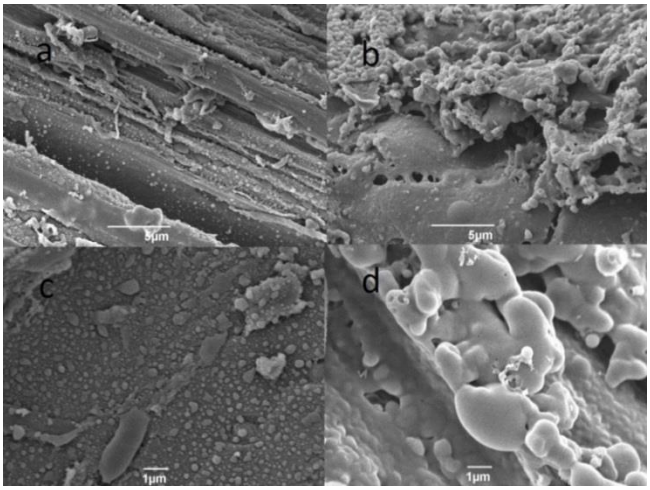


Figure 48 Surface images obtained by SEM on *Miscanthus* treated with water and 0.2 M H_2SO_4 pretreatment at 200 ºC; microwave power: 300 W. a. Water pretreatment; magnification bar scale is 5 µm; b. 0.2 M H_2SO_4 pretreatment; magnification bar scale is 5 µm ; c. Water pretreatment; magnification bar scale is 1 µm; d. 0.2 M H_2SO_4 pretreatment; magnification bar scale is 1 µm

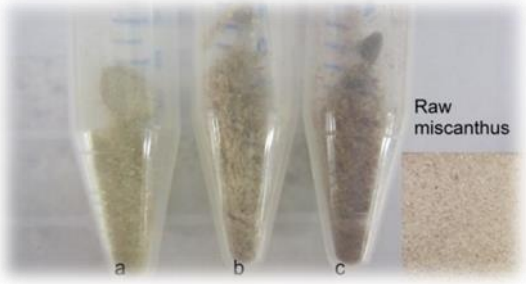


Figure 49 Biomass appearance after conventional heating pretreatment. a. H_2O; b. NaOH; c. H_2SO_4(temperature: 180 ºC; hold time: 40 Min)

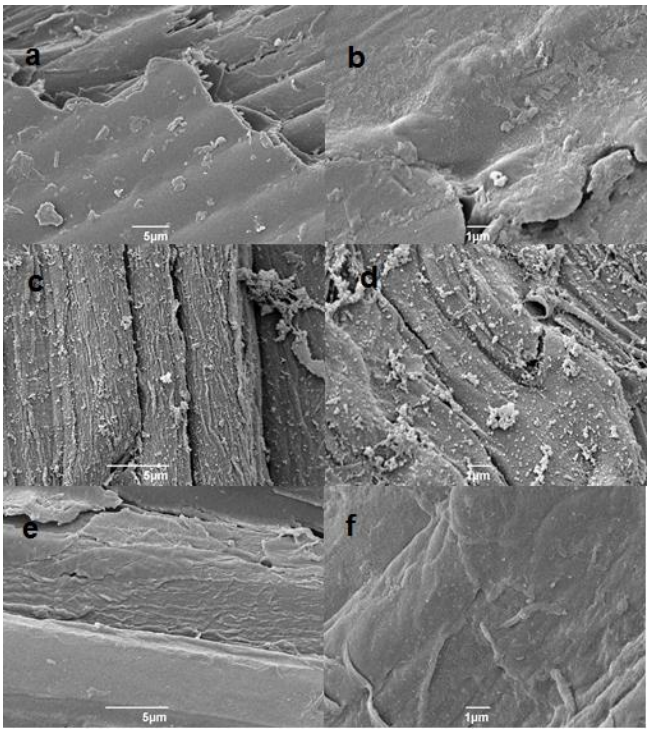


Figure 50 Surface images obtained by SEM on *Miscanthus* treated with conventional heating method at 180 ºC; a. Water pretreatment; magnification bar scale is 5 µm; b. Water pretreatment; magnification bar scale is 1 µm c. 0.2 M NaOH pretreatment; magnification bar scale is 5 µm; d. 0.2 M NaOH pretreatment; magnification bar scale is 1 µm; e. 0.2 M H_2SO_4 pretreatment; magnification bar scale is 5 µm; f. 0.2 M H_2SO_4 pretreatment; magnification bar scale is 1 µm.

Figure 49 shows biomass appearance after conventional heating pretreatment and the colour is slightly changed under each condition assayed here. The biomass pretreated with H_2SO_4 turns into brown colour rather than total black. Figure 50 presents the SEM images of biomass pretreated under conventional heating condition. As can be seen, water pretreatment has a mild influence on biomass surface, as the surface is smooth and flat. However, under NaOH pretreatment condition, parallel strips appear and biomass surface is covered with lignin deposits, which is similar to that of microwave heating pretreatment when temperature is 160 ºC. When H_2SO_4 is used as the pretreatment media, the biomass surface still keeps smooth and compact structure.

Hence, similar to that of microwave heating condition, water and H_2SO_4 have a mild effect on biomass surface when the temperature is 180 ºC. Carbonization of biomass at 200 ºC could be a consequence of the temperature achieved with the microwave, since in under conventional heating condition with longer time (40 Min), carbonization was not observed[12]. The hold time must play an important role, as Chen *et al.* also used

microwave to facilitate biomass pretreatment and their results showed no carbonization at 130-190 °C for 10 Min by using 0.2 M H_2SO_4.

2.6 Sugar degradation study

As was shown in Figure 29 and Figure 33, H_2SO_4 pretreatment led to promising yield of reducing sugars and selectively produced glucose, when holding time is 20 Min. However, it is known that sugar dehydration also is facilitated by H_2SO_4, leading to the formation of furfural, formic acid, hydroxymethylfurfural (HMF) and levulinic acid, which are classified as inhibitors for fermentation [199]. Hence, the sugar dehydration products during microwave assisted pretreatment were studied. *Miscanthus* was pretreated in 0.2 and 0.4 M H_2SO_4 for 20 Min. After the pretreatment, the liquor fraction was separated from biomass solid fraction and then it was extracted with ethyl acetate (3 × 15 ml). It is worth mentioning that the liquor fraction smell very different, which probably due to the production of acetaldehyde. When ethyl acetate was removed by rotary evaporation, the final product was a viscous dark brown substance. It was analysed by GC and NMR.

Figure 51 and Figure 52 show the GC spectra of the sugar degradation substance by using 0.2 and 0.4 M H_2SO_4 respectively as pretreatment media. From GC analysis, levulinic acid (LA) was suggested as the predominant sugar degradation product during the microwave assisted acid pretreatment and very small amount of furfural was also obtained. There are negligible amounts of other chemicals as well. The yield of LA was quantified by using anisole as an external standard chemical. See Table 4, about 68-71 mg organic products are obtained from 400 mg biomass, in which LA is 25-31 mg. The conversion of LA from biomass is between 6-8% under 0.2 M or 0.4 M H_2SO_4 conditions.

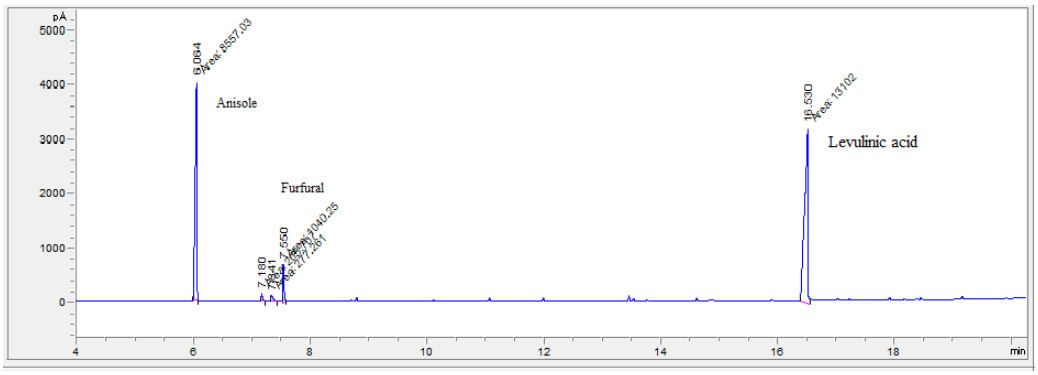


Figure 51 GC spectrum of sugar degradation product analysis (0.2 M H_2SO_4, 20 Min, microwave assisted pretreatment)

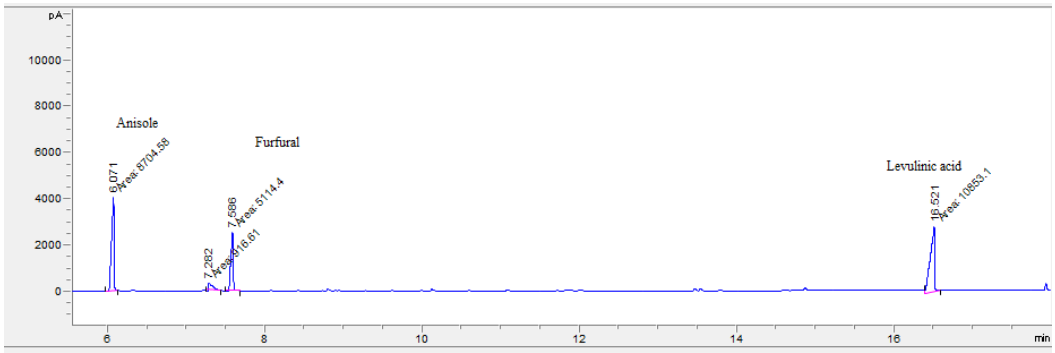


Figure 52 GC spectrum of sugar degradation product analysis (0.4 M H_2SO_4, 20 Min, microwave assisted pretreatment)

In order to confirm levulinic acid is the major product. The sugar degradation products obtained from 0.4 M H_2SO_4 pretreatment was analysed by NMR, see Figure 53. The chemical shifts from the spectrum are listed in Table 17. From NMR, we can confirm that levulinic acid was the predominant degradation product, with small amount of furfural. Because ethyl acetate was used for extraction, it also presents in the NMR spectra. It is noticed that there is a gap of mass balance here, possibly because of the residue of ethyl acetate solvent. Secondly, during the step of re-dissolving organic products with ethyl acetate to make NMR samples, a black insoluble substance presented, which could be humins. Overall, there is a potential of using microwave technology to selectively produce LA from hexose or biomass under diluted H_2SO_4 conditions, which would be of great interest of biorefinery concept.

Table 16 Organic products during microwave assisted H_2SO_4 pretreatment

Conditions (H_2SO_4, 20 Min)	Organic products (mg)	LA (mg)	Furfural (mg)	LA/ biomass (%)
0.2 M	71 ± 8	31 ± 10	5 ± 3	8 ± 3
0.4 M	68 ± 0.4	25 ± 5	1 ± 0.2	6 ± 1

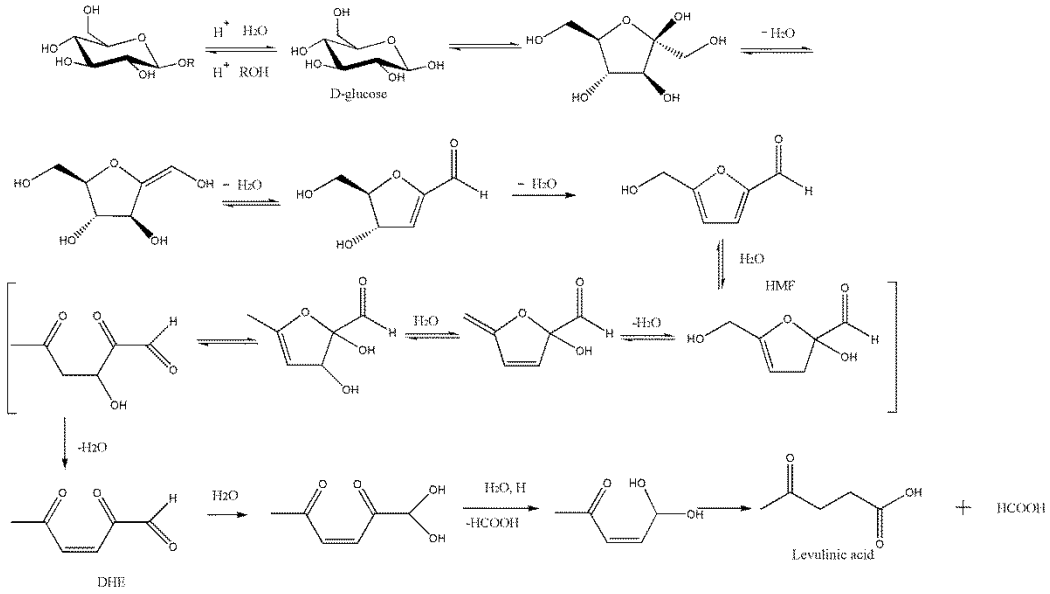
*Each condition was done in triplicates and the results shown here were average with standard deviation

Table 17 Assignments of ^{1}H NMR spectrum

Proton positions	Chemical shifts (ppm)
	Furfural
1	8.00 (d), J=2.38 Hz, 1H
2	6.566, 6.575, 6.575, 6.579, (dd), J=2.38, 2.1 Hz, 1H
3	7.23,7.24(d), Ξ=H
4	9.61 (s), 1H
	Levulinic acid
5	2.16(s), 3H
6	2.56, 2.58, 2.59 (t), J = 6.07 Hz, 2H
7	2.70, 2.72, 2.73 (t), J = 6.83 Hz, 2H

	Ethyl acetate
8	2.00 (s), 3H
9	4.05, 4.07, 4.08, 4.10, (q), J = 7 Hz, 2H
10	1.19, 1.21, 1.23, (t), J= 7 Hz, 3H

Levulinic acid is produced from biomass through the pathway of HMF (see Scheme 5). It is widely accepted that 2,5-dioxohex-3-enal (DHE) is transformed from HMF, although no intermediates were identified. Through this pathway, formic acid is also generated.[200]



Scheme 5 Pathway of converting glucose into levulinic acid under acid condition [200]

2.7 Conclusions and future work

Miscanthus is one of the most promising energy crops in Europe and processing alternatives for second generation biofuel production. In order to produce bioethanol more efficiently, pretreatment of biomass is necessary. Several factors will influence the pretreatment process, such as pretreatment methods, temperature, holding time and catalysts. In this chapter, *Miscanthus* was pretreated with NaOH or H_2SO_4 under microwave condition and the influence of temperature (130 ^oC to 200 ^oC) was studied. Firstly, the sugar yield from *Miscanthus* during pretreatment process was studied and it firstly rose then declined with the increasing of temperature. 180 ^oC is the optimal temperature to efficiently remove sugars from biomass during our pretreatment process. In general, H_2SO_4 contributed to better sugar yield than water and NaOH under each same temperature condition. NaOH and water pretreatments only broke down hemicellulose in the biomass, giving rise to xylose as major sugar component in the

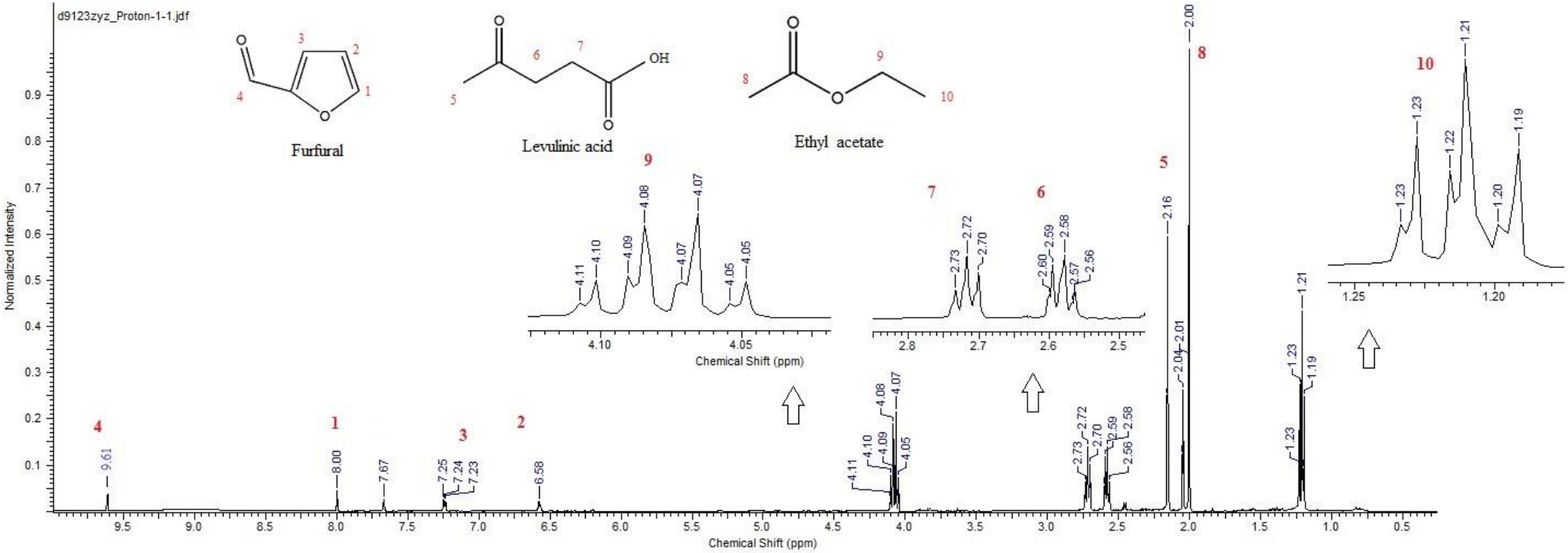


Figure 53 NMR spectrum of sugar degradation product analysis

biomass, giving rise to xylose as major sugar component in the pretreatment media, whereas H_2SO_4 not only degraded hemicellulose but also had a strong influence on crystalline cellulose. The maximum sugar yield from available carbohydrate (73%) was obtained by using 0.2 M H_2SO_4 at 180 ^oC. Meanwhile, maximum production of glucose from available carbohydrate (47%) was achieved. Maximally, the reducing sugar release from microwave assisted pretreatment is 12.5 times more than that of conventional heating pretreatment. Secondly, chemical compositions before and after pretreatment were studied. Hemicellulose was efficiently removed by H_2SO_4. Crystalline cellulose percentage is largely influenced by pretreatment media, holding temperature and pretreatment heating method. Temperature had a strong influence on the lignin content, as different form of lignin deposits were observed from SEM images of biomass surface. NaOH has a strong delignification effect. Last but not least, digestibility was strongly influenced by pretreatment conditions as well. Compare to water and H_2SO_4 pretreatment, NaOH pretreatment significantly enhanced *Miscanthus* digestibility, which was maximally 10 times higher than that of untreated *Miscanthus*, due to effectively removal of lignin and hemicellulose. With the increasing temperature, the digestibility of NaOH pretreated biomass firstly increased then declined slightly.

In comparison with classic conventional heating pretreatment, the reducing sugar release from MW pretreatment is 12.5 times more than that of conventional heating pretreatment, probably due to the unique microwave effects on biomass and the temperature condition achieved by microwave. In conventional heating process, temperature of subject is increased because of convection, conduction and radiation of heat. In contrast, under microwave condition, subject is heated because of dipole rotation and ionic conduction. As one of the major components of biomass, crystalline cellulose plays an important role in the microwave assisted pretreatment process and it is able to act as an active microwave absorber, promoting the overall biomass decomposition. Lignin removal process is strongly influenced by microwave effect, it could be due to ester linkages between lignin and hemicellulose are broken. It was highlighted that microwave is able to effectively degrade biomass and breakdown polysaccharides into their monomers under much lower temperature (130 ^oC) within shorter time, showing the great potential of using microwave assisted thermal-chemical pretreatment for biomass in bioethanol process.

Sugar degradation of microwave assisted biomass pretreatment was studied. The results showed that levulinic acid was selectively produced during the pretreatment, as well as a small amount of furfural. The about 6-8% biomass was converted into levulinic acid,

under the influence or diluted H_2SO_4. Hence, in the future, it would be interesting to optimize the production of LA from biomass by using microwave technology. The results from this chapter had a brode temperature range study and provide valuable information for choose temperature for other biomass as well. By varying pretreatment conditions, selectively production of xylose, glucose and levulinic acid have been achieved here, which has not been mentioned in other study before, showing promising application of MW assisted pretreatment.

Regarding to fact that a promising amount of reducing sugars are released into pretreatment media, it is of great interests to investigate the fermentability of the pretreatment media. Energy save of bioethanol derived from different feedstock was assumed due to previous research has been done by others,[65] but a detailed energy balance assessment is necessary to ensure microwave processing of biomass offering improved energy efficiency over conventional process. Meanwhile, life cycle assessment of the sustainability and toxic release inventory of bioethanol derived from *Miscanthus* with microwave assistance should be investigated.

Chapter 3: Microwave assisted acid and alkaline pretreatment for using *Miscanthus* biomass

Aspect of work described in this chapter has been published in:

Microwave-enhanced formation of glucose from cellulosic waste

Jiajun Fan, Mario De bruyn, Zongyuan Zhu, Vitaliy Budarin, Mark Gronnow, Leonardo D. Gomez, Duncan Macquarrie, James Clark, *Chemical Engineering and Processing: Process Intensification*, 2013, **71**, 31-42

Microwave assisted acid and alkali pretreatment of Miscanthus biomass for biorefineries

Zongyuan Zhu, Rachael Simister, Susannah Bird, Simon J. McQueen-Mason, Leonardo D. Gomez,Duncan J. Macquarrie, AIMS Bioengineering, , 2015, 2(4): 449-468.

3.1 Introduction

The results in the previous chapter showed that 180 ºC is the optimal temperature to achieve maximum sugar yield from *Miscanthus* during the pretreatment process under all the experimental conditions assayed. In this chapter, the effect of treatment time from 5- 30 Min, using a fixed temperature of 180 ºC, is examined. In addition, the use of H_2SO_4 or NaOH is also examined using varied concentrations from 0.2 - 1 M. The following discussions illustrate the effects of hold time and pretreatment media concentration on the pretreatment process.

3.1.1 Effect of catalyst and hold time on sugar release

Hold time and catalyst concentration can have a strong effect on the release of sugars from *Miscanthus* during pretreatment. Figure 54 shows that, when H_2O is used as pretreatment medium using a hold time of 5 minutes, a small amount of sugars are released into the pretreatment media. Increasing the hold time increases the yield of reducing sugars from the available carbohydrate. The maximum sugar removal was achieved with a hold time of 20 Min producing up to 1.25 µmol of reducing sugars per mg of biomass, corresponding to a sugar yield of 28.7% (the yield in this study corresponds to total carbohydrate in biomass).

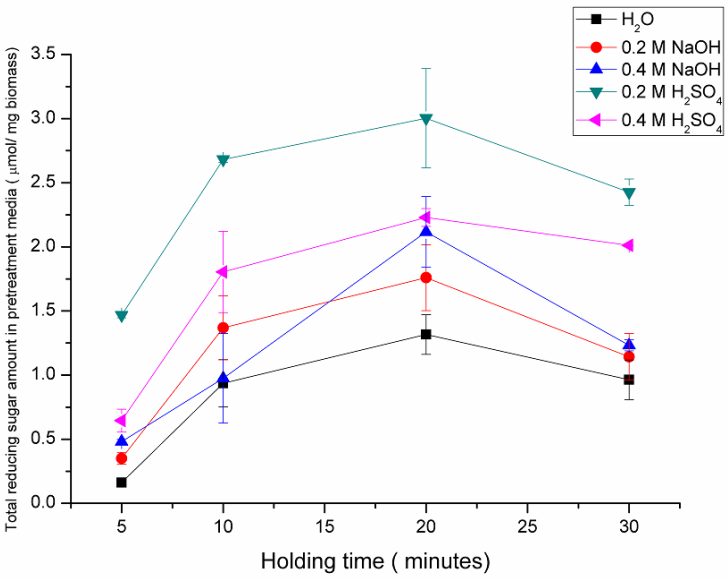


Figure 54 Total reducing sugar amounts present in the pretreatment liquors (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Funazukuri studied the conventional hydrothermal degradation of different cellulosic materials, including cotton cellulose, filter paper and cellulose powder, at higher

temperature (543 K).[182] His results showed that with increasing hold time from 0-60 Min, increasing total sugar yields (maximum 48-65%) were obtained and all of the cellulose was completely degraded by the end of the reaction. High temperature water can liberate acidic components from biomass, predominantly acetic acid and promote the dissociation of acetyl linkages between the sugar rings in biomass during hydrolysis.[201] The pH of liquid hot water pretreated biomass is generally within in the range of 4 to 5 without any addition of base or buffer solution, due to the self-buffering of biomass.[101] Therefore, the hydronium ions generated from both water and generated acid can promote hemicellulose depolymerization to oligosaccharides and monosaccharides.[202, 203]

The amount of reducing sugar released into the pretreatment medium using alkaline or acid solutions was also measured, with a hold time of between 5-30 Min (Figure 54). A similar pattern is observed for all pretreatment conditions. Increasing the holding time from 5 to 10 Min increased the reducing sugar yield sharply, with a further slight increase at 20 Min, followed by a reduction when the holding time is increased to 30 Min. This could be explained by the further degradation of the produced sugars (major components, such as xylose or glucose) under extended pretreatment conditions, which has been discussed in Chapter 2. Also, Li *et al.* studied liquid hot water pretreatment for *Miscanthus* and reported that the initially produced hexose and xylose can be further degraded to hydroxylmethylfurfural (HMF) and furfural, the yields of which increased as the pretreatment severity increased.[103] In our study, the biomass is carbonized under severe acid conditions (see the following SEM discussion Figure 70), showing typical spheres characteristic of biomass carbonization.[198] At the same time, as was discussed in chapter 2, levulinic acid was produced as the major sugar degradation product. H_2SO_4 produces a higher sugar release than NaOH. When the acid concentration is increased from 0.2 M to 0.4 M, the total sugar production declined, due to the stronger acid condition facilitating further degradation of the produced sugars.[204] The maximum sugar production is up to 3 μmol/mg of biomass, corresponding to a yield of 75.3% from available carbohydrate when *Miscanthus* is pretreated with 0.2 M H_2SO_4 for 20 min. Increasing the concentration of NaOH from 0.2 M to 0.4 M does not significantly increase the amount of sugar released. When the hold time was 5 min, both concentrations of NaOH produced very low sugar yields, 0.35 and 0.48 μmol/mg of biomass respectively (yields from the available carbohydrates of 7.8% and 10.6%) for 0.2 M and 0.4 M NaOH pretreatments. The sugar yield reaches a maximum of 1.32 and 1.76 μmol /mg of biomass (giving yields from the available carbohydrates of 43% and 50.7%) when 0.2 M NaOH and 0.4 M NaOH are applied for

20 Min. Overall, 0.2 M H_2SO_4 is the most efficient condition at all the concentrations of H_2SO_4/NaOH tested. As 0.2 M H_2SO_4 leads to optimum reducing sugar release, it can be predicted that maybe a milder condition can have better reducing sugar release. Hence, shorter hold time (2 Min) and 0.1 M H_2SO_4 are tested in this case.

Figure 55 shows the reducing sugar amount released into pretreatment media. Firstly, 0.2 M H_2SO_4 pretreatment still gives better sugar release than 0.1 M and 0.4 M H_2SO_4 and glucose is the major product with all three concentrations assayed here. Interestingly, highest xylose yield is achieved when 0.2 M H_2SO_4 was used, indicating hemicellulose is effectively broken down to xylose by using 0.2 M H_2SO_4, but 0.4 M H_2SO_4 led to xylose degradation. Figure 56 shows reducing sugar release when 0.1 M H_2SO_4 is used as pretreatment media. As can be seen, 5 Min gives rise to highest amount of reducing sugar release and increasing hold time decreases the reducing sugar yield. Interestingly, 5 Min is the best at 0.1 M H_2SO_4 condition. Nevertheless, from the previous discussion we know that at higher concentrations, 10-20 Min is the best. The reason could be the sugar decomposition rate is higher than the biomass depolymerisation rate when 0.1 M H_2SO_4 is applied. When higher concentration acid is used, the biomass depolymerisation rate is higher than sugar decomposition rate. Overall, compared to the results above, milder conditions lead to less reducing sugar release. Hence the following analysis will be focussed on the conditions listed in Figure 54.

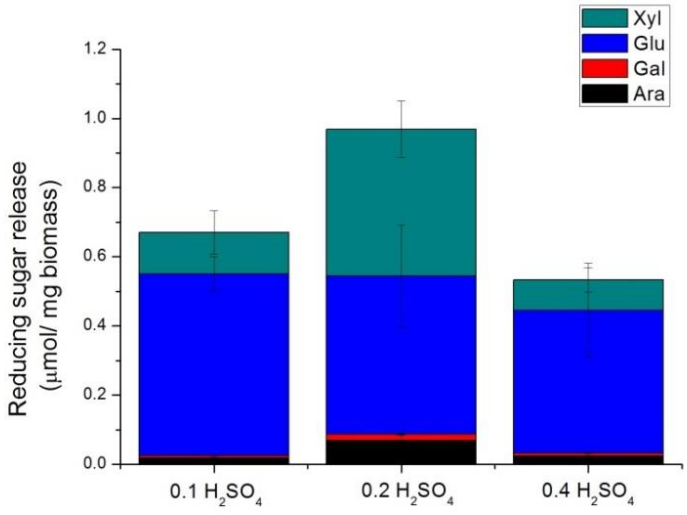


Figure 55 Reducing sugar release when hold time is 2 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

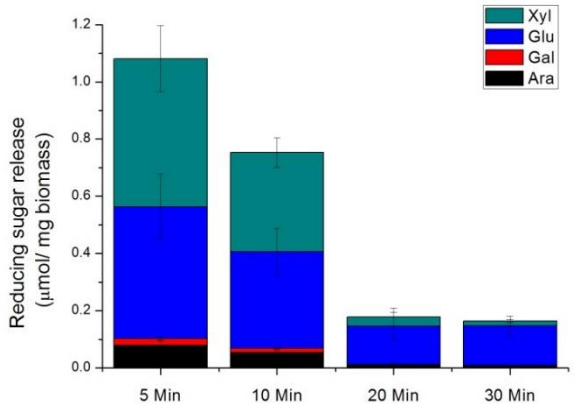


Figure 56 Reducing sugar release when 0.1 M H_2SO_4 is used as pretreatment media (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

3.1.2 Effect of catalyst and hold time on monosaccharides in pretreatment media

The hold time and catalysts have a strong influence on the monosaccharide composition of the sugars released. In the case of water and alkaline pretreatment, xylose is the major monosaccharide released, showing that hemicelluloses are broken down (Figure 57 to Figure 60). Arabinose is the second major sugar constituent, followed by glucose and galactose, with minor quantities of mannose detected. These results indicate that the water and NaOH pretreatment extracted hemicelluloses fractions. The proportion of each monosaccharide does not change significantly across the conditions assayed.

Using a 5 Min hold time, a very small amount of sugar is released by water or using a low concentration of NaOH and 1 M NaOH produces higher sugar amounts (sugar yield is 24% from available carbohydrate) (Figure 57). However, under acidic conditions, 0.2 M H_2SO_4 results in a higher level of observed sugar than 0.4 M and 1 M H_2SO_4, with glucose and xylose being the major sugar components, due to effective degradation of crystalline cellulose and hemicellulose (see following discussion of hemicellulose and crystalline cellulose: Figure 62 and Figure 63). More concentrated acid gives rise to a lower level of sugar production, due to the further degradation of the produced sugar under acidic conditions. When the hold time was 10 min (Figure 58), the sugar production was greatly enhanced compared to that of 5 min. Almost equal amounts of glucose and xylose are produced by using 0.2 M H_2SO_4. However, when 0.4 M H_2SO_4 is applied as the pretreatment medium, xylose yield is significantly reduced and glucose

becomes the only major product, indicating that xylose is degraded under severe acid condition and cellulose starts to be broken down into glucose. In our work, as was discussed in Chapter 2 (page 90, Sugar degradation study), significant amounts of levulinic acid and small amount of furfural were identified in the pretreatment liquor fraction when the biomass was pretreated with H_2SO_4 under microwave conditions at 180 ^oC. Hence, it suggested xylose was degraded into furfural in our study.

