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Pretreatment methods for the production of biofuels from lignocellulosic biomass

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Summary

1. Introduction

Public awareness on climate change, environmental problems and energy security is continuously growing and government cannot ignore the necessity to adopt urgent and sustainable (both economic and environmental) solutions to evolve our style of life.

One of the solutions that is emerging is bio refinery: the conversion of biomass into valuable by-products, from bio-fuel to various chemicals. In a larger vision, it can be defined as a facility that optimizes the integrated production of heat, power, transportation fuels, materials, chemicals, feed and food from biomass.

From the last few years, biorefinery is a promising sector with a considerable potential to capitalize the various lignocellulosic materials into a variety of relevant bio-products in industrial scale. Interestingly, a bio-refinery has an enormous potential to use and monetize all types of biomass-based sources that includes agricultural, agro-industrial, municipal, etc. Moreover, the materials produced by those sources are the most promising feedstock as they are a natural, abundant, and renewable resource essential to the functioning of industrial societies and critical to the development of a sustainable global economy.

The field of biofuel energy has evolved considerably in the last few years. Innovations in the bio refinery world are providing a portfolio of sustainable and eco-compatibly products able to compete the market presently dominate by the petroleum-based products. Effort in the large scale translation of update technologies of conversion of biomass to biofuels have to be done in way to optimize the economy of conversions.

There is no doubt that biofuels have the ability to fulfil the energy demand of the world, and also have the ability to reduce the carbon emissions, but accumulated bioenergy production on agricultural/cultivable land may rise a severe threats for the daily use of edible food crops. Thus, in recent years, kind of biomass considered as wastes have gained considerable importance owing to their novel characteristics that include renewability, recyclability and sustainability, words that are emphasized in growing scientific knowledge and environmental awareness.

Lignocellulosic feedstock represents an extraordinarily large amount of renewable bio resource available in surplus on Earth and it is a suitable raw material for vast number of applications for human sustainability. Some of the major factors are the recalcitrance of the plant cell wall due to integral structural complexity of lignocellulosic fractions and strong hindrance from the inhibitors and by-products that are generated during pretreatment.

This work is particularly focused on the pretreatment methods used to overcome these obstacles and to enhance the otherwise low effective conversion into valuable product. An overview of biomass, its distribution and uses is given, followed by a description of the building structure of lignocellulosic biomass. A panning on the available pretreatment methods used in these last years is given, and only the most common will be analysed in depth in the subsequent chapter. A proposal of optimization methods concludes this work, with some experimental results present in literature.

2. Lignocellulosic biomass

2.1 Biomass

2.1.1 What is biomass?

In scientific fields the word *biomass* includes the set of materials of organic origins, while in the strictly energetic context it designates any organic matter of plant or animal origin from which energy can be obtained through thermo-chemical or biochemical processes.

Biomass is considered as waste from the agricultural and agro-food sector, forestry products, organic products derived from the biological activity of humans and animals, organic fraction of municipal solid waste and all that is directly or indirectly lead to activity of chlorophylline photosynthesis process.

Biomasses are considered a renewable source as they have a relatively short period of regeneration. The use of biomass in the energy sector is considered to be null in terms of carbon dioxide production related to greenhouse effect issues.

In fact, biomass can be thought of as a temporary CO_2 storage. Through the chlorophylline photosynthesis process plants fix carbon of the atmosphere in organic compounds, predominantly carbohydrates, thanks to solar energy.

Using biomass, and its by-products, as fuel the same amount of CO_2 absorbed throughout its life, assuming a complete combustion, is now released into the atmosphere. It would also be released into the atmosphere by decomposition of the biomass following its death. Therefore, the so-called "carbon cycle" is considered as a closed loop.

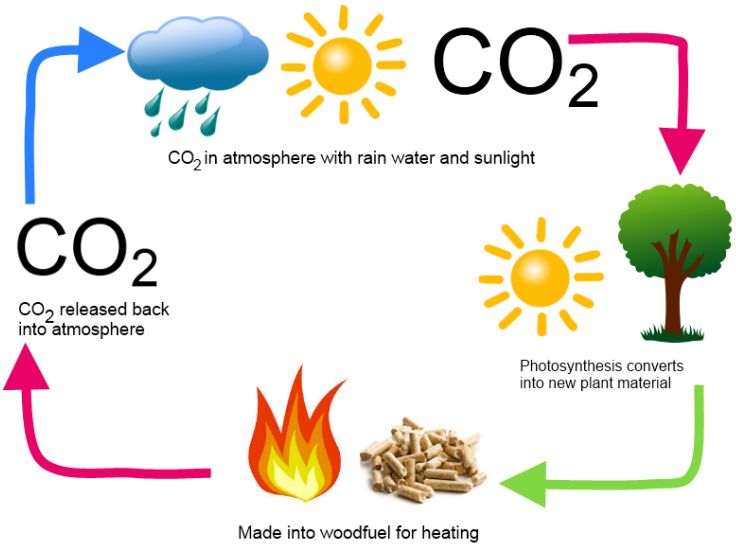


Figure 2.1 – Carbon cycle

2.1.2 Types of biomass

Biomass resources can be used directly as a fuel or converted to another form or energy product. These resources are available on a renewable basis and they are commonly referred to as feedstocks [2.1]. Biomass feedstocks are of several types: they include dedicated

energy crops, agricultural crops, forestry residues, algae, biomass processing residues, municipal waste, and animal waste. Here a brief description of them:

- *Dedicated energy crops* are non-food crops that can be grown on marginal land specifically to provide biomass. These break down into two general categories. Herbaceous energy crops are perennials that are harvested annually after taking a certain period to reach full productivity. These include such grasses as switchgrass, miscanthus (also known as elephant grass or e-grass), bamboo, sweet sorghum, tall fescue, kochia, wheatgrass, and others. Short-rotation woody crops are fast-growing hardwood trees that are harvested within 5 to 8 years of planting. These include hybrid poplar, hybrid willow, silver maple, eastern cottonwood, green ash, black walnut, sweetgum, and sycamore.
- *Agricultural crops* include currently available commodity products such as corn starch and corn oil, soybean oil and meal, wheat starch, and vegetable oils. They generally yield sugars, oils, and extractives, although they can also be used to produce plastics as well as other chemicals and products. Agriculture crop residues include biomass materials, primarily stalks and leaves, that are not harvested or removed from fields in commercial use. Examples include corn stover (stalks, leaves, husks, and cobs), wheat straw, and rice straw.
- *Forestry residues* include biomass not harvested or removed from logging sites in commercial hardwood and softwood stands as well as material resulting from forest management operations such as pre-commercial thinning and removal of dead and dying trees. Examples include tree tops, limbs, and other woody material.
- There are a variety of *aquatic biomass resources*, such as algae, giant kelp, other seaweed, and marine microflora. Algae are a diverse group of primarily aquatic organisms, often fast growing and able to live in freshwater, seawater, or damp oils. They may be unicellular and microscopic or very large, as in the giant kelps. Certain algae produce hydrogen and oxygen, while others manufacture hydrocarbons and other products.
- *Biomass processing residues* are all processing yields by-products and waste streams that have a significant energy potential. For example, the processing of wood for products or pulp produces unused sawdust, bark, branches, and leaves/needles. These residues can then be burned for heat and energy, or converted into additional by-products. Because these residues are already collected at the point of processing, they can be convenient and relatively inexpensive sources of biomass for energy.
- *Municipal wastes* is any organic matter, including sewage, industrial, and commercial wastes, from municipal waste collection systems. Plant-derived organic material makes up a significant fraction of residential, commercial, and institutional post-consumer waste. However, municipal waste does not include agricultural and wood wastes or residues.
- *Animal wastes* from farms and animal-processing operations are a complex mixture of organic materials that can pollute the environment if left unprocessed. Through biochemical conversion processes like anaerobic digestion, these wastes can be used to make many products, including energy.

2.1.3 Uses of biomass

Regarding the use of biomass, it can be divided into two main branches: a traditional use and a modern use [2.2]. "Traditional use" means that the technologies involved have been known and used for a long time, while "modern use" means that the technologies have been developed in recent years. Since the "cave man" discovered the fire, the humanity used biomass, firstly for heating, cooking, lighting and safety purposes, then, a few thousand years later, at the beginning of the industrial revolution, to move machine thanks to the steam engine. Finally, for the last 150 years, our societies have used the biomass to generate electrical power, but in a small amount compared to fossil fuels.

Today biomass is used for cooking and heating especially in the developing countries and rural or isolated zones. The scale of its use is too difficult to measure to any degree of accuracy. According to International Energy Agency (IEA), 10% of global energy consumption is due to the use of traditional biomass for heating and cooking. The main issue with the use of traditional biomass is that equipment are usually inefficient with consequences such as health problems, pollution, deforestation and safety. Replacing old equipment can be a solution to decrease local pollution, especially particulate emissions. However, for many people across the world, traditional biomass remains the only viable energy option as it tends to be readily available, free, simple and easy to use.

Biomass using modern technology differs from traditional biomass in two key characteristics: firstly, that the source of organic matter should be sustainable and secondly, that the technology used to obtain the energy should limit or mitigate emissions of flue gases and account for ash residue management. Also, the efficiency of conversion is higher leading to less use of fuel. Modern biomass is largely used in some regions, notably in northern Europe and parts of North America. Modern biomass technologies include liquid biofuels used to power automobiles and to produce heat in boilers, industrial and residential cogeneration and bio-refineries used in generating electricity, liquid biofuels and pellet heating systems.

The largely used technology is *combined heat and power* (CHP), also called cogeneration, that produces simultaneously heat and electricity. CHP, particularly together with district heating and cooling (DHC), is an important part of greenhouse gas emission reduction strategies, due to higher efficiency and a reduced need for fuels in comparison to stand-alone systems. Electricity production can be fuelled by solid, liquid or gaseous biofuels, with the biggest fraction of bio-power today being produced using solid biofuel.

Thermochemical Biomass Gasification is a high temperature process that produces a fuel gas, which, after cleaning, can provide a good environmental performance and high flexibility in applications. The process is used to convert biomass (solid biomass, wastes) into a combustible gas that can be used for different purposes. Typical feedstock for gasification is cellulosic biomass such as wood chips, pellets or wood powder, or agricultural by-products like straw or husks.