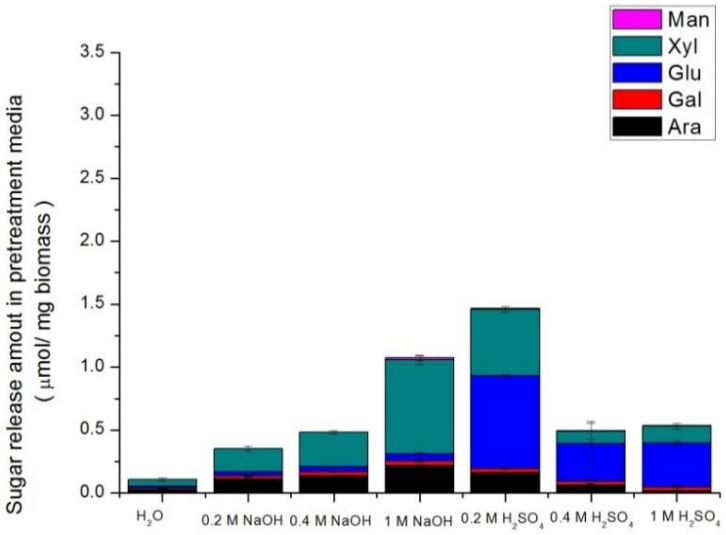


Figure 57 Monosaccharide amount after various pretreatments using a hold time of 5 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

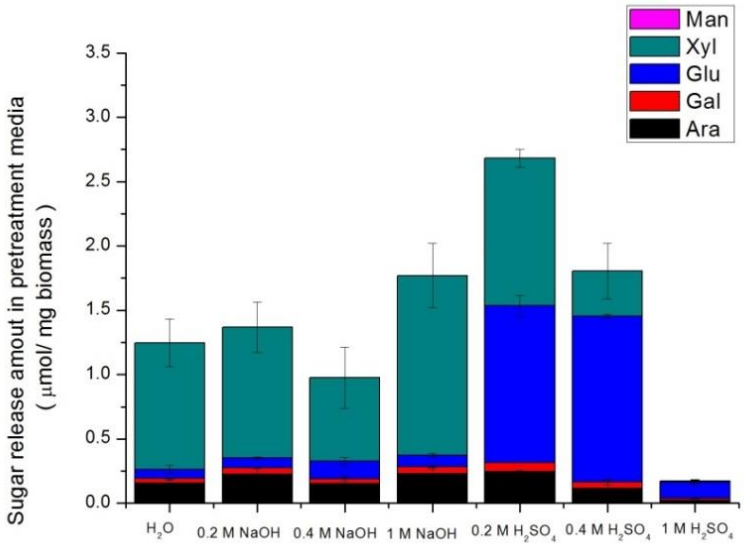


Figure 58 Monosaccharide amount after various pretreatments using a hold time of 10 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

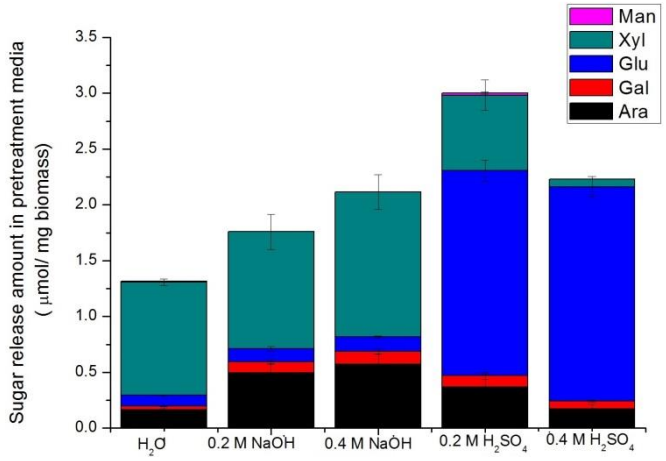


Figure 59 Monosaccharide amount after various pretreatments using a hold time of 20 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

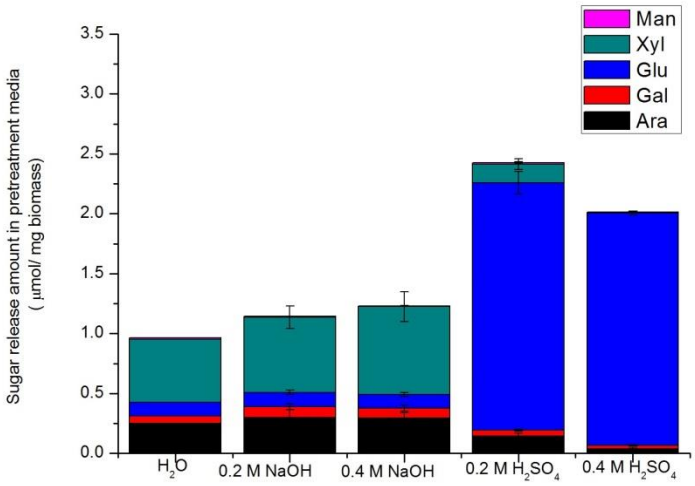


Figure 60 Monosaccharide amount after various pretreatments when hold time is 30 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

In Figure 59 it can be observed that a 20 Min hold time in combination with 0.2M H_2SO_4 produces maximum sugar yield (3.0 μmol / mg biomass; yield: 75.3%). When 0.2 M and 0.4 M H_2SO_4 are applied, glucose, derived from both cellulose and glucans, is the major monosaccharide. The glucose yield is 47.6% and 50% respectively when 0.2 M and 0.4 M H_2SO_4 are used as a pretreatment media. Xylose yield decreases, which could be due to its further degradation under acidic conditions. In contrast, 0.4 M NaOH gives rise to the maximum production of xylose (1.3 μmol/mg of biomass; yield: 28%). Similar results can be observed when the hold time is 30 Min (Figure 60). In the case of water and NaOH media, xylose production also declines, but it is still the major component.

From the results here, it can be seen that at low H_2SO_4 concentrations, both xylose and glucose can be produced by the breakdown of hemicellulose and cellulose in *Miscanthus* biomass. However, xylose quickly decomposes into furfural under severe acidic conditions, thus glucose becomes the major component in the pretreatment media.[205] On the other hand, alkaline and water pretreatments tend to break down just hemicelluloses and give rise to high yields of xylose, which is more stable under neutral or higher pH conditions.

Various pretreatments has been studied on *Miscanthus* previously, Brosse *et al.* used an H_2SO_4 assisted ethanol organosolv system to pretreat *Miscanthus*. The results showed 0.14-9.08% glucan removal under similar temperatures (170-180 °C) with a longer hold time (60 Min).[151] Yu *et al.* pretreated *Miscanthus* by using an aqueous ammonia/

hydrogen peroxide system at lower temperatures (90-150°C) with longer hold times (1-4 h), with lower cellulose degradation occurring during the pretreatment (2.4-19.1%).[206] Haverty *et al*. studied peroxide/formic acid assisted pretreatment of *Miscanthus* with the results showing 0.3-4.37% cellulose removal across the conditions assayed.[207] In our study, highly efficient cellulose and hemicellulose removal from *Miscanthus* are achieved by using microwave acid or alkaline pretreatments, and the optimal sugar yield from carbohydrate is 75.3% within shorter time. Overall, compared to other pretreatment methods, microwave assisted acid pretreatment can lead to better sugar release during pretreatment. In the future, the extraction of sugar can be tuned by varying the pretreatment conditions. For example, a sequential treatment involving e.g. 15 Min pretreatment by using water to extract xylose and the other 5 Min by using H_2SO_4 to extract glucose could be very interesting to study.

3.1.3 Lignin content

Lignin has been considered as one of the main factors behind biomass recalcitrance.[208] Alkaline and oxidative treatments, such as alkaline peroxide and lime and oxygen, have been utilized to remove lignin.[18, 80, 116] Figure 61 shows the amount of lignin remaining in the biomass solid fraction after various pretreatments. The lignin content in the initial 400 mg of untreated *Miscanthus* was 112 mg (83% of total lignin amount in biomass material). By using water as pretreatment media, 31-51.5 mg (28.5–46%) lignin was removed. After NaOH pretreatment the amount of lignin in the samples decreased sharply, in good agreement with previous studies showing that NaOH conditions have a significant delignification effect. [12, 21, 80] 0.2 M NaOH can remove 84 mg lignin after 20 Min pretreatment. When the hold time was increased to 30 Min, up to 93 mg lignin were removed. 0.4 M NaOH is more effective than 0.2 M NaOH and 105.5 mg (94.2%) lignin is removed after 20 Min hold time. The lignin content also decreased during H_2SO_4 pretreatments, however, not as effectively as NaOH pretreatment. When 0.4 M H_2SO_4 was used for pretreatment, a hold time of 10 Min resulted in a greater lignin removal (79 mg presented in the biomass) than for 5 Min (90 mg presented in the biomass). However, when the hold time was increased from 10 Min to 20 Min, less lignin is removed (89 mg presented in the biomass, which is 79% of total lignin amount). As it has been mentioned in the previous chapter, it could be explained by lignin extraction from the inner regions of the cell wall and subsequent condensations and re-deposition on the surface. Li *et al.* reported that depolymerisation and subsequent re-polymerization of lignin occurs, with increasing severity of steam pretreatment of aspen wood.[187] Acetic acid assisted pretreatment of aspen wood also leads to similar results.[187] Overall, NaOH had better and more efficient lignin removal ability.

Increasing concentrations of NaOH or H_2SO_4 had little influence on the lignin removal. However, changing hold time influenced the lignin removal or lignin redistribution process in the case of H_2SO_4 pretreatment. The chemical structure of the biomass is studied by FT-IR (Figure 66) and the results show that the lignin structure is modified by the pretreatment methods. Lima *et al.* studied conventional pretreatment for biomass and the results showed that H_2SO_4 pretreatment has little influence on lignin removal during pretreatment when the temperature ranged from 50 to 180 °C. In contrast, the results in this study show lignin removal was improved under H_2SO_4 condition and microwave assistance has a positive impact on lignin removal.[209] Due to the nature of microwave heating, compared to polysaccharides, lignin is less polar and is therefore expected to have less interaction with microwave energy. Therefore, microwave performance on lignin is not as direct as it is on cellulose or hemicellulose. However, it is worth noting that the ester linkages between lignin and hemicellulose could be largely influenced by microwave (see FT-IR discussion 3.1.7), which may further lead to a lignin removal or redistribution on the biomass.

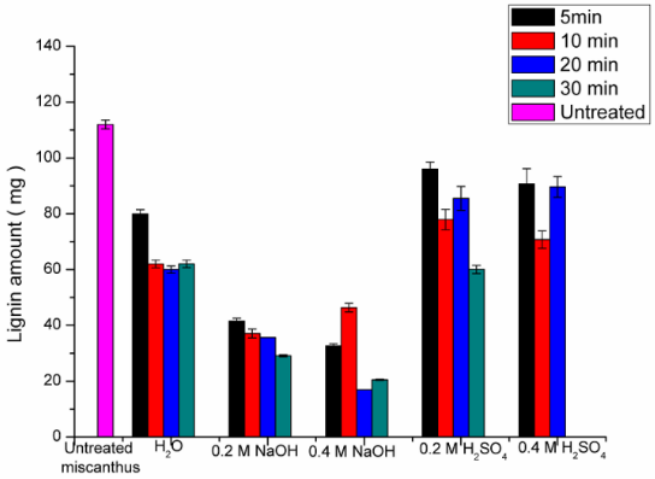


Figure 61 Lignin content changes after varied pretreatments (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

3.1.4 Hemicellulose composition

Hemicellulose was measured using a protocol described by Foster *et al.*[168] The hemicellulose is hydrolysed into its monomers by using 2 M TFA (Trifluoroacetic acid) treatment, then the monosaccharides are measured by using HPAEC (Dionex IC 3000) on a Dionex Carbopac PA-20 column with integrated amperometry detection. Therefore, by measuring the hydrolysed monosaccharides we can calculate back and quantify the

hemicellulose content in the biomass sample. The hemicellulose present in *Miscanthus* undergoes different degrees of removal depending on the pretreatment conditions. Untreated *Miscanthus* contains 42% hemicellulose, comprising arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid, with xylose and glucose as the major components. Figure 62 shows the hemicellulose percentages in the biomass material before and after pretreatment using a hold time of 5 Min. Water pretreatment decreased the hemicellulose content to 39%. Using a NaOH pretreatment, the hemicellulose decreased further in a concentration dependent fashion. By using 1 M NaOH, the hemicellulose percentage in the biomass decreased to 22.5%. In contrast, H_2SO_4 pretreatment is effective in removing hemicellulose from the biomass, with it dropping sharply to 5% by using 0.2 M or 0.4 M H_2SO_4. Almost all of the hemicellulose is removed when 1 M H_2SO_4 is applied for pretreatment.

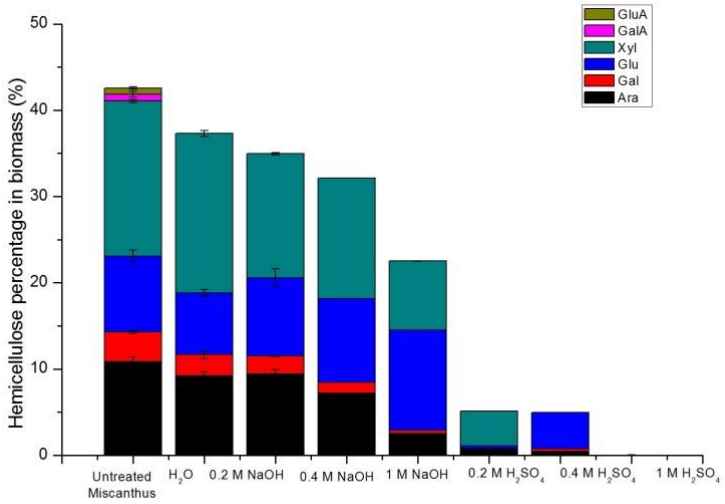


Figure 62 Hemicellulose percentage changes after varied pretreatments (hold time is 5 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Combining the results of monosaccharides analysis in pretreatment media, we can conclude that hemicellulose was efficiently broken down under mild acid conditions, which contribute to a promising sugar release, when a hold time is only 5 Min, (see Figure 57). Lima *et al.* pretreated various biomass at 180 ^oC for 40 Min and a similar level of hemicellulose removal was obtained by using NaOH or H_2SO_4.[209] In contrast, microwave assisted pretreatment in this study is 8 times faster in removing hemicellulose.

3.1.5 Crystalline cellulose analysis

Crystalline cellulose percentage is a vital property for biomass, as it provides mechanical rigidity and toughness for the composite material. It also influences wall extensibility, which is an important determinant of plant growth and differentiation.[191] As was mentioned in Chapter 2, crystalline cellulose percentage of biomass sample is of great importance during pretreatment process. In this study, crystalline cellulose percentage is measured not only for the purpose of measuring how crystallized the biomass is, but also how much crystalline cellulose is available for enzyme to break down. The results from this part would be related to the results from saccharification of the biomass (see Figure 64), because the crystallinity of biomass has a significant influence on biomass digestibility. Highly crystallised biomass has lower digestibility.

As can be seen from Figure 63, in general, the microwave assisted alkaline pretreatment was less effective than water and acid pretreatment in terms of decreasing the crystalline cellulose percentage. H_2SO_4 can reduce cellulose crystallinity, while NaOH reacts with biomass derived acids such as formic acid, acetic acid and glycolic acid, leading to partial neutralisation and less reduction of crystalline cellulose.[103] However, NaOH can also alter the cellulose structure and increase the amorphous cellulose content in the biomass, making it more disordered. Mittal *et al.* studied NaOH pretreatment on α–cellulose, cotton linters and corn stover at 25 °C and the results showed that the amorphous content increased as much as two fold [17]

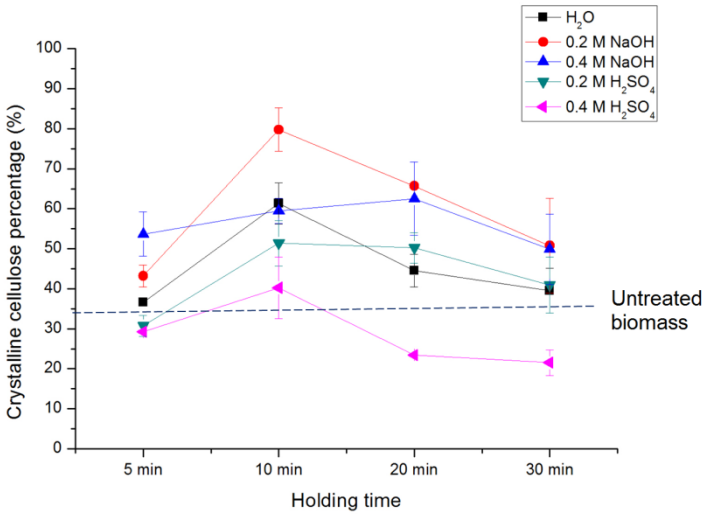


Figure 63 Crystalline cellulose percentage changes after varied pretreatments (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

In this study, crystalline cellulose percentages in biomass before and after various microwave assisted pretreatments are investigated. The crystalline cellulose percentage in untreated *Miscanthus* is 34%. In comparison with untreated biomass, pretreatment

generally increased crystalline cellulose percentages in the biomass samples, apart from 0.4 M H_2SO_4 pretreatment. Using short retention times in acid and water pretreatments, there is little change when it is compared with that of untreated biomass. However, when NaOH is used, crystalline cellulose percentage goes up, especially in the case of 0.4 M NaOH, due to the efficient removal of hemicellulose. When hold times are increased to 10 Min, the crystalline cellulose percentages are all increased to different extents, due to lignin and hemicellulose being removed and the solid residue becoming more crystalline. The highest crystalline cellulose percentage is 80% when 0.2 M NaOH is used for 10 Min. Meanwhile, 0.4 M NaOH gives rise to a lower crystalline cellulose percentage, which could be due to the higher NaOH concentration altering the crystalline cellulose and making it more amorphous. Using 0.4 M H_2SO_4 leads to lower crystalline cellulose percentage than 0.2 M H_2SO_4, which is expected. When hold times are further increased to 20 Min, crystalline cellulose percentages are still higher than that of untreated biomass. However, they are lower than that of 10 Min, due to the crystalline cellulose undergoing hydrolysis under acid or water conditions, or changing into the amorphous form under alkaline conditions. Longer hold time (30 Min) leads to even lower crystalline cellulose percentage. It is worth noting that neither concentration of NaOH brings a significant difference to the content of crystalline cellulose. It could be explained by the fact that maximum amount of lignin and hemicellulose are removed at this time condition. Water and 0.2 M H_2SO_4 lead to similar crystalline cellulose percentages and 0.4 M H_2SO_4 gives least crystallised biomass. In the future, it is worth checking the performance of water and 0.2 M H_2SO_4 on commercial crystalline cellulose, because rather different glucose yields are released into pretreatment media by using these two pretreatment media; at the same time, similar crystalline cellulose percentage presented in the biomass samples. Hence, it can be predicted that, due to the acidic condition of water pretreatment, crystalline cellulose is changed to a more amorphous form, while 0.2 M H_2SO_4 can complete degrade crystalline cellulose and release good yield of glucose into pretreatment media.

3.1.6 Digestibility analysis

Digestibility of un/pretreated biomass was measured as it was measured in Chapter 2. As can be seen, *Miscanthus* digestibility of solid fraction is increased after all the pretreatments, albeit to widely differing extents. The digestibility of untreated *Miscanthus* is rather low, with only 10.25 nmol sugar per mg biomass per hour digestion. This result is expected as due to the rigid structure of biomass the enzyme is not able to hydrolyse the polysaccharides efficient. When water is used as the pretreatment media,

the biomass digestibility gradually increases with increasing hold time, reaching 48 nmol sugar per mg biomass after 1 h digestion when a pretreatment hold time of 20 Min had been used. NaOH remarkably enhances biomass digestibility, with the sugar concentration produced remaining unaltered irrespective of changing pretreatment conditions. The highest digestibility (92 nmol/mg/h sugar) is obtained by using 0.4 M NaOH for 30 Min. On the other hand, H_2SO_4 only slightly improves *Miscanthus* digestibility. For 0.2 M H_2SO_4, the hold time has negligible effect on digestibility. Whilst when 0.4 M H_2SO_4 is used, the digestibility is marginally increased using a 5 Min hold time and thereafter declines.

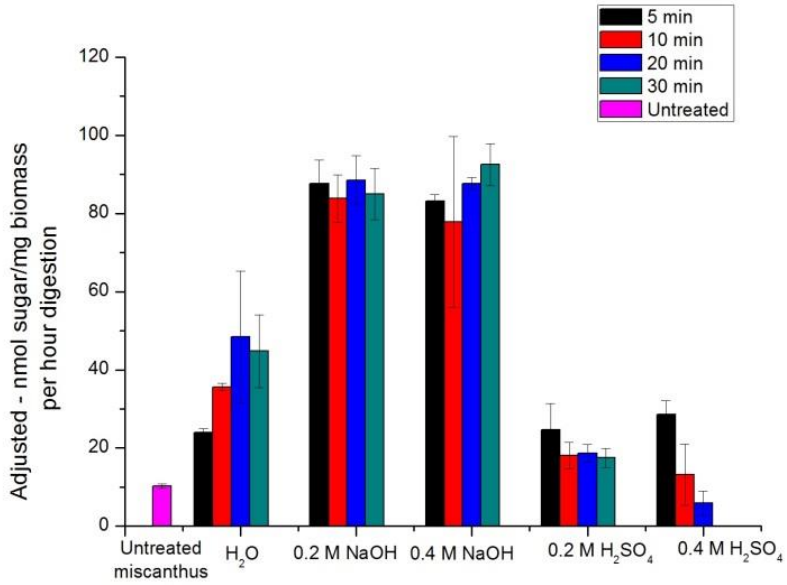


Figure 64 *Miscanthus* digestibility after various pretreatments (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

As we know, hemicellulose has a positive and dominant effect on biomass digestibility.[11] Polysaccharides in plants are protected in the cell walls by lignin, due to the fact that they are also the food source for microbes. Therefore, lignin plays synergistic and negative roles in sugar production by enzymatic hydrolysis after chemical pretreatment.[11] An ideal pretreatment would satisfy a condition that alongside low levels of removal of hemicellulose and cellulose which should not be degraded (except some partial chain scission) as these are the sugar producing moieties, lignin is efficiently removed and biomass structure is more open for enzyme to break down.

The higher saccharification after alkaline pretreatment is consistent with the loss of significant amounts of lignin during pretreatment. As discussed before, NaOH has a strong delignification effect. Meanwhile, abundant crystalline cellulose is presented in

the biomass, which leads to promising glucose production, despite the fact that high crystalline cellulose is not as easy to degrade as amorphous cellulose. In contrast, fairly low digestibility is obtained using H_2SO_4 pretreatment, which can be explained by the fact that the easily hydrolysed sugars are released into the pretreatment liquor, reducing the amount of sugars available for enzymatic digestion. Several factors affecting the absolute enzymatic hydrolysis rate were outside the scope of the present study and were not optimised (solids loading; enzyme loading; the effect of various additives; the enzyme cocktail used; or the temperature and duration of digestion).

It is worth mentioning that the total overall sugar available for fermentation is the sum-up of reducing sugar release during pretreatment and fermentable sugars available from biomass residue. Table 18 shows the potential optimal condition for NaOH and H_2SO_4 pretreatment. However, depending on whether the major sugar is xylose, two different yeasts may need to be used in the case of NaOH pretreatment, while only one yeast will be needed in the case of H_2SO_4 pretreatment.

Table 18 potential total sugar available for fermentation

	NaOH	H_2SO_4
Reducing sugar release during pretreatment (liquid fraction)	322 μg/mg biomass (xylose =195 μg; glucose = 3.8 μg; arabinose = 86 μg)	393 μg/mg biomass (glucose = 345 μg; xylose = 9.89 μg; arabinose = 26 μg)
Glucose from enzymatic hydrolysis (solid fraction)	13-16 μg/ mg biomass per hour digestion	1.0 – 5.2 μg/ mg biomass per hour digestion

3.1.7 FT-IR analysis

Chemical changes on the surface of the samples were qualitatively analysed by ATR-FTIR (Attenuated total reflectance Fourier transform infrared) spectroscopy. Figure 65 shows sharp peaks at 897 cm^{-1} and 1159 cm^{-1} in the spectra, which are attributed to C-O-C stretching at the β-glycosidic linkage between the sugar units. [5] The absorbance at 897 cm^{-1}, 1033 cm^{-1}, 1065 cm^{-1} and 1108 cm^{-1} can also be associated with cellulose. [210, 211] Strong peaks at 1065 cm^{-1} and 1033 cm^{-1} relate to C-O stretching at C-3, C-C and C-O stretching at C-6.[210] When the concentration of NaOH was increased, a peak at 1065 cm^{-1} appeared. This indicates that removing hemicellulose and lignin enhances cellulose-associated signals.[208, 211] According to previous research, crystalline cellulose has a characteristic C-O vibration absorbance at 1098 cm^{-1}.[212-214] However,

this peak is not observed here. The peak at 1108 cm^{-1} appeared after NaOH pretreatments and we consider that it relates to the crystalline cellulose, whose appearance suggests that the biomass is more crystalline.[212] This result is in good correlation with previous crystalline cellulose percentage discussion. Lignin has absorbance around 1424 cm^{-1}, 1512 cm^{-1} and 1604 cm^{-1} .[211, 215] Figure 65 shows that lignin associated peaks appeared at similar positions and they almost disappear after NaOH pretreatment. The absorption at 1424 cm^{-1} could be related to methyl groups present in lignin.[157] However, it also could be attributed by CH_2 symmetric bending of crystalline cellulose and its disappearance during alkaline treatment can also because of disruption of crystalline cellulose.[216] As discussed above, the percentage of crystalline cellulose in NaOH pretreated *Miscanthus* is higher than that of untreated *Miscanthus* at this condition and the peak at 1108 cm^{-1} is stronger (indicating the biomass is more crystallised). Here, the peak at 1424 cm^{-1} disappears in a similar manner to the 1512 cm^{-1} and 1604 cm^{-1}, it can be predicted that the peak at 1424 cm^{-1} is related to lignin. The absorption at 1512cm^{-1} is related to the phenolic ring vibrations of lignin.[208] The signal at 1604 cm^{-1} is also related to the aromatic ring in lignin. The peak at 1239 cm^{-1} (C-O stretching of acetyl groups from hemicellulose) disappears after pretreatment. [217] The peak at 1731 cm^{-1} represents the ester bond (C=O) between hemicelluloses and lignin.[25] After pretreatment, these two signals become very weak, indicating that the hemicellulose is successfully deacetylated, or the ester bonds between hemicellulose and lignin are broken. The peak at 1634 cm^{-1} is attributed to the bending mode of the absorbed water.[27] C-H deformation in cellulose and hemicellulose at 1370 cm^{-1}; [217] C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives at 1320 cm^{-1}. [218]

The peak at 1033 cm^{-1} (associated with cellulose) is less pronounced after 0.2 M H_2SO_4 pretreatment, whilst it becomes stronger after 0.4 M and 1 M H_2SO_4 (Figure 66). Two peaks around 1055 cm^{-1} and 1103 cm^{-1} appeared after 0.4 M and 1 M H_2SO_4 pretreatment, which is contributed by C-O vibrations of cellulose and glucose ring stretch from cellulose. [212] Similar to NaOH pretreatment, the hemicellulose peak around 1239 cm^{-1} disappeared after 0.2 M and 0.4 M H_2SO_4 pretreatment. Lignin absorbance around 1424 cm^{-1}, 1512 cm^{-1} and 1604 cm^{-1} barely show any changes, suggesting that H_2SO_4 has little influence on lignin structure. The peak around 1731 cm^{-1} reduced in intensity and shifted after pretreatment, suggesting that at least the ester linkages between hemicellulose and lignin are partially broken.

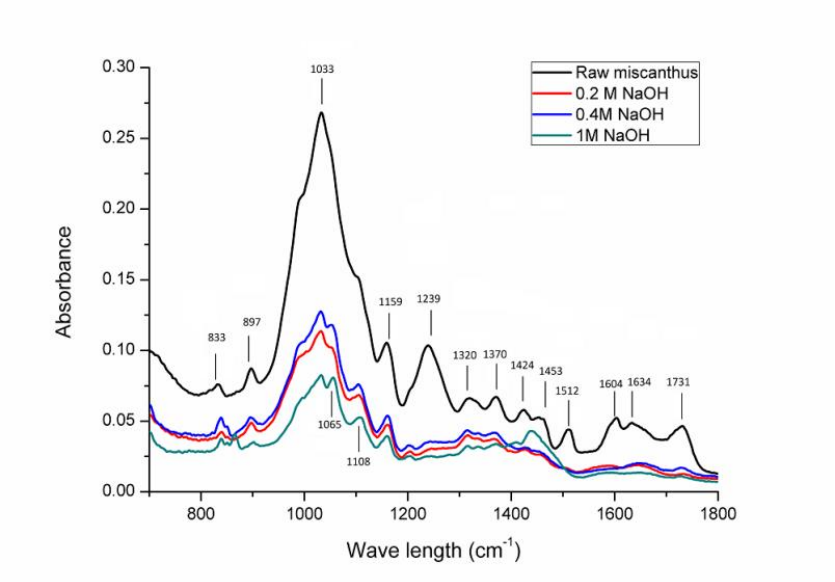


Figure 65 FT-IR analysis of Miscanthus after microwave assisted NaOH pretreatments when hold time was 5 min

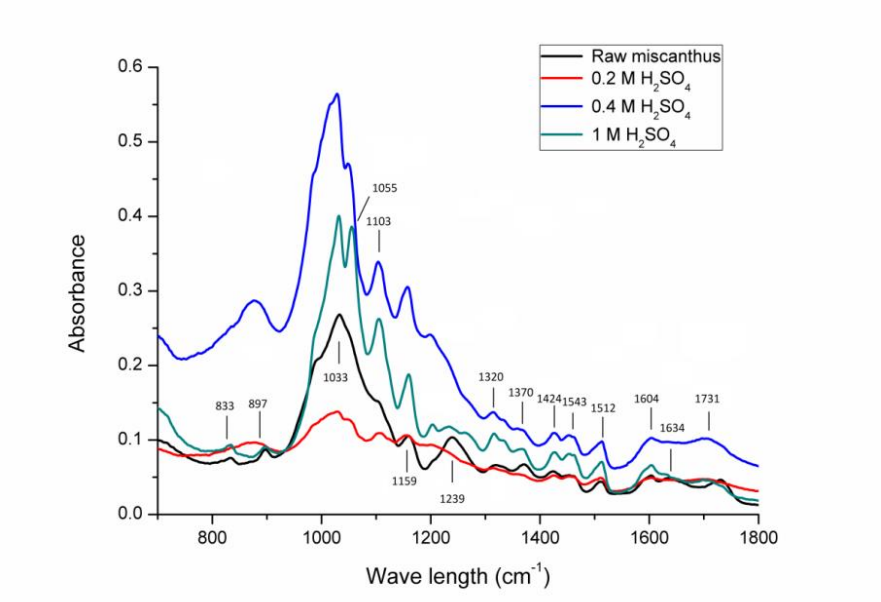


Figure 66 FT-IR analysis of Miscanthus after microwave assisted H_2SO_4 pretreatments when hold time was 5 Min

Table 19 Chemical composition changes in biomass after pretreatments

peak position (cm^{-1})	Assignment
897	β-glycosidic linkage between the sugar units
1033	C-O stretching
1055	Related to polysaccharides content appearance
1065	C-C stretching
1108	Relate to crystalline cellulose
1159	C-O-C stretching at the β-glycosidic linkage

1239	Acetyl C-O stretching of hemicellulose
1320	C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives
1370	C-H deformation
1424	Stretching of O-CH_3
1512	Phenolic ring vibrations of lignin
1604	Aromatic ring stretching of lignin
1634	Bending mode of the absorbed water
1731	Acetyl groups on hemicellulose

3.1.8 SEM analysis

Scanning electron microscopy is a useful technique to study the morphological changes of biomass. Untreated *Miscanthus* presents vascular elements packed in bundles (Figure 67a and b) with relatively flat and clean surface (Figure 67c). Figure 68 to Figure 70 present biomass surface characteristics produced by microwave assisted pretreatment of *Miscanthus* when the hold time was 20 Min.

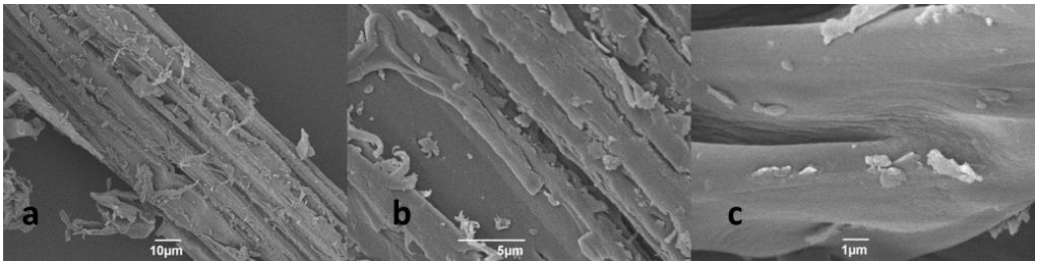


Figure 67 Surface images of the untreated *Miscanthus* obtained by SEM. (a) general view of a fibre surface, bar scale: 10 µm; (b) flat surface of a fibre showing, bar scale: 5 µm and (c) amplification of the surface, bar scale: 1 µm

Figure 68 shows *Miscanthus* pretreated using water. When compared to untreated *Miscanthus*, water pretreatment causes few changes when observed under lower resolution, however, a rough and striped surface is observed at maximum magnification (Figure 68c), indicating that water treatment has a mild effect on the biomass surface. In contrast, NaOH has a pronounced effect on the biomass surface structure. Figure 69a-c present the images from 0.2 M NaOH pretreatment. Firstly, the surface coating that can be observed in Figure 67 is damaged and the biomass surface becomes rough with the appearance of parallel strips. Additionally, some of biomass fibres start to become exposed. With the application of 0.4 M NaOH, the effect of the alkaline treatment is more obvious. The biomass surface coating is totally removed and it is covered with exposed biomass fibres (Figure 69 d-f). The exposed fibre size is smaller after 0.4 M

NaOH pretreatment, compared to that of 0.2 M NaOH, which could be due to the higher concentration NaOH having a stronger influence on biomass surface. The tightly packed fibres in the raw *Miscanthus* start to dismantle and are exposed due to lignin removal from the interstices between the fibres bundle, which is in agreement with the great decrease in lignin content (Figure 61).

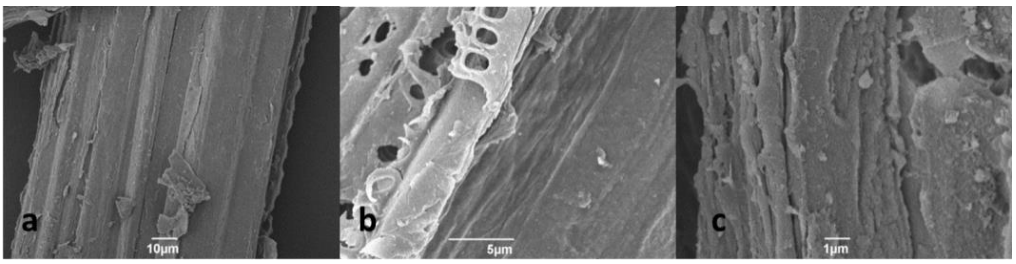


Figure 68 Surface images obtained by SEM on *Miscanthus* treated with water pretreatment, under a 300 W microwave power and three different magnifications with scale bars between 10 µ m, 5 µm and 1 µm

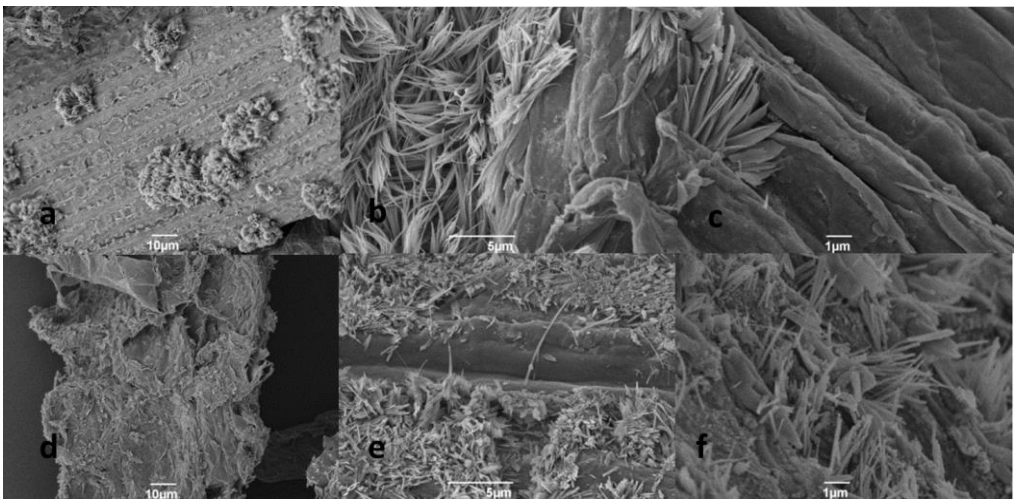


Figure 69 Surface images obtained by SEM on *Miscanthus* treated with 0.2 M and 0.4 M NaOH. Three different magnifications with scale bars between 10 µm, 5 µm and 1 µm. a-c: 0.2 M NaOH pretreatments; d-f: 0.4 M NaOH pretreatments

The microwave assisted pretreatments under acid conditions using 0.2 and 0.4 M H_2SO_4 result in distinct morphological changes, when compared to the effects of the NaOH pretreatment. Figure 70a-c and Figure 70d-f shows the surface of *Miscanthus* treated with 0.2 M and 0.4 M H_2SO_4 respectively. The samples undergoing 0.2 M H_2SO_4 pretreatment show a very similar morphology to the untreated *Miscanthus*, indicating that the acid treatment is too mild under these conditions. At higher magnification, samples treated with acid present a tight and compact structure of cellulose microfibrils, similar to the raw bagasse and very different from the alkali effect on the ultrastructure of the cell wall. This seems strange as this material showed a high removal of hemicellulose. Possibly the biomass is degraded without breaking down the surface.

Samples treated with 0.4 M H_2SO_4 were carbonized, as the samples become a black powder macroscopically and under microscope it presents a degraded aspect, with spherical particles typical of hydrothermally carbonised samples (Figure 70d-f) and showing a carbonized structure that keeps the general aspect of the fibre conducting bundles on the sample. Catalysed degradation of sugars to furans and levulinic acid and subsequently to hydrothermal carbon explains the very low amount of sugar present in solution from the bagasse samples treated with higher concentration acid.[198] A similar type of structure was previously observed during to the formation of hydrothermal carbon via acid catalysed conversion of biomass derived sugars to hydroxymethylfurfural to its polymer at similar temperatures.[198] Yao *et al.* proposed pathways to produce carbon sphere from fructose and glucose dehydration in closed system under different temperature without acid (see Scheme 6), which could explain our carbon sphere occurred in H_2SO_4 pretreatment of *Miscanthus*.[219]

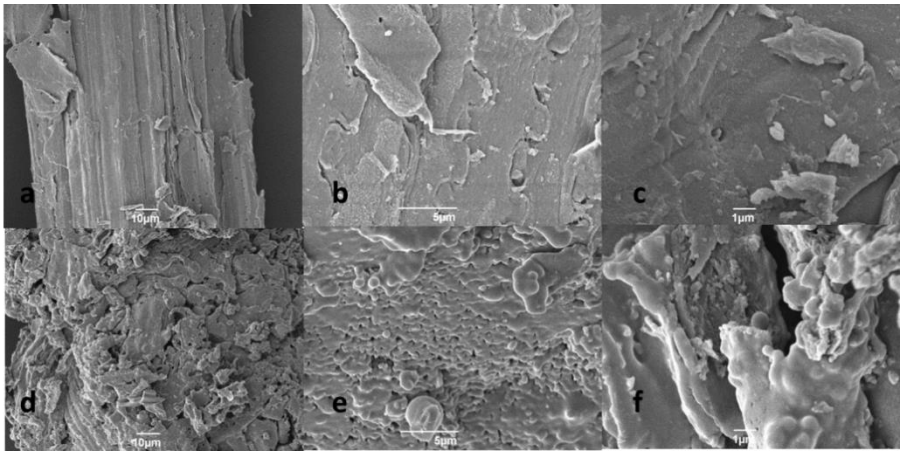
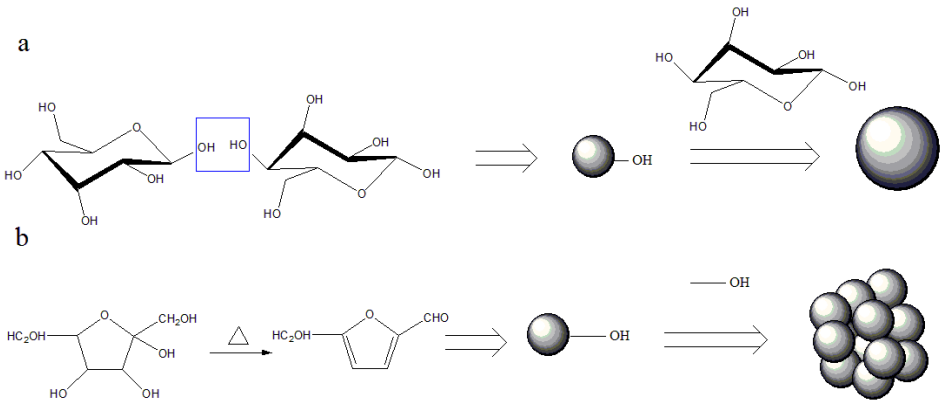


Figure 70 Surface images obtained by SEM on *Miscanthus* treated with 0.2 M and 0.4 M H2SO4. Three different magnifications with scale bars between 10 μm and 1 μm are shown. a-c: 0.2 M H_2SO_4 pretreatments; d-f: 0.4 M H_2SO_4 pretreatments



Scheme 6 The dehydration and carbonization process of (a) Glucose and (b) Fructose [219]

3.1.9 Simultaneous saccharification fermentation study (SSF)

After pretreatment, the biomass residue can be converted into monomeric sugars via enzymatic hydrolysis. The sugars are then fermented into ethanol by yeast. Three enzyme-catalysed processes have been investigated for the conversion of lignocellulosic biomass into ethanol: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and direct microbial conversion (DMC). Enzymatic hydrolysis and fermentation can be performed sequentially, which is referred to as separate hydrolysis and fermentation (SHF). However, these two steps can be performed simultaneously, which is referred to as simultaneous saccharification and fermentation (SSF). It was firstly developed for lignocellulosic biomass by researchers at Gulf Oil Company in 1974.[220] The most important benefit of the SSF process is its ability to convert sugars into ethanol rapidly. Glucose produced by the hydrolysis process is immediately consumed by microorganisms, which reduces cellulose inhibition. Hence, sugar production rate, concentration and yields are increased and enzyme loading requirement decreased.[220] Overall, SSF requires less capital cost and higher ethanol concentration is achieved than SHF. It also eliminates the possibility of contamination by unwanted organisms that are less ethanol tolerant than the microbes selected for fermentation, which is favourable for continuous operations of commercial interest.[221] Nevertheless, the yeast is difficult to reuse, as the lignin residue is mixed together with the yeast. In addition, the optimal temperature for enzyme hydrolysis (50 ^{o}C) and yeast fermentation (30 ^{o}C) is different, meaning the conditions used in SSF cannot be optimized for both processes.[222] Direct microbial conversion is a method of converting lignocellulosic biomass directly to ethanol, in which both ethanol fermenting and all required saccharification enzymes are produced by a single microorganism. Hence, it combines all three processes (cellulase production, cellulose hydrolysis and fermentation) in one step. It is cost-effective, due to the reduced number of reactors. However, the ethanol yields are very low, because of the production of metabolic by-product and the organisms usually have low ethanol tolerance. [220] Therefore, in this work, SSF were conducted to investigate the ethanol production from *Miscanthus* with microwave pretreatment.

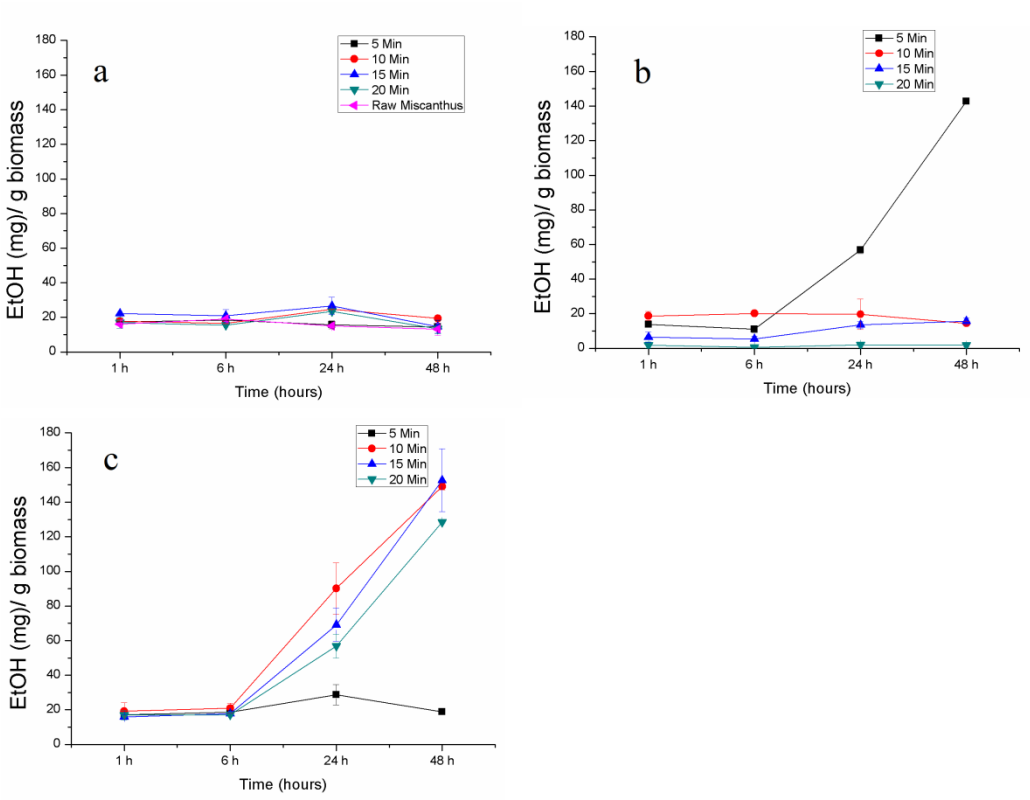


Figure 71 Ethanol production of untreated/ pretreated Miscanthus over 48 hours incubation time; a. water pretreatment; b. H_2SO_4 pretreatment;c. NaOH pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

For this work, microwave pretreatment was scaled up, with CEM MARS 6. The pretreatment temperature controlled at 180 ºC using a hold time of 20 Min. Similar to Chapter 2, liquid fraction and solid fraction were obtained by separating biomass and liquid pretreatment media. The SSF was performed on the biomass solid fraction. Figure 71 shows the time course of ethanol production in SSF process of sugarcane bagasse with or without microwave assisted pretreatment. As can be seen from Figure 71a, compared to untreated *Miscanthus*, water pretreated *Miscanthus* gives rise to a very low ethanol production, regardless of the increasing pretreatment time and incubation time. In the case of H_2SO_4 pretreatment, distinctive differences can be observed when the hold time is increased from 5 min to 20 min. Ethanol production was 143 mg/g biomass when the pretreatment hold time was 5 min. Longer holding time reduced ethanol production drastically. It could be due to the inhibitors produced during the pretreatment process, such as levulinic acid and furfural.[223] The other explanation is that the majority of digestible sugars are released during the pretreatment process, meaning the remaining biomass residue is less digestible. The biomass was washed with ethanol in order to remove possible inhibitors produced in the pretreatment process prior to the SSF process, therefore, the first explanation is less probable. The result here is in agreement with the

previous results of digestibility study that H_2SO_4 pretreated biomass material is less digestible (Figure 64). However, NaOH has a completely different effect. As can be seen from Figure 71c, when pretreatment time is 5 Min, a very small amount of ethanol is produced, however, with longer pretreatment times significant amount of ethanol are produced. The highest ethanol production (152 mg/g biomass) was achieved when the pretreatment time was 15 Min using an incubation time of 48 h. The results are in good agreement with the previous digestibility study and the biomass morphological study showing that biomass is more digestible due to the effect of NaOH on the biomass structure (Figure 69). Overall, promising ethanol yields are achieved by microwave assisted pretreatment and it can be optimized by changing pretreatment media and pretreatment time.

3.1.10 Glucose decomposition study

As was presented in previous section (section 3.1.2), the sugar yields during severe acid pretreatment are rather low. Hence, the glucose decomposition under microwave condition was briefly studied.

400 mg glucose was hydrolysed in 0.2 and 0.4 M H_2SO_4 solution for 10 or 20 Min with microwave assistance. After hydrolysis, the pretreatment media and solid carbonaceous material were separated by filtration. Sugars remaining in the liquor were quantified by HPEAC and organic product was extracted from liquor with ethyl acetate (3×16 ml) and analysed. Under microwave condition, glucose was converted into mannose, organic products and carbonaceous material. Table 20 shows the mass balance of the glucose conversion process. As can be seen from Table 20, when the glucose was under the condition of 0.2 M H_2SO_4 for 10 Min, about 26% glucose was left in the pretreatment media. Very small amount of mannose and carbonaceous material was identified. The organic product yield is about 38 mg, which was analysed by GC (anisole was used as an external standard to quantify Levulinic acid (LA)) and NMR. Commercial LA sample is used as a standard. As can be seen from Figure 72, the major peak around 11 Min is the predominant peak of the organic product GC spectrum, which is contributed by levulinic acid. In order to confirm the result, ^{1}H-NMR was performed for the organic product. As can be seen from Figure 73, the chemical shift at 2.1859 ppm is contributed by CH_3 and chemical shifts around 2.6 ppm are contributed by CH_2 (B) and chemical shifts at 2.7 ppm are contributed by CH_2 (C). The chemical shift of COOH does not present in the spectrum, because the H on COOH is exchanged with D in the solvent, hence it was absent in the spectrum. LA is the major component in the organic product, which is the same result as in Chapter 2.

When the acid condition is stronger, the amount of remaining glucose in the pretreatment media is less, whilst the yield of organic product is higher. For instance, when the 0.4 M H_2SO_4 was used for 20 Min, the glucose is only about 3 mg, while 72 mg organic product yielded from glucose. The increasing of pretreatment severity leads to the increasing carbonaceous material and organic product yield. Overall, a significant amount of levulinic acid is obtained from this microwave assisted process and the yield is between 8-10%. However, there is a great mass loss that is absent from the analysis here, which possibly could be contributed by the release of formic acid, acetic acid and other volatile product. However, more study should be done to find out the mass loss.

Table 20 Mass balance of glucose hydrolysis

Hydrolysis conditions	Sugars in liquid(mg)		Carbonaceous material (mg)	Organic product (mg)	LA from organic product (mg)	Mass loss
	Glucose	Mannose				
0.2M H_2SO_4 10min	104.7	4.65	0.17	38.08	35	252.38
0.2M H_2SO_4 20min	57.3	4.69	0.28	56.2	33	281.53
0.4M H_2SO_4 10min	30.8	2.99	1.53	54.45	40	310.23
0.4M H_2SO_4 20min	2.77	3.14	2.52	72.1	42	319.47

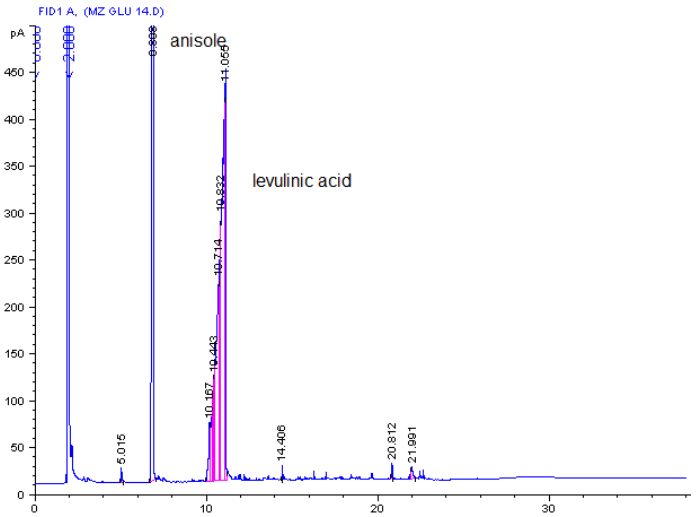


Figure 72 Typical GC spectrum of organic products from glucose (0.2 M H_2SO_4, 20 Min); Anisole is used as an internal standard

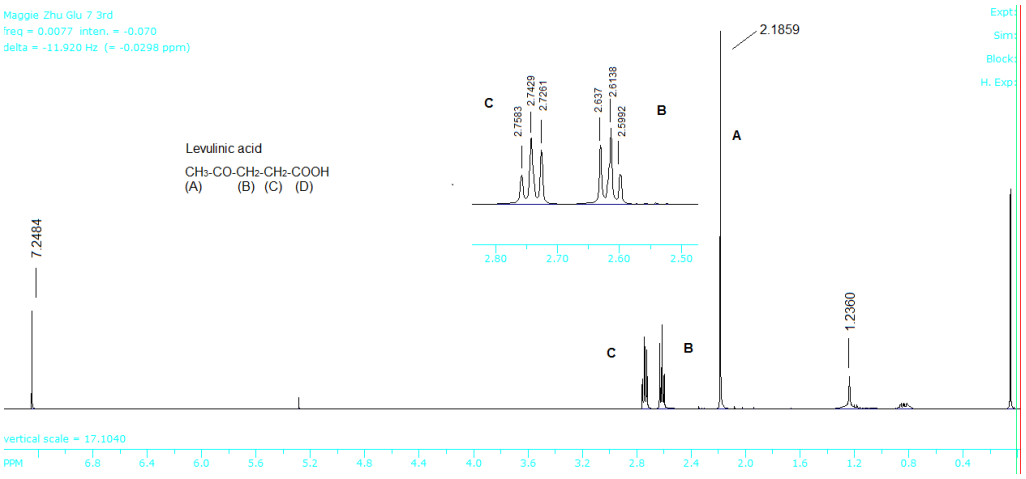


Figure 73 Typical ^{1}H-NMR spectrum of organic product (use $CDCl_3$ as solvent); condition: (0.2M, H_2SO_4, 20 Min)

Therefore, the glucose decomposition led to the significant yield of levulinic acid. Due to the limited amount of time, the decomposition of xylose is not studied. In the future, it would be interesting to study the decomposition products from xylose as well.

3.1.11 Potential gel formation

As was mentioned in experimental section (Chapter 7), the liquid fraction was neutralized by NaOH or HCl before the monosaccharides analysis by Dionex. During the neutralization of NaOH samples by 1M HCl, a gel product was formed (see Figure 74A). This was found in most of the NaOH pretreatment samples and very strong H_2SO_4 pretreatment (see Figure 74B). Microwave assisted pretreatment lead to an effective break down of hemicellulose, contributing to an abundance of sugars in the pretreatment media. These isolated heteropolysaccharides are able to form a dense macromolecular network with low mobility, which can explain this gel product here.

Due to its gel properties, there is an increasing interest on extracting hemicellulose and forming film from it.[224-226] Therefore, in order to find more information about this gel product, 10 runs of microwave assisted pretreatment were performed under the condition of 180 ^{o}C, 0.2 M NaOH for 10 Min in the CEM Discover microwave machine. The liquor fraction was separated from biomass solid fraction by filtration and collected together in order to get enough liquor to film casting. Two groups of experiments were prepared. Figure 75 shows the result of the film casting from the gel product. As can be seen, the film presents a yellow colour and the texture is soft, while it has a low ductility.

10min samples		20min samples	
0.2M NaOH	22 ✓	25	×
	23 ×	26	×
	24 ✓	27	✓
0.4M NaOH	34 ✓	37	✓
	35 ✓	38	✓
	36 ×	39	✓
1M NaOH	55 ✓	58	✓
	56 ✓	59	✓
	57 ✓	60	✓
0.2MH2SO4	67 ×	70	×
	68 ×	71	×
	69 ×	72	×
0.2MH2SO4	82 ×	82	×
	83 ×	83	×
	84 ×	84	×
1MH2SO4	97 ×	103	✓
	98 ×	104	✓
	99 ×	105	✓

A B

Figure 74 Gel product from neutralization procedure. The numbers are sample names. Tick means the gel product appeared. Cross means gel product being absent.

Conventional plastic packages create a significantly burden to the environment, for instance, a plastic bottle can take 450 years and plastic bag can take 10-20 years to be decomposed.[227] Moreover, wildlife habitat could be impacted by these abandoned plastic items. Therefore, it is of great interest and necessity to form a new type of biodegradable plastic material. Our gel product during pretreatement process is a promising candidate for bio-based plastic packing material. In the future, it would be interesting to scale up the pretreatment by using other microwave machine e.g. CEM Mars, improve its mechanical properties and test its biodegradability .

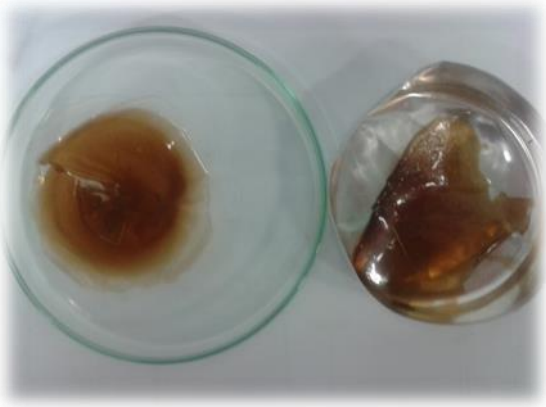


Figure 75 Film obtained from pretreatment media

3.1.12 Energy balance calculation and predication

Energy input and output were briefly studied. The experiments were done in CEM Mars, in which 12 samples can be pretreated at the same time under same energy input. There experiments were done in 2 groups and the conditions are presented in the Table 21. According to the results from SSF, we get maximal ethanol production at the condition of 0.2 M NaOH pretreatment, at 180 ^{o}C for 20 Min. The energy output of sample A is 13 kJ. The energy absorbed by water was measured by just adding water in the reactor and

calculated from power profile. We assume all the energy was used. The energy absorbed by water in this case is 20 kJ, which is the energy used to heat up and keep water temperaute at 180 ^{o}C for 20 Min. When both biomass and water were put in reactor, the energy consumption is 48 kJ. Therefore, the energy absorbed by biomass is 28 kJ (9.33 kJ/g biomass). So, the total energy balance is – 15 kJ per sample. Therefore, the biomass loading was tested in order to improve the energy efficiency, see Table 21. The higher biomass loading can lead to a lower energy input on each gram of biomass without changing/ little changing of pretreatment media volume. Therefore, 12 g biomass was immersed in 70 ml solution and pretreated under H_2SO_4 or NaOH condition. The biomass samples were well mixed in the pretreatment solution. Firstly, considering H_2SO_4 can break down biomass more efficiently than NaOH, so short holding time was used (5 Min). Similarly, 70 ml water was used to test the energy absorbed only water. When holding time is 5 Min, energy absorbed by water is 12 kJ. The results show that less energy was absorbed by biomass, which is only 12 kJ per sample (1 kJ/ g biomass). When NaOH was used for pretreatment, longer holding time (20 Min) was applied. The energy absorbed by water is 23 kJ, which was used to heat up the media to 180 ^{o}C and keep it for 20 Min. The energy absorbed by each sample is 19 kJ (1.6 kJ/ g biomass). Hence, in comparison with 3 g biomass loading, 12 g loading remarkably improved energy efficiency, which is 5.8 to 9.3 times higher under same conditions. It is worth mentioning that in real situation, a substantial amount of heat would be extracted from the hot biomass samples and resused (e.g. to heat water for the nest batch, which would reduce the energy input of the microwave).

Table 21 Energy balance test

	Conditions	Loading	Energy input per sample (kJ)	Energy output (kJ)	Energy biomass absorbed (kJ)	Energy balance for biomass
Sample A for SSF	0.2 M NaOH 20 Min 180 ^{o}C	3 g *Miscanthus* 60 ml	48	13	48-20=28	-15
Energy absorbed by water	H_2O 20 Min 180 ^{o}C	60 ml	20			
Sample B for energy input test 1	0.2/0.4 M H_2SO_4 5 Min 180 ^{o}C	12 g *Miscanthus* 70 ml	24	-	24 – 12=12	--

Energy absorbed by water	H_2O 5 Min 180 °C	70 ml	12			
Sample C for energy input test 2	0.2/0.4 M NaOH 20 Min 180 °C	12 g *Miscanthus* 70 ml	42	-	42-23=19	--
Energy absorbed by water	NaOH 20 Min 180 °C	70 ml	23			

Table 22 shows the biomass mass balance of the pretreatment. 0.4 M H_2SO_4 led to a good weight loss during pretreatment. Due to the limited amount of time, the fermentation of these conditions were not conducted. However, combining the SSF results and these results indicate that improved loading and using 0.2 M/0.4 M H_2SO_4 within a short period has good potential to give a promising energy balance.

Table 22 Sample weight loss during pretreatment

Samples	Test 1 0.2 M NaOH	Test 1 0.4 M NaOH	Test 2 0.2 M H_2SO_4	Test 2 0.4 M H_2SO_4
Mass loss	0.3 g	0.5 g	1.4 g	5.2 g

3.2 Conclusions and future work

Miscanthus is one of the most promising energy crops in Europe and improvements in the processing of this species can contribute to realise second generation biofuels. In this work, microwave assisted pretreatment of *Miscanthus* with water, H_2SO_4 (0.2 M, 0.4 M or 1 M) and NaOH (0.2 M or 0.4 M or 1 M) was performed at various hold times (5 Min to 30 Min) under 180 °C. Different analysis techniques have been used to assess the efficiency of the pretreatment. Firstly, the reducing sugar release was measured. Increasing the hold time firstly increased and then decreased the reducing sugar yield. 20 Min hold time can remove the largest amount of sugars using various pretreatment media. By varying the pretreatment media, xylose or glucose was selectively produced. The maximum sugar yield from the available carbohydrates is 75.3% by using 0.2 M H_2SO_4 pretreatment for 20 Min and glucose yield from available carbohydrate is 46.7% under this condition. However, severe acid conditions can lead to the further degradation of sugar products and biomass carbonization. Water and NaOH have a similar influence on sugar release during the pretreatment, giving rise to xylose as the major sugar

component because of hemicellulose degradation. Optimal xylose yield (28%) was achieved by using 0.4 M NaOH pretreatment for 20 Min. Secondly, changes of biomass major compositions (crystalline cellulose, hemicellulose and lignin) were evaluated and compared. The results showed that H_2SO_4 led to efficient decomposition of both hemicellulose and cellulose, but it gives lower digestibility afterwards, because most of the digestible sugars are released during the pretreatment step. These digestible sugars are able to be converted into ethanol. Hence, a different approach of SSF needs to be developed for the pretreatment liquid fraction, because the pretreatment media (where the sugar presents) has either too low or too high pH condition. Lignin was largely removed from biomass with NaOH, 94.2% of which was removed from *Miscanthus* by using 0.4 M NaOH for 20 Min. NaOH has a stronger influence on the biomass surface, leading to exposed biomass fibres. Hence, digestibility of *Miscanthus* pretreated with NaOH was 8 to 9 times higher than that of untreated *Miscanthus*, due to the efficient removal of hemicellulose and lignin and a more open biomass structure. Thirdly, SSF was used to investigate the potential bioethanol production of pretreated biomass solid fraction. Promising ethanol production was obtained by using NaOH pretreated biomass, which was about 7 times higher than that of untreated biomass material. Energy balance was briefly studied and the result showed higher biomass loading potentially led to a better energy efficiency.

Overall, this chapter studied microwave assisted H_2SO_4 or NaOH pretreatment of *Miscanthus* at 180 ^oC. Compared to the previous study on pretreatment methods, the results in this chapter shown great sugar yields and selective productions of glucose or xylose during short reaction time. At the same time, bio-based film showing the potential of bioderived plastic in the future, which showing great importance in the concept of biorefinery.

The beneficial effect of microwave heating is that it is volumetric and selective towards the polar parts of lignocelluloses, which eventually facilitates the disruption of their recalcitrant structures. However, further work is needed to scale up the system (larger batch or continuous process). Also, a critical assessment of the cost and benefits of this approach are needed because the initial capital investment and operation costs of microwave heating are significant. From the results of this chapter, there are several potential improvements of pretreatment which can be tested in the future. 1. The pretreatment media can be tuned to selectively remove xylose or glucose, e.g. we can use microwave assisted water pretreatment for 20 Min to extract xylose and the other 5 Min to extract glucose. 2. Water and 0.2 M H_2SO_4 give rise to rather different reducing

sugar release, but they also lead to very similar crystalline cellulose percentage. Test water pretreatment pH would be very useful to find more information here. 3. The gel product for film casting could be scaled up by using other microwave machine e.g. CEM Mars and more study should be done to improve its mechanical properties. 4. SSF of energy balance test samples could be conducted.

Chapter 4: Microwave assisted acid and alkaline pretreatment for using *Sugarcane bagasse*

4.1 Introduction

Sugarcane belongs to the grass family and it is common in tropical and subtropical countries throughout the world. It can be as tall as 8 to 20 feet and is generally about 2 inches thick (Figure 76).[228] It is cultivated in about 195 countries and Brazil is the world's largest sugarcane producer. Sugarcane is used for the production of sugar, Falernum, molasses, soda and ethanol for fuel.[229] As we all know, sugarcane is an important bioenergy crop, where its juice has been successfully used for the production of bioethanol. Brazil, India, China, Mexico, Thailand and Pakistan are major sugarcane production countries. The reason is that the warm weather condition is beneficial to sugarcane growth.[230]

Figure 76 Sugarcane

Figure 77 Sugarcane bagasee

Sugar processing begins when the cane plants arrives at the sugar mill, where the juice is extracted from cane by rotating knives, shredders and crushers. The fibrous residue of cane stalk after crushing and extraction of juice from the sugarcane is called bagasse (see Figure 2). [231] In this chapter, sugarcane bagasse was obtained from a farm in Sao

Paulo, Brazil and was used as the feedstock for microwave assisted pretreatment study here.

Sugarcane bagasse is able to be converted into bioethanol via biotechnological route without jeopardizing food need. Various pretreatment methods have been done on sugarcane bagasse. For instance, Chen *et al.* studied the effect of microwave assisted H_2SO_4 pretreatment for sugarcane bagasse and revealed that at 190 ^oC the fragmentation of particles become very pronounced and almost all hemicellulose was removed and the crystalline structure of cellulose disappeared.[232] Vivekanand studied steam explosion pretreatment of sugarcane bagasse and the results show that increasing severity led to an accumulation of lignin in the pretreated samples, which is primarily contributed to the production of pseudo-lignin from xylan degradation products. Meanwhile, increasing severity of treatment leads to increasing glucose yields.[233] Krishnan *et al.* reported that AFEX (ammonia fibre expansion) pretreatment improved the accessibility of cellulose and hemicellulose in sugarcane bagasse during enzymatic hydrolysis by breaking down the ester linkages and other lignin carbohydrate complex bonds. The maximum glucan conversion of the AFEX pretreated bagasse is 85% and the xylan conversion is 95-98%. [234]

Figure 78 Sugarcane bagasse sample

In this chapter, sugarcane bagasse sample used here was already knife milled into powder (see Figure 78) and is pre-treated in CEM Discover Microwave machine in the presence of H_2SO_4 or NaOH and the temperature was controlled at 180 ^oC; as the study on *Miscanthus* suggested 180 ^oC was the optimal temperature condition. Holding time was in the range of 5 to 20 Min. The same analysis techniques which were used for *Miscanthus* were used here as well, in order to compare whether biomass has an influence on the pretreatment process and its subsequent digestibility.

4.2 Monosaccharides analysis

Figure 79 to Figure 81 show the reducing sugar release during pre-treatment process. As can be seen, in the case of water and alkaline pre-treatment, xylose is the major monosaccharide, suggesting that the hemicellulose is broken down in preference to cellulose and that there is a high proportion of xylan. Arabinose is the second major sugar constituent. Perceptible amount of glucose and galactose and minor quantity of mannose were detected.

When holding time is 5 Min (see Figure 79), water and 0.2 M NaOH pretreatment give rise to similar total sugar productions, which is 0.95 and 1.0 μmol reducing sugar/ mg biomass respectively (yield: 24% and 26%. The yields here all correspond to carbohydrate mass in biomass). However, when NaOH concentration is increased to 0.4 M, the sugar production declined considerably to 0.67 μmol reducing sugar/ mg biomass. 0.2 M and 0.4 M H_2SO_4 can give similar total sugar yield, which is 0.63 and 0.69 μmol reducing sugar/ mg biomass(yields: 16% and 18%). It is worth mentioning that instead of xylose, glucose becomes the major product in the acid pretreatment media, indicating that cellulose starts to be broken down into glucose. Compared to 0.2 M H_2SO_4, 0.4 M H_2SO_4 is able to yield better glucose production, suggesting that higher concentration H_2SO_4 has stronger performance on breaking down cellulose.