Pellets are another form of modern bioenergy source. Pellet is a term used for a small particle of cylindrical form produced by compressing an original material. At present, pellets

are mainly produced from wood residues, though the volume of pellets produced from agricultural by-products such as straw, husks of sunflower seeds and stalks and corn leaves etc. is increasing. A key advantage of pellets compared to unprocessed biomass is the high density and high energy content per unit volume, which is convenient for long distance transportation.

The goal of CO_2 emissions reductions and the subsequent renewable energy incentives have led some power plant operators to broaden their fuel palette to include various carbon-neutral biomass fuels. *Co-firing* of fossil fuels and various types of biomass is a mature technology and is currently being successfully practiced globally. With technological advances, many limitations associated with it have been overcome. Many coal-fired plants have been converted or retrofitted to accommodate co-firing with limited impact on efficiency, operations or lifespan. However, there is much more to co-firing than simply adding a secondary fuel. Boiler technology and design remain critical issues when evaluating the maximum share of biomass that can be used without compromising boiler performance or the lifetime of the boiler components.

The conversion of bioenergy crops such as corn and sugar cane, into *biofuels*, synthetic equivalents for oil products such as gasoline or diesel, has a long-established history. Production of ethanol from crops dates back as far as the development of the automotive industry and it was in use as a mix additive to oil derived fuels, until the low price of gasoline led it to dominate after the Second World War. Biofuels returned to commercial-scale use in the 1970s, triggered by the oil crises of the decade when Brazil took the lead in the production of ethanol from sugar cane. Liquid biofuels for transport are part of important strategies to improve fuel security, mitigate climate change and support rural development. Conventional biofuels (also referred to as first generation biofuels) are being produced globally with a current production volume of more than 100 billion litres annually. To complement the conventional biofuels, recent advances are focused on the next generation of biofuels. Advanced biofuels, generally referred to as second or third generation biofuels are produced from a broad spectrum of predominantly non-edible biomass feedstock. Some of these are “drop-in” biofuels that can be applied in existing distribution infrastructure and engine platforms. By-products of advanced biofuel production include bio-electricity, bio-heat, bio-chemicals and protein based feed. Apart from the technologies of fermentation (ethanol) and esterification (biodiesel), there are various alternative pathways. For example, in biomass gasification, biomass is converted into a combustible gas. The gas can be used, after upgrading, as a transportation fuel or can be further processed into liquid biofuels. Pyrolysis is another technique where oxygen starved environment leads to the conversion of biomass into bio char, bio liquids and non-condensable gases. The bio liquids can then be refined to be used as transportation fuels, heating fuels, or for production of chemicals. Finally, algal biofuels (otherwise called as third generation biofuels) are being explored as a sustainable alternative to fossil fuels. Algae are an alternative feedstock that uses sunlight, carbon dioxide, nutrients and water to produce oils that can be used as feedstock for biofuel production. However, the technology is not yet cost competitive and production is energy intensive.

Biogas is a gas produced by anaerobic fermentation of different forms of organic matter and is composed mainly of methane (CH_4) and carbon dioxide (CO_2). Typical feedstock for

biogas production are manure and sewage, residues of crop production (i.e., straw), the organic fraction of the waste from households and industry, as well as energy crops including maize and grass silage. Biogas is supplied to a variety of uses or markets, including electricity, heat and transportation fuels. In many countries, the gas is used for direct combustion in household stoves and gas lamps are increasingly common. However, producing electricity from biogas is still relatively rare in most developing countries. In industrialized countries, power generation is the main purpose of most biogas plants; conversion of biogas to electricity has become a standard technology. Leading countries in producing biogas include Germany, India and China. The use of biogas for transport is dominant in the EU region.

Another proven conversion technology for obtaining biogas is the creation of synthesis gas (known as syngas) via thermochemical gasification. The economics for biomass to syngas conversion will depend greatly on demand levels, regional gas markets and also the pace of growth for gas demand in those same markets. With technological advances and ongoing research and development to commercialise the use of biogas for power generation, the generation market is looking to take some positive steps in the next years.

Advanced biofuels are generally referred to as second or third generation biofuels. The feedstock includes lignocellulose-based ethanol, hydrogenated vegetable oil (HVO), algae based biofuels and biogas. However, due to uncertainty in biofuel and fossil oil markets, and in policy domains, a number of large-scale facilities are reportedly idle at the current time.

Dispatchable energy sources are those sources that can be dispatched at the request of power grid operators or of the plant owner, meaning they can be ramped up or shut down in a relatively short amount of time based on the current need for energy. The global energy supply is currently in transition, with increasing amounts of weather dependent renewable energy sources connected to grid. Need for balancing and adjusting the supply could refer to time intervals of a few seconds up to a couple of hours, in addition to demand side management. The role of grid management is expected to increase in the future to ensure that customers will continue to receive the required amount energy at the required time, and therefore technical solutions for production, grid management and supply are needed. The need for balancing the production and use of energy is relevant on wide temporal variation and scale requirements. Biomass can renewably address the whole spectrum of requirements, from frequency control solutions and reserve capacity to seasonal storage of energy. Bioenergy, as the only currently existing large-scale dispatchable form of renewable energy, in addition to hydropower, can be used as a climate friendly option to store energy and to make grid operation more stable on system level. In addition to the ability of balancing electric grid with large shares of weather dependent renewable electricity production, it brings carbon neutral balancing elements also to heating and transport sectors. Bioenergy as energy carrier is stable and therefore the use in power generation will have new roles for peak demands and adjusting generation for secure and reliable grid operation. Bioenergy can be used to balance the grid in existing installations, and especially existing CHP infrastructure to operate as peak and balancing power plants. This brings an additional benefit of balancing the electric grid with bioenergy. Biomass can also be utilised as a refined energy carrier, such as bio-methane or bio-liquid, and used in gas turbines, engines, fuel cells or dedicated burners for peak demand. The use in thermal power plants also

increases possibilities for balancing the system in the form of turndown ratio and also using thermal storage.

2.1.4 Feedstock and distribution of biomass

Bioenergy data globally is insufficient due to numerous reasons [2.3]. Lack of knowledge on data collection and gathering, lack of capacity (e.g. finances), and the complexity of the bioenergy system are some of reasons for low availability of updated bioenergy data. One thing for sure is that bioenergy is an important energy source and will play a crucial role in the future energy mix.

According to WBA Global Bioenergy Statistics, in 2014, the total primary energy supply of biomass was 59.2 EJ which is 10.3% of all the supply of energy globally of 573 EJ. Since 2000, the biomass supply grew at an average annual growth rate of 2.3%. Biogas and liquid biofuels had the highest increase at 11.2% and 15.6% respectively.

Biomass supply comes from a variety of feedstock. Forestry is the mainstay of the bioenergy sector: the forestry industry generates more than 87% of all the biomass feedstock for bioenergy in the form of fuelwood, charcoal, forestry and wood industry residues, recovered wood and black liquor. The agriculture sector contributes 10% with animal and agricultural by-products. Energy crops producing bioethanol and biodiesel are included in the agricultural sector. Finally, municipal solid waste and landfill gas cover the remaining 3% of the biomass feedstock sources.

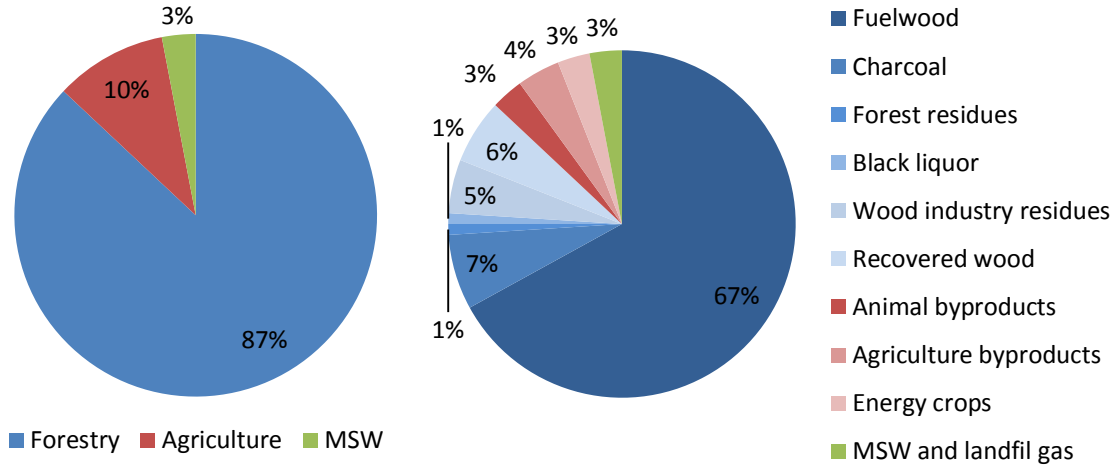


Figure 2.2 – Biomass feedstock sources [2.3]

The supply of biomass varies among continents. Most of the bioenergy produced from municipal solid waste is in Europe. Americas is a world leader in producing bioenergy from liquid biofuels. For biogas as a source, Europe again leads. However, half of the biomass source globally is via solid biomass use in Asia.

Land use is a critical factor for bioenergy development. Unlike other renewable energy sources, biomass requires significant land for growing forests, agriculture crops etc. to supply the bioenergy demand globally.

Forestry sector, as mentioned earlier, is the largest contributor to biomass supply globally. American continent including North, Central and South America holds the largest amount of forest land globally. Close to 40% of the forest area is in that region. Europe follows second at 25% largely due to the high concentration of forests in Russia. Asian continent has the highest share of planted forests. The top 10 countries with highest forest land area (excluding EU - 28) are led by Russia, Brazil and Canada. One third of all the forest area is in two countries, Russia and Brazil. Primary forests are those with no visible human activities: these are the highest in Russia, Brazil and Canada. Among planted forests, China has planted 77.8 million ha of forests.