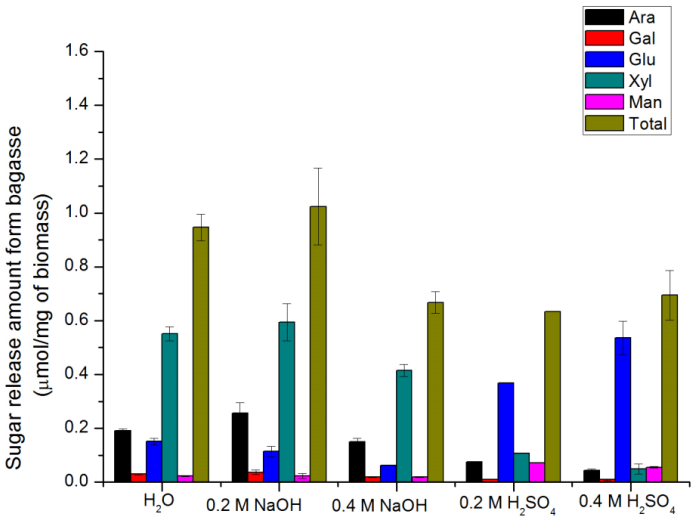


Figure 79 Monosaccharide amount after various pre-treatments when holding time is 5 Min

(each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Figure 80 presents sugar production when holding time is increased to 10 Min, water pretreatment can give rise to 0.77 μmol reducing sugar/ mg biomass (yield: 20%). With

help of NaOH pretreatment, total sugar release is remarkably increased and the xylose productions are 0.52 and 0.57 μmol /mg biomass (yields: 14% and 15%). In the case of H_2SO_4 pretreatment, 0.2 M H_2SO_4 can give better sugar yield than 0.4 M H_2SO_4. The glucose production is 0.59 μmol / mg biomass respectively and 0.26 μmol/ mg biomass respectively. As was mentioned in Chapter 3, less sugar is produced when higher concentration acid is applied, because severe acid conditions push further degradation of produced sugars. [174] Glucose was dehydrated into 5-Hydromethyl-2-furaldehyde which further is converted into levulinic acid and formic acid; Xylose could be dehydrated into furfural, These furanic products will react with sugars via condensation reaction to form humic substances or humins. [235]

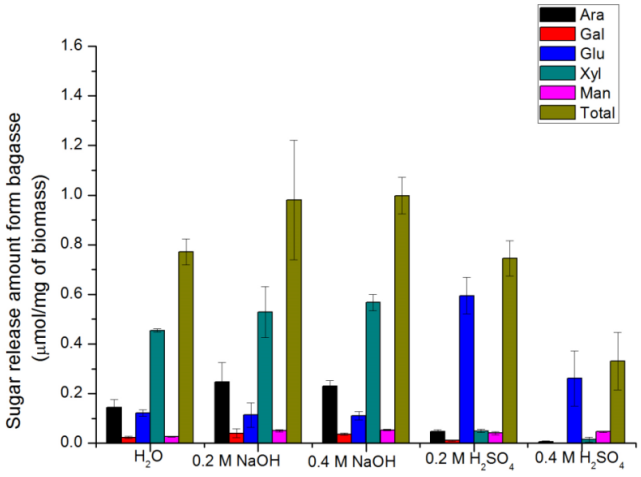


Figure 80 Monosaccharide amount after various pretreatments when holding time is 10 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

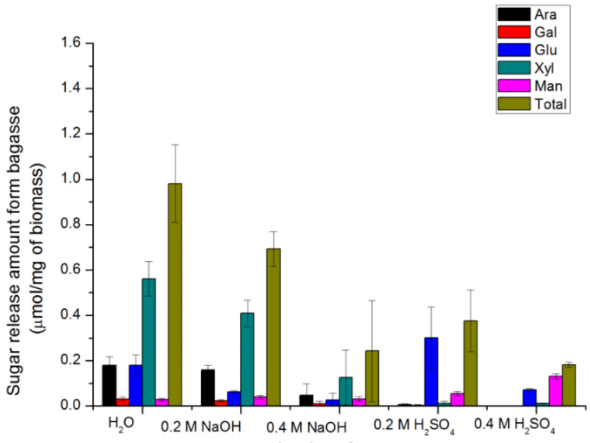


Figure 81 Monosaccharide amount after various pretreatments when holding time is 20 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

From a previous study on *Miscanthus* (Chapter 2), it was known that 20 Min is the optimum holding time to release the maximum amount of sugar into pre-treatment media. Hence, the holding time is further increased up to 20 Min here (see Figure 81). When water is applied as pretreatment media, the total sugar release is 0.98 μmol/ mg biomass (yield from carbohydrate: 24%), with significant amount of xylose produced. However, the sugar yield declines sharply when NaOH concentration is increased from 0.2 M to 0.4 M. By using 0.2 M and 0.4 M H_2SO_4, total sugar productions dropped significantly as well. The glucose production is only 0.30 μmol/mg biomass and 0.07 μmol/mg biomass respectively when H_2SO_4 and 0.2 M and 0.4 M are used.

Apart from catalyst, holding time also has a significant effect on sugar production. Comparing Figure 79 to Figure 81, it can be found that when water and 0.2 M NaOH are used as pretreatment media, changing holding time from 5 Min to 20 Min has a relatively milder influence on sugar production. In the case of 0.4 M NaOH pre-treatment, the sugar yield from available carbohydrate sharply drops from 25% to 6.2% when holding time is increased from 10 Min to 20 Min. In contrast, holding time has a significant impact on sugar production when sulphuric acid is used as pretreatment. It can be observed that total sugar amount firstly increased and then dropped down when 0.2 M H_2SO_4 is applied and 10 Min is the optimal holding time. However, in the case of 0.4 M H_2SO_4, total sugar amount gradually decreases when holding time is increasing from 5 Min to 20 Min. It is possibly because 0.4 M H_2SO_4 has a higher pH and it effectively broke down cellulose and hemicellulose when holding time was as short as 5 Min. With longer holding time it contributed to further degradation of produced sugars.

According to previous results on *Miscanthus*, H_2SO_4 generally released more reducing sugars, such as arabinose, galactose, glucose, xylose and mannose, into pretreatent media than NaOH within short hold time (5 to 20 Min). However, the results here show that H_2SO_4 actually gave rise to lower yield of reducing sugars than NaOH or even H_2O. It would be interesting to shorten the time to 2 Min or 1 Min and see if higher reducing sugar yields can be obtained. It could be predicted that different biomass materials would have different optimal holding time or even temperature condition to achieve best sugar production from pretreatment procedure, because: 1. they have different ratios of cellulose, hemicellulose and lignin; 2. their biomass architectural structure could be different.

Conventional heating pretreatment under similar conditions (180 ºC for 40 Min) was also investigated; Figure 82 shows the reducing sugar release from sugarcane bagasse when a conventional heating method is used for pretreatment. As can be seen, H_2SO_4 gives better sugar production than water and NaOH and glucose is the major component. However, the reducing sugar release results are rather lower than that of microwave assisted pretreatment. Maximally, the reducing sugar yield of microwave heating pretreatment is 5.4 times higher than that of conventional heating pretreatment within less than half the time. Therefore, better reducing sugar release is obtained within shorter period by microwave assisted pretreatment.

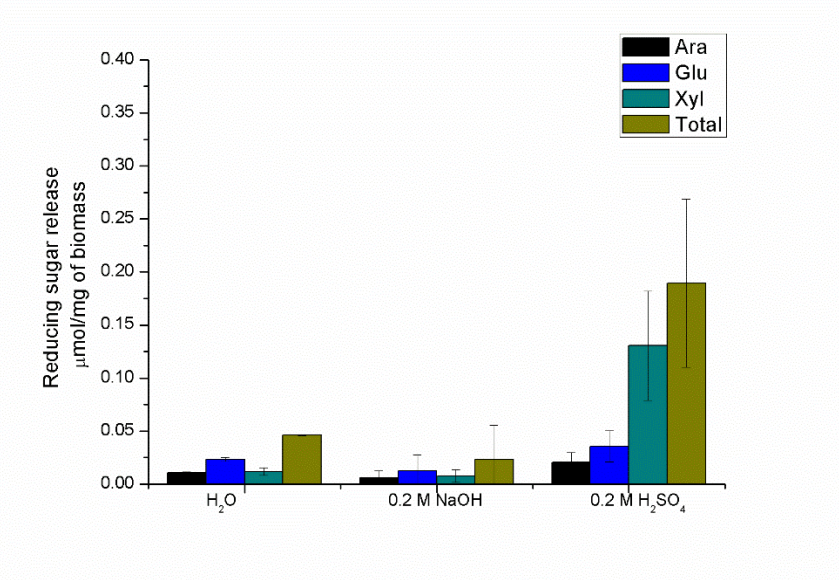


Figure 82 Reducing sugar release from conventional heating pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

4.3 Lignin analysis

When using lignocellulosic biomass as feedstock for second generation bioethanol, lignin is one of main factors behind the biomass recalcitrance.[208] Therefore, it is very important to remove lignin during pretreatment procedure and improve biomass digestibility in the following hydrolysis step. The lignin percentage in sugarcane bagasse is 31%. In this experiment, 400 mg bagasse is used, so the untreated sugarcane bagasse has about 124 mg lignin content after various pretreatments. As can be seen from Figure 83, all the lignin content after pretreatment decreased variedly. After water pretreatment, the amount of lignin present in biomass is 61 to 71 mg. NaOH pretreatments remarkably reduced lignin content, which is in good agreement with previous studies that alkaline

conditions have a delignification effect.[12, 21, 80] 0.2 M and 0.4 M NaOH pretreatments have similar lignin removal performance when holding time is 5 Min or 10 Min, during which 92 - 97 mg lignin is removed from biomass. It is worthy to mention that when bagasse is pretreated with 0.4 M NaOH for 20 Min, only 20 mg lignin is left behind in the biomass. However, H_2SO_4 is less effective than NaOH, regardless the changing of concentration from 0.2 to 0.4 M. When the hold time is 5 Min and 10 Min, 34 mg to 40 mg lignin presents in the biomass samples. However, when holding time increased from to 20 Min, the lignin amount in biomass is higher than that of 5 Min and 10 Min, which could be due to the released lignin particle redepositing back onto biomass surface. Lignin is fluidized at temperature in the range of 120 ^{o}C-200 ^{o}C.[208] Hence, a hypothesis was put forward that when high temperature pretreatment is applied, the fluidized lignin accumulates into small particles, separate from cellulose and migrates from native cell wall to the bulk liquid phase. Fluidized lignin will be eventually solidified and redeposit on biomass surface, leading to an enriched surface lignin.[184, 196] Figure 84 shows the scanning electronic microscope image of bagasse pretreated with 0.2 M H_2SO_4 for 20 Min. As can be noticed, 'lignin deposit' particles appeared on the biomass surface and they are smaller and less regular than the typical spherical particles which are due to biomass carbonization. This observation is in good agreement with our inference. Li *et al.* reported that depolymerisation and subsequent re-polymerisation of lignin occurs, with increasing severity of steam pretreatment of aspen wood. [187] Acetic acid assisted pretreatment of aspen wood also led to similar increasing amount of lignin amount. In comparison to *Miscanthus*, lignin in bagasse proved easier to remove. Under water and NaOH pretreatment, a similar amount of lignin is removed from *Miscanthus* and bagasse. Under H_2SO_4 condition 10-52 mg lignin is removed from *Miscanthus*, whereas 66-89 mg lignin is removed from bagasse.

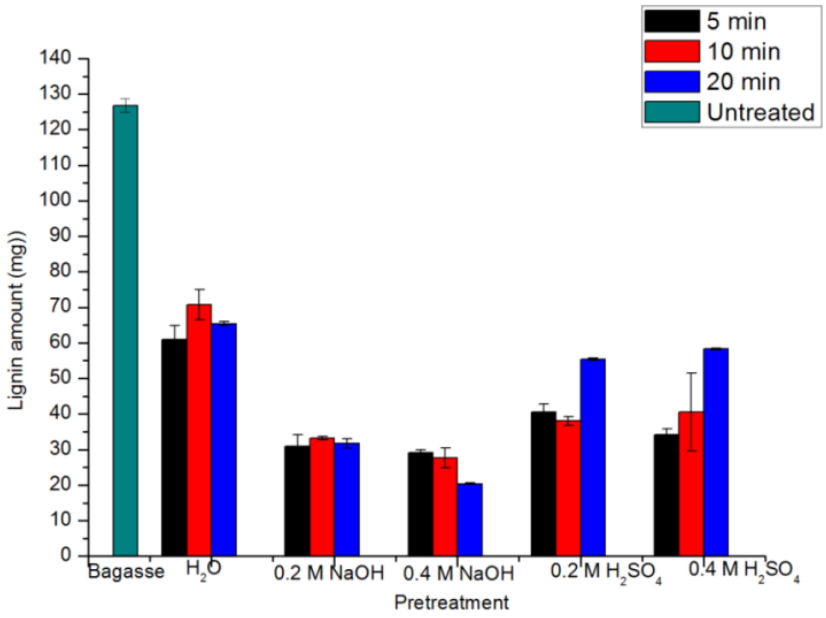


Figure 83 Lignin amount in the sugarcane bagasse samples with microwave assisted pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

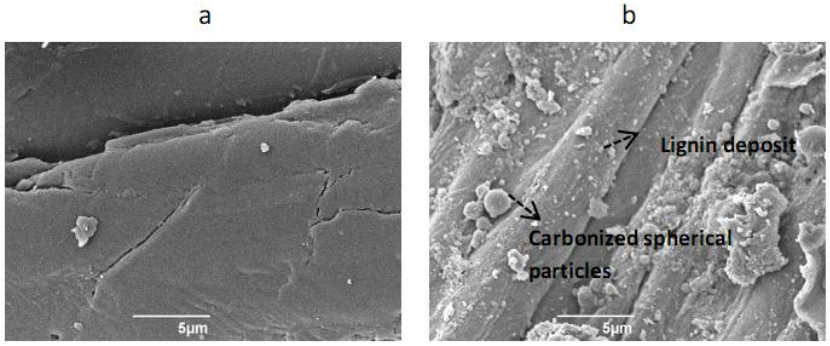


Figure 84 SEM image of (a) raw bagasse (b) 0.2 M H_2SO_4 pretreated bagasse (holding time is 20 Min) at same magnifications with scale bars of 5 μm

Lignin amounts in the biomass samples pretreated by conventional heating method were also measured (see Table 23). As can be seen, NaOH still is more effective in removing lignin from biomass samples. Under H_2O and H_2SO_4 pretreatments, similar lignin amounts present in the biomass samples. In comparison with conventional heating pretreatment, microwave assisted pretreatment are more effective in removing lignin from biomass. Lignin is a highly branched three-dimensional crosslinked polymer, which means it has poor ability to interact with microwave in a traditional mode. [236] In other words, lignin is a poor microwave absorber. However, the linkages between lignin and hemicellulose are polar groups, which could be largely influenced by microwave and further these linkages could be broken under H_2SO_4 / NaOH influence.

Table 23 Lignin amount in 400mg biomass samples pretreated by conventional heating methods

	H_2O	NaOH	H_2SO_4
Lignin content (mg)	96 ± 4	56 ±1.2	97 ± 4.8

4.4 Hemicellulose analysis

The hemicellulose percentages in biomass were measured by using same method used in previous chapters. Biomass samples measured here are samples pretreated for 5 Min. As can be seen from Figure 85, Hemicellulose comprises arabinose, galactose, glucose, xylose, mannose, galactic acid and gluconic acid, with xylose and glucose as major component. The hemicellulose percentage is 46% in untreated sugarcane bagasse, it decreases variedly after different concentration of acid or alkaline pretreatment. Water and NaOH pretreatments are able to remove similar amount hemicellulose from biomass. When 0.4 M NaOH is used as pretreatment media, the hemicellulose percentage in biomass dropped to 25%. In contrast, H_2SO_4 is significantly more efficient in removing hemicellulose from biomass. The residual hemicellulose percentage is only 5% and 3% respectively after 0.2 M H_2SO_4 and 0.4 M H_2SO_4 pretreatments. Therefore, H_2SO_4 is more effective in extracting hemicellulose from biomass than NaOH and water. It is in agreement with former results of monosaccharides analysis of pretreatment medium that water and NaOH have similar performance on reducing sugar release with xylose as major component (see Figure 79). In the case of H_2SO_4, due to the efficient hemicellulose removal, cellulose is effectively broken down, contributing to high yield of glucose (see Figure 79). Compared to *Miscanthus*, more hemicellulose is removed from sugarcane bagasse under same NaOH conditions. The hemicellulose percentage of *Miscanthus* decreased from 42% to 33% by using 0.4 M NaOH for 5 Min, but it decreases from 46% to 25% at the same condition for bagasse. With H_2SO_4 as pretreatment media, significant amount of hemicellulose is removed, regardless of the acid concentration change and biomass type. In the future, it is worth to try shorter holding time or more dilute pretreatment media for bagasse pretreatment and ideally to achieve a more energy efficient pretreatment process.

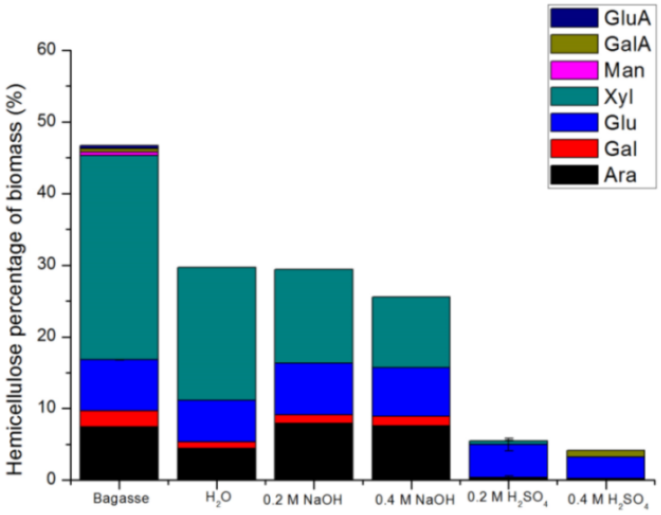


Figure 85 Hemicellulose percentages from biomass samples with microwave assisted pretreatment (holding time: 5 Min; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Hemicellulose percentage of sugarcane bagasse pretreated with conventional heating method is also measured. As can be seen from Figure 86, almost all of the hemicellulose is removed under this conventional condition (180 ^{o}C, 40 Min). It is worth mentioning that even when a significant amount of hemicellulose is depolymerised, the amount of detectable sugars in the pretreatment media is relatively small (Figure 82). Similar results were obtained when *Miscanthus* was used as the feedstock. The reason could be these hemicellulose were depolymerized. However, the produced sugars could then be degraded into other chemicals. It would be interesting to check the pretreatment liquid fraction to see if there is any degradation product present.

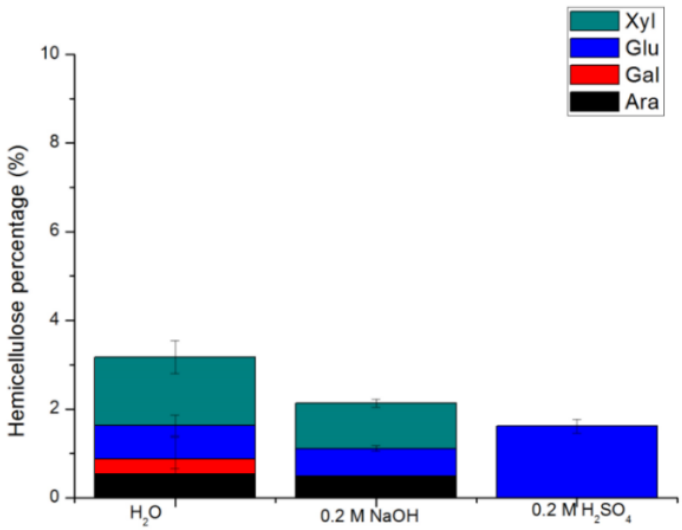


Figure 86 Hemicellulose percentage of biomass samples pretreated with conventional heating method (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

4.5 Crystalline cellulose analysis

As has been mentioned in previous chapters, completely disordered or amorphous cellulose is able to be hydrolysed at a much faster rate than partially crystalline cellulose.[17] Therefore, crystalline cellulose percentage of biomass is a crucial property in hydrolysis processing. With pretreatment, cellulose becomes more amenable and accessible to cellulose enzymes, thereby enhancing glucose production.[17] In this section, the crystalline cellulose percentage of un/pretreated sugarcane bagasse is measured (Figure 87). It is 25% in untreated sugarcane bagasse. As can be seen, water and NaOH pretreatment can increase the crystalline cellulose percentage in a varied manner. The residual biomass material has a higher percentage of crystalline cellulose, because hemicellulose is easier to decompose than cellulose. With the increasing holding time, it gradually declines, because longer holding time has a stronger effect on crystalline cellulose. In the case of H_2SO_4, when the holding time is 5 Min, the crystalline cellulose percentage of 0.2 M and 0.4 M H_2SO_4 pretreatment is slightly higher than that of untreated bagasse. However, when holding time increased to 10 Min, the crystalline percentage from 0.2 M H_2SO_4 pretreatment is increased up to 36%, due to the removal of hemicellulose and lignin. However, the crystalline cellulose percentage of 0.4 M H_2SO_4 pretreatment is sharply dropped from 37% to 9% when holding time increased from 5 Min to 10 Min, indicating sever pretreatment condition can effectively breakdown crystalline cellulose structure. When the holding time is further increased to 20 Min, both 0.2 M and 0.4 M H_2SO_4 can lead to outstanding decomposition of crystalline cellulose and their crystalline cellulose percentage is only 11% and 10% respectively. Therefore, the results here are in good agreement with previous results, NaOH is able to remove lignin and hemicellulose without major decomposition of crystalline cellulose, whereas H_2SO_4 not only removes hemicellulose and lignin but also decomposes crystalline cellulose efficiently when the condition is severe. When *Miscanthus* is used as feedstock, the crystalline cellulose percentage starts to increase when the holding time was 10 Min, because hemicellulose and lignin were removed effectively when holding time is about 10 Min. However, for sugarcane bagasse, the crystalline cellulose becomes higher when the holding time is as short as 5 Min, which again suggest that hemicellulose and lignin in sugarcane bagasse are easier to remove than *Miscanthus*.

Conventional pretreated biomass underwent the same analysis for crystalline cellulose (see Table 24). For water and NaOH pretreatment, the biomass samples present higher crystalline cellulose percentage than untreated biomass. However, H_2SO_4 give rise to lower crystalline cellulose. The results here are similar to that of microwave assisted

pretreatment, except microwave assisted pretreatment leads to higher crystalline cellulose. The reason could be that conventional heating pretreatments have longer times than microwave assisted pretreatment, which may lead to more crystalline cellulose change into amorphous cellulose.

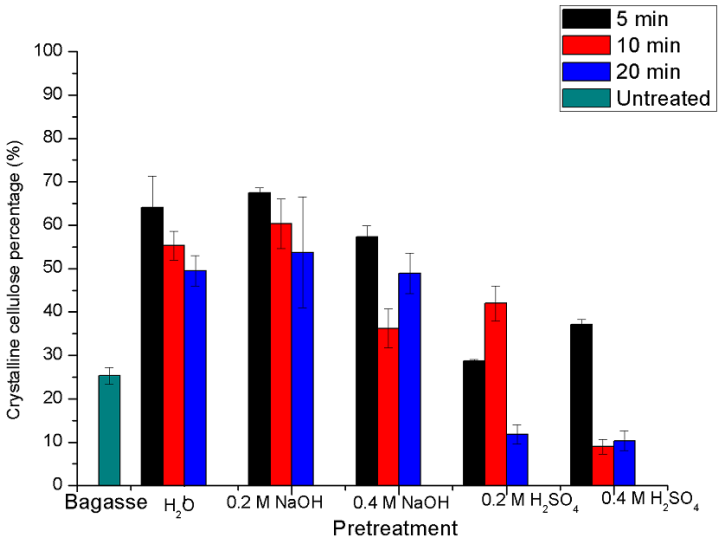


Figure 87 Crystalline cellulose percentage of un/pretreated biomass by using microwave assisted pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Table 24 Crytalline cellulose percentage of biomass after using conventional heating pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

	H_2O	H_2SO_4	NaOH
Crystalline cellulose (%)	29.6 ± 4.1	20.8 ± 3.2	37.7 ± 2.5

4.6 Digestibility of sugarcane bagasse after pretreament

As was mentioned before, digestibility is a crucial property to measure after the biomass has been pretreated. The digestibility of untreated bagasse is 113 nmol glucose/mg biomass × hour (nmol glucose /m.b.h), meaning 113 nmol glucose can be derived from 1 mg biomass per hour (the total enzymatic hydrolysis is 4 hour in total). As can be seen from Figure 88, bagasse digestibility varied after all the pretreatments. When holding time is 5 Min, water pretreatment has little influence on digestibility. However, it is significantly enhanced when holding time is increased to 10 Min and 20 Min, which are 175 and 177 nmol glucose/m.b.h respectively. With the NaOH pretreatments, the

biomass digestibilities are similarly improved, regardless the changing of holding time and alkaline concentration. Nevertheless, H_2SO_4 leads to very low biomass digestibility, especially in the case of 0.4 M H_2SO_4. It is only 19 nmol glucose/ m.b.h and 15 nmol glucose/m.b.h when holding time are 10 Min and 20 Min respectively. Under the mild saccharification condition, the release of sugar produced during the acid pretreatment process limits the availability of substrates for subsequent enzyme hydrolysis. The second possibility could be that hot acid treatment contribute to the formation of inhibitors of enzyme hydrolysis, furans such as furfurals, 5-HMF and acetic acid, carboxylic acid, formic acid, levulinic acid and phenolic compounds, and therefore reduce sugar release.[100, 237] In this work, the substrates were rinsed after pretreatment prior to enzyme saccharification. Hence, the later explanation seems unlikely. Lignin plays a synergistic and negative role in sugar production by the enzymatic hydrolysis after chemical pretreatment.[11] Due to the delignification effect of NaOH, NaOH pretreated sugarcane bagasse with low lignin percentage and higher crystalline cellulose percentage possesses higher digestibility. Nevertheless, because acid pretreated bagasse has a relatively higher lignin content and lower crystalline cellulose percentage (see Figure 83 and Figure 5.b), less glucose is produced by enzyme saccharification of biomass solid fraction after pretreatment. In comparison with *Miscanthus*, sugarcane bagasse has a remarkably higher digestibility without any pretreatment (10.25 nmol glucose/m.b.h vs 113 nmol glucose/m.b.h). From previous discussion, it was suggested that lignin and hemicellulose in sugarcane bagasse is easier to remove, leading to a high crystalline cellulose percentage within a shorter holding time. These results together suggest that even though the chemical compositions of *Miscanthus* and sugarcane bagasse are similar, their architectural structure must be different and polysaccharides in sugarcane bagasse are easier to approach and degrade than those of *Miscanthus*.

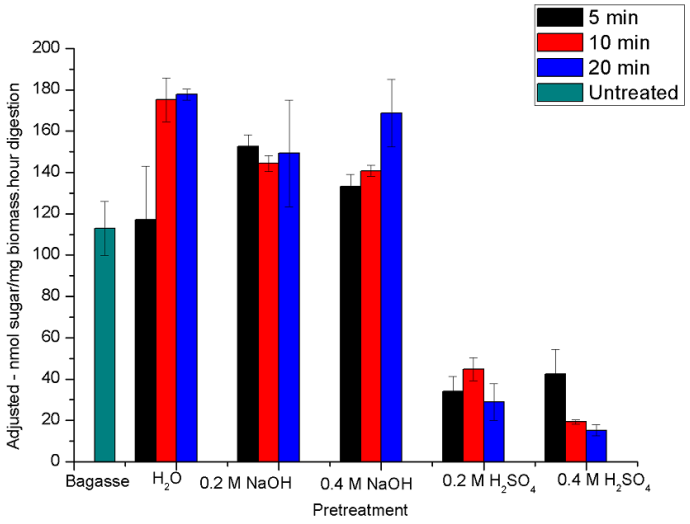


Figure 88 Digestibility of un/pretreated sugarcane bagasse samples with microwave assistance (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Digestibility of conventional pretreated sugarcane bagasse is also measured. As can be seen from Table 25, in comparison to H_2O pretreatment, biomass pretreated with NaOH has higher digestibility, which is similar to that of microwave assisted pretreatment. Overall, microwave assisted pretreatment lead to higher digestibility of biomass samples than conventional heating method.

Table 25 Digestibility of un/pretreated sugarcane bagasse samples under conventional heating method (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

	H_2O	NaOH	H_2SO_4
Digestibility of biomass	60.62 ± 4.0	165.25 ± 5.7	50.96 ±1.8

4.7 FT-IR analysis

Chemical changes in the surface of samples are qualitatively analysed by ATR-FTIR spectroscopy. Untreated, H_2O, NaOH and H_2SO_4 pretreated sugarcane bagasse were also analysed by ATR-FTIR. Here are spectra of pretreated sugarcane bagasse when holding time is 5 Min.

As was mentioned before, cellulose is a homopolysaccharide composed of β-D-glucopyranose units linked together by (1->4)- glycosidic bonds. Figure 89 shows sharp peaks at 898 cm^{-1} and 1159 cm^{-1} in the spectra, which are attributed to C-O-C stretching at the β-glycosidic linkage between the sugar units.[5] The absorbance at 1033 cm^{-1} and 1101cm^{-1} can be associated with cellulose.[210, 211] Strong peaks at 1033 cm^{-1} relates to C-O stretching at C-6.[210] As can be seen from Figure 89, water

pretreatment has very little impact of the biomass chemical compositions, since all the peaks are similar to those of raw bagasse. It is reported that lignin has absorbance around 1422 cm^{-1}, 1512 cm^{-1} and 1600 cm^{-1} [211, 215]. As is depicted in Figure 89, lignin has obvious absorbance at 1425 cm^{-1}, 1516 cm^{-1} and 1604 cm^{-1}. The peaks almost disappear after NaOH pretreatment, which is consistent with former discussion that NaOH has a strong delignification effect. The absorption at 1425 cm^{-1} is proposed to be concerned with the methyl group presenting in lignin.[157] The absorption at 1516cm^{-1} is related to the phenolic ring vibrations of lignin.[208] The absorption at 1604cm^{-1} is contributed by aromatic compounds. At the same time, the peak at 1457 cm^{-1} is also related to lignin and it disappeared after NaOH pretreatment.[211] As we know, acetate can be easily broken down. The peak at 1241 cm^{-1} (acetylation C-O stretching of hemicellulose) is diminished totally after NaOH pretreatment, suggesting that acetyl groups are effectively removed from hemicellulose by pretreatment.[217] The peak at 1727 cm^{-1} represents the ester bond (C=O) between hemicelluloses and lignin.[25] After pretreatment, the signal becomes very weak, again indicating that the linkages were broken. C-H deformation in cellulose and hemicellulose is present at 1371 cm^{-1} [217]; C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives can be seen at 1321 cm^{-1}.[218]

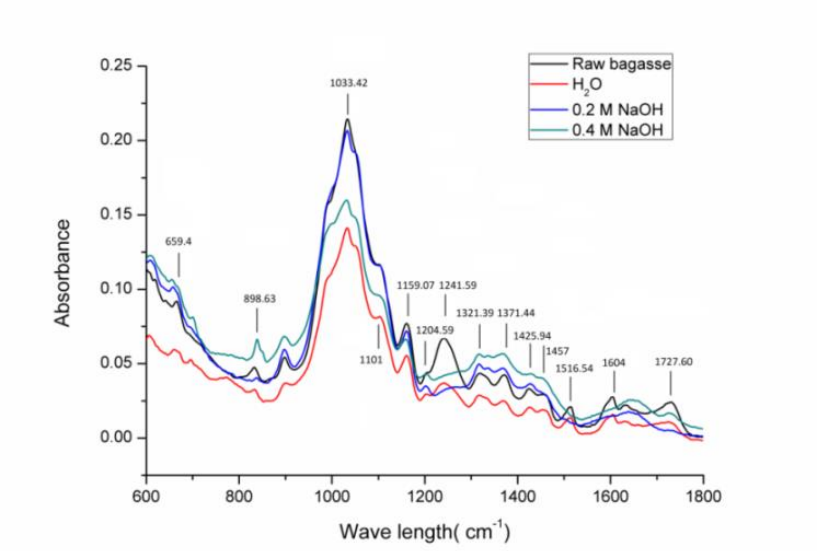


Figure 89 FT-IR analysis of sugarcane bagasse after water/ NaOH pretreatment when holding time is 5 Min

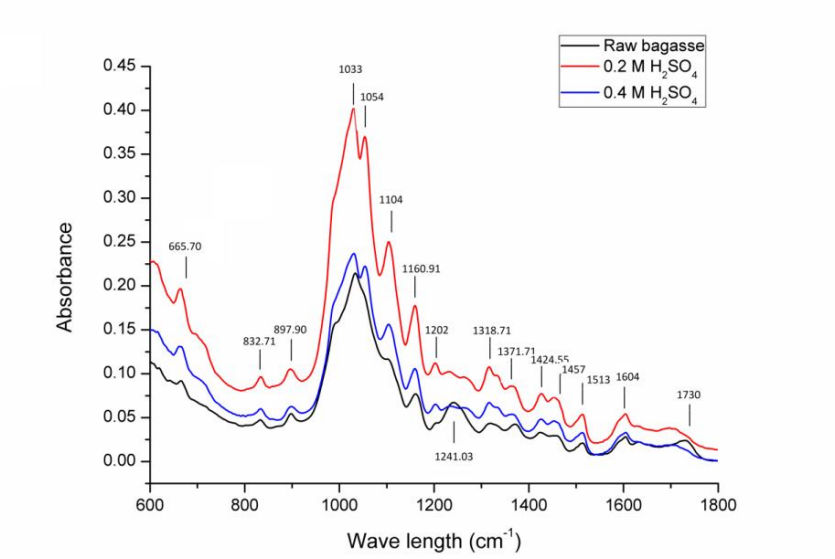


Figure 90 FT-IR analysis of sugarcane bagasse after water/ NaOH pretreatment when holding time is 5 Min

Two peaks around 1054 cm-1 and 1104 cm-1 appeared after H2SO4 pretreatment, which are contributed by polysaccharides content.[25] The hemicellulose acetyl group peak around 1241 cm-1 disappeared after 0.2 M and 0.4 M H2SO4 pretreatment. Lignin absorbance around 1424 cm-1, 1457 cm-1, 1513 cm-1 and 1604 cm-1 show little changes, indicating H2SO4 has little influence on lignin structure. The peak around 1730 cm-1 disappeared after pretreatment, suggesting that linkages between hemicellulose and lignin are broken

Table 26 Chemical composition changes in biomass after pretreatments

peak position (cm^{-1})	Assignment
897	β-glycosidic linkage between the sugar units
1033	C-O stretching
1104	Related to polysaccharides content appearance
1108	Relate to crystalline cellulose
1159	C-O-C stretching at the β-glycosidic linkage
1239	acetyl C-O stretching of hemicellulose
1321	C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives
1371	C-H deformation
1457	Related to lignin
1424, 1425	Stretching of O-CH_3
1513, 1516	Phenolic ring vibrations of lignin
1604	Aromatic ring stretching of lignin
1727,1730	Ester linkages between hemicellulose and lignin

4.8 SEM analysis

Knife milled bagasse samples present two main morphological features: fibres and pith particles (Figure 91). Fibres come from sugarcane conducting vessels while pith particles are mainly from parenchyma tissue. The fibre surface is structured with parallel stripes and is partially covered by residual material. Pith is a more fragile and fragmented structure containing pits, which are small pores connecting neighbouring cells on the surface of the walls (Figure 91 (B)). Both structures were imaged in this work, but bagasse fibres are preferentially presented to simplify comparisons.