Woodfuel is wood used for fuel purposes: cooking, heating or power production. It includes wood harvested from main stems, branches and other parts of trees. It is the major contributor to biomass supply globally. Globally, 1.87 billion m^3 of wood fuel is produced, mostly in Asia and Africa. Considering the average growth since 2000, Europe wood fuel production increased by 3% annually while Asia reduced its production growth rate by 0.7%. India is the largest producer of woodfuel globally – 307 million tonnes in 2015. The country has seen a 11% increase in production in 15 years while in contrast, China decreased its production by 24%. All African countries have increased their production.

Agriculture sector is a significant contributor to the biomass supply in terms of energy crops for biofuels production and heat and electricity along with the use of residues. It is crucial to understand the sector in terms of area, crops production and yields, residues potential etc. Agriculture crops have varying uses in supply of biomass for energy purposes. Maize, sugarcane and oil seed crops are converted to produce liquid bioethanol and biodiesel. Other cereals and sugar crops can be used for producing advanced biofuels via the use of residues like straw, husk and stalk etc. Globally, the largest crops in terms of area harvested include wheat (220 million ha), maize (185 million ha) and rice (163 million ha) which are predominantly produced in Asia, Americas and Asia respectively. It is important to note that not all the area is available for biofuel production and only a fraction is used. Yields are a crucial part of the food and fuel debate in the biofuels sector. There is significant potential to producing more crops for food, feed and fuel by increasing the crop yields in various regions. Comparing yields of major crops across all continents, Africa has the lower yields than the world average in all major crops. The agricultural sector contributes 10% of the global biomass supply. However, it has significant potential to increase the supply of biomass. Unused land can be used for agricultural purposes to produce both energy and food. Crop yields could be increased to produce more tonnes per ha of land. The use of agricultural residues can be a major source of energy generation.

Waste to energy is an important part of the bioenergy supply. Waste obtained from municipalities and industries contributes to the increasing supply of biomass globally. Currently, the total share of waste sector in biomass supply is only 3%. This sector is classified into municipal waste (renewable and non-renewable) and industrial waste. Municipal waste consists of products obtained from households, industry, hospitals etc. which are collected by local authorities for processing. On the other hand, industrial waste is waste consisting of solid and liquid products (e.g. tyres) processed directly in specialized plants. Large part of the waste to energy conversion occurs in Europe (55% of the global)

while the rest is in Asia and Americas. Although there might be waste conversion facilities in Africa and Oceania, they are not in the scale occurring in the rest of the regions.

2.2 Lignocellulosic biomass

Lignocellulosic biomass refers to plant biomass that is composed mainly of cellulose, hemicellulose, and lignin [2.4]. Their percentages vary according to the species. In the table below [2.5] are presented some examples.

	Lignin [%]	Hemicellulose [%]	Cellulose [%]
Sugar cane bagasse	20	25	42
Sweet sorghum	21	27	45
Hardwood	18-25	24-40	40-55
Softwood	25-35	25-35	45-50
Corn cobs	15	35	45
Corn stover	19	26	38
Rice straw	18	24	32
Nut shells	30-40	25-30	25-30
Newspaper	18-30	25-40	40-55
Grasses	10-30	25-50	25-40
Wheat straw	16-21	26-32	29-35
Banana waste	14	15	13
Bagasse	23	17	55
Sponge gourd fibres	15	17	67

2.2.1 Cellulose

Cellulose is a polysaccharide formed by glucose molecules linked by β (1 → 4) glucosidic bonds [2.4]. The building block of cellulose is cellobiose, since the repeating unit in cellulose is a two-sugar unit.

The number of glucose units in a cellulose molecule is referred to as the degree of polymerization (DP). Cellulose molecules are randomly oriented and have a tendency to form intra- and inter-molecular hydrogen bonds. As the packing density of cellulose increases, crystalline regions are formed. Most wood-derived cellulose is highly crystalline and may contain as much as 65% crystalline regions. The remaining portion has a lower packing density and is referred to as amorphous cellulose. The molecular chains are packed in layers, which consist of parallel chains of anhydroglucopyranose units. The chains are held together by intermolecular hydrogen bonds, the so called Van Der Waals forces. There are also intramolecular hydrogen bonds between the atoms of adjacent glucose residues. This structure is referred to as cellulose I or native cellulose.

Cellulose I is insoluble in most solvents including strong alkali. Alkali will swell cellulose but not dissolve it. Cellulose dissolves in strong acids such as 72% sulfuric acid, 41% hydrochloric acid, and 85% phosphoric acid, but degradation occurs rapidly. It is difficult to isolate cellulose from wood in a pure form because it is intimately associated with lignin and hemicellulose.

Cellulose II is another important type of cellulose used for making cellulose derivatives. It is not found in nature. Cellulose II is obtained by mercerization and regeneration of native cellulose. Mercerization is treatment of cellulose I with strong alkali. Regeneration is treatment with carbon disulphide to form a soluble xanthate derivative. The derivative is converted back to cellulose and reprecipitated as cellulose II.

There is also a cellulose III structure, which is formed by treatment of cellulose I with liquid ammonia at about –80ºC followed by evaporation of the ammonia. Alkali treatment of cellulose III gives cellulose II [2.4]. Cellulose IV is formed by heating cellulose III in glycerol at 260ºC. There are other types of cellulose, based on the method of extraction from wood.

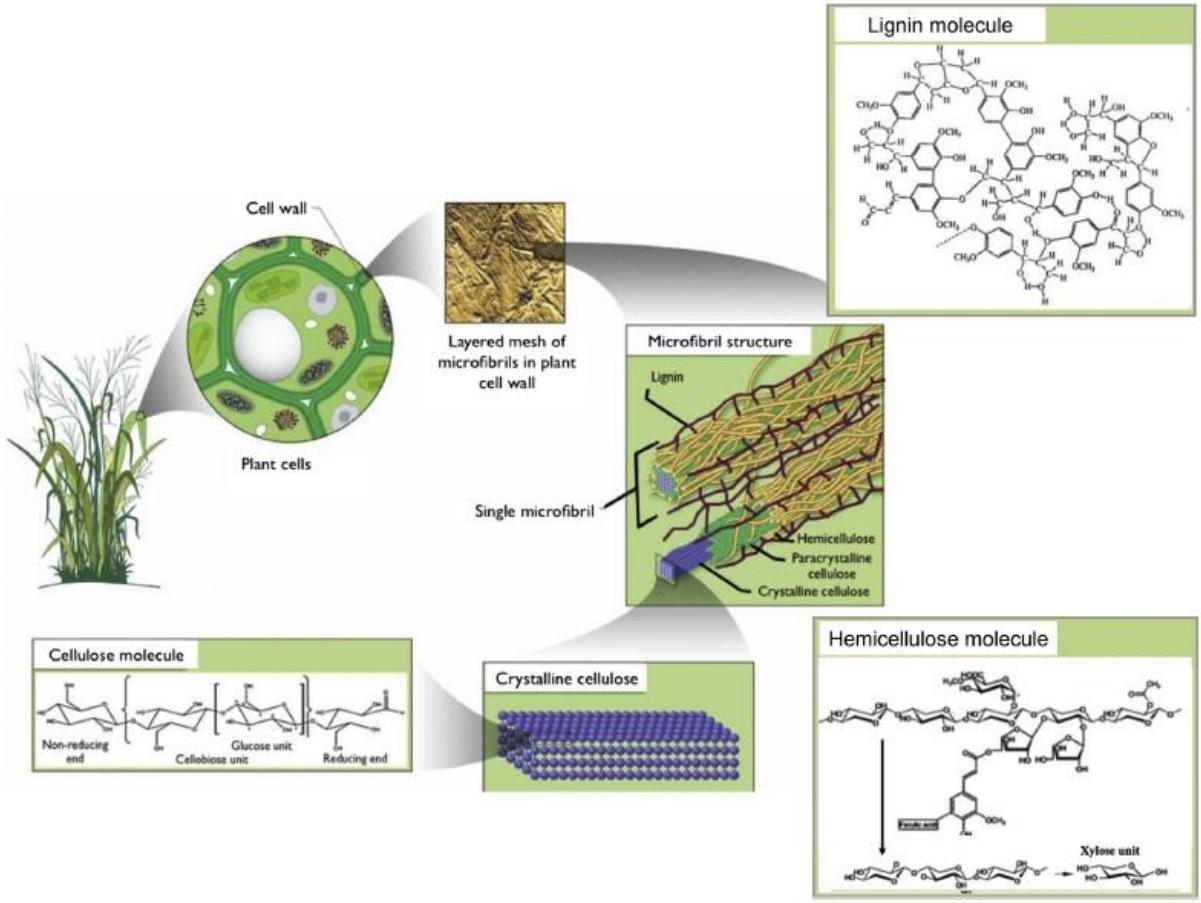


Figure 2.3 - Cell wall composition [2.17]

Another categorization of cellulose is its availability to water, microorganisms, etc [2.4]. The cellulose is divided in accessible and non-accessible. The surfaces of crystalline cellulose are accessible but the rest of the crystalline cellulose is non-accessible. Most of the non-crystalline cellulose is accessible but part of the non-crystalline cellulose is so covered with both hemicelluloses and lignin that it becomes non-accessible. Concepts of accessible and non-accessible cellulose are very important in moisture sorption, pulping, chemical modification, extractions, and interactions with microorganisms.

2.2.2 Hemicellulose

Hemicellulose consists of a collection of polysaccharide polymers with a lower DP than cellulose (average DP of 100–200) and containing mainly the sugars D-xylopyranose, D-glucopyranose, D-galactopyranose, L-arabinofuranose, D-mannopyranose, D-glucopyranosyluronic acid, and D-galactopyranosyluronic acid with minor amounts of other sugars [2.4]. Hemicelluloses are intimately associated with cellulose and contribute to the

structural components of the plant. Some hemicelluloses are present in very large amounts when the plant is under stress.

Hemicelluloses usually consists of more than one type of sugar unit and are sometimes referred to by the sugars they contain, for example, galactoglucomanan, arabinoglucuronoxylan, arabinogalactan, glucuronoxylan, glucomannan, etc. The hemicelluloses also contain acetyl- and methyl- substituted groups. Hemicelluloses are soluble in alkali and are easily hydrolysed by acids.

The hemicelluloses can then be precipitated from the alkaline solution by acidification using acetic acid. Further treatment of the neutralized solution with a neutral organic solvent such as ethyl alcohol results in a more complete precipitation.

2.2.3 Lignin

Lignins are amorphous, highly complex, mainly aromatic polymers of phenyl propane units [2.4]. Lignin acts like a glue by filling the gap between and around the cellulose and hemicellulose complexion with the polymers. It is present in all plant biomass; therefore, it is considered as a by-product or as a residue in bio-ethanol production process. The three-dimensional polymer is made up of C–O–C and C–C linkages. The precursors of lignin biosynthesis are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These phenyl-propanes denoted as 0, I, II methoxyl groups attached to rings give special structure I, II and III. These groups depend on the plant source which they are obtained. Structure I exist in plants (grasses) and structure II found in the wood (conifers) while structure III present in deciduous wood.

2.3 Lignocellulosic biomass pretreatments

Pretreatment is an important step for the recovery of cellulosic content from lignin based biomass as compare to the starchy materials [2.5]. While dealing with lignocellulosic biomasses, pretreatment is also required to break down the lignin barrier to recover cellulose, which is further subjected to enzymatic hydrolysis to convert into fermentable sugars. During the past few decades, several pretreatment approaches have been developed for generating cost-effective fermentable sugar from most of the agricultural cellulose and hemicellulose containing lignocellulosic materials.

An effective pretreatment is characterized by several criteria: preserving hemicellulose fractions, to yield maximum fermentable sugar contents, limiting the loss of carbohydrate, to minimize the formation of inhibitors due to deg33radation products, minimizing energy input, and the process is economically efficient as well as cost-effective [2.5]. The result of the pretreatment must not only defend but also justify its impact on the cost of downstream processing steps and the trade-off between operating costs, capital costs, biomass costs, etc. While, comparing various pretreatment options, all of the above mentioned criteria should be comprehensively considered as a basis to achieve maximal end product of interest.

Lignocellulosic biomass pretreatment methods are broadly classified into two groups: non-biological and biological [2.7]. Non-biological pretreatment methods do not involve any microbial treatments and are roughly divided into different categories: physical, chemical, and physico-chemical methods.

2.3.1 Physical pretreatment

Mechanical extrusion is the most conventional method of biomass pretreatment where the feedstock materials are subjected to heating process (>300 °C) under shear mixing [2.7]. This pretreatment process results mainly in production of gaseous products and char from the pretreated lignocellulosic biomass residues. Due to the combined effects of high temperatures that are maintained in the barrel and the shearing force generated by the rotating screw blades, the amorphous and crystalline cellulose matrix in the biomass residues is disrupted. However, this method requires significant amount of energy making it a cost intensive method and difficult to scale up for industrial purposes.

Although the sugar yields are high, mechanical extrusion cannot alone suffice pretreatment of a range of lignocellulosic feedstocks with varied cellulose, hemicellulose, and lignin contents. Thus, it needs better pretreatment methods for higher sugar yields. Besides, sugar recovery is also significantly influenced by the properties of the biomass.

Glycerol and acetic acid are by-products that are formed during the pretreatment of lignocellulosic feedstocks. However, the by-product formation was significantly lower because in mechanical extraction only physical interactions were observed between the feedstock and the barrel blades. Studies clearly demonstrate that mechanical extrusion treatment had a significant effect on breakdown of cellulose and hemicelluloses fractions from a wide variety of lignocellulosic feedstocks; however, when combined with other pretreatment methods, mechanical extrusion performs better and might enhance the overall yield of the reducing sugars.

Mechanical grinding (milling) is used for reducing the crystallinity of cellulose [2.7]. It mostly includes chipping, grinding, and/or milling techniques. Chipping can reduce the biomass size to 10–30 mm only while grinding and milling can reduce the particle size up to 0.2 mm. However, studies found that further reduction of biomass particle below 0.4 mm has no significant effect on rate and yield of hydrolysis. Chipping reduces the heat and mass transfer limitations while grinding and milling effectively reduce the particle size and cellulose crystallinity due to the shear forces generated during milling. The type and duration of milling and also the kind of biomass determine the increase in specific surface area, final degree of polymerization, and the net reduction in cellulose crystallinity. Different milling methods such as two-roll milling, hammer milling, colloid milling, and vibratory milling are used to improve the digestibility of the lignocellulosic materials. Compared to ordinary milling process, vibratory ball milling is found to be more effective in reducing cellulose crystallinity and improving the digestibility of spruce and aspen chips. Also, wet disk milling has been a popular mechanical pretreatment because of its low energy consumption. Disk milling enhances cellulose hydrolysis by producing fibres and is more effective as compared to hammer milling which produces finer bundles.

Microwave irradiation is a widely used method for lignocellulosic feedstock pretreatment because of various reasons such as easy operation, low energy requirement, high heating capacity in short duration of time, minimum generation of inhibitors, and degrades structural organization of cellulose fraction [2.7]. Moreover, addition of mild-alkali reagents is preferred for more effective breakdown.

Sonication is relatively a new technique used for the pretreatment of lignocellulosic biomass [2.7]. However, laboratory studies have found sonication a feasible pretreatment option. Ultrasound waves produce both physical and chemical effects which alter the morphology of lignocellulosic biomass. Ultrasound treatment leads to formation of small cavitation bubbles which rupture the cellulose and hemicellulose fractions thereby increasing the accessibility to cellulose degrading enzymes for effective breakdown into simpler reducing sugars. The maximum cavitation was formed at 50 °C which is also the optimum temperature for many cellulose degrading enzymes. The ultrasonic field is primarily influenced by ultrasonic frequency and duration, reactor geometry and its type and solvent used. Furthermore, biomass characteristics, reactor configuration, and kinetics also influence the pretreatment through sonication. Duration of sonication has maximum effect on pretreatment of biomass. However, prolonging sonication beyond a certain limit has no additional effect in terms of delignification and sugar release. Besides duration, the frequency of sonication directly determines the power of sonication, which is also an important factor affecting the lignocellulosic feedstock pretreatment. Most of the researchers have used ultrasound frequency of 10–100 kHz for the pretreatment process which has been enough for cell breakage and polymer degradation. However, higher sonication power level is reported to adversely affect the pretreatment process. Therefore, power and duration of sonication should be optimized based on the biomass and slurry characteristics to meet the desired pretreatment objectives.

Pyrolysis has also been employed for the pretreatment of lignocellulosic biomass in bio-refinery processes [2.7]. Unlike bioethanol applications, pyrolysis treatment is used for production of bio-oil from lignocellulosic feedstocks. In brief, pyrolysis is a thermal

degradation process where biomass was subjected to high-temperature treatment, generally operated at 500–800 °C in the absence of oxidizing agent. At this temperature, cellulose rapidly decomposes leading to formation of end products such as gaseous substances, pyrolysis oil, and charcoal. Pyrolysis is divided into slow and fast pyrolysis based on the heating rate. The amount of each end product varies depending on the type of pyrolysis, biomass characteristics, and reaction parameters. Besides production of high value energy-rich products, pyrolysis is adapted by thermal industries due to easy transport management, storage, combustion, and retrofitting and is flexible in production and marketing. Pyrolysis is found to be more efficient when carried out in the presence of oxygen at lower temperatures. Biomass to liquid (BtL) route is used for the production of transportation fuels from biomass which includes conversion of biomass to syngas to high-quality Fischer-Tropsch (FT) fuels. The most efficient and commercially feasible route was found to be based on torrefaction followed by pyrolysis and pelletization.

Pulsed-electric field (PEF) pretreatment exposes the cellulose present in the biomass by creating the pores in the cell membrane thereby allowing the entry of agents that will break the cellulose into constituent sugars [2.7]. In PEF pretreatment, the biomass is subjected to a sudden burst of high voltage between 5.0–20.0 kV/cm for short durations (100 μs). The advantages of PEF are low energy requirement due to very short duration of pulse time and the treatment can be carried out at ambient conditions. Also, the PEF instrument is simple in design due to lack of moving parts.

2.3.2 Chemical pretreatment

Acid treatment is the most commonly used conventional pretreatment method of lignocellulosic feedstocks, although it generates high amount of inhibitory products such as furfurals, 5-hydroxymethylfurfural, phenolic acids, and aldehydes [2.7]. The corrosive and toxic nature of most acids requires a suitable material for building the reactor which can sustain the required experimental conditions and corrosive nature of acids. Still it is the most widely employed pretreatment method on industrial scale. Based on the type of end application, two types of acid pretreatments are developed: high temperature (>180 °C) for short duration (1–5 min) and low temperature (<120 °C) for long duration (30–90 min). In some cases, enzymatic hydrolysis step could easily be avoided as acid itself hydrolyses the biomass into fermentable sugars. However, extensive washing is necessary to remove acid before fermentation of sugars. Different types of reactors such as percolation, plug flow, shrinking-bed, batch, flow-through, and counter current reactors have been developed. The concentrated acid must be recovered after hydrolysis in order to make the process economically feasible. Different acids have been used for the pretreatment of a variety of biomass. The most common commercially used acid is dilute sulphuric acid (H_2SO_4), especially thanks to its low cost. Therefore researchers have carried out the pretreatment of lignocellulosic biomass through various other acids such as oxalic acid and maleic acid. Dicarboxylic organic acids are more efficient for carrying out the hydrolysis of the substrate over a range of temperature and pH values. Furthermore, oxalic acid is less toxic to yeasts and other microorganisms than sulfuric and acetic acids, does not hamper glycolysis and does not produce odour.

In contrary to acid treatment, *alkali pretreatment* methods are in general performed at ambient temperature and pressure [2.7]. The most commonly used alkali reagents are the hydroxyl derivatives of sodium, potassium, calcium, and ammonium salts. Among these hydroxyl derivatives, sodium hydroxide was found to be most effective. Alkali reagents degrade the side chains of esters and glycosides leading to structural modification of lignin, cellulose swelling, cellulose decrystallization, and hemicellulose solvation. As compared to acid pretreatment, the solubility of cellulose and hemicellulose is very low with the alkali pretreatment. The solubility improves on increasing the internal surface area of cellulose, decreasing the degree of polymerization and crystallinity, and disrupting the lignin structure. The conditions for mild alkali pretreatment are less harsh as compared to other pretreatment methods especially acid pretreatment method. Further, a neutralizing step is required to remove the inhibitors as well as lignin. Being an inexpensive pretreatment method, the only drawback of alkali treatment is its high downstream processing cost because the process utilizes a large quantity of water for removing the salts from the biomass.