A B

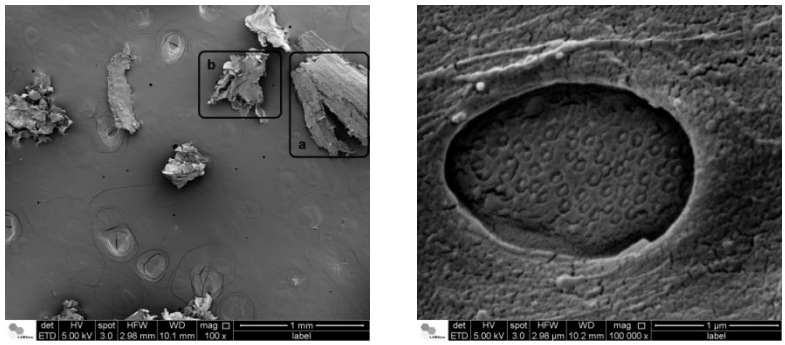


Figure 91 Surface images of the untreated sugarcane bagasse obtained by scanning electron microscopy. (A). General view of the sample showing fibres and pith (a and b); (B) surface of pith showing pits.

Figure 92 a, d and g show micrographs of the surface of untreated bagasse particles under variable magnification. Untreated bagasse presents a relatively flat and clean surface, as shown in Figure 92 (a, d and g), with conducting fibre packed in bundles (Figure 92 (d)). Figure 92 (b, e and h) are obtained from bagasse pretreated with water at 180 ^{o}C for 5 Min. As can be noticed, the biomass fibre bundles are combined together tightly. Under higher magnification, biomass surface is flat and smooth. Therefore, water treatment is very mild and has little influence on biomass structure, which is in good agreement with previous IR analysis. Nevertheless, pretreatment signs are visible when bagasse is pretreated with 0.2 M NaOH (see Figure 92(c, f and i)). Under low magnification, the biomass surface coating that can be observed in untreated bagasse (Figure 92(a)) is removed and the fibre bundles which were tightly packed in the untreated bagasse start to dismantle under the NaOH action. This effect had been previously observed for bagasse samples treated with NaOH without the microwave action and alkali concentrations 10 times higher.[12] Under medium magnification (see Figure 92(f)), the neighbouring fibres bundles have lower adhesion between them. As discussed previously (section 4.5), water pretreatment and 0.2 M NaOH pretreated bagasse have similar crystalline percentage when holding time is 5 Min. However, the digestibility of NaOH pretreated biomass is higher than that of water pretreated samples

(see Figure 88), which could be due to these two pretreatments having distinctive actions on biomass. NaOH pretreatment not only removed more lignin from biomass than water pretreatment (see Figure 83), but also made cellulose fibres more exposed and accessible for enzyme to attack. Higher magnification is applied in order to have a better understanding of biomass surface characteristics change (see Figure 92(i)). As can be noticed, there is a significant amount of residual material deposited on the fibre surface. These are probably lignin aggregates, formed by lignin extraction from the inner regions of the cell wall, followed by condensations and re-deposition on the surface. Lignin redeposition has been observed in other lignocellulosic samples treated under alkaline conditions.[28, 196, 238]

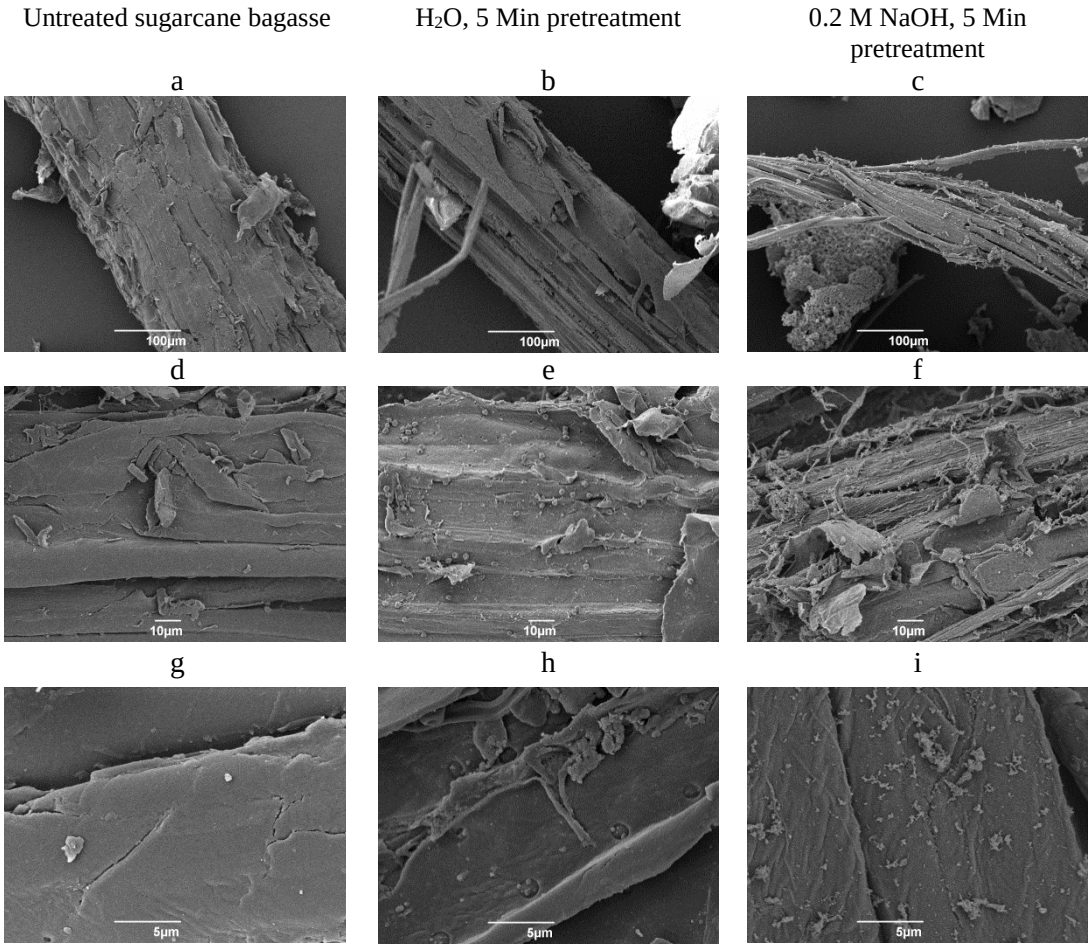


Figure 92 Surface images obtained by JEOL on sugarcane bagasse samples. Raw bagasse (a.x250; d.x1000; and g. x 5000), H_2O pretreated bagasse (b. x250; e. x1000; h.x5000) and 0.2 M NaOH pretreated bagasse(e. x250; f. x1000; i. x5000); holding time is 5 Min.

a b c

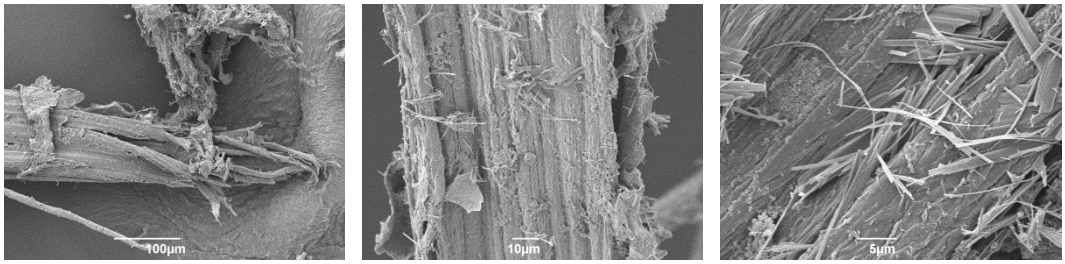
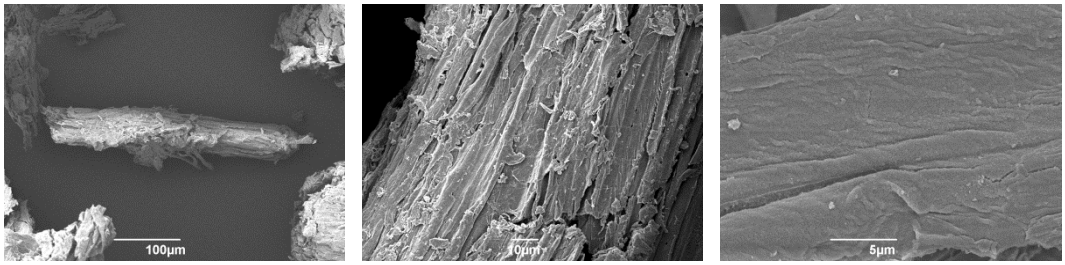


Figure 93 Surface images obtained by JEOL on sugarcane bagasse samples pretreated with 0.4 M NaOH (a. x 250; b. x 1000; c. x 5000); holding time is 5 Min

Figure 93 presents the 0.4 M NaOH pretreated bagasse surface characteristics information under different magnification scales. As can be noticed, the effect of 0.4 M NaOH is similar to that of 0.2 M NaOH and cellulose bundles are partly detached from each other (compare Figure 93 (a) to Figure 92). Cellulose bundles are exposed and covered with small size fibres (see Figure 93(b)). However, the surface characteristics are very distinctive from that of 0.2 M NaOH pretreatment when higher magnification is applied (see Figure 93(c)). 0.4 M NaOH can effectively penetrated biomass and make the cellulose fibres significantly exposed and small deposits are observed on the biomass surface.

Figure 94 presents biomass surface changes after 0.2 M H_2SO_4 pretreatment when holding time is 5 Min and 10 Min respectively. When 0.2 M H_2SO_4 is applied for 5 Min, the biomass surface coating is damaged and a number of parallel strips appear on biomass surface. With higher magnification, the biomass shows a tight and compact structure, which is similar to that of untreated bagasse (compare Figure 94(c) to Figure 92(g)). Hence, 0.2 M H_2SO_4 pretreatment have a mild performance on bagasse surface when holding time is 5 Min. Nevertheless, when holding time is increased to 20 Min, the biomass shows a completely differently features. Macroscopically, the samples become a black powder like coal. Under microscope, it can be observed from Figure 11 (e), the biomass coating is completely removed and fibre bundles are combined together with spherical particles aggregating together and surrounding on them. With higher magnification we can clearly see the typical spherical particles showing biomass is becoming carbonized (see Figure 94(f)).[198]

a b c



d e f

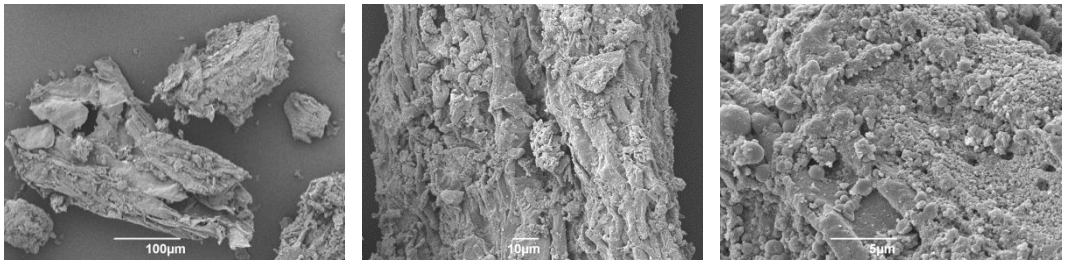


Figure 94 Surface images obtained by JEOL on bagasse pretreated with 0.2 M H_2SO_4 for 5 Min(a. x250; b. x1000; c. x5000) and 20 Min(d.x 250; e. x1000; f. x 5000); holding time is 5 Min

Therefore, NaOH and H_2SO_4 have completely different performance on biomass. NaOH is able to remove the biomass surface coating and make cellulose fibres bundles more exposed and accessible for enzyme to attack. At certain conditions lignin deposits appeared on the biomass surface, probably due to the temperature or pH change. H_2SO_4 pretreatment with short holding time (5 Min) has mild influence on biomass and biomass presents a smooth surface and compact network. However, when holding time is up to 20 Min, the bagasse sample is completely carbonised with a still tight biomass structure. In other words, H_2SO_4 pretreatment tends to degrade instead of fractionate the components and lead to biomass carbonisation.

4.9 Conclusion

In this chapter, sugarcane bagasse is used as feedstock for microwave assisted H_2SO_4 or NaOH pretreatments and conventional heated pretreatments were performed in order to compare. Different analysis techniques were used to evaluate the pretreatment process. In comparison to conventional pretreatment, microwave assisted pretreatment shows great potential. In general, the results are similar to that of *Miscanthus*. Firstly, promising reducing sugar release was obtained from pretreatment process within shorter holding time by using microwave assisted pretreatment. Production of xylose and glucose were obtained by using NaOH or H_2SO_4 as pretreatment media. Maximally, the reducing sugar release from microwave assisted pretreatment is 5.4 times higher than that of conventional heating method and the shorten the holding time 8 times. Longer holding time will facilitate further degradation of produced sugars. Secondly, hemicellulose is effective broken down within short period of holding time by using H_2SO_4. 74-83% lignin is effectively removed by NaOH during pretreatment, whereas 67-73% lignin is removed by H_2SO_4. Due to the efficient removal of lignin and hemicellulose, as well as more exposed cellulose bundles under NaOH performance, bagasse solid fraction pretreated by NaOH has a promising digestibility. Carbonization of bagasse was also observed when H_2SO_4 pretreatment condition is severe (longer holding time: 20 Min), further contributing to relatively low digestibility of pretreated

biomass. However, due to the different chemical compositions ratio and potentially different biomass architecture structure, optimal pretreatment conditions for *Miscanthus* and sugarcane bagasse are different. Hemicellulose and lignin in sugarcane bagasse are easier to remove than that of *Miscanthus*. The optimal pretreatment time is as short as 10 Min, which meaning less energy input and less sugar degradation during pretratment process.

Overall, in this chapter promising sugar production was achieved during very short amount of time, suggesting sugar cane bagasse is a very promising candicate for pretretment. It is worthwhile to conduct SSF process to evaluate the further potential of using sugarcane bagasse as the feedstock for bioethanol production.

Chapter 5: Microwave assisted acid and alkaline pre-treatment for using *Maize* biorefineries

5.1 Introduction

Maize is a tropical grass and it needs warm temperatures (see Figure 95). Optimal temperature lies between 20-24 ^{o}C and the temperature at night should not below 14 ^{o}C. However, maize is cultivated on every continent except Antarctica. Depending on the variety and climate condition, it requires 70 to 210 days for its full development.[239]

Figure 95 Maize

There are 835379- 991291 thousand metric tons maize produced world-wide (see Table 1). United States is the biggest maize producing country, contributing to 32%-40% of world maize production. It grows intensively in Southeast Asia, especially in China (20%-23% world maize production). It also widely distributed in Europe, Africa and South America.

Table 27 World maize production (source: USDA —Foreign Agricultural Service)[240]

Production (thousand metric tons)	2019 /11	2011 /12	2012 /13	2013 /14	2014/15 Feb	2014/15 Mar
Argentina	25200	21000	27000	26000	23000	23500
Brazil	57400	73000	81500	80000	75000	75000
Canada	12043	11359	13060	14194	11500	11500
China	177245	192780	205614	218490	215500	215500
Ethiopia	4895	6069	6158	7451	6500	6500
European Union	58272	68123	58896	64259	74160	74160
India	21730	21760	22260	24260	22500	22500

Indonesia	6800	8850	8500	9100	9200	9200
Mexico	21058	18726	21591	22880	23200	23200
Nigeria	8800	9250	7630	7700	7500	7500
Philippines	7271	7130	7261	7532	7900	7900
Russia	3075	6962	8213	11635	11500	11500
Serbia	6800	6400	3750	6400	6850	6850
South Africa	10924	12759	12365	14982	13500	11500
Ukraine	11919	22838	20922	30900	28450	28450
Others	86329	88368	90084	92553	93940	93810
Subtotal	519761	575374	594804	638336	630200	628570
United States	315618	312789	273192	351272	361091	361091
World Total	835379	888163	867996	989608	991291	989661

Maize can be divided into silage maize and grain maize, according to their utilization. Silage maize is cultivated for feed and is predominantly used on-farm. However, grain maize not only can be used for feed (poultry, corn-cob-mix for pigs), but also for food (maize-meal-products, snacks, cornflakes) or for industrial purpose (starch, paper industry).). Temperature and precipitation play significant roles in its production. In general, silage maize is more suitable to grow in north-western European regions, due to the shorter and wetter climatic conditions and can be harvested for this purpose while still unripe. Nevertheless, grain maize production dominates in dryer and warmer regions of central and southern Europe. [239] According to the data from US Department of Agriculture Economic Research, 79% of maize is used for the biofuel production (see Figure 96). Only 29% of maize is used for food and manufacturing. Therefore, we can assume that there is little food competition here, since there is enough maize for food and other purposes.

A number of pretreatment methods have been studied on maize. Schell *et al.* studied dilute H_2SO_4 (0.5-1.41%) pretreatment of corn stover under conventional heating condition (165-183 ^oC). They obtained a high xylose yield (70-77%) and the cellulose conversion yields in SSF of 80-87%. The kinetic modelling results suggested that low pH was required to achieve the highest xylose yield and higher temperature promotes higher yield, while shorter residence times are required. [241] Nikolic *et al.* studied microwave pretreatment for corn and the results show that the glucose concentration in

pretreatment liquor was increased 8.48% and percentage of theoretical ethanol yield of 92.27% were achieved after 44 h of the simultaneous saccharification and fermentation (SSF) process of corn meal[130].

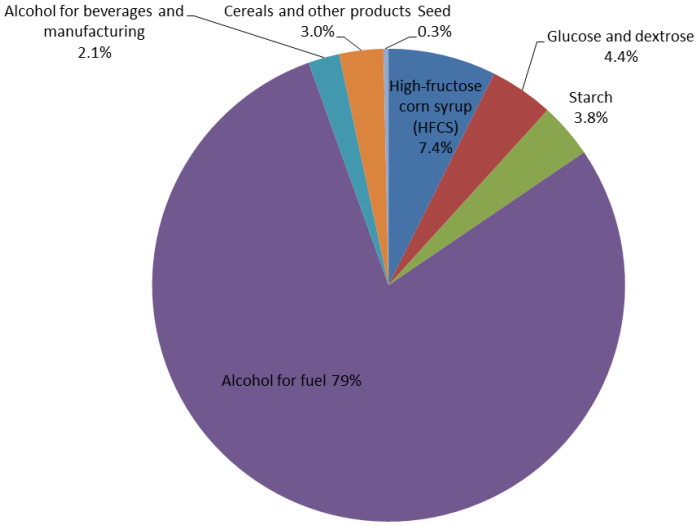


Figure 96 US maize usage segment (2014-2015 Sep-Aug)[242]

In this chapter, maize (obtained from Lousignan, France) is used as the feedstock for the microwave assisted pretreatment (see Figure 97). CEM Discover Microwave machine is used as microwave source. The maize was pretreated in the presence of H_2SO_4 or NaOH and the temperature controlled at 180℃, as the study on *Miscanthus* suggested 180 ºC is the optimal temperature condition. Holding time was in the range of 5 to 20 Min. The same analysis techniques which have been used for *Miscanthus* and sugarcane bagasse were used for maize as well, in order to compare the pretreatment process and its subsequent digestibility. Conventional heating pretreatment was performed at the temperature of 180 ºC for 40 Min, in order to compare with the results of microwave assisted pretreatment.

Figure 97 Maize sample used in this study

5.2 Monosaccharides analysis of pretreatment liquid fraction

Monosaccharides released from maize during pretreatment process are measured by HPEAC (High-Performance Anion-Exchange Chromatography) (see Figure 98 to Figure 100). Arabinose, galactose, glucose, xylose and mannose presented in the pretreatment media. As can be seen from Figure 98, when holding time was 5 Min, similar to the results of *Miscanthus*, 0.2 M H_2SO_4 gave rise to best sugar release (1.25 μmol/ mg biomass) with a promising yield of glucose (0.75 μmol/ mg biomass). An increase of acid concentration from 0.2 M to 0.4 M led to a reduction of sugar release to 0.86 μmol/ mg biomass. In the case of H_2O and NaOH pretreatment, similar sugar profiles were obtained and H_2O pretreatment led to better sugar production than NaOH pretreatments. An increase of NaOH concentration has little influence on sugar production and compositions. The reducing sugar production is 0.66 μmol/ mg biomass and 0.60 μmol/ mg biomass respectively for 0.2 M and 0.4 M NaOH pretreatments. Under NaOH and H_2O conditions, similar amounts of xylose and glucose are obtained from liquor fraction of maize pretreatment, which is different from that of *Miscanthus* and sugarcane bagasse (they presented a reducing sugar mixture composed high amount of xylose). It suggests that the hemicellulose composition and structure of maize is different from that of *Miscanthus* and sugarcane bagasse. As was discussed in Chapter 3 and 4, xylan is the major composition of hemicellulose material in *Miscanthus* and sugarcane bagasse. Considering maize has a good yield of glucose under H_2O and NaOH conditions, both glucan and xylan could be the major compositions of hemicellulose fraction of maize.

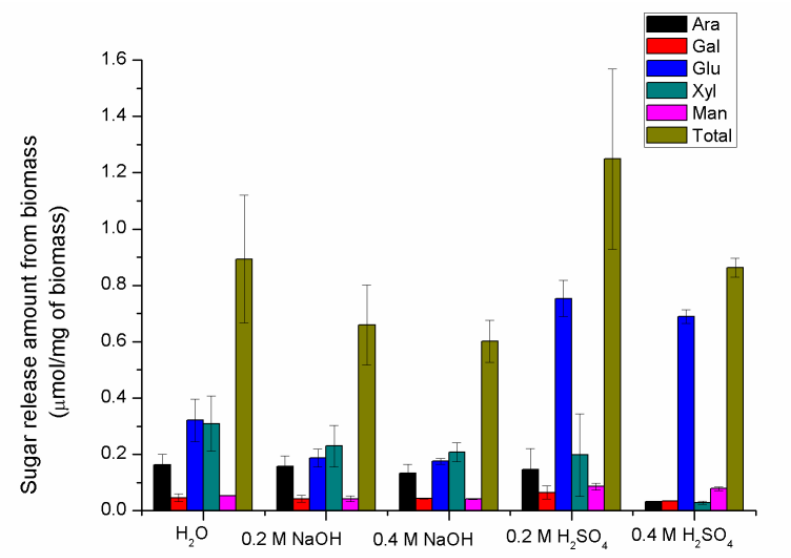


Figure 98 Monosaccharide amount after various pretreatments when holding time is 5 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Figure 99 shows the reducing sugar release in pretreatment media when holding time is 10 Min. Similar to the results of 5 Min, 0.2 M H_2SO_4 pretreatment led to better sugar production than NaOH and H_2O pretreatments and the reducing sugar release was 1.08 μmol/ mg biomass with high production of glucose (0.78 μmol/mg biomass). When acid concentration increased from 0.2 M to 0.4 M, the reducing sugar release dropped to 0.32 μmol/ mg biomass, indicating a large amount of reducing sugars are degraded into other chemicals. 0.2 M NaOH leads to a sugar production of 0.87 μmol/ mg biomass, with xylose, glucose and arabinose as major products. When NaOH concentration increased to 0.4 M, the sugar yield reduced to 0.63 μmol/mg biomass, which was probably due to sugar degradation. [243]

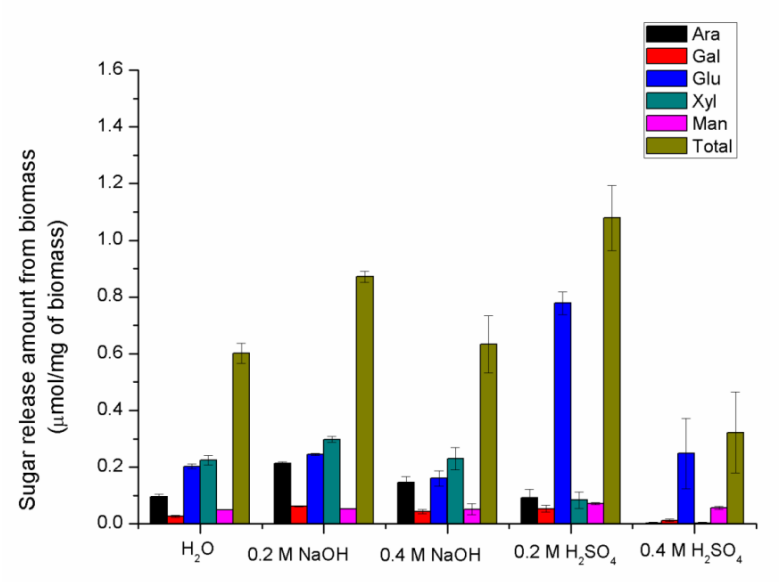


Figure 99 Monosaccharide amount after various pretreatments when holding time is 10 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

When holding time increased to 20 Min (see Figure 100), the reducing sugar release of 0.2 M and 0.4 M H_2SO_4 pretreatments dropped to 0.39 μmol/ mg biomass and 0.24 μmol/ mg biomass respectively, due to the degradation of produced sugar under strong acid conditions, as was suggested in Chapter 3. H_2O and NaOH pretreatments led to better sugar release than H_2SO_4 pretreatments and the reducing sugar production is 0.85 μmol/ mg biomass and 0.77 μmol/mg biomass respectively. An increase of NaOH concentration decreases the reducing sugar release to 0.52 μmol/ mg biomass.

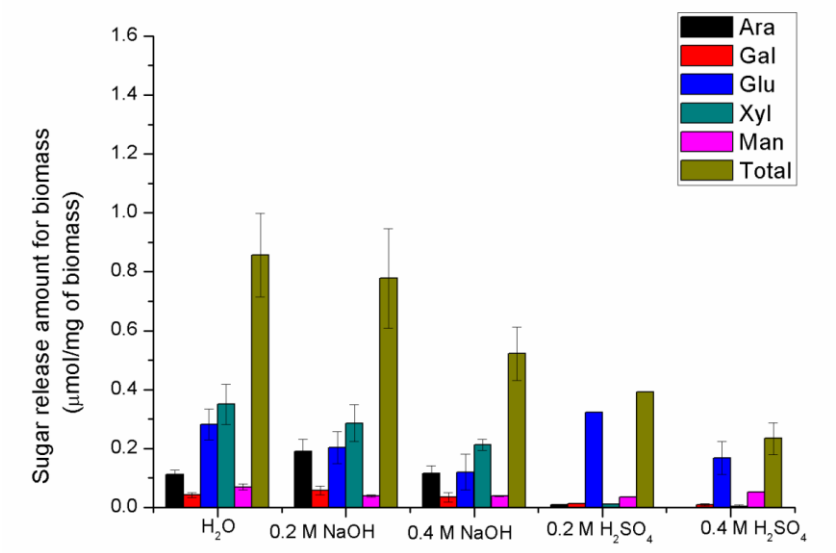


Figure 100 Monosaccharide amount after various pretreatments when holding time is 20 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Overall, comparing Figure 98 to Figure 100, it can be seen that H_2SO_4 efficiently breaks down biomass (both hemicellulose and cellulose) and leads to promising reducing sugar release with high yield of glucose. However, it also contributes to a fast degradation of produced sugars, leading to a low reducing sugar release when the condition is severe (high acid concentration or long holding time). Best sugar release amount is up to 1.25 μmol/ mg biomass (yield: 21.3%) when 0.2 M H_2SO_4 is only used for 5 Min. In comparison with the optimal condition of *Miscanthus* (20 Min, 0.2 M H_2SO_4), less reaction time is needed here. As we discussed before, xylan is the major component of hemicellulose in *Miscanthus*, whereas glucan and xylan are the major components of hemicellulose in maize. Different hemicellulose composition could be the reason of their different optimal holding time. From the following discussion of hemicellulose (see Figure 103), we know that maize has a higher hemicellulose percentage than *Miscanthus* (52% and 42% respectively). Hence, the optimal holding time is shorter. H_2O and NaOH broke down hemicellulose in preference to cellulose and gave rise to xylose and glucose as major products during pretreatment process. Increase of NaOH concentration reduced the sugar yield presenting in the pretreatment media, whereas increasing holding time had little influence on sugar release if the NaOH concentration is fixed. This result is different from previous results on *Miscanthus* and sugarcane bagasse where both NaOH concentration and the holding time play important roles of sugar release and degradation.

Conventional heating pretreatment was performed at 180 ^oC for 40 Min in order to compare with microwave assisted pretreatment. Figure 101 presents the result of reducing sugar release in the liquor fraction by using conventional heating pretreatment. As can be seen, 0.2 M H_2SO_4 lead to better sugar yield (0.29 μmol/ mg biomass) than NaOH and H_2O, which is in agreement with the results of microwave assisted pretreatments. Under conventional heating pretreatment, almost equal amount of glucose and xylose are obtained by using acid, while only glucose presented as major product when H_2O and NaOH are used as pretreatment media. Under microwave condition, both glucose and xylose are obtained as major products by all the pretreatment media. Moreover, in comparison with conventional heating pretreatment, 4.3 times more sugar is achieved during 8 times less holding time. Therefore, microwave assisted pretreatment is more effective and efficient.

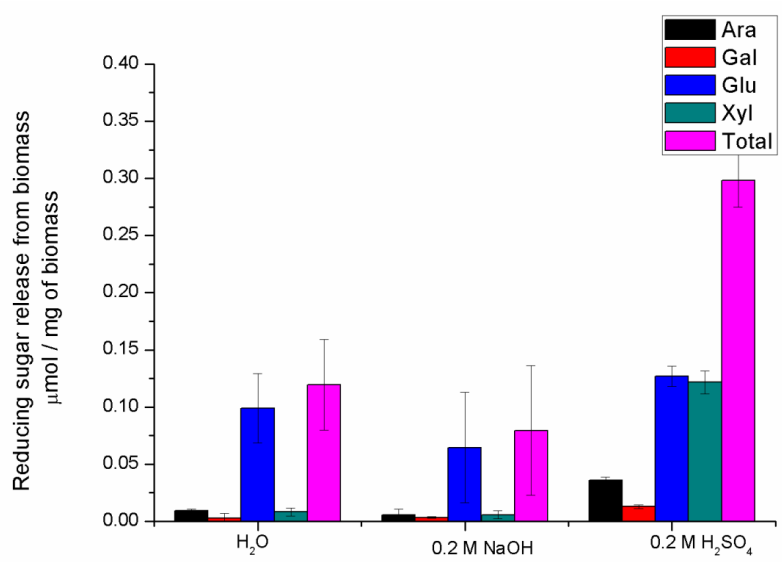


Figure 101 Monosaccharide amount after conventional heating pretreatment (40 Min, 180 ºC; each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

5.3 Lignin content analysis of biomass solid fraction

Lignin amount in maize was also measured by using same methods used in previous chapters. Figure 102 shows the lignin content present in biomass solid fraction after pretreatment. The lignin amount in 400 mg untreated biomass is 119 mg. After various pretreatment, lignin is effectively removed. When H_2O is used as pretreatment medium, increasing amount of lignin is removed from biomass with the longer holding time. In the case of NaOH and H_2SO_4, lignin is effectively removed when the holding time is as short as 5 Min. Only 9-16 mg of lignin presented in the biomass after NaOH/ H_2SO_4 conditions assayed here. When *Miscanthus* was used as feedstock, NaOH presented a remarkably stronger delignification effect than H_2O and NaOH. However, here NaOH and H_2SO_4 showed similar delignification effect. The reason could be lignin in maize is easier to remove than that of *Miscanthus*, which probably due to their less strong crosslinked lignin structure.

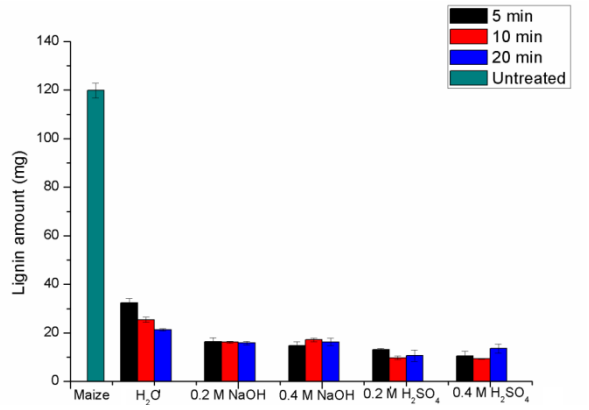


Figure 102 Lignin amount in the pretreated maize under different conditions (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Table 1 presents the lignin content in the biomass solid fraction after conventional heating pretreatment. As can be seen, similar amount of lignin is removed by H_2O and 0.2 M H_2SO_4, whereas 0.2 M NaOH lead to a better lignin removal process. In comparison with conventional heating pretreatment, lignin is removed from biomass more efficiently within shorter holding time. Therefore, microwave assistance facilitates the lignin removal process. As we discussed in chapter 1, lignin has an aromatic structure which has little interaction with microwave. However, the results here suggest lignin removal is largely promoted by microwave and the reason could be the ester bonds interact with microwave and promote the lignin removal process. In comparison with *Miscanthus* and sugarcane bagasse, lignin in maize is easier to remove.

Table 1. Lignin amount in biomass samples pretreated by conventional heating methods(each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

	H_2O	0.2 M NaOH	0.2 M H_2SO_4
Lignin amount (mg)	88 ± 6.8	58 ± 2.64	88 0.56

5.4 Hemicellulose percentage analysis of biomass solid fraction

The hemicellulose percentages in biomass were measured by using the same method used in previous chapters. Biomass samples measured here are maize pretreated under microwave assistance for 5 Min. Each condition was repeated 3 times and the results below are the average values with standard deviations (the standard deviations are very small and couldn't be seen in the figure). As can be seen from Figure 103, hemicellulose percentage in untreated maize is 52%. The hemicellulose material in maize is composed of a range of monosaccharides, namely fructose, arabinose, galactose, glucose, xylose,

mannose, Galacturonic acid and Glucoronic acid. Arabinose, glucose and xylose are the major constituents. With H_2O and NaOH pretreatments, similar amounts of hemicellulose (29%-33%) were removed from biomass material. In agreement with previous results of monosaccharides analysis, a remarkable amount of hemicellulose is removed by H_2SO_4. The hemicellulose percentage dropped to 8.2% by using 0.2 M H_2SO_4.When the acid concentration increased to 0.4 M, the hemicellulose in biomass is completely removed.