Ozone treatment is mainly used for reducing the lignin content of lignocellulosic biomass as it mainly degrades lignin but negligibly affects hemicellulose and cellulose [2.7]. Ozonolysis is performed at ambient temperature and pressure. Also, it does not produce any toxic inhibitors therefore is environment friendly and does not affect the post-pretreatment processes like enzymatic hydrolysis and yeast fermentations. The important factor which affects the ozone pretreatment is the moisture content of the biomass: higher the moisture content, lower the lignin oxidization. Although ozonolysis is an effective pretreatment method, the high amount of ozone required makes it an expensive pretreatment method, making it a less suitable option for pretreatment at industrial scale.

The *organosolv process* involves addition of aqueous organic solvents such as ethanol, methanol, ethylene glycol, acetone etc. to the biomass under specific condition of temperature and pressure [2.7]. Commonly, this process takes place in the presence of an acid, base or salt catalyst. Temperature in organosolv pretreatment depends on the type of biomass and catalyst involved and may reach up to 200 °C. This process is mainly used for the extraction of lignin which is a value-added product. Apart from lignin, cellulose fraction and hemicellulose syrup of C5 and C6 sugars are also produced during the course of organosolv pretreatment. Removal of lignin from the biomass exposes the cellulose fibres for enzymatic hydrolysis leading to higher conversion of biomass. The physical characteristics of pretreated biomass such as fibre length, degree of cellulose polymerization, crystallinity etc. depends upon variable factors such as temperature, reaction time, solvent concentration and catalyst used. High temperatures, high acid concentrations, and long reaction time have led to the formation of inhibitors of fermentation. The main drawback of this process is the high cost of the solvents, though this drawback can be minimized by recovering and recycling solvents through evaporation and condensation. Removal of solvents is very important because the solvent may cause negative effect on growth of microorganisms, enzymatic hydrolysis and fermentation. Organosolv is less preferred due to high risk involved in handling organic solvents that are highly flammable.

Ionic liquids (ILs) have received great attention in last decade for the pretreatment of lignocellulosic biomass [2.7]. Ionic liquids are comparatively a new class of solvents which are entirely made of ions (cations and anions), have low melting points (<100 °C), negligible

vapour pressure, high thermal stabilities, and high polarities. Imidazolium salts are the most commonly used ILs. ILs are assumed to disrupt hydrogen bonding of lignocellulosic components. Ionic liquids has been able to effectively pretreat lignocellulosic biomass, however, there are certain challenges that need to be addressed such as high cost of ILs, difficulty in recycling and reuse, inhibitor generation etc.

Deep eutectic solvents (DESs) are a relatively new class of solvents having many characteristics similar to ionic liquids [2.7]. A DES is a fluid generally composed of two or three cheap and safe components that are capable of self-association, often through hydrogen bond interactions, to form a eutectic mixture with a melting point lower than that of each individual component. Although DESs are similar to ILs in terms of physical behaviour and physical properties, DESs cannot be considered as ionic liquids due to the fact that DESs are not entirely composed of ionic species and can be obtained from non-ionic species.

In the recent past, a large number of natural products have been brought into the range of ILs and DES [2.7]. These products include choline, urea, sugars, amino acids, and several other organic acids. Such solvents obtained from natural sources are termed as *Natural Deep Eutectic Solvents (NADES)*. Unlike ILs, NADES are cost effective, easier to synthesize, non-toxic, biocompatible, and highly biodegradable. Moreover, many studies recovered and reused these novel solvents with high efficiency. Foreseeing the potentiality of NADES in several applications, these solvents are regarded as the solvents for the twenty first century. Despite having a lot of potential for the extraction of natural products, the high viscosity of NADES is an obvious disadvantage.

2.3.3 Physico-chemical pretreatment

Steam pretreatment is one of the most commonly used physico-chemical methods for pretreatment of lignocellulosic biomass [2.7]. Due to the changes that occur during this process, this method is also called “auto hydrolysis”. Steam pretreatment is typically a combination of mechanical forces (pressure drop) and chemical effects (autohydrolysis of acetyl groups of hemicellulose). In this process, biomass is subjected to high pressure (0.7–4.8 MPa) saturated steam at elevated temperatures (between 160 and 260 °C) for few seconds to minutes which causes hydrolysis and release of hemicellulose. The steam enters the biomass expanding the walls of fibres leading to partial hydrolysis and increasing the accessibility of enzymes for cellulose. After this, the pressure is reduced to atmospheric condition. During this pretreatment, the hydrolysis of hemicellulose into glucose and xylose monomers is carried out by the acetic acid produced from the acetyl groups of hemicellulose: hence this process is also called as autohydrolysis. The factors that affect steam pretreatment are temperature, residence time, biomass size and moisture content. The efficiency of steam pretreatment can be effectively enhanced in the presence of catalysts such as H_2SO_4, CO_2 or SO_2. Acid catalyst has been found to most successful in terms of hemicellulose sugar recovery, decreased production of inhibitory compounds and improved enzymatic hydrolysis. Limited use of chemicals, low energy requirement, no recycling cost and environment friendly are some of the advantages of steam pretreatment method. On the other hand, the possibility of formation of fermentation inhibitors at high temperature,

incomplete digestion of lignin-carbohydrate matrix and the need to wash the hydrolysate which decreases the sugar yield by 20% are few disadvantages associated with steam pretreatment.

Liquid hot water method, also called hot compressed water, is similar to steam pretreatment method but as the name suggests, it uses water at high temperature (170–230 °C) and pressure (up to 5 MPa) instead of steam [2.7]. This leads to hydrolysis of hemicellulose and removes lignin making cellulose more accessible. This also avoids the formation of fermentation inhibitors at high temperatures. LHW can be performed in three different ways based on the direction of flow of water and biomass into reactor:

- Co-current pretreatment, in which both the slurry of biomass and the water is heated to the required temperature and held at the pretreatment conditions for controlled residence time before being cooled.
- Counter current pretreatment, in which the hot water is pumped against the biomass in controlled conditions.
- Flow through pretreatment where the biomass acts like a stationary bed and hot water flows through the biomass and the hydrolysed fractions are carried out of the reactor.

Low-temperature requirement, minimum formation of inhibitory compounds and low cost of the solvent are some of the advantages associated with LHW. However, it requires large amount of energy in downstream processing due to large amount of water involved.

Wet oxidation is one of the simple methods of lignocellulosic pretreatment where the air/oxygen along with water or hydrogen peroxide is treated with the biomass at high temperatures (above 120 °C for 30 min) [2.7]. Earlier this method as also used for waste water treatment and soil remediation. This method is most suitable for lignin enriched biomass residues. The efficiency of wet oxidation is dependent on three factors: oxygen pressure, temperature, and reaction time. In this process, when the temperature is raised above 170 °C, water behaves like an acid and catalyses hydrolytic reactions. The hemicelluloses are broken down into smaller pentose monomers and the lignin undergoes oxidation, while the cellulose is least affected by wet oxidation pretreatment. Besides these, studies report that the addition of chemical agents like sodium carbonate and alkaline peroxide in wet oxidation reduces the reaction temperature, improves hemicellulose degradation and decreases the formation of inhibitory components such as furfurals and furfuraldehydes. This pretreatment method is unlikely to reach industrial scale of biomass pretreatment because of the high cost of the hydrogen peroxide and the combustible nature of the pure oxygen.

Sulphite pretreatment to overcome recalcitrance of lignocellulose (SPORL) is a popular and efficient pretreatment method for lignocellulosic biomass [2.7]. It is carried out in a combination of two steps. First, the biomass is treated with calcium or magnesium sulphite to remove hemicellulose and lignin fractions. In the second step, the size of the pretreated biomass is reduced significantly using mechanical disk miller. SPORL pretreatment has been popular in the recent times because of its versatility, efficiency, and simplicity. It reduces the energy consumption to 1/10 required for the reduction of size of biomass. It has very high conversion rate of cellulose to glucose and maximizes hemicellulose and lignin

removal and recovery. It has the capacity to process a variety of biomass and has excellent scalability for commercial production by retrofitting into existing mills for production of biofuels. However, certain issues such as sugar degradation, requirement of large volumes of water for post-pretreatment washing and high cost of recovering pretreatment chemicals need to be addressed for making SPORL a cost effective pretreatment technology.

Methods that use liquid ammonia for the pretreatment of lignocellulosic biomass are *ammonia fiber explosion* (AFEX), *ammonia recycle percolation* (ARP) and *soaking aqueous ammonia* (SAA) [2.7]. In AFEX, lignocellulosic biomass is heated with liquid ammonia (in 1:1 ratio) in a closed vessel at temperature 60–90 °C and pressure above 3 MPa for 30–60 min. After holding the desired temperature in vessel for 5 min, valve is opened which explosively releases the pressure leading to evaporation of ammonia and drop in temperature of the system. It is similar to steam explosion but ammonia is used instead of water. Lignocellulosic biomass when treated with ammonia at high pressure and given temperature causes swelling and phase change in cellulose crystallinity of biomass leading to increase in the reactivity of leftover carbohydrates after pretreatment. The lignin structure gets modified which increases the water holding capacity and digestibility. Unlike other pretreatment methods, AFEX treatment does not produce inhibitors, which is highly desirable for downstream processing. Besides, the overall cost of the pretreatment process is significantly low due to the absence of additional steps like water washing, detoxification, recovery, and reuse of large quantities of water.

CO_2 explosion process carries out the pretreatment of biomass through supercritical CO_2 which means the gas behaves like a solvent [2.7]. The supercritical CO_2 is passed through a high pressure vessel containing the biomass. The vessel is heated to the required temperature and kept for several minutes at high temperatures. CO_2 enters the biomass at high pressure and forms carbonic acid which hydrolyses the hemicellulose. The pressurized gas when released disrupts the biomass structure which increases the accessible surface area. This pretreatment method is not suitable for biomass having no moisture content. Higher the moisture content in the biomass, higher the hydrolytic yield. Low cost of carbon dioxide, low temperature requirement, high solid capacity, and no toxin formation makes it an attractive process. However, high cost of reactor which can tolerate high pressure conditions is a big obstacle in its application on large scale.

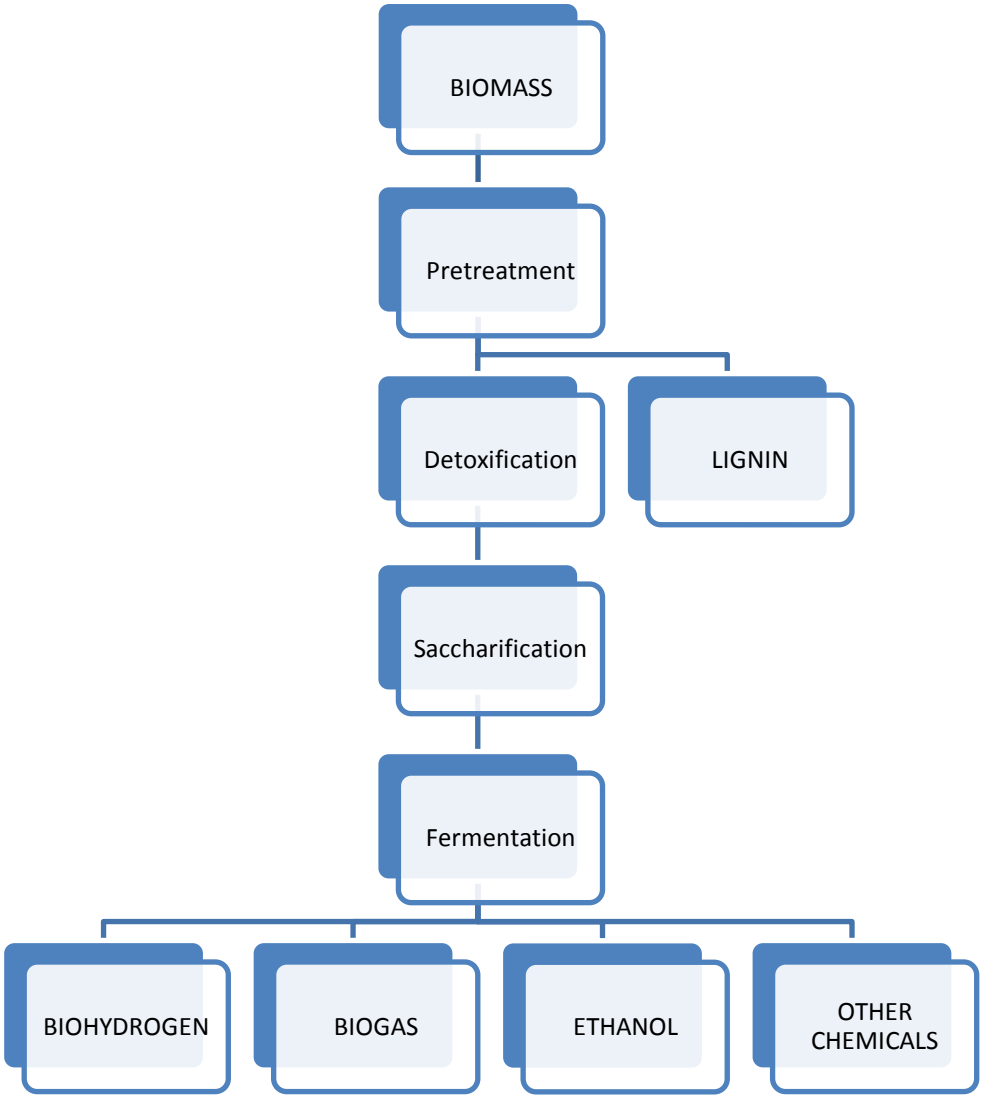
Oxidative pretreatment involves treatment of lignocellulosic biomass by oxidizing agents such as hydrogen peroxide, ozone, oxygen or air [2.7]. A number of chemical reactions such as electrophilic substitution, side chain displacements, and oxidative cleavage of aromatic ring ether linkages may take place during oxidative pretreatment. This process causes delignification by converting lignin to acids, which may act as inhibitors. Therefore, these acids need to be removed. A major drawback of oxidative pretreatment is that it damages a significant amount of hemicellulose making it unavailable for fermentation. The most commonly employed oxidizing agent is hydrogen peroxide. It has been found that hydrolysis of hydrogen peroxide leads to formation of hydroxyl radicals which are responsible for degradation of lignin and production of low molecular weight products. Removal of lignin from lignocellulose exposes cellulose and hemicellulose leading to increased enzymatic hydrolysis.

2.3.4 Biological pretreatment

In comparison to conventional chemical and physical pretreatment methods, biological pretreatment is considered as an efficient, environmentally safe and low-energy process [2.7]. Nature has abundant cellulolytic and hemicellulolytic microbes which can be specifically targeted for effective biomass pretreatment. Biological pretreatments are carried out by microorganisms such as brown, white, and soft-rot fungi which mainly degrade lignin and hemicellulose and little amount of cellulose. Degradation of lignin by whiterot fungi occurs due to the presence of peroxidases and laccases (lignin degrading enzymes). Although the biological pretreatment is highly intriguing, the rate of hydrolysis of lignocellulosic fractions is too slow which severely hampers to be foreseen as a potential pretreatment method at an industrial scale. In order to make biological pretreatment at par with other pretreatment methods, more basidiomycetes fungi should be tested for its ability to delignify the biomass effectively at a faster rate. Studies have found that a combination of another pretreatment process with biological pretreatment process is more effective as compared to a single pretreatment process.

2.4 Products of pre-treatment step and subsequent processing

The intermediate goal of the energetic conversion process is the saccharification of pretreated biomass, from which several biofuels or chemicals can be obtained. Saccharification is the process of breaking up the complex carbohydrates into theirs monosaccharide components. In order to have a good enzymatic digestibility of cellulose, the operational conditions are set to remove hemicellulose and/or lignin from the lignocellulosic matrix. However, while maximizing the goal other factors are also affected, for example the degradation of the solubilized fragments as a results of severe conditions. The amount and nature of the formed degradation products are related to the pretreatment process and conditions. The percentage of biomass components depends, as seen, on the species of feedstock, and it have a major impact on the formation of inhibitors during pretreatment process.



Lignin is harder to convert than cellulose and it can be recovered after pretreatment stage because it still contains a lot of useful functionality: the simpler use is to burn it to produce process heat. Lignin have also the potential to be a chemical feedstock via gasification to produce syngas, and then to produce methanol, dimethyl ether, olefins, and mixed alcohols.

Alternatively there are different hydrocracking, hydrogenation or oxidation methods that can be used to convert lignin into aromatic hydrocarbon. Hence, lignin can be a secondary valuable product.

Detoxification process is fundamental to guarantee a correct concentration of degradation compounds, generated during pretreatment step, to carry out efficiently the subsequent processes. Acid/alkaline hydrolysis or enzymatic hydrolysis are used to break polysaccharides of cellulose and hemicellulose into C5 and C6 sugars.

2.4.1 Products of pre-treatment process

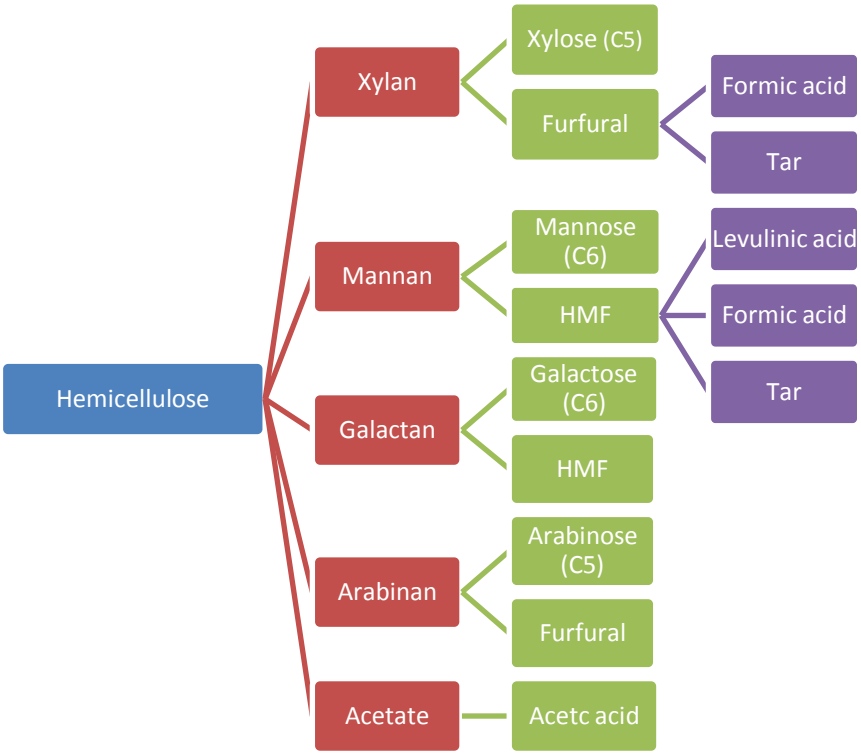
In case of chemical or physico-chemical pretreatment process, biomass undergoes hydrolysis also during this step, producing several products [2.8].

As seen before, cellulose is formed by a sequence of glucose molecules, called cellobiose. A percentage of cellulose, that depends on the pretreatment process conditions, reacts and generates glucose. Oxidative conditions leads to the formation of gluconic and glucaric acids.