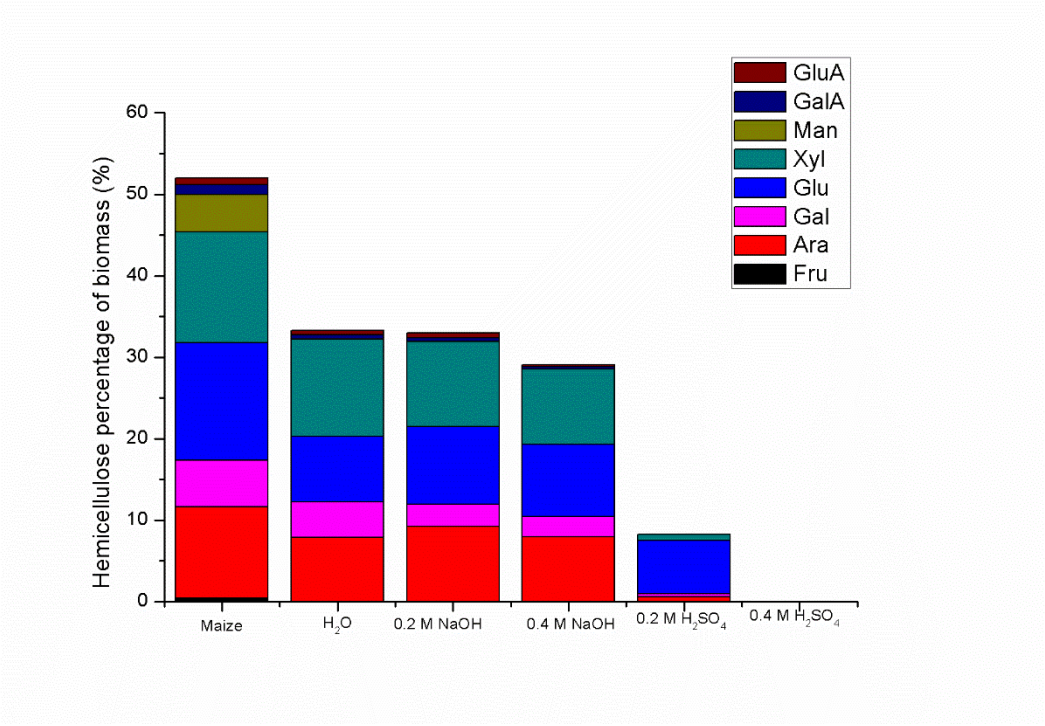


Figure 103 Hemicellulose percentages from biomass solid fraction with microwave assisted pretreatment (holding time: 5 Min; (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Hemicellulose percentage of biomass solid fraction from conventional heating pretreatment (180 ^oC, 40 Min) was also measured (See Figure 104). As can be seen, hemicellulose is effectively removed from maize, there is only 1.5%-3% hemicellulose present in the solid fraction. In this case, 505 mg – 520 mg hemicellulose should be present in the liquid fraction in the other shorter chains form or degradation chemicals. However, combining the results from monosaccharides, only 0.3 μ mol reducing sugar was produced from 1 mg biomass, which is equivalent to 49 mg reducing sugar. Therefore, a large amount of hemicellulose was successfully extracted from biomass. However, they are absent from the monosaccharides analysis in the liquid fraction. It can be assumed that they could be degraded into other chemicals. However, further confirmation should be made by analysing organic products in the liquid fraction of conventional pretreatment. In comparison with the results of microwave assisted

pretreatment results, conventional heating led to a more effective hemicellulose removal, which could be mostly due to its longer holding time. However, this long holding time possibly led to a significant degradation of produced sugars. In contrast, microwave assisted pretreatment is more effective in breaking down hemicellulose into its constituent monosaccharides, as a good yield of reducing sugars was achieved (1.25 μmol/ mg biomass= 213 μg / mg biomass, at the condition of 0.2 M H_2SO_4 and 5 Min) and possibly less sugar degradation.

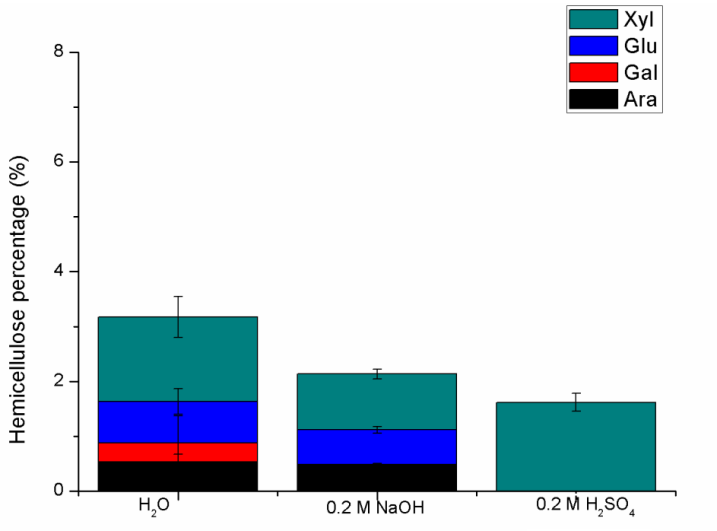


Figure 104 Hemicellulose percentages from biomass samples with conventional heating pretreatment (180 ºC; holding time: 5 Min; (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

5.5 Crystalline cellulose percentage of biomass solid fraction

In this section, the crystalline cellulose percentage of un/pretreated maize is measured by using the same method used in previous studies on *Miscanthus* and sugarcane bagasse. As can be seen from Figure 87, the crystalline cellulose percentage in untreated maize is 20.8%. When H_2O is used, the crystalline cellulose percentage of biomass increases with the increasing pretreatment time (5 Min to 20 Min). The result is expected, because the hemicellulose and lignin are increasingly removed, leaving crystalline cellulose as the major component. When 0.2 M NaOH is used as pretreatment media, the crystalline cellulose percentage kept around 55%, regardless the holding time changes. However, when NaOH concentration is increased to 0.4 M, the crystalline cellulose percentage increased up to 52% during a holding time of 5 Min. It immediately dropped to 21% and 26% when holding time increased to 10 Min and 20 Min respectively. This result can be explained by the fact that hemicellulose and lignin were firstly removed during 5 Min of holding time, leading to a high proportion of crystalline cellulose in biomass material. As we know, NaOH can change crystalline cellulose form to amorphous form. When the

pretreatment condition is severe (longer holding time), the crystalline cellulose starts to change into amorphous form, leading to a decrease of crystalline cellulose percentage in biomass.[17] Similar results were reported in Chapters 3 and 4. When 0.2 M H_2SO_4 was used as pretreatment media, crystalline cellulose percentage similarly goes up initially, because hemicellulose and lignin is removed. With severe condition, the crystalline cellulose percentage drops off, due to degradation of crystalline cellulose. It is worth mentioning that the performance of NaOH and H_2SO_4 on biomass and crystalline cellulose are distinctive. It can be assumed that NaOH tend to change crystalline cellulose form and make it more amorphous, whereas H_2SO_4 could lead to a direct degradation of crystalline cellulose.

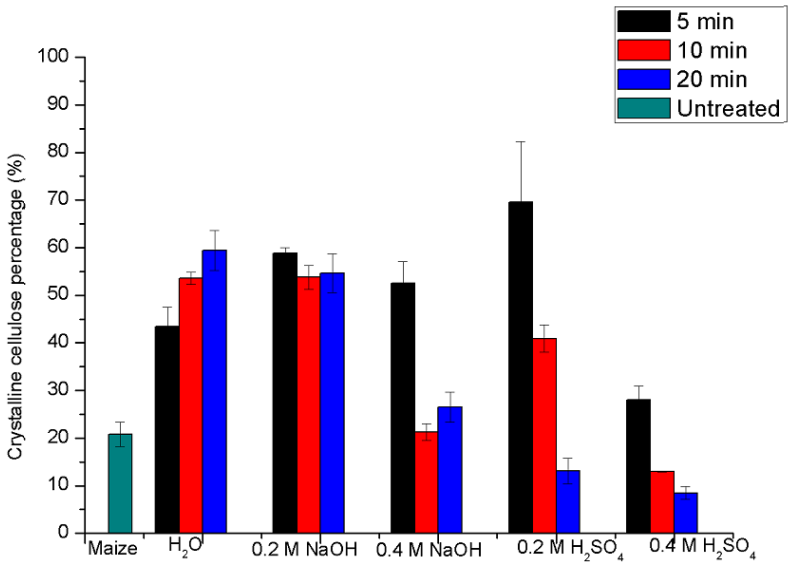


Figure 105 Crystalline cellulose percentage of un/pretreated biomass solid fraction by using microwave assisted pretreatment

The crystalline cellulose of maize pretreated under conventional heating pretreatment is also measured (see Table 28). As can be seen, crystalline cellulose percentage of H_2O pretreatment remained almost the same as the untreated maize. Combining to the result of lignin and hemicellulose, if the crystalline cellulose form keeps as crystallised as it was in the untreated biomass, the crystalline cellulose percentage should be higher than untreated biomass. However, the crystalline cellulose percentage for H_2O pretreatment shows little change. It indicates the crystalline cellulose form may change into amorphous form under the pretreatment condition, hence the proportion of crystalline cellulose and the other two major components (lignin and hemicellulose) keeps the same under this conventional heating condition (180 ^oC, 40 Min). When 0.2 M NaOH is used pretreatment, the crystalline cellulose percentage increased up to 32.9%. The increased crystalline cellulose percentage is resulted from lignin and hemicellulose removal during

pretreatment process. However, when 0.2 M H_2SO_4 is used for pretreatment, crystalline cellulose percentage decreased to 16%. The result is similar to that of microwave assisted pretreatment when 0.2 M H_2SO_4 was used for 20 Min.

Table 28 crystalline cellulose percentage in biomass solid fraction after conventional heating pretreatment

	H_2O	0.2 M NaOH	0.2 M H_2SO_4
Crystalline cellulose percentage in biomass solid fraction	20.02 ± 2.7	32.9 ± 5.1	16.33 ± 1.64

5.6 Digestibility of maize solid fraction

As it was mentioned before, digestibility of biomass solid fraction is an important property to measure. See Figure 106, for untreated maize, it is 128 nmol sugar/mg biomass.hour digestion, meaning 128 nmol glucose is produced from 1 mg biomass during 1 hour enzymatic digestion (the total enzyme digestion is 4 hours). When H_2O is used as pretreatment media, the biomass digestibility gradually increases up to 168 nmol sugar/mg biomass.hour digestion with the increasing holding time. 0.2 M and 0.4 M NaOH pretreatments lead to similar biomass digestibility, which is between 150 to 171 nmol sugar/mg biomass.hour digestion. However, in the case of H_2SO_4 pretreatment, the biomass digestibility rapidly decreased when holding time is 5 Min. With further increase of holding time, the biomass digestibility further decreased. When holding time is 20 Min, the biomass digestibility of 0.2 M H_2SO_4 pretreated maize is only 17 nmol sugar/mg biomass.hour digestion. It is further reduced when H_2SO_4 concentration is 0.4 M. The results here are similar to that of *Miscanthus* and sugarcane bagasse that H_2O and NaOH improved biomass digestibility. Biomass digestibility is improved, because hemicellulose and lignin are efficiently removed during pretreatment process and cellulose in biomass solid fraction is more accessible for enzyme. The low biomass digestibility of acid pretreated maize is due to the significant sugar release during pretreatment process.

The digestibility of maize pretreated under conventional heating condition is also assayed (see Table 29). As can be seen, after H_2O pretreatment, the biomass digestibility has little change considering the standard deviation. Similar to microwave assisted pretreatment, 0.2 M NaOH pretreatment increase the biomass digestibility up to 160 nmol sugar/mg biomass.hour digestion. In the case of 0.2 M H_2SO_4 pretreatment, the

biomass digestibility is only 80 nmol sugar/mg biomass.hour digestion, which is similar to the result of microwave assisted pretreatment when holding time is 5 Min. Overall, microwave assisted pretreatment and conventional heating pretreatment lead to similar results, but the former one is faster.

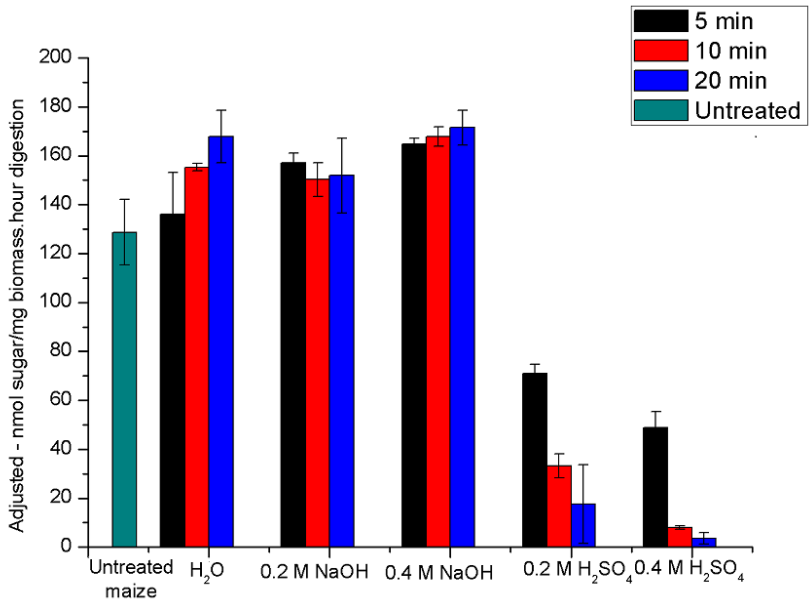


Figure 106 Digestibility of un/pretreated maize samples with microwave assistance

Table 29 Digestibility of conventional heating pretreated maize (180 ºC, 40 Min)

	H_2O	0.2 M H_2SO_4	0.2 M NaOH
Digestibility of biomass fraction	111.58 ± 3.0	80.01 ± 2.65	159.9 ± 3.13

5.7 FT-IR analysis of biomass

Chemical changes in the surface of samples are qualitatively analysed by ATR-FTIR spectroscopy. Cellulose is a homopolysaccharide composed of β-D- glucopyranose unit linked together by (1->4)-glycosidic bonds[5]. Figure 107 shows IR spectrum of biomass solid fraction after microwave assisted H_2O and NaOH pretreatment when holding time is 5 Min. After H_2O pretreatment, all the peaks positions are similar to that of untreated maize, suggesting that very little change of the biomass chemical compositions. Figure 12 and 13 show sharp bands at 898 cm^{-1} and 1159 cm^{-1} in the spectra, which is contributed by C-O-C stretching at the β-glycosidic linkage between the sugar units[210]. The absorbance at 1030 cm^{-1} and 1101 cm^{-1} can be associated with cellulose[210, 211]. In Figure 13 and Figure 108 , the aforementioned peaks are identified. Strong peaks at 1033 cm^{-1} relate to C-O stretching at C-6[210]. As can be seen from Figure 107, peak at 1101 cm^{-1} becomes more pronounced after NaOH pretreatment, indicating the

characteristics of cellulose are enhanced because hemicellulose and lignin are removed [232]. There is a peak around 1204 cm^{-1} which appeared after NaOH pretreament and which could be concerned with cellulose and hemicellulose core structure, because lignin is removed and they are more exposed[14]. The peak at 1241 cm^{-1} (C-O stretching of acetyl and other ester groups of hemicellulose) is diminished totally after NaOH pretreatment, implying that hemicellulose is effectively deacetylated and ester links between hemicellulose and lignin are also cleaved.[217]. Also, the peak at 1727 cm^{-1} represents linkages between hemicellulose and lignin, such as ester-linked acetyl, feruloyl and p-coumaroyl groups[25]. After NaOH pretreatment there is no absorbance at this position, indicating that the linkages were broken. C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives are at 1321 cm^{-1} [218]; C-H deformation in cellulose and hemicellulose at 1371 cm^{-1} [217]; lignin has absorbance around 1425 cm^{-1} , 1516 cm^{-1} and 1606 cm^{-1} [211, 215]. These lignin absorptions can be identified in the untreated maize and H_2O pretreated bagasse. The absorption at 1425 cm^{-1} is proposed to be concerned with the methyl group present in lignin. [157]. The absorption at 1515 cm^{-1} is related to aromatic stretch[208]. The absorption at 1605cm^{-1} has a contribution from aromatic compounds. Peaks at 1516 cm^{-1} and 1606 cm^{-1} disappear after NaOH pretreatment, indicating the removal of lignin. However, the peak at 1425 cm^{-1} can still be seen. It could because some OCH_3 still remain after NaOH pre-treatment. As we know, various phenolates can be released during treatment, such as p-coumaric acid, ferulic acid, vanillin, syringic acid and p-hydroxybenzoic acid, among which syringic acid has two $–OCH_3$ groups[14]. It will be difficult for base to remove the second one of these, as the first removal of $–OCH_3$ group will create a negative charge on the ring.

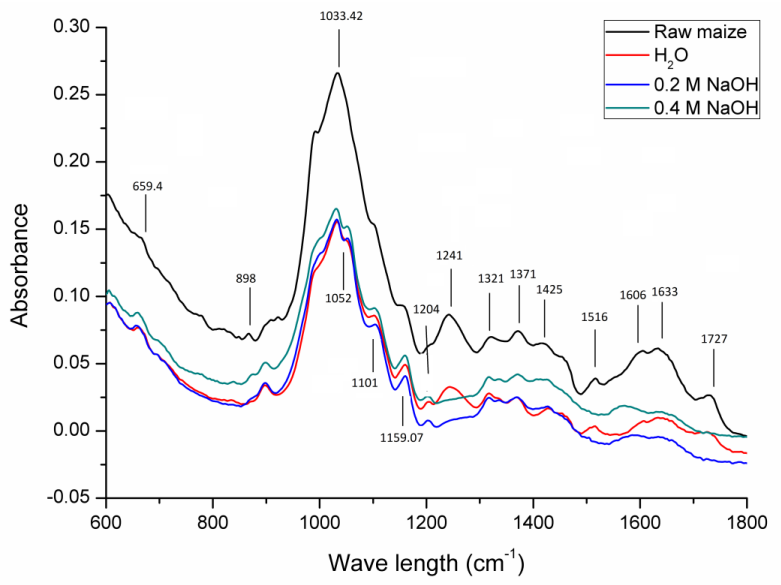


Figure 107 FT-IR analysis of maize after water/ NaOH pretreatment when holding time is 5 Min

Figure 108 shows IR spectra of maize samples after microwave assisted H_2SO_4 pretreatment when holding time is 5 Min. As can be seen, the peak at 898 cm^{-1} is stronger after pretreatment, suggesting cellulose characteristic is enhanced. Three peaks around 1052 cm^{-1}, 1101 cm^{-1} and 1158 cm^{-1} appeared after H_2SO_4 pretreatment, which is contributed by cellulose, suggesting a more exposed cellulose in biomass solid fraction. As mentioned before (see Figure 87), when maize was pretreated with 0.2 M H_2SO_4 for 5 Min, the crystalline cellulose percentage in biomass solid fraction is 70%, which is in agreement with the strong peaks here. A peak around 1200 cm^{-1} appeared after H_2SO_4 pretreatment, which could be concerned with cellulose and hemicellulose core structure[14]. Similar to NaOH pretreatment, peaks at 1241 cm^{-1} and 1727 cm^{-1} also disappear, indicating acetyl groups on hemicellulose are removed and the linkage between hemicellulose and lignin are broken. However, the peaks concerned with lignin (1424 cm^{-1}, 1512 cm^{-1} and 1604 cm^{-1}) are still shown in the spectrum after H_2SO_4 pretreatment, indicating acid has small influence on lignin structure.

Therefore, the results are similar to *Miscanthus* and sugarcane bagasse that both H_2SO_4 and NaOH can efficiently remove acetyl groups in hemicellulose and broke ester linkages between lignin and hemicellulose. Lignin is effective removed by NaOH, while H_2SO_4 has little influence on lignin structure. However, Figure 102 showed that lignin is effectively removed by both NaOH and H_2SO_4 pretreatments under microwave condition. The reason for these different findings could be that NaOH tends to break down lignin and removes it completely from biomass, whereas H_2SO_4 is likely to depolymerise lignin and they start to repolymerise again and probably redeposit back on

the biomass. These repolymerisation products presumably couldn't be quantified by using acetyl bromide method due to their different structures. Nevertheless, because they redeposit back on biomass, their corresponding aromatic and OCH_3 peaks can be shown up in IR spectrum.

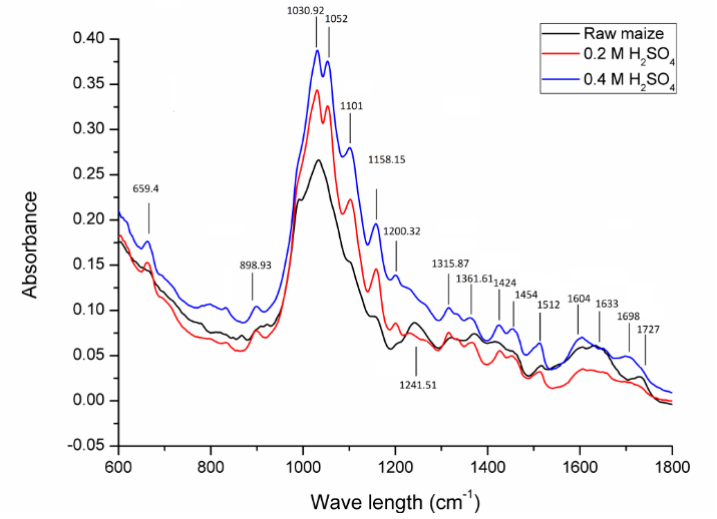


Figure 108 FT-IR analysis of maize after H_2SO_4 pretreatment when holding time is 5 Min

5.8 Morphological study of biomass

Figure 109 and Figure 110 present images obtained from solid fraction of microwave assisted un/pretreated maize by scanning electronic microscope. Figure 109 a-c shows biomass characteristics of untreated maize. As can be seen in Figure 109a, the parallel cellulose bundles are tightly packed together. With higher resolution, biomass presents a flat and smooth surface (Figure 109 b and c). Figure 109 d-f shows biomass surface after maize being pretreated with 0.2 M NaOH for 5 Min. Compared to untreated maize, the biomass structure starts to detach (see Figure 109d). Figure 109e shows that the biomass surface coating is considerately damaged, we can see that parallel strips appeared on the biomass surface after pretreatment. However, with higher resolution (see Figure 109f), the biomass surface characteristics are similar to that of untreated maize.

As was studied before, when holding time is too short, H_2SO_4 has little influence on biomass surface. Therefore, here the SEM images presented here are obtained from biomass samples undergoing microwave assisted 0.2 M H_2SO_4 pretreatment for 20 Min (see Figure 110). After H_2SO_4 pretreatment, compare to untreated maize, the cellulose bundles also start to dismantle and there are voids between each bundle (see Figure 15 a). With higher resolution (see Figure 110b), fractured biomass fragments appeared on the surface, but the surface coating is not removed (see Figure 110 b and c). Therefore, similar to previous discussion, NaOH and H_2SO_4 have different influence on biomass surface. NaOH tends to remove biomass coating and also and make cellulose fibres

detached from each other and more exposed, but H_2SO_4 tend to break parts of biomass into fragments.

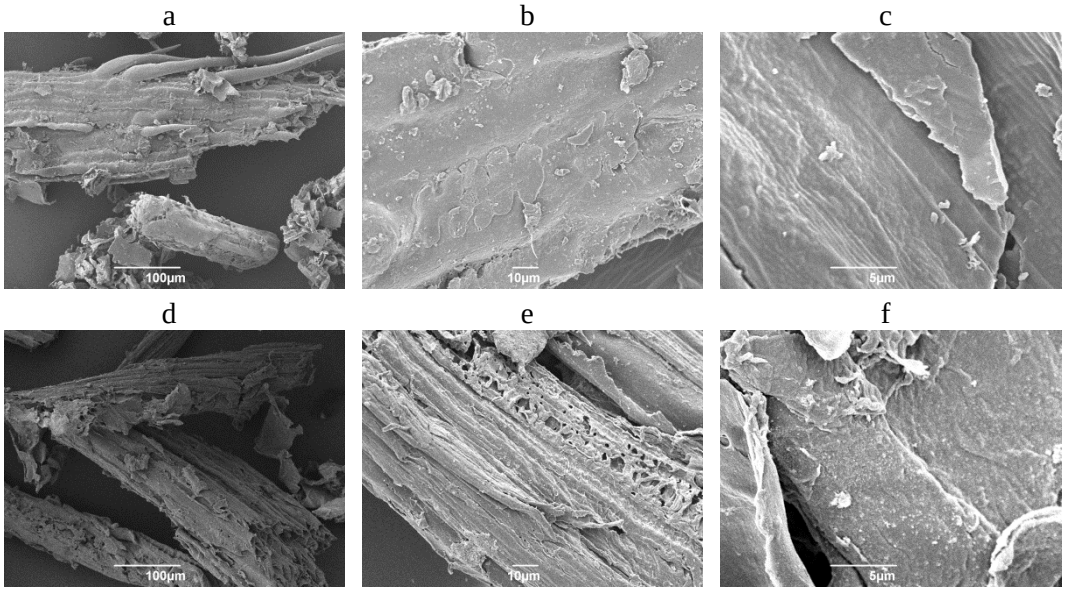


Figure 109 SEM images obtained from untreated maize (a. x250; b. x1000; c. x5000) and 0.2 M NaOH pretreated maize (d. x250; e. x1000; f. x5000) ; holding time is 5 minutes.

Therefore, alongside the difference between crystalline cellulose percentage of NaOH and H_2SO_4 pretreated biomass solid fraction, their distinctive morphology characteristics could also explain their different digestibility.

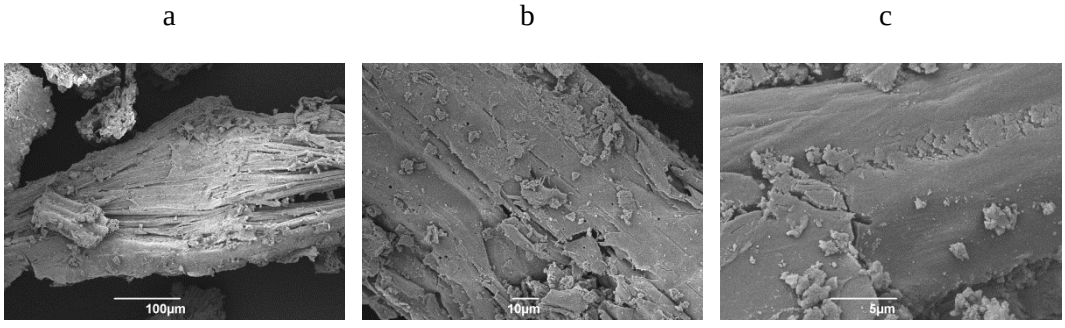


Figure 110 SEM images obtained from 0.2 M H_2SO_4 pre-treated maize(a. x 250; b. 1x1000; c. x5000); holding time is 20 minutes

5.9 Conclusions

In this chapter, maize is used as feedstock for microwave assisted H_2SO_4 or NaOH pretreatments. Different analysis techniques were used to evaluate the pretreatment process. In comparison to conventional pretreatment, microwave assisted pretreatment shows great potential. Firstly, monosaccharides release during pretreatment process were

studied and compared. Similar to the results from *Miscanthus* and sugarcane bagasse, microwave assisted pretreatment of maize lead to a faster sugar release process. Compared to conventional heating pretreatment, microwave assisted pretreatment led to 4.3 times more sugar during 8 times less holding time. Selective production of glucose is also obtained by using H_2SO_4. Different from *Miscanthus* and sugarcane bagasse, both xylose and glucose are major sugar components during H_2O and NaOH pretreatment for maize, suggesting both glucan and xylan are major hemicellulose material. The optimal holding time for maximal sugar release is shorter than that of *Miscanthus* and sugarcane bagasse, indicating the hemicellulose of maize is easier to remove. Secondly, lignin content of un/pretreated maize is compared. Different from the results of *Miscanthus* and sugarcane bagasse, rather than only NaOH showing strong delignification effect, lignin is effectively removed under all the microwave assisted conditions assayed here. However, from FTIR results, the lignin removing processing during NaOH and H_2SO_4 are different. The former one indicated the absence of lignin related peaks, while the later one still presented lignin related peaks. It can be assumed that NaOH break lignin and released them into pretreatment media, while H_2SO_4 probably depolymerised lignin and they re-polymerised again and then re-deposited back on biomass. These re-polymerisation products were not quantified by using acetyl bromide method. Thirdly, similar to the results of *Miscanthus* and sugarcane bagasse, hemicellulose is effectively removed by H_2SO_4 pretreatment. Due to its longer holding time, more hemicellulose is removed by conventional heating pretreatment than microwave assisted pretreatment. It is assumed that conventional heating pretreatment leads to serious sugar degradation. The supportive evidence is that a large amount of hemicellulose was removed, but a very low reducing sugar release yield was obtained, indicating these released sugars could have been degraded into other chemicals. However, further study need to be done to confirm this assumption. Thirdly, similar to the results of *Miscanthus* and sugarcane bagasse, NaOH pretreated maize showed increased digestibility, due to the efficient removal of lignin and hemicellulose. H_2SO_4 led to lower digestibility due to the biomass degradation. Conventional heating led to similar results, apart from a reduced biomass degradation effect of H_2SO_4. Finally, SEM results suggest that NaOH and H_2SO_4 have different influence on biomass surface. NaOH tends to remove biomass coating and also make cellulose fibres detach from each other and become more exposed, but H_2SO_4 tend to break parts of biomass into fragments. Hence, apart from different changing of chemical compositions, different morphological features led to different biomass digestibility.

Overall, the effective pretreatment for maize was achieved during very short amout of time (5 Min). Sugarcane bagasse and maize have the similar amount of chemical components, but their effective pretreatment time are quite different. In short, the optimal pretreatment conditions can be predicted by their chemical compositions and morphological characteristics. It would be interesting to find information about their internal structure and inorganic content. These properties could have an influence on pretreatment process. It would be interesting to study the types of hemicellulose in *Miscanthus*, sugarcane bagasse and maize, because the type of hemicellulose could play an important role deciding the optimal pretreatment holding time. Sugar degradation products in conventional heating pretreatment should be studied, in order to confirm the assumption that sugar degradation results in low sugar yield in pretreatment media. In this work, 5 Min is shown as the optimal holding time for the microwave assisted H_2SO_4 pretreatment here. It is remarkably short compared to previous research studied before. The short pretreatment probabaly lead to less sugar degradation. Therefore, in the future it would be interesting to study the relation bwtween sugar degradation and pretreatment time.

Chapter 6 Microwave assisted Ferric chloride pretreatments for C4 plants

6.1 Introduction

Biomass material has very low glucose yield without pretreatment, because of its recalcitrant structure. As was mentioned before, the pretreatment step is carried out to reduce biomass recalcitrance and biomass depolymerisation and hemicellulose solubilisation were involved. Lewis acid has been proposed to improve fractionation through acidolysis. For instance, Constant *et al.* studied the influence of Lewis acid ($FeCl_2$, $CuCl_2$. $FeCl_3$) on wheat straw during organosolv pretreatment and the results showed that a large amount of soluble phenolic-derived oligomers were produced.[244] He *et al.* studied the effects of metal chlorides ($CaCl_2$, $MgCl_2$, $FeCl_3$, NaCl and $AlCl_3$) on the solubility of lignin during pretreatment of Masson pine and the result shows that $FeCl_3$ has remarkable lignin removal ability. [245] $FeCl_3$ behaves as Lewis acid. Additionally, it is an oxidation reagent. Therefore biomass depolymerisation occurs during $FeCl_3$ pretreatment.[120] At the same time, it is also a good oxidising agent, which will often lead to lignin oxidation. It is less corrosive to equipment than inorganic acid [246]. Liu *et al.* pretreated corn stover with $FeCl_3$. The results showed hemicellulose can be effectively removed and almost all the ether linkages and some ester linkages between lignin and carbohydrates were disrupted without any effect on delignification. When corn stover was pretreated by $FeCl_3$ at 160 ^oC for 20 minutes, an optimum hydrolysis yield of 98% was achieved. This yield was significantly higher than that of untreated one (22.8%).[120]

From the above literature review, $FeCl_3$ was chosen and used as pretreatment media. *Miscanthus*, maize and sugarcane bagasse were used as the feedstock for the microwave assisted pretreatment. CEM Discover Microwave machine was used as the microwave source. The temperature was maintained at 180 ^oC, in order to compare with the results of NaOH and H_2SO_4 pretreatments studied in previous chapters. Holding time was 5 Min and 10 Min respectively. The same analysis techniques and methods applied to NaOH and H_2SO_4 pretreatments were used here as well.

Miscanthus, maize and sugarcane bagasse were obtained from Netherlands, Brazil and France respectively. Table 30 shows their biomass compositions and particle size. As can be seen, in comparison to bagasse and maize, *Miscanthus* has higher percentage of crystalline cellulose, while maize has a high proportion of hemicellulose. In general, ash content is very low and the bagasse has a relatively larger particle size. These properties possibly have influence on the pretreatment process.

Table 30 Biomass compositions of raw *Miscanthus*, bagasse and maize (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

Biomass	Crystalline cellulose (%)	Hemicellu -lose (%)	Lignin (%)	Ash (%)	Particle Size (μm)
Miscant-hus	34 ± 2.5	42% ± 2.8	28 ± 2%	0.83 ± 0.03	100 × 57
Bagasse	25 ± 2.7	48 ± 2.3%	31 ± 1.2%	3.68 ± 0.46	625 × 188
Maize	20 ± 1%	52 ± 2%	30 ± 1.2%	4.92 ± 0.14	350 × 98

6.2 Monosaccharide analysis

Figure 111 and Figure 112 show the monosaccharides composition of pretreatment media when holding time is 5 Min. As can be seen, arabinose, galactose, glucose, xylose and mannose presented in the pretreatment media, suggesting hemicellulose is efficiently depolymerised into its monosaccharides. Miscanthus, sugarcane bagasse and maize lead to similar yields and compositions of reducing sugars. The total reducing sugar is in the range of 0.79 to 0.84 μmol/ mg biomass. As glucose is the predominant constituent, it indicates that cellulose was efficiently broken down under $FeCl_3$ conditions. Liu et al. pretreated corn stover with a number of inorganic salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeSO_4$, $Fe_2(SO_4)_3$) and the results show that $FeCl_3$ remarkably increased hemicellulose degradation at temperatures ranging from 140 to 200 ^oC, with high xylose recovery and low cellulose removal.[120] López-Linares et al. studied the conventional heating $FeCl_3$ pretreatment of olive tree biomass and the results showed that best glucose recovery in liquid fraction is 43.8% at a temperature of 160 ^oC for 30 Min. More severe conditions (180 ^oC) lead to significant glucose degradation.[121] In this study, the maximum glucose yield is 0.7 μmol/ mg biomass, which is 37% glucose recovery from cellulose composition in the biomass within just 5 Min.