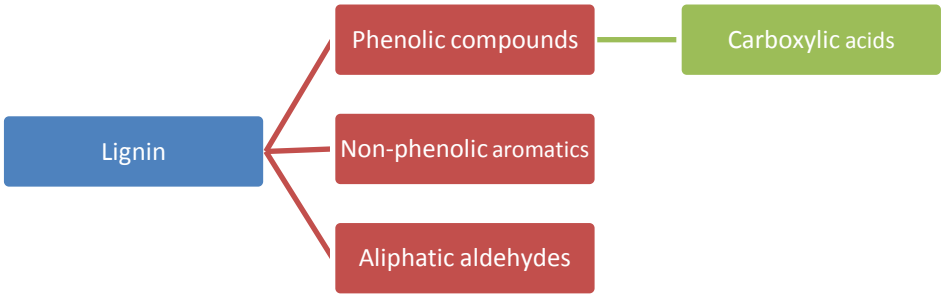
Hemicellulose is a compound of polysaccharides. It has a low degree of polymerization and it is easy to hydrolyse [2.8]. Hydrolysis of backbone of hemicellulose leads to the formation of pentoses, hexoses and uronic acids. However under acid conditions uronic acid and pentoses undergo dehydration with formation of 2-furaldehyde, known also as furfural, while the hexoses are dehydrated to 5-hydroxymethyl-2-furaldehyde, also known as HMF. Under severe conditions, HMF is further degraded to levulinic and formic acids. Furfural is also affected to further degradation to formic acid and to condensation reactions with formation of resins. The acetyl group present in hemicellulose undergoes hydrolysis and generates acetic acid. The hydrolysates produced during annual plants pretreatment contains also phenolic acids.

Although in lower amounts than in acid pretreatments most of the above-mentioned products can also be formed during hydrothermal pretreatments, which generally start at a pH that is close to neutrality but get acidified as the reaction proceeds and acetic acid and uronic acids are released.

Under alkaline conditions the carbohydrates are better preserved than at low pH values, but some degradation also occurs leading to the formation of carboxylic acids. The peeling reactions occurring during alkaline treatments lead to endwise degradation of polysaccharides with formation of saccharinic acids, and also some amounts of lactic acid, formic acid and different dihydroxy and dicarboxylic acids. Acetic acid, formed by saponification of the acetyl groups, is another typical product of alkaline treatments. Under alkaline wet oxidation, the furan aldehydes are oxidised to furoic acid.



Lignin macromolecules reactions generate a high number of phenolic compound which differ depending on the sort of biomass and treatment conditions [2.8]. Together with the phenolic compounds, there are also non-phenolic aromatics that are the phenylic constituents of the lignocellulosic hydrolysates. Apart from furan aldehydes and phenylic aldehydes, it is likely that small aliphatic aldehydes form during pretreatment. Recent researches indicate that small aliphatic aldehydes are ubiquitous in biomass after pretreatment under acid conditions. Phenolic compounds are also formed during alkaline processes. Under alkaline wet oxidation the phenolic compounds are oxidised to different carboxylic acids.



Metal ions can also be formed during acidic processing of biomass [2.8]. Acid conditions can cause corrosion of pretreatment equipment, resulting in the liberation of heavy metal ions, such as copper, nickel, chromium and iron, which can be inhibitory to fermenting microorganisms. Other cations, such as sodium, calcium and magnesium, can come from pretreatment chemicals or from adjustment of the pH.

2.4.2 Inhibitory by-products

After pretreatment step, the conversion of cellulosic fraction into fermentable sugars requires that pretreated biomass undergo detoxification, to minimize the sugars degradation and the subsequent formation of inhibitors for microbial metabolism [2.8]. The inhibitory compound can be divided into four groups:

- Substances released from the hemicellulose structure, such as acetic acid, which originates in the deacetylation of xylan;
- phenolic compound and other aromatic compound derived from partial degradation of lignin;
- furan derivatives, furfural and HMF resulting from degradation of pentoses and hexoses;
- metals ions leached from equipment.

These compounds affect the physiology of fermenting microorganism, therefore it is essential to eliminate these compounds or decrease their concentration to certain tolerances.

Carbohydrate degradation products such as the common aliphatic carboxylic acids (acetic acid, formic acid, and levulinic acid, and the furan aldehydes furfural and HMF) exhibit relatively low toxicity, but can be present in high concentrations depending on the pretreatment conditions and the feedstock. Formation of formic acid and levulinic acid occurs at the expense of sugars and it is therefore desirable to use pretreatment conditions in which the formation of these acids is minimized. For these reasons the concentrations of aliphatic carboxylic acids in pretreated biomass may be low enough to stimulate rather than inhibit ethanol formation.

Aromatic carboxylic acids are found within the group of phenylic compounds, which include both phenolic aromatic carboxylic acids, such as for example ferulic acid and 4-hydroxybenzoic acid, and non-phenolic aromatic carboxylic acids, such as cinnamic acid [2.8]. In contrast with the carbohydrate-derived aliphatic carboxylic acids mentioned previously, each of the aromatic carboxylic acids are present in relatively low concentrations in lignocellulosic hydrolysates, and their inhibitory effect is typically stronger than that of the aliphatic carboxylic acids. For example, the inhibitory effect of ferulic acid would tend to occur at concentrations that are two order of magnitudes lower than those of the common aliphatic carboxylic acids acetic acid, formic acid, and levulinic acid.

Other inhibitory compounds that can tentatively form through pretreatment under acidic conditions include quinones and small aliphatic aldehydes [2.8]. Although the presence of these groups of compounds in lignocellulosic hydrolysates warrants further attention: that compounds, such as benzoquinone, are strongly inhibitory to yeast.

The catalytic action of cellulolytic enzymes can be inhibited by non-productive binding to constituents of the solid fraction, such as lignins and residual hemicelluloses [2.8]. Inhibition of cellulases is also caused by soluble carbohydrates and aromatic substances in the pretreatment liquid. Product inhibition of cellulolytic enzymes by monosaccharides, such as glucose, and disaccharides, such as cellobiose, is a well-known problem. More recently, the inhibitory effects of oligosaccharides derived from xylan and mannan have been investigated. The presence of such oligosaccharides is dependent on the pretreatment

method, and also on the potential inclusion of enzymes that degrade hemicellulose-derived oligosaccharides in the enzyme preparation.

Solubilized aromatics, such as phenolics, may also affect enzymatic saccharification negatively [2.8]. Another finding that supports the significance of aromatic substances as enzyme inhibitors is that inhibition of cellulolytic enzymes can be alleviated through addition of sulphur oxyanions, such as sulphite and dithionite, which react with many aromatic compounds but not with sugars. Furthermore, when sodium borohydride was used for detoxification rather than sulphite or dithionite, inhibition of the fermenting microorganism was alleviated, but not inhibition of the cellulolytic enzymes. Treatment with sulfur oxyanions results in sulfonation of aromatic compounds making them less reactive, negatively charged and strongly hydrophilic, while treatment with sodium borohydride makes them less reactive without changing the hydrophilicity very much.

Efforts to commercialize bioconversion of lignocellulosic feedstocks could potentially focus on feedstocks with relatively low recalcitrance, which makes it possible to perform pretreatment under mild conditions [2.8]. These condition led to low concentrations of furan aldehydes and phenols. Feedstock engineering targeting components such as lignin, hemicellulose, and pectin is another approach to decrease recalcitrance and thereby reduce inhibitor release. By selecting or engineering plants with low acetyl content, the risk for formation of inhibitory concentrations of acetic acid can potentially be minimized. These strategies are of interest mainly with regard to short-rotation crops dedicated to bio-refining through a sugar platform process.

2.4.3 Detoxification

There are several methods of detoxification [2.9]. Different lignocellulosic hydrolysate have different degrees of inhibition and different microorganism have different inhibitor tolerances, so the method used to detoxify depends on biomass feedstock and on type of microorganism used. Detoxification methods can be divided into physical, physico-chemical, chemical or biological treatments.

Physical treatments are evaporation and use of membranes. Evaporation leads to a reduction of volatile compounds (such as acetic acid, furfural…) but it has the disadvantage of increasing the concentration of non-volatile toxic compound. Membranes have surface functional groups attached on their internal pores, which eliminate metabolic inhibitors.

Physico-chemical treatments can be summarized in: ion exchange resins, neutralization, overliming, activated charcoal and extraction with organic solvents.

Ion exchange resins methods is one of the most efficient detoxification method. It removes lignin derived inhibitors, acetic acid, furfurals, improving the fermentation yield. These resins can be reactivated and reused, so increasing its efficiency. However, this process leads also to a significant loss of fermentable sugars.

Neutralization method is used with low pH hydrolysate. Using calcium and sodium hydroxide, phenolics and furfurals are separated by precipitation. Adding $Ca(OH)_2$ there is also the precipitation of $CaSO_4$. It is desirable to remove precipitates by centrifugation.

Overliming process exploits the precipitation of toxic components and the instability of some inhibitors at high pH. It is the most used method thanks to its high efficiency to remove inhibitors and its low cost. After the treatment the pH of hydrolysate is reduced to desirable values.

Activated charcoal adsorption is widely used detoxification method, with low costs and efficient removal of inhibitors compounds.

Biological treatment method uses specific enzymes or microorganisms that act on the inhibitors compound of the hydrolysate and change them. Little waste is generated during this process and it could be performed directly in the fermentation vessel before fermentation step. This method is more feasible, environmental friendly, with fewer side reactions and less energy requirement. However, it present a long process time.

2.4.4 Saccharification

Saccharification step, also indicated as hydrolysis, brakes the remaining long chain of polysaccharides of hemicellulose and cellulose into the corresponding monosaccharides. This process can be carried out in two different ways: chemical hydrolysis and enzymatic hydrolysis. Chemical hydrolysis needs a detoxification process subsequently, while biological hydrolysis needs it previously. Chemical hydrolysis couldn't need a pretreatment step.

Acid hydrolysis is the oldest technology for converting biomass [2.11]. There are two basic types of acid hydrolysis process commonly used: diluted acid and concentrated acid hydrolysis. The diluted acid process is conducted under high temperature and pressure for a time order of minutes. The acid used are H_2SO_4 and HCl, but sulfuric acid is more efficient. The percentage of acid is in the order of 1%. Sugar yield are up to 90%, but most used processes are limited to an efficiency of 50% in order to be an economically viable industrial process. The temperature-pressure conditions lead to significantly relevant degradation of sugars.