Figure 112 shows the reducing sugar release when holding time is 10 Min. The total sugar production decreased to 0.65-0.68 μmol/ mg biomass, which is lower than that of 5 Min. The reason could be the degradation of produced sugar. As can be seen, glucose is still the major component of the sugar mixture in the liquid fraction of biomass pretreatment. Similar to H_2SO_4 pretreatment in previous chapters, $FeCl_3$ also selectively produced glucose. However, H_2SO_4 was more efficient in breaking down cellulose and led to better glucose production (e.g. *Miscanthus*: 0.75, 1.22 and 1.83 μmol glucose/ mg biomass respectively for 5, 10 and 20 Min; Temperature: 180 ^oC). Therefore, $FeCl_3$

pretreatment is able to depolymerise cellulose and produce glucose as the major product. However, it is less acidic than H_2SO_4 pretreatment, thus less glucose is produced.

According to previous results of H_2SO_4 pretreatments in chapter 2 and 3, xylose was produced when the pretreatment conditions were mild (lower concentration or shorter holding time). However, in the current $FeCl_3$ pretreatment study, selective production of xylose was not observed. In the future, it would be interesting to shorten the reaction time to 2-3 Min and increase $FeCl_3$ concentration from 0.4 to 1 M to find out if xylose could be selectively obtained.

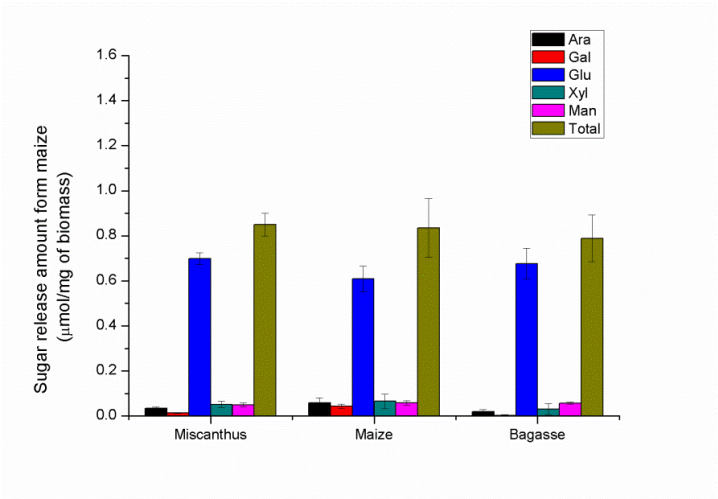


Figure 111 Monosaccharide amount after various pretreatments when holding time is 5 Min (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

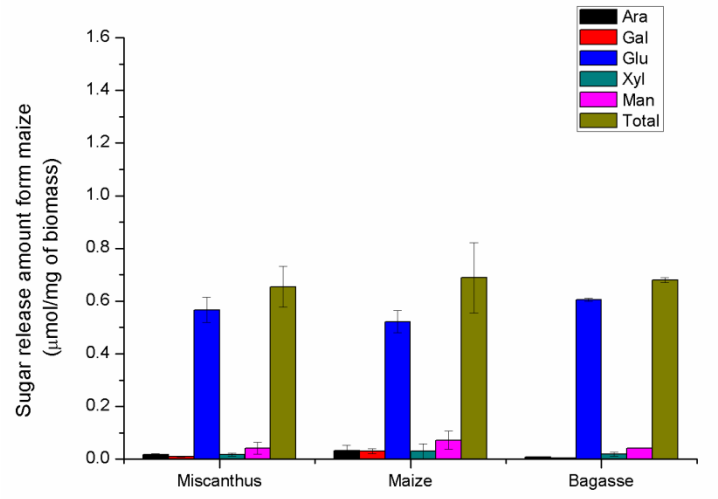


Figure 112 Monosaccharide amount after various pretreatments when holding time is 10 Min(each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

6.3 Lignin content analysis after $FeCl_3$ pretreatment

Lignin amount in maize is measured by using same methods used in previous chapters. Each condition was repeated 3 times and the results shown below are the average values with standard deviations. Figure 113 shows the lignin content remaining in the biomass. As can be calculated from Table 30, the lignin content is 112 mg, 120 mg and 124 mg in 400 mg untreated biomass material. The results here show that $FeCl_3$ has a remarkable delignification effect, as it significantly dropped to 10 -22 mg within short holding time. Lu *et al*. studied microwave assisted $FeCl_3$ pretreatment of rice straw. In their study, the lignin percentage in the solid fraction decreased from 16.2% to 9.8% ($FeCl_3$: 0.14 mol/l, Temperature = 160 ^oC, Holding time = 19 Min). [247] According to Liu *et al*., $FeCl_3$ can remove ether linkages and some ester linkages between lignin and carbohydrates in corn stover by conventional pretreatment and the lignin percentage increased from 21.3% to 47.8% (Temperature 180 ^oC, 20 Min), due to the removal of hemicellulose.[120] Lü *et al.* also reported lignin is efficiently removed (39.5% of the total lignin in untreated biomass material) by $FeCl_3$ (160 ^oC, 19 Min, 0.14 M $FeCl_3$). Therefore, in comparison with conventional heating method, microwave assisted $FeCl_3$ pretreatment has better ability to remove lignin. Compared with these three pretreatment methods, the results in the current study show more efficient lignin removal during shorter reaction times. As we know, microwave is dielectric heating, the ester linkages between lignin and carbohydrate could be largely influenced by the microwave effect, which probably accelerates lignin removal. Therefore, microwave assisted pretreatment led to very effective delignification effect, making the biomass more accessible for the subsequent enzymatic hydrolysis.

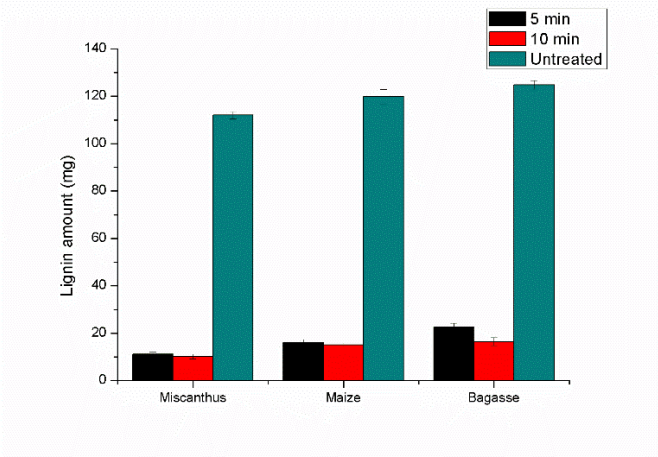


Figure 113 Lignin amount remaining in the solid fraction after $FeCl_3$ pretreatment(each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

6.4 Crystalline cellulose analysis

Crystalline cellulose percentage of biomass is a significant property for hydrolysis processing. In this chapter, it was measured by using the same method employed in previous chapters.

Figure 114 shows crystalline cellulose percentage of biomass solid fraction after $FeCl_3$ pretreatment when holding time is 5 Min or 10 Min. Crystalline cellulose percentage of untreated *Miscanthus* is 34%. It decreased to 31% and 22% respectively, suggesting that more severe conditions lead to greater crystalline cellulose degradation. In the case of untreated maize, the crystalline cellulose percentage is 21%. It increases to 29% when holding time was 5 Min. Then it decreases to 20% when holding time was 10 Min. For sugarcane bagasse, the crystalline cellulose percentage decreases to 22% when holding time is 5 Min and then it drops to 17% when holding time is 10 Min. Increasing crystalline cellulose percentage has been previously reported, which indicates that $FeCl_3$ pretreatment removed amorphous components in the biomass material during pretreatment and leaving the biomass solid fraction more crystallised.[120, 247] With longer holding time, the exposed crystalline cellulose starts to degrade under the $FeCl_3$ effect, which is similar to that of H_2SO_4.

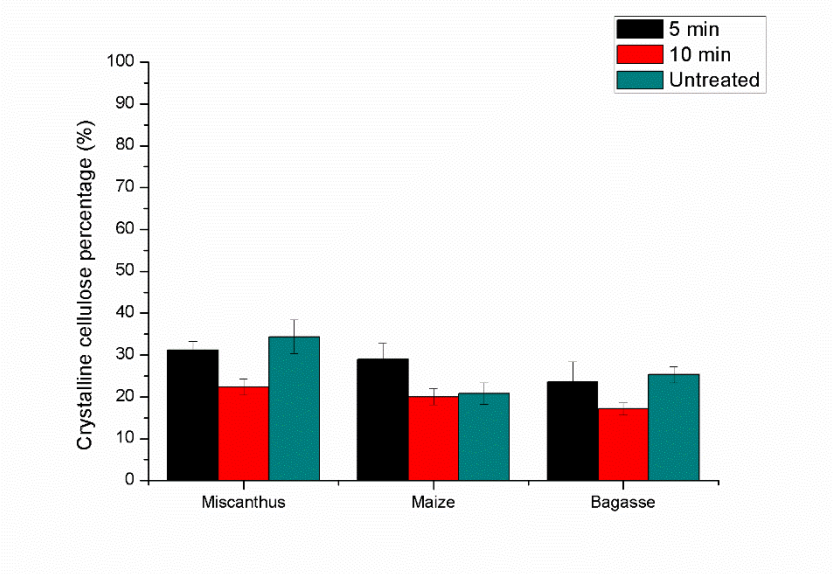


Figure 114 Crystalline cellulose percentage of solid fraction of biomass after $FeCl_3$ pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

6.5 Digestibility analysis

Digestibility of pretreated biomass was measured by using the same methods employed in previous chapters. Biomass solid fraction was washed with ethanol (3×10 ml) before the measurement. Figure 115 shows the biomass digestibility after $FeCl_3$ pretreatments. As can be seen, untreated *Miscanthus* has very low digestibility (10 nmol glucose/ mg biomass). After $FeCl_3$ pretreatment, the biomass digestibility is still very low, with a marginal increase for 5 and 10 min of 23 and 20 nmol glucose/ mg biomass respectively. For maize and bagasse, the digestibility of untreated biomass is rather high, 128 and 113 nmol glucose/ mg biomass. However, after $FeCl_3$ pretreatment, the biomass digestibility dropped significantly. Increasing holding time decreases biomass digestibility. When holding time is 10 Min, it is only 28 and 16 nmol glucose/ mg biomass respectively for maize and bagasse. The results from Liu *et al*. show that enzymatic hydrolysis of corn stover was enhanced by $FeCl_3$ pretreatment by removing almost all of the hemicellulose present and increasing the accessibility of cellulase to cellulose.[120] The low digestibility of $FeCl_3$ pretreated biomass here could be explained from two aspects. Firstly, a high yield of glucose was released during pretreatment process (see Figure 111 and Figure 112), suggesting a large amount of cellulose was broken down during pretreatment process. Therefore, cellulose available for enzymatic hydrolysis to produce glucose is reduced. Secondly, despite the fact that the biomass was washed with ethanol (3 × 10 ml), the biomass material remains very darkly coloured. The very low digestibility could be due to inhibitor effect of remaining $FeCl_3$ as well. However, it is worthy to check the remaining $FeCl_3$ amount on biomass after washing step.

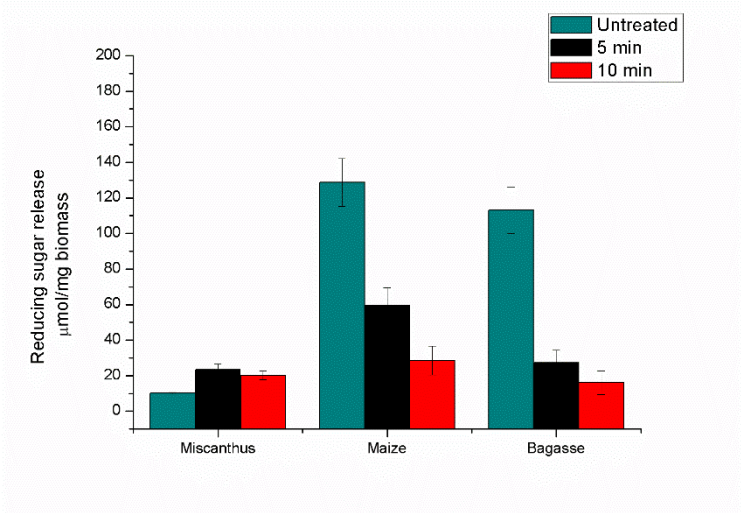


Figure 115 Biomass digestibility after $FeCl_3$ pretreatment (each condition was repeated in triplicates and average value was reported here; error bar was reported as standard deviation)

6.6 FT-IR

ATR-FTIR spectroscopy is used to qualitatively determine the chemical change of biomass samples. Figure 116 shows FTIR spectra of untreated and $FeCl_3$ pretreated *Miscanthus* and Table 31 listed the major peaks and their corresponding chemical groups. As can be seen, similar to the effect of H_2SO_4, two peaks appeared around 1056 cm^{-1} and 1106 cm^{-1}, which are contributed by cellulose.[212] The result is expected, because hemicellulose and lignin were efficient removed, leading to a more exposed cellulose structure. The peak at 1239 cm^{-1} is completely diminished, indicating the acetyl groups on hemicellulose are effectively broken down. The lignin absorbance (1421 cm^{-1}, 1456 cm^{-1}, 1508 cm^{-1} and 1598 cm^{-1}) remain the same. However, in the section of lignin analysis (0see section 6.3) the results showed that lignin was efficiently removed from the biomass. The possibly explanation is that the OH group on lignin was oxidised by $FeCl_3$ into C=O, meaning this oxidised product cannot react with acetyl bromide. (see Scheme 7) Therefore, the acetyl bromide lignin amount is very low, but the aromatic still gave the aromatic relevant peaks. A new peak appeared at 1700 cm^{-1}, which is the lignin oxidation peak corresponding to a conjugated C=O stretch (see Scheme 7). Alkyl ester peak at 1731 cm^{-1} disappeared, suggesting the ester linkages between lignin and hemicellulose are broken. More evidence for the breakdown of the esters and acetyl groups after $FeCl_3$ pre-treatments, is found with the absence of peak 1242 cm^{-1}. Similar changes can be observed when sugarcane bagasse and maize is used for pretreatment (see Figure 117 and Figure 118). Lignin relevant peaks (1421/1424 cm^{-1}, 1454/1455 cm^{-1}, 1513/1516 cm^{-1} and 1603/1606 cm^{-}) keep unaltered. In Figure 118, the peak intensity of 1033 cm^{-1} is remarkably lower, suggesting the effective degradation of cellulose. However, a peak around 1056 cm^{-1} appeared after $FeCl_3$ pretreatment, which could be contributed by polysaccharides. Compared to *Miscanthus* and sugarcane bagasse, the peak at 1731 cm^{-1} in the un/pretreated maize is absent, which probably due to low level of alkyl ester groups of hemicellulose in the biomass samples.

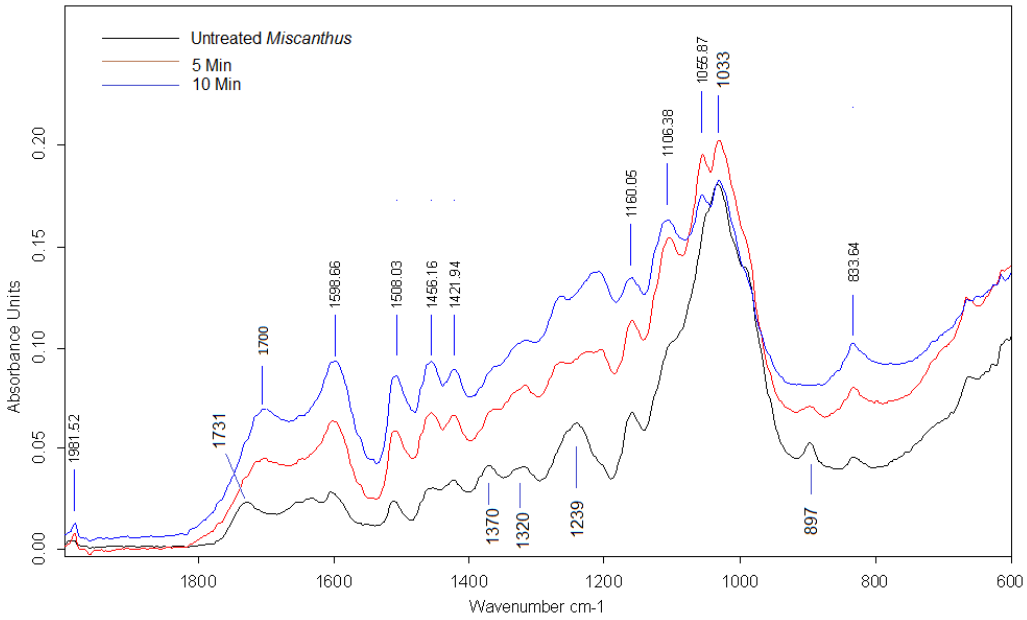
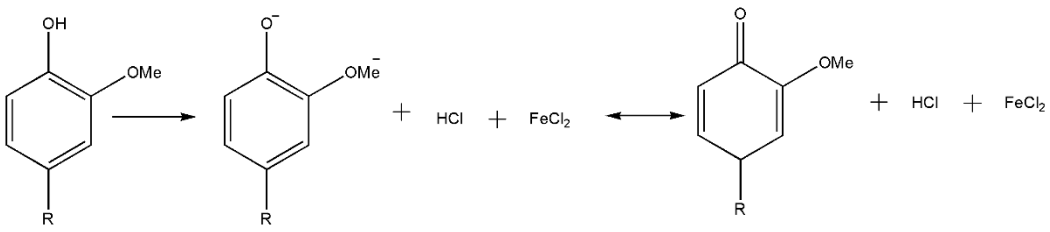


Figure 116 FT-IR spectrum of untreated and 0.2 M $FeCl_3$ pretreated *Miscanthus* for 5 minutes and 10 minutes



Scheme 7 Oxidation reaction of phenol and $FeCl_3$

Table 31 Chemical composition changes in biomass after pretreatments

peak position (cm^{-1})	Assignment	Reference
897	β-glycosidic linkage between the sugar units	[248]
1033	C-O stretching	[212]
1056	C–O vibrations of cellulose	[212]
1106	C–O vibrations of crystalline cellulose; glucose ring stretch from cellulose	[212]
1160	C-O-C stretching at the β-glycosidic linkage	[249]
1239	acetyl C-O stretching of hemicellulose	[217]
1320	CH_2- wagging vibrations in the cellulose and hemicellulose	[249]
1370	C–H stretch of cellulose	[212]
1421	Stretching of O-CH_3	[156]
1456	Aromatic C-H deformation; asymmetric stretching in –CH_3 and	[248]

	$-CH_2-$	
1508	Phenolic ring vibrations of lignin	[212]
1698	Aromatic ring stretching of lignin	[212]
1731	Alkyl ester from cell wall hemicellulose C=O; strong carbonyl groups in branched Hemicellulose	[212]

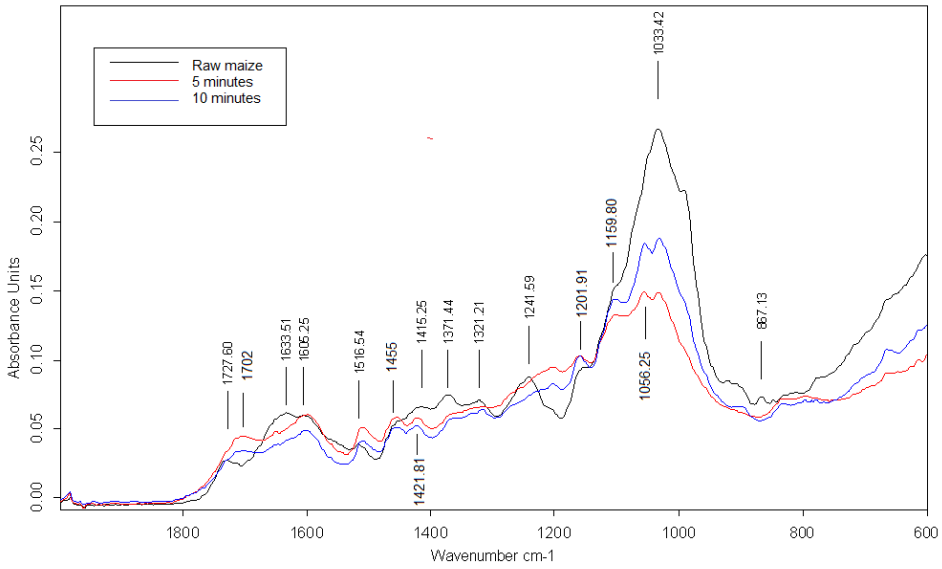


Figure 117 19 FT-IR spectrum of untreated and 0.2 M $FeCl_3$ pretreated maize for 5 minutes and 10 minutes

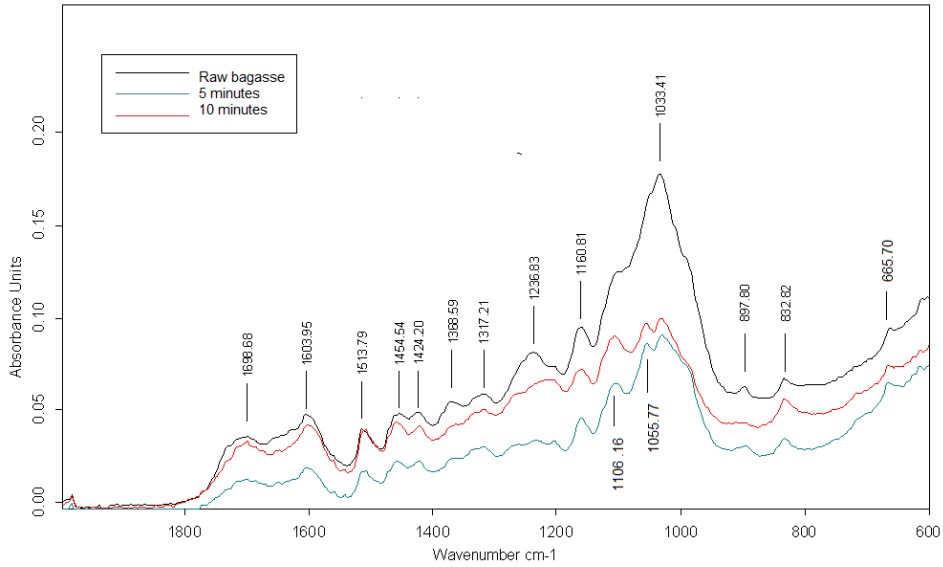


Figure 118 19 FT-IR spectrum of untreated and 0.2 M $FeCl_3$ pretreated bagasse for 5 minutes and 10 minutes

6.7 SEM analysis

According to the analysis results mentioned above, *Miscanthus*, maize and sugarcane bagasse gave rise to similar sugar release during pretreatment and chemical compositions in the solid fractions. In this section, only *Miscanthus* is used as a sample to study the morphological change after $FeCl_3$ pretreatment. Figure 119 a-c present SEM images of *Miscanthus* obtained under different magnification. As can be seen from Figure 119b, the biomass surface of untreated *Miscanthus* is rough and parallel cellulose bundles can be observed. When the magnification is increased (Figure 119 c), the smooth surface coating is presented. Figure 119 d-f show the images of *Miscanthus* pretreated with $FeCl_3$ for 5 minutes. The biomass surface is covered with small biomass fragments alongside the parallel cellulose bundles. We can see this feature better when magnification is increased (Figure 119 e). Overall, the biomass presents an erosion-like characteristic. When the magnification is further increased (Figure 119 f), the biomass surface is alike to that of untreated *Miscanthus*, showing a smooth surface. Therefore, in comparison with NaOH and H_2SO_4, the performance of $FeCl_3$ on biomass surface is milder.

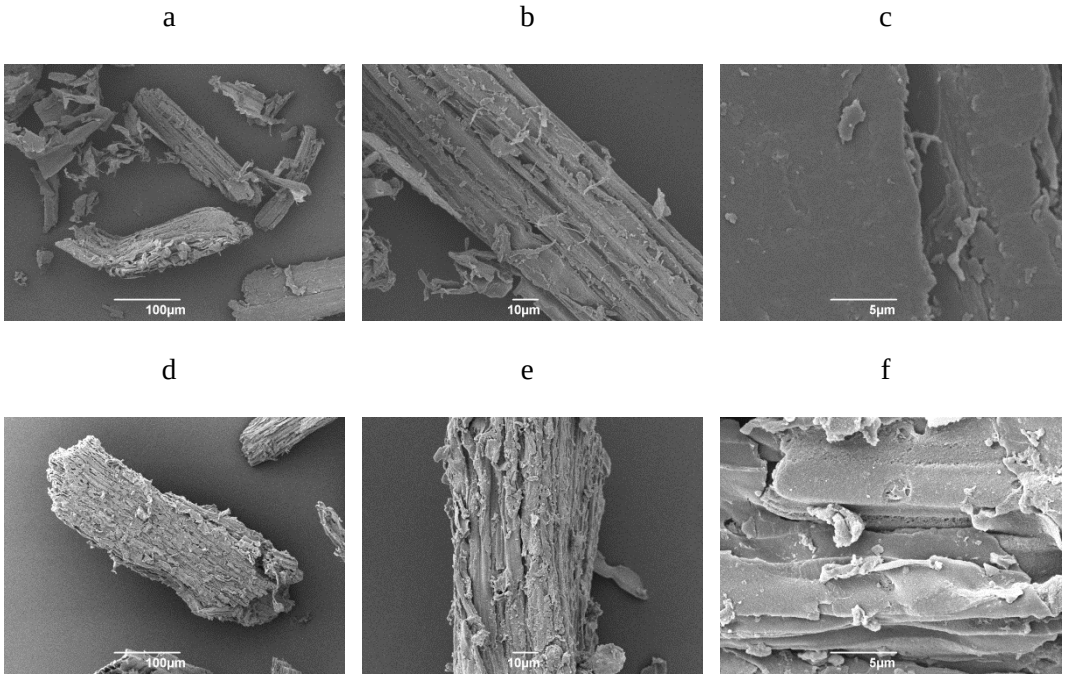


Figure 119 SEM images for untreated Miscanthus (a. x250; b. x1000; c. x5000) and 0.2 M $FeCl_3$ pre-treated miscanthus (d. x250; e. x1000; f. x5000); holding time is 5 minutes

6.8 Conclusion

In this chapter, $FeCl_3$ is used to pretreatment *Miscanthus*, maize and sugarcane bagasse. The temperature was controlled at 180 ^oC and holding time was controlled at 5 Min and 10 Min respectively. The results show that $FeCl_3$ behaved similar to H_2SO_4 and crystalline cellulose was efficiently broken down into glucose over a short time. In the

case of *Miscanthus*, the maximal glucose is 0.7 μmol/ mg biomass, which is 37% glucose recovery from available cellulose within just 5 Min. However, the production of glucose is not as good as that of H_2SO_4. Acetyl bromide lignin amount is rather low after $FeCl_3$ pretreatment. Combing the result of FTIR, the possible reason is lignin is effectively oxidised by $FeCl_3$. According to previous research by others, hemicellulose was efficiently broken down into xylose under the effect of $FeCl_3$.[120, 250] Due to the limited time, hemicellulose was not directly measured in this study. However, in this study, xylose has a very low yield. The possibly explanation is that xylose is degraded into other chemicals.

The results of this work indicates that $FeCl_3$ has an interesting performance on biomass. Both acidity and oxidation effect were identified and the later effect has never being reported before so far. At the same time, the selective production of glucose shown great interest of $FeCl_3$ as a metal catalyst for biomass pretreatment in the art of bioethanol production.

There are several aspects which would be interesting to study in the future. It would be interesting to measure the hemicellulose percentage of $FeCl_3$ pretreated biomass. Very low xylose yield was obtained during pretreatment process and it would be worth studying xylose degradation under microwave assisted $FeCl_3$ conditions. It would also be interesting to shorten the reaction time to 2-3 Min and increase $FeCl_3$ concentration from 0.4 to 1 M to find out if xylose could be selectively obtained. It would be of interest to carry out $FeCl_3$ pretreatment under conventional heating conditions to compare with the results obtained from microwave assisted pretreatment. According to previous research, $FeCl_3$ pretreated biomass material has promising digestibility, whereas the digestibiligy in this study is very low.[121, 122, 251] Therefore, further study should be done to investigate the reasons behind our low digestibility. At the same time, high glucose presented in the pretreatment media, but the residue $FeCl_3$ in pretreatment media is the major limitation for further fermentation step. Future work need to be done to remove $FeCl_3$ in the pretreatment media.

Chapter 7 Experimental Methods

7.1 Material and reagents

Biomass materials used in this study are *Miscanthus*, Sugarcane bagasse and Maize, which were obtained from York, Sao Paulo and Lousignan respectively (See Table 32). The biomass were harvested and dried in the oven (50 ^{o}C, 40 hours) following milling process. The biomass sample used for pretreatment was powder sample (see Figure 120). Samples were then kept at atmospheric conditions before use.

Table 32 Biomass used in this study (each test was done in triplicates and the standard deviation was calculated)

Biomass	**Source**	**Particle size (μm)**	**Cellulose (%)**	**Hemice -llulose (%)**	**Lignin (%)**	**Ash (%)**
Miscanthus	York, North Yorkshire, UK	Hammer milled 100 × 57	34 ± 2.5	42 ± 2.8	28 ± 2	0.83 ± 0.03
Sugarcane bagasse	Cosan Mill, Sao Paulo Brazil	Knife milled 625 × 188	25 ± 2.7	48 ± 2.3	31 ± 1.2	3.68 ± 0.46
Maize stover	Lousignan, France	Knife milled 350 ×98	20 ± 1	52 ± 2	30 ± 1.2	4.922 ± 0.143

Figure 120 Biomass samples

Table 33 listed the standard chemicals and reagents used in this study.

Biomass samples	**source**	**Properties**
Acetic acid	Fisher	99.5%
Acetyl bromide	Sigma Aldrich	99%
Agar	FORMEDIUM	Undescribed
Arabinose	Sigma Aldrich	≥99.9%
Athrone	Sigma Aldrich	97%
$Ba(OH)_2$	Sigma Aldrich	95%
Bacto-peptone	Becton, Dickson and company	Undescribed
Celluclast	nonozymes	Undescribed
Dichloromethane	Fisher	95%
dDMSO	Sigma Aldrich	≥99.9%
Ethanol Absolute	VWR Chemicals	≥99.5%
$FeCl_3$	Sigma Aldrich	≥97%

Fucose	Sigma Aldrich	≥99.9%
Galactose	Sigma Aldrich	≥99.9%
Galacturonic acid	Sigma Aldrich	≥99.9%
Glacial acetic acid	Fisher Scientific	≥99.7%
Glucose	Fisher scientific	≥99.9%
Glucuronic acid	Sigma Aldrich	≥99.9%
Glycerol	Sigma Aldrich	≥99%
HCl	VWR Chemicals	32%
H_2SO_4	Fisher Scientific	95%
Hydroxylamine HCl	Sigma Aldrich	99%
Mannose	Sigma Aldrich	≥99.9%
3-methyl-2-benzothiazolinonehydrozone (MTBH)	Aldrich	97%
NaCl	Sigma Aldrich	99.5%
NaOH	Fisher Scientific	≥99.9%
Nitric Acid	Sigma Aldrich	65%
Novozyme 188	nonozymes	Undescribed
Propan-2-ol	Sigma Aldrich	99.8%
Rhamnose	Sigma Aldrich	≥99.9%
Sodium acetate	Fisher	Undescribed
Trifluoroacetic acid (TFA)	Sigma Aldrich	99%
Xylose	Sigma Aldrich	≥99.9%
Yeast extract	FORMEDIUM	Undescribed

7.2 Microwave pre-treatment

CEM Discovery SP Microwave, 300 W, 2.45 GHz

The pre-treatment was conducted in the CEM monomode microwave machine (CEM Discover SP-D, US). 35 ml Pyrex® vial was charged with 0.4 g of biomass (*Miscanthus*/ maize/ sugarcane bagasse) and 16 ml H_2O, H_2SO_4 (0.2M, 0.4 M and 1M), NaOH (0.2M, 0.4 M and 1M), or 0.2 M $FeCl_3$ solution. A magnetic stir bar was put in the sample tube to make sure the pretreatment condition is homogeneous. Pretreatment was performed at various temperatures (130-180 ^oC) within a range of reaction times (5 to 30 Min). Each pretreatment condition was done in triplicates. Figure 121a shows the Microwave machines with an auto-sampler and Figure 121b shows microwave reaction tube with biomass sample and pretreatment. Before each group of experiments, the IR temperature probe was calibrated at 180 ^oC using glycerol according to the CEM operating instructions. Glycerol and a stir bar were placed in a round bottom flask, which was put in CEM. The machine has a calibration program in which it heats up the sample to a target temperature (for instance 180 ^oC). A thermometer was used to check to see if it accurately measuered and then entered the measured temperature in the machine. The machine will give a slope nearby 1.05-1.10. If the slope is above 1.10, the calibration need to be done again.

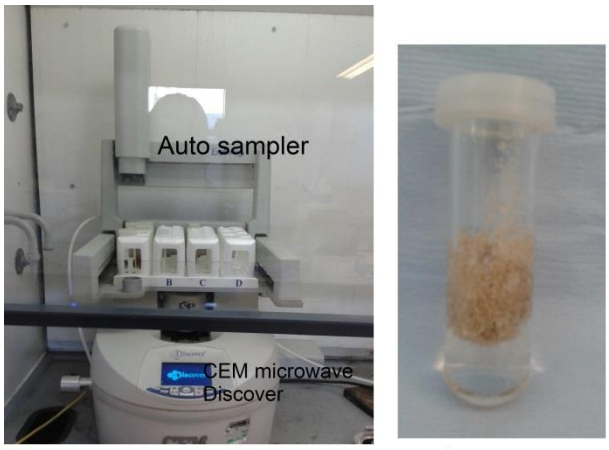


Figure 121 a. Microwave machines with an auto-sampler; b. 35 ml Pyrex® vial with corresponding silicon cap charged with biomass sample and pretreatment media

Figure 2 shows typical microwave assisted pretreatment temperature, pressure profile and power density. The temperature here was 180 ºC and holding time was 10 Min. As can be seem, the ramping stage was about 2-3 Min and cooling stage was about 5 Min. In order to maintain stable temperature, the power density started from 300 W. It instantly dropped to 70- 80 W when the aim temperature was achieved.

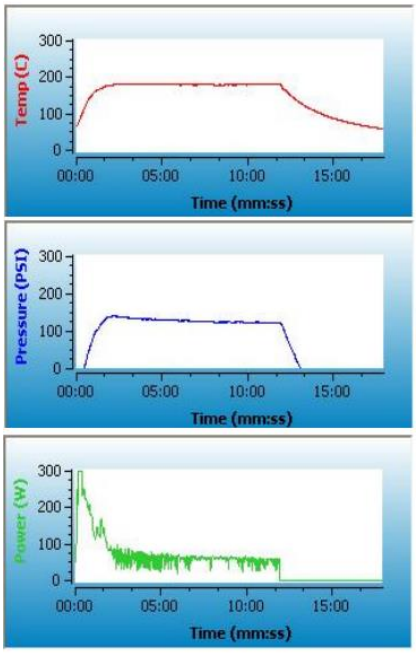


Figure 122 Typical heating profile for biomass microwave assisted pre-treatment

CEM MARS 6 Microwave, 1800 W, 2.45 GHz

The microwave assisted pretreatment was scaled up by using CEM MARS with One Touch™ Technology, using Easyprep™ Plus Easy Prep Teflon 100 ml closed vessels

(Figure 123). The machine was used in power dynamic mode. A dual IR probe (within the microwave cavity) and a fibre optic probe (positioned in a glass insert on the control reactor) are used in the machine for accurate temperature measurements following automatic calibration.

Figure 123 a. CEM Mars; b. Mars sample vessel 100 ml

7.3 Conventional heating pretreatment method

Conventional heating pretreatment was conducted in an acid digestion vessel (Parr Instruments, Moline, IL). The acid digestion vessel was charged with 0.4 g biomass with 16 ml pretreatment media (0.2 M NaOH or H_2SO_4). The temperature was controlled at 180 ^oC and hold time was 40 Min. Same separation and samples preparation procedures for analysis were carried out as was mentioned above for microwave assisted pretreatment. Each pretreatment condition was done in triplicate.

Figure 124 Conventional pretreatment acid digestion vessel (Parr Instruments, Moline, IL)

7.4 Sample preparation and analysis

Figure 125 shows overall pretreatment and analysis process after pretreatment. The liquid fraction was separated from solid biomass fraction by centrifuge. Liquid samples were neutralized to pH 7 by 150 mM $Ba(OH)_2$ or 1 M HCl. Solid fraction was rinsed with ethanol (3×10 ml) and dried at 50 ^oC overnight. Dionex was used to analysis monosaccharides released into the pretreatment media during pretreatment (section 7.4.1). Chemical compositions (cellulose, hemicellulose, lignin and ash) were measured by Dionex, UV and mass balance method. The detailed procedure could be found in the following sections (7.4.2 to 7.4.5). Biomass digestibility was measured by using a saccharification robot (see section 7.4.6). Scanning morphological images were obtained from JEOL scanning electron microscope (see section 7.4.8). Simultaneous saccharification and fermentation (SSF) process was conducted to investigate the potential ethanol production of pretreated biomass and the detail procedure is described in the following section (see Section 7.4.11).

7.4.1 Analysis of carbohydrates in liquid fraction

The monosaccharide analysis of the pre-treatment liquid was carried out using High Performance Ion Exchange Chromatography, using a DionexICS-3000PC, Thermo scientific, USA, equipped with electrochemical detector to quantify the corresponding sugar content[252]. 100 mM of nine monosaccharides mixture was prepared (arabinose, fucose, galactose, galacturonic acid, glucose, glucoronic acid, mannose, rhamnose and xylose). 2 ml of liquor sample was neutralised to pH 7 by 1 M NaOH or 150 mM $Ba(OH)_2$. 500 µl neutralised sample, as well as standard monosaccharides (250, 500 and 750 µl) were taken into a new tube and dried in speedvac under 50 ^oC. 500 µl of 2M TFA was added in the tube and dry Argon was flushed, with hydrolysis condition of 100 ^oC for 4 hours. TFA was completely evaporated by speed evaporator (Thermal Scientific, SAVANT SPDB1DDA, US), attached with a refrigerated vapour trap (Thermal Scientific, SAVANT RVT4104, US). Dark brown sugar solid appeared in the tube bottom, which was washed with isopropanol (2× 200 µl). Then the monosaccharides mixture was mixed and re-suspended in 150 µl ultra purified water, followed by 20 times dilution. The liquor sample was filtered with 0.45µm PTFE filters, using a 1ml syringe, into HPLC vials with pre-slit septa caps. Then the samples were run on Dionex.

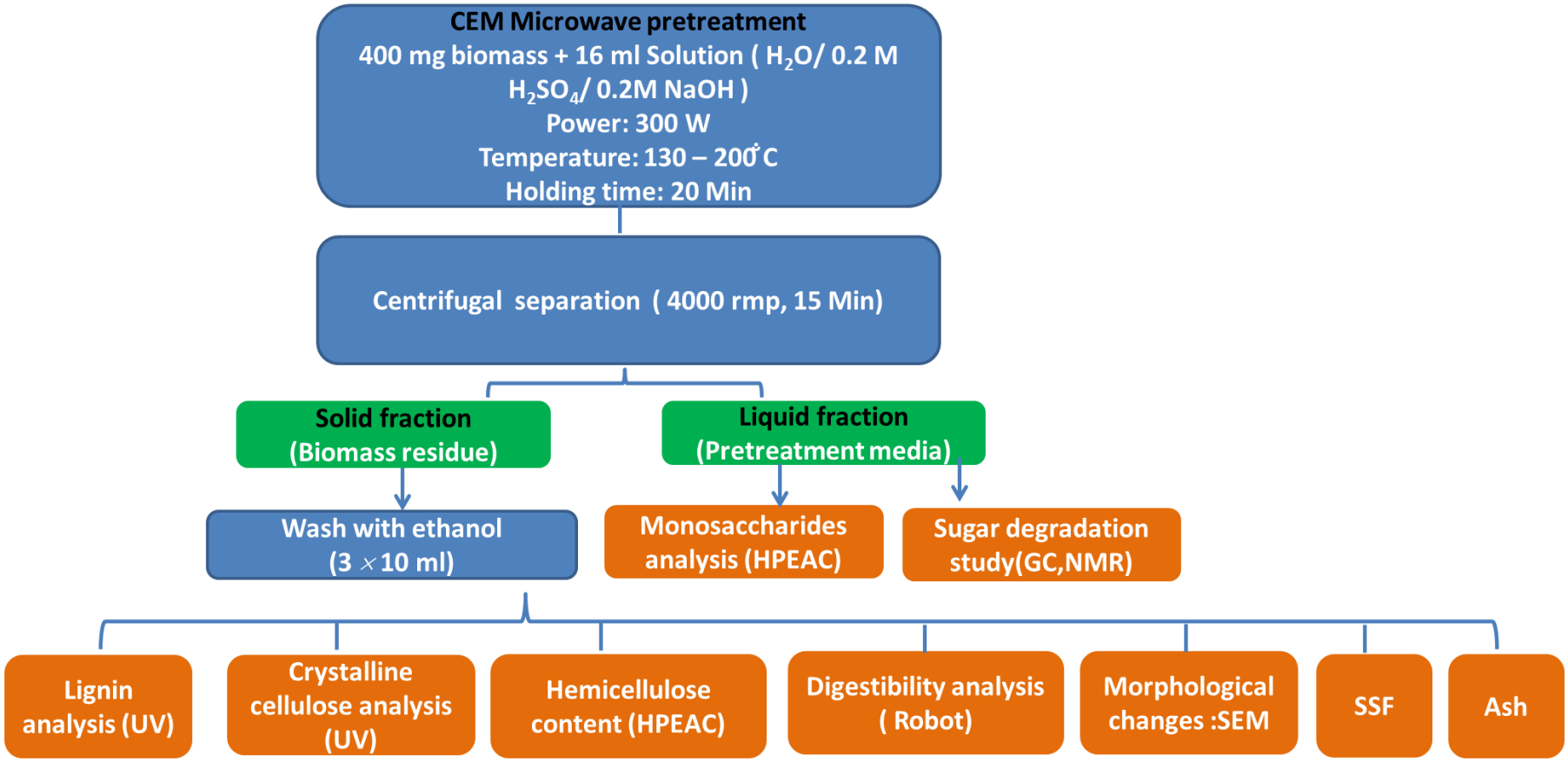


Figure 125 Pre-treatment method and analysis diagram

7.4.2 Lignin quantification

Lignin was quantified as follows: 3.5 mg of biomass was dissolved in acetyl bromide solution (25% v/v acetyl bromide/glacial acetic acid), then 1 ml 2 M NaOH and 175 µl hydroxylamine HCl in a 5 ml volumetric flask were added. The solution was taken to 5 ml with acetic acid and diluted 10 times. The absorbance was read at 280 nm on UV (VARIAN 50Bio) and the percentage of lignin calculated using the following formula[170]:

ABSL%= {abs/(coeff × pathlength)} × {(total volume × 100%)/ biomass weight}

Coefficient = 17.75; Pathlength =1 cm; Total volume= 5 ml; biomass= 3.5 mg.

7.4.3 Hemicellulose analysis

4 mg biomass was hydrolyzed by adding 0.5 ml 2 M trifluoroacetic acid (TFA). Dry argon was flushed into the vials, followed by 4 hours hydrolysis under 100 ^{o}C for 4 hours (mixed several times during hydrolysis). The vials were cooled at room temperature and TFA was completely evaporated by speed evaporator. Biomass was washed with isopropanol (2×200 µl), which was also evaporated by speed evaporator. Then the biomass sample was re-suspended in 200 µl ultra purified water. After mixing, the biomass residue and liquid were separated by centrifuge. The supernatant was taken into a new tube and diluted 20 times, which was re-suspended in 150µl ultrapure water. The liquor was filtered with 0.45 µm PTFE filters, using a 1ml syringe, into HPLC vials with pre-slit septa caps. Then the monosaccharides in hemicellulose were measured on DionexICS-3000PC.

7.4.4 Analysis of crystalline cellulose

To determine the percentage of crystalline cellulose in biomass, 10 mg untreated or pre-treated biomass was hydrolysed by 500 µl of 2M TFA at 100 ^{o}C for 4 h. The solids residue was subsequently hydrolysed using 1 ml acetic acid: nitric acid: water (8: 1: 2 v/v) at 100 ^{o}C for 30 Min. The resulting residue was crystalline cellulose, which was hydrolysed into glucose by 175 µl 72% H_2SO_4 at room temperature for 45 Min and then diluting the H_2SO_4 to 3.2% and heating the samples at 120 °C for 2 h. After centrifuge, there was brown substance appeared at the bottom of sample bottle. The liquor was diluted 10 times and Anthrone Reagent (2 mg anthrone/ ml concentrated H_2SO_4) was used to quantify corresponding glucose[168]. A standard curve of glucose was made by 1mg/ml glucose stock. 2 ml samples tubed were used and corresponding liquor was

added in (see Table 34), which were heated under 80 ºC for 30 Min in heating block. Glucose and Anthrone resulted in green-blue colour complex. The samples were mixed well and 200 µl was taken into an optical dish (see Figure 126), which was read at 620 nm on Tecan Sunrise plate reader.

Table 34 Anthrone test standard curve and sample test

	Sample (µl)	dH2O (µl)	Anthrone Reagent (µl)
Blank	0	400	800
Std 0.5	2	398	800
Std 1	4	396	800
Std 2	8	392	800
Std 4	16	384	800
Std 6	24	376	800
Std 8	32	368	800
Std 10	40	360	800
Sample	40	360	800

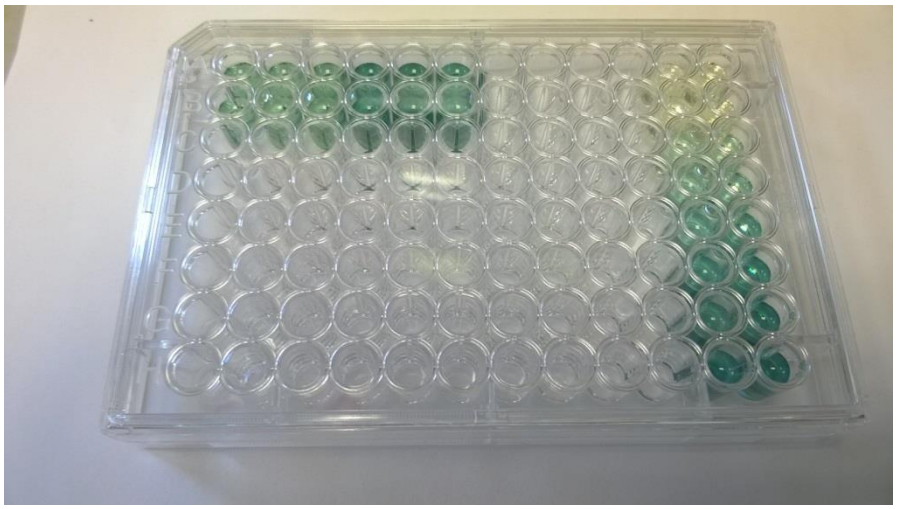


Figure 126 Anthrone test with commercial glucose and biomass samples

7.4.5 Ash content measurement

1 g biomass was burned in air in an oven (CARBOLITE AFA1100) at 540 ºC for 4 hours. The ash content equates to the residue which was weighed.

7.4.6 Analysis of saccharification

The saccharification of biomass was investigated by using a high throughput saccharification assay which is based on a robotic platform that can carry out the enzymatic digestion and quantification of the released sugars in a 96-well plate format. Enzymatic hydrolysis was carried out using an enzyme cocktail with a 4:1 ratio of Celluclast and Novozyme 188 (cellobiase from Aspergillus niger; both Novozymes, Bagsvaerd, Denmark). The enzymes were filtered using a Hi-Trap desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) before use. 0.1 mg biomass was hydrolysed for 8 hours with 250 µl enzyme cocktail, in 250 ml of 25 mM sodium acetate buffer at pH 4.5, at 30 ^{o}C. Determination of sugars released after hydrolysis was performed using a modification of the method by Anton and Barrett using 3-methyl-2-benzothiazolinonehydrazone (MBTH) method [192].

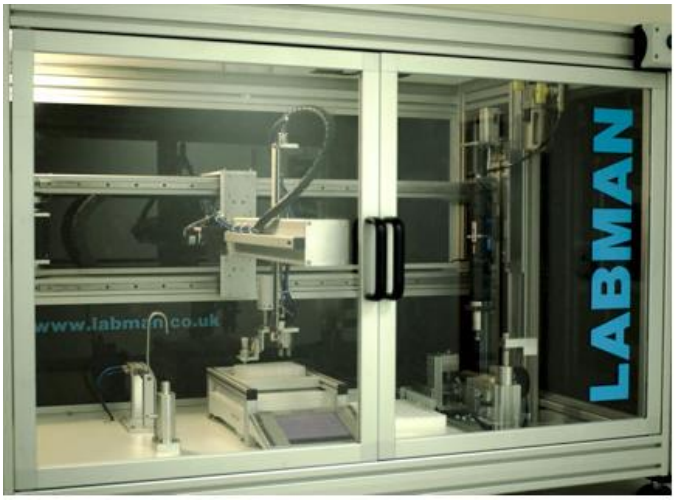


Figure 4 High throughput saccharification robort

7.4.7 Chemical analysis of the solid residues

The chemical composition of solid residues before and after the pre-treatments were analysed by Fourier transformed infrared spectrometry (FT-IR) (VERTEX 70, Bruker).

Attenuated total reflection–Fourier transformed infrared spectroscopy (ATR-FTIR) was conducted using a Bruker Optics Vertex system (VERTEX 70, Bruker) with built-in diamond-germanium ATR single reflection crystal. Untreated and pretreated samples were pressed firmly against the diamond surface using a screw-loaded anvil. Sample spectra were obtained under 64 scans between 650 cm^{-1} to 2000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Air was used as background for untreated and pre-treated biomass.

7.4.8 Morphological studies

Morphological characteristics of un/pre-treated biomass residue were studied using a scanning electron microscope fitted with tungsten filament cathode (JEOL, JSM-6490LV, Japan). Samples were sputter-coated with 7 nm Au/Pd to facilitate viewing by SEM. Images were obtained under vacuum, using a 5 kV accelerating voltage and a secondary electron detector.

7.4.9 Sugar degradation analysis

The liquid fraction from filtration step was extracted with ethyl acetate (3×15 ml). The solvent was removed by using rotary evaporator. The organic product was viscous dark brown substance, which was weighed and re-dissolved into ethyl acetate and $CDCl_3$ to conduct GC and NMR analysis respectively.

A HP 6890 GC equipped with FID detector was used to quantify the levulinic acid and furfural. The flow rate for He was 1.3 ml/ Min. A Stabilwax column (30 m × 0.25 mm × 0.25 μm) was used. Oven temperature was programmed to rise from 45 ^oC to 250 ^oC at 10 ^oC/ Min. The injection column was 0.4 μL.

Proton NMR experiments were carried out in a Jeol NMR 400 Spectrometer at Proton frequency of 399.78 MHz.

7.4.10 Microwave assisted pretreatment and sample preparation for SSF(Simultaneous saccharification and fermentation) study

Biomass sample for SSF process was prepared by using CEM Mars microwave machine (CEM Mars 6, US). Mars microwave reactor vessel (100 ml) was charged with 3 g of biomass (*Miscanthus*) and 60 ml H_2O/H_2SO_4 (0.2M)/ NaOH (0.2M). Pretreatment was performed at 180 ^oC for 20 Min. Each pre-treatment condition was done in triplicates. Figure 123 shows the Microwave machines and sample vessel.

When pretreatment was finished, biomass solid fraction and liquid fraction was separated by filtration. Biomass solid fraction was washed with ethanol (3 × 60 ml), which was dried in the oven for 24 hours at 50 ^oC. This dried biomass solid fraction was used in the following SSF process.

7.4.11 Simultaneous saccharification and fermentation of pretreated *Miscanthus*

All the containers, solutions and biomass material (except the enzyme) were autoclaved before fermentation. All the experiments were carried in flow hood.

Prepare yeast solution

Yeast (*Saccharomyces* cerevisiae, NO. ATCC 200062) strand was used for fermentation of 6 membered sugar rings in this experiment. Yeast extract 10g, Bacto-peptone 20g, Glucose 20g and Bacto agar 20g was added in 1L deionised water. Then it was autoclaved and stored at room temperature. Each agar plate was prepared by adding 20 ml of agar solution to petri dish. In order to grow yeast, yeast was streaked onto plates to achieve single colonies, stored in 30 ^{o}C room for 48 hours and checked after 24 hrs. The plate was sealed with Micropore and stored at 4 ^{o}C for up to 2 months (see Figure 127). Each dot was a single colony.

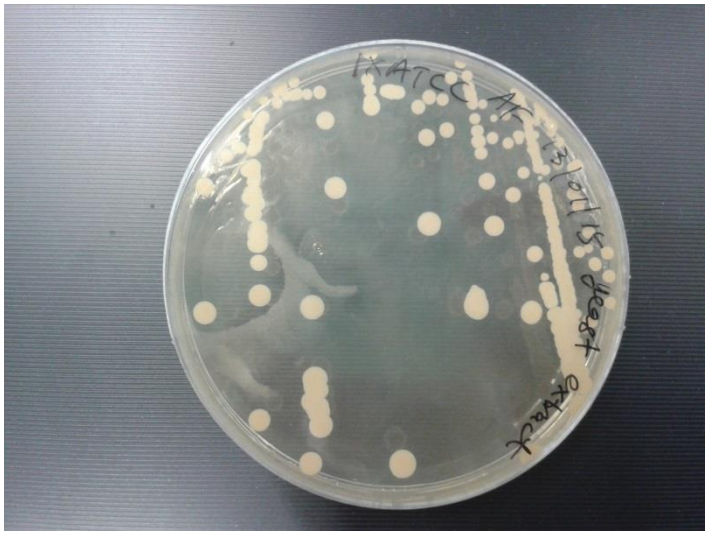


Figure 127 Yeast dish

1x ATCC solution was yeast growing media, which was made by adding 10 g Yeast extract, 20 g Bacto-peptone, 20 g glucose and 1L deionised water. After autoclave, it was stored at 4 ^{o}C.

There were two stages of yeast growing. Firstly, yeast was taken by taking one single colony from the yeast plate into a falcon tube and 10 ml 1x ATCC was added in each falcon tube. 3 falcon tubes were prepared in this way and then they were sealed with Micropore tape and put on the shaker for 16-18 hours under 30 ^{o}C. Secondly, 20 falcon tubes were taken for second stage of growing yeast. 20 ml 1x ATCC and 0.5 ml 1st stage yeast mixture was added into each falcon tube. After sealing them with Micropore tape, they were put on the shaker for 24 hours under 30 ^{o}C. Then yeast solutions were removed into centrifuge bottle. After mixing well, optical density (OD) was measured by UV (VARIAN 50Bio). If the OD is below 5, the yeast was centrifuged down (1500 rpm, 5 Min; Thermo Scentific, Heraeus Megafuge 40 R Centrifuge), liquor would be

removed. Then the yeast was re-suspended in autoclaved ultrapure water to get 5 OD. This 5 OD yeast solution was used for fermentation.

Simultaneous saccharification and fermentation of *pretreated Miscanthus*

10 x ATCC solution: 50g of Yeast extract and 100g of Bacto-peptone were added to 500 ml deionised water.

SSF was conducted in a conical flask (see Figure 128; water stopper to ensure the fermentation environment is anaerobic). 1g biomass samples were added into 100 ml flasks and autoclaved before SSF. 10.75 sterile water, 0.25 ml NaOAc buffer (pH 4.5), 1 ml enzyme solution (4:1 v/v ratio of Celluclast and Novozyme 188; both Novozymes, Bagsvaerd, Denmark), 10 x ATCC solution and 200 µl yeast solution were added in the autoclaved flask.

Figure 128 SSF flask

After 1 hour fermentation, the sample was collected in to GC tube containing 500 µL of 1M NaCl and 0.04% 1-propanol. Samples were collected after 6, 24, 48 hours and stored at -80 ^{o}C for analysis.

Ethanol measurement

For ethanol measurement, analysis was carried out by Tony Larson (Department of Biology, University of York) by using GC method. The GC was an Agilent 6890N equipped with a Gerstel CIS-4 septumless injection system and Gerstel MPS-2 autosampler and fitted with a SGE BP-1 column (25m x 0.15mm ID, 0.25µm film) with

a 5m retention gap. The MS was a Leco Pegasus IV. The sample was adsorbed onto a Supelco pink SPME fibre for 1 min, then desorbed into the CIS4 (Cooled Injection System) liner at 250°C for 0.1min and then baked out for 7min before the next sample cycle (autosampler program: Alex_Lanot_SPME_07) . The GC was run with He as carrier at 1mL/min constant flow with 200:1 split ratio, with a temperature ramp as follows: 70°C 2.5min, ramp 65°C/min to 200°C and hold for 1min, then cool at 70°C/min to 70°C and hold for 1min (GC program: Alex_Lanot_SPME_08). The MS was set to collect masses between 10-300m/z at 20 scans/s. The ion source was 230°C and transfer line temperature 250°C (MS program: Alex_Lanot_SPME_01). After data processing, EtOH is reported as the area under m/z 31 at ~112s and the IS as the area under m/z 59 at ~120s

The standard curve was obtained with a series of ethanol concentration between 0.1 and 10 % (v/v). Using a ratio of area ethanol/1-propanol, the standard curve was linear within this range ($R^2 \geq 0.99$).

The matrix of the sample (presence of medium or dead yeast or sugar) and the incubation at 100 ^{o}C before storage did not affect the results.

7.5 Glucose decomposition products analysis

CEM microwave reactor vessel (30 ml) was charged with 200 mg glucose and 16 ml H_2SO_4 (0.2M or 0.4M H_2SO_4). Hydrolysis was carried out at 180 ^{o}C for 10 Min or 20 Min. After hydrolysis, carbonised black residue was separated from liquor by filtration. 2 ml liquid sample was neutralized with 150 mM $Ba(OH)_2$ and remaining sugars was quantified by Dionex. Degradation products were extracted from remaining liquor by adding ethyl acetate (3×10ml). Organic layers were collected and ethyl acetate is removed by using rotary evaporator. Brown viscous organic product was observed at the bottom of bottle, which is further analysed by GC, GC-MS and NMR.

7.5.1 Gas chromatography analysis

The degradation product was weighted and re-dissolved in 2 ml DCM (dichloromethane). Anisole was used as an external standard (15 mg/ml) for GC analysis. A HP 6890 GC equipped with FID detector was used to quantify the levulinic acid and furfural. The flow rate for He was 1.3 ml/ Min. A Stabilwax column (30 m × 0.25 mm × 0.25 μm) was used. Oven temperature was programmed to rise from 45 ^{o}C to 250 ^{o}C at 10 ^{o}C/ Min. The injection column was 0.4 μl.

7.5.2 ^{1}H NMR and ^{13}CNMR of degradation products

Approximately 10 mg of degradation product was dissolved in 2 ml $CDCl_3$ for NMR analysis. Proton NMR experiments were carried out in a Jeol NMR 400 Spectrometer at Proton frequency of 399.78 MHz. The spectrum was reintegrated in the chosen range using Spinworks 3 software.

7.5.3 Gel product from *Miscanthus* by microwave assisted NaOH pre-treatment

CEM microwave reactor vessel (30 ml) was charged with 0.4 g of biomass (*Miscanthus*) and 16 ml 0.2 M NaOH solution. The temperature was controlled at 180 ^{o}C and the power was 300 W. Holding time was 5 Min. After microwave reaction, biomass and pre-treatment media were separated by centrifugal (3500 rpm, 10 Min). The experiment was repeated 10 times and all the liquid fraction was collected in order to have enough substrate for gel formation.

160 ml liquor was neutralized by using 1M HCl until pH 7. Then, 260 ml ethanol was added to yield gel product. Gel product was separated by filtration and dried in fume cupboard until a constant value was obtained. About 0.4 g gel was obtained from 4 g biomass.

Gel substance was re-dissolve in 10 ml, 80 ^{o}C deionised water, stirring for 40 Min (keep temperature at 80 ^{o}C). The film was formed on the petri dishes with a diameter of 9 cm. Then the film was dried and aged for 4-5 days upon drying at ambient conditions with a temperature of 21 ± 1.5 ^{o}C, at 42 ± 3% relative humidity. This condition is controlled by using saturated $BaCO_3$ solution in a dessicator.

Chapter 8 Conclusion and Future Work

The main aim of this study was to use microwave technology to improve the processing of promising energy crops in Brazil and Europe, including *Miscanthus*, sugarcane bagasse and maize, in order to realise second generation bioethanol.

Pretreatments were able to alter the structure of lignocellulosic biomass and made it more accessible for enzymes. Additionally, it selectively removed hemicellulose and lignin from the lignocellulosic matrix, leaving the biomass more digestible. In this work, H_2SO_4 (0.2 M, 0.4 M or 1 M), NaOH (0.2 M or 0.4 M or 1 M) and 0.2 M $FeCl_3$ were used as pretreatment media. The influences of hold time (5 Min to 30 Min) and pretreatment temperature (130 to 200 ^oC) were investigated. Different analysis techniques have been applied to assess the efficiency of the pretreatment.

Firstly, the reducing sugar release during pretreatment was measured by Dionex. The results showed that in comparison with conventional heating pretreatment, microwave assisted pretreatment was significantly efficient and effective. For example, the maximal reducing sugar release of *Miscanthus* was 12.5 times higher than that of conventional heating pretreatment under same conditions (180 ^oC, 0.2 M H_2SO_4) with half less pretreatment time.

Secondly, temperature, holding time and pretreatment media played significant role in the pretreatment process. Due to the nature of microwave heating and biomass structure, the most productive pretreatment was achieved at the temperature of 180 ^oC. Above this condition, polar parts of the lignocellulosic material effectively interacted with microwave field, leading to increasingly far-reaching biomass decomposition. Holding time was varied from 2 Min to 40 Min. The results showed increasing holding time firstly improved reducing sugar release and then it contributed to increasing sugar degradation. Xylose and glucose were selectively produced by using H_2SO_4 and NaOH or changing holding time, because hemicellulose was easier to be decomposed than cellulose. For instance, when *Miscanthus* was used as feedstock, maximum sugar yield from the available carbohydrates is 75.3% by using 0.2 M H_2SO_4 pretreatment for 20 Min and glucose yield from available carbohydrate is 46.7% under this condition. Optimal xylose yield from available carbohydrate (28%) was achieved by using 0.4 M NaOH pretreatment for 20 Min. In the case of $FeCl_3$, glucose was selectively produced, possibly because it behaved as Lewis acid, leading to depolymerisation of cellulose. Sugarcane bagasse and maize had similar reducing sugar yields, despite the fact that

their optimal conditions were different, which is likely due to their different biomass compositions percentages and morphological structures.

Thirdly, chemical compositions and morphological changes of un/pretreated biomass were investigated and compared. The results showed that hemicellulose was easier to remove than cellulose. NaOH has a strong delignification effect then H_2SO_4. $FeCl_3$ has a strong influence on hemicellulose and cellulose, while it reacted with lignin without largely removing it.

The biomass morphological characteristics were studied by SEM. The results demonstrated that biomass cellulose bundles were generally more exposed and more accessible after NaOH pretreatment. However, little changes were brought by mild H_2SO_4 pretreatment; severe H_2SO_4 led to biomass carbonisation. The significant removal of hemicellulose and lignin, as well as more exposed fibre structure led to enhanced bioethanol conversion via SSF process (simultaneous saccharification fermentation). The bioethanol production from microwave pretreated *Miscanthus* is 7 times higher than that of untreated biomass, suggesting promising future of using microwave pretreated biomass for bioethanol production.

Three types of biomass materials including *Miscanthus*, sugarcane bagasse and maize were used in this work. It is highlighted that the optimal conditions of maximal sugar yield from biomass during pretreatment are influenced by their chemical compostions. In general, these three types of biomass material have similar lignin percentage. Their ash contents are all low, about 0.83-5%. In comparison with *Miscanthus*, sugarcane bagasse and maize are less crystalline and their hemicellulose is the largest component in the biomass (about 48% to 52% of the biomass). Therefore, their effective pretreatment was achieve is very short time (5-10 Min) compared to that of *Miscanthus* (20 Min). At the same time, it is worth mentioning that the morphological characteristics of these three biomasses are different. Compared to *Miscanthus* and sugarcane bagasse, maize presented a more flat and smooth surface, which possibly led to a different digestibility. However, both maize and sugarcane bagasse gave better digestibility under same conditions. Hence, effective pretreatment can be choosen according to their crystalline cellulose percentage and morphological characteristics. The outcome of this work indicates the potential optimised microwave pretreatment conditions for different biomass material, based on their chemical composition and morphological characteristics. A strong correlation between lignin and biomass digestibility was observed in this work. The results shown that higher biomass digestibility were obtained from lower lignin content biomass material. Therefore, using pretreatment to remove

lignin is a significant method to improve biomass digestibility, which was proved in NaOH pretratment in this study. Hence, from choosing biomass subject to optimise pretretment condition. Last but not least, a significant amount of digestible sugars were released into pretreatment media during pretreatment by tuning the pretreatment media system. It is worth to mention that compared to conventional heating pretreatment, selective productions of glucose and xylose were obtained by using H_2SO_4 or xylose.

In the future, it would be interesting to study the fermentability of the pretreatment media. Energy balance of microwave assisted pretreatment were briefly studied and the results suggested that higher loading led to better energy efficiency. Hence, it would be worth to do a more detailed energy balance of study of microwave pretreatment by changing biomass loading and pretreatment media volume. In our work, a gel product was formed during NaOH pretreatment, which could be a promising candidate for bio-plastics. A study of this gel product is of great interest of green chemistry concept. Microwave assisted $FeCl_3$ pretreatment leads to an effective break down of polysaccharides, contributing to promising yield of glucose. However, due to the limited amount of time, conventional heating $FeCl_3$ pretreatment was not studied. In the future, it would be interesting to study the conventional heating method of $FeCl_3$ pretreatment. The three biomass internal structure should be compared, which probably could be an important factor influencing pretreatment condtion optimising.

Microwave heating is drawing attentation due to its dielectric heating mechanism, which is much faster than conventional heating. Hence, biomass decomposition process was significantly influenced. Althought the induscrial scale up of microwave technology is still under research, the outstanding amount of sugar release during microwave pretreatment and selective glucose or xylose productions in the current work proved microwave technology had a significant performance on biomass pretreatment process. Future work need to be done to scale up the microwave assisted pretreatment process studied in this study. In contrast to previous study, the optimal pertreatment time was sharply reduced from several hours to only 5-20 Min. Overall, the result of this study shown microwave technology provided a significantly efficient way to assist the thermo-chemical conversion for biomass and it has a promising potential in the process of 2^{nd} energy generation biofuel production.

Abbreviations

Ara	Arabinose
BuOH	Butanol
DMC	Direct Microbial Conversion
EPA	US Environmental Protection Agency
EtOH	Ethanol
FT-IR	Fourier Transform Infrared
Fuc	Fucose
Gal	Galactose
galA	Galacturonic acid
GC	Gas chromatography
GHG	Green House Gases
GL	Gigaliter
Glu	Glucose
gluA	Glucuronic acid
HMF	Hydroxymethlyfurfural
HPEAC	High Performance Anion Exchange Chromatography
HPLC	High performance liquid chromatography
LA	Levulinic acid
LCA	Life Cycle Assessments
IEA	International Energy Agency
Man	Mannose
MW	Microwave
NMR	Nuclear Magnetic Resonance
Rha	Rhamnose
SEM	Scanning Electron Microscopy
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TFA	Trifluroacetic acid
Tg	Teragrame

UV	Ultraviolet
XyG	Xyloglucans
Xyl	Xylose
DMSO	Dimethyl Sulfoxide

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