Concentrated acid hydrolysis provides a complete and rapid hydrolysis with little degradation [2.11]. This process uses mild temperatures and ambient pressure. Sulfuric acid is used in percentages from 30% to 70%. Sugar yield is around 90%.

In conclusion, acid hydrolysis have high sugar recovery and high reaction rates but its disadvantages are environmental and corrosion problem, high cost for acid recovery and, in case of diluted acid method, high utility cost for elevated temperatures.

Alkaline hydrolysis is effective in removing lignin and acetyl groups [2.11]. It is a slow process and it is suitable for herbaceous crops and agricultural residues and not suited for woody biomass due to its higher lignin content. The reaction rates are higher than acid processes but it is very difficult to obtain high yields of sugars because of alkalis attack the sugars. Sodium hydroxide is usually used and yields are about 30%. Resident time is in the order of 1h. Thanks to its capacity of disrupting lignin structure, alkaline hydrolysis is used more efficiently as pretreatment method of lignocellulosic biomass.

To degrade cellulose a number of enzymes are needed [2.12]. They are divided into three types: endoglucanases, cellobiohydrolases and β-glucosidase. These three group work synergistically. To breakdown hemicellulose, several enzymes such as xylanase, b-xylosidase, glucuronidase, acetylesterase, arabinanase, galactomannanase and glucomannanase are required. The most used bacteria that produce cellulase enzymes are Aspergillus and Trichoderma. To improve the substrate digestibility, removal of lignin is desirable. Enzymatic hydrolysis has an efficiency of 75%-95%, with residence time of the order of days. Its advantages are the high sugar yields and mild process conditions. On the other hand it requires pretreatment and detoxification steps and the cost of enzymes is significant.

2.4.5 Fermentation and purification

Fermentation step can be perform separately or together saccharification [2.13]. These processes get the name of Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). Fermentation step has characteristic time of the order of days. Fermenting microorganism are used to convert monomeric sugars to alcohols (such as ethanol) or other chemicals (such as hydrogen, methane or lactic acid). Different organism are used, such as bacteria, yeasts and fungi, but the most frequently used agent is Saccharomyces cerevisiae, the common bakery yeast. Pure culture of microorganism is used for bioethanol production, while mixed culture is use both for biogas and biohydrogen production.

Industrial bioethanol fermentation is carried out with selected yeast strains [2.14]. Numerous efforts have been reported in strain improvement, aimed at improving the strain stability, pH-, ethanol-, osmo- and temperature tolerance, productivity and suppression of the respiratory chain in yeasts. The alternative use of bacteria has been investigated intensively but, due to several issues, yeasts are preferable to bacteria for a large-scale ethanol fermentation. The use of genetically modified organisms (GMO) in bio-ethanol production is restricted to laboratory scale investigations. From a practical point of view, proper substrate selection, efficient ethanol recovery, and environmentally friendly by-product management (stillage, CO_2) are considered essential.

Different from bio-ethanol fermentation, the formation of bio-methane (through anaerobic digestion) is induced by a multi-strain mixed bacterial culture, that depends fundamentally on the substrate applied and on the fermentation conditions [2.15]. Hydrolysis step and fermentation step can be carried out simultaneously, with some microorganism responsible for degradation of monomers into acids (the process called acidogenesis) and others responsible of methane formation (methanogenesis). Most important environmental parameters are temperature, pH, alkalinity, and redox conditions. Important fermentation conditions are substrate concentration, nutrient ratio, substrate volatile solid loading rate, hydraulic residence time, as well as metabolite and by-product formation. These parameters have to be properly controlled during the start-up of fermentation.

With the same features of methane production, the process can lead to product hydrogen changing some parameters, like pH, to deactivate the methanogenesis organism and

inserting hydrogenesis bacteria [2.13]. H_2 production is carried out at higher temperature (>80 °C) with respect to methane, with efficiency of almost the theoretical maximum. This process is called also dark fermentation.

All products need a type of purification before been used [2.16]. Distillation is the simplest method to separate ethanol from the fermentation substrate. A maximum of 95% purity can be reached. About biogas, the purification is done to remove water and dust through a physical process, such as cyclone separator, or impurities, like H_2S and CO_2, through physico-chemical processes, such as membranes or adsorption. As hydrogen concentration increases in the bioreactor, H_2 synthesis decreases and metabolic pathways of the whole cell biocatalysts shift toward the formation of by-products such as lactate and other solvents. Consequently, systems should properly be designed and operated to reduce H_2 partial pressure and thus the concentration of H_2 dissolved in the fermentation broth before it leads to the repression of its generation. Many technologies have been applied for hydrogen separation such as pressure swing adsorption, solvent adsorption, cryogenic recovery and membrane. The separation and selective purification of hydrogen via membrane technology appear to be promising to replace the conventional hydrogen separation system.

3. Analysis of pretreatment methods

The aim of this chapter is to give an in-depth explanation of the three most common pretreatment methods: steam explosion, chemical hydrolysis and enzymatic hydrolysis. For each method we will analyse the physical and chemical laws that regulate it, the variable parameters, the most used technologies, the most common systems and the different sugars yields with respect to type of biomass and process settings.

3.0.0 Feedstock key parameters

In addition to the process conditions, pretreatment efficiency is primarily related to the intrinsic properties of the lignocellulosic biomass.

Storage is the upstream operations for cellulosic bioconversion [3.17]. Efficient storage will significantly improve the efficiency of pretreatment and hydrolysis, and hence reduce the economic cost of the biomass utilization. Several storage methods are used, but dry storage is the most common method used after biomass harvest. Wet storage is used to preserve the newly collected agricultural residues with moisture higher than 60%. Wet storage let to avoid the drying step and improved feedstock susceptibility to enzymatic hydrolysis. The chemical composition of feedstock during the storage period changes, and the modification are linked directly to the storage method. Loss of water-extractable sugars is likely due to respiration of plant cells and microbial metabolism during storage and they obviously decreased with increasing storage time. Structural components (glucan, xylan and lignin) don't vary significantly after dry and wet storage. Although lost more non-structural components, wet stored biomass obtain higher sugar conversions and yields compared with dry stored biomass.

Results of pretreatment processes highly depends on *particle size* [3.5]. Size reduction of biomass before pretreatment is an energy-intensive and expensive process, but it is necessary for the bioconversion. The size reduction is strictly related to another parameter: the grindability [3.13]. It is define as the material resistance to grind. Standard grindability test are used largely for coal and petroleum coke, but there aren't standard tests on biomass yet. Energy consumption for grinding depends on initial and final biomass particle size, moisture content, material properties, mass feed rate and machine variables. Biomass particle size obviously impacts on the design of handling, transportation and conversion facilities [3.5]. Suitable particle size means to improve the efficiency of the pretreatment due to the high efficient heat and mass transfer.

For a group of biomass particles, the *particle density* is defined as the mass of all particles divided by the volume which the particles occupy excluding the pore space volume [3.13]. For biomass particles that cannot have a well geometrically defined volume, their volume can be estimated by pressure difference with a known quantity of pressurized gas flowing from a reference volume into a cell containing biomass particles. Particle density of biomass is usually used in the computational fluid dynamics simulation of biomass thermochemical conversion reactors.

Moisture sorption of biomass is important for biomass harvest, handling, transport and storage [3.13]. Therefore, proper drying and storage operations are required to preserve the quality of biomass feedstock. The Equilibrium Moisture Content (EMC) is a key parameter to characterize the water sorption behaviour of biomass and it is defined as the moisture content of a material in equilibrium in a particular environment with respect to temperature and relative humidity. The EMC of biomass depends on its composition, porosity, microstructure and specific surface area.

The thermal properties of biomass heavily influence its thermochemical conversion characteristics [3.13]. When biomass is heated, biomass particles are subjected to heat conduction along and across their fibers, which in turn influences their thermochemical conversion behaviour. Thermal conductivity of biomass depends on moisture, porosity, density, temperature and heating direction: in fact, biomass is an anisotropic material. Thermal conductivity can be estimated through semi empirical formulas. Also the specific heat, which is dependent on moisture content and temperature, can be assessed with semi empirical formulas.

For the logistics an important physical property of biomass particles is the *bulk density* [3.13]. It is defined as the ratio of the mass of biomass particles to the total volume of biomass particles including the pore space volume between and within the biomass particles. It is a key parameter in designing biomass handling and transport. Furthermore, the flow properties of biomass feedstocks play an important role in transportation, storage and handling. *Flowability*, as a foremost flow property, is a measure of how well biomass flows from one point to another point. Several parameters are commonly reported to characterize the flowability of biomass: the angle of repose, cohesion coefficient, compressibility index and flow index

The *heating value* (HV) of biomass is an important parameter [3.9]: it is a representative of the energy content that lignocellulosic biomass feedstock possess as potential biofuel. The HV of a lignocellulosic biomass type can vary significantly with the climate and soil conditions [3.13]: consequently the HV is presented as a range of values. Normally woody biomasses have slightly higher values than herbaceous biomasses. The HV of biomass materials can be determined experimentally using an adiabatic bomb calorimeter or it can be estimated through mathematical models based on the chemical composition, proximate and ultimate analysis [3.9].

The chemical composition analysis is usually performed according to the National Renewable Energy Laboratory (NREL) standards [3.10]. NREL supply guides and spreadsheets to help scientists and analysts understand more about the chemical composition of raw biomass feedstocks and process intermediates for conversion to biofuels.

Lignocellulosic biomass is composed, as seen in the previous chapter, primarily of cellulose, hemicellulose and lignin [3.13]. These components are associated with each other and vary depending on the type of lignocellulosic biomass. In biochemical or thermochemical conversion processes, the performance of cellulose, hemicellulose and lignin are different. Accurate chemical composition analysis of lignocellulosic biomass enables evaluation of conversion yields and process economics, in particular for biochemical conversion. The biodegradation of cellulose and hemicellulose is greater than that of lignin, thus, the overall

conversion of biomass with higher content of cellulose will be greater than biomass with high content of lignin. Forest and agricultural residues are suitable for combustion, gasification and pyrolysis, while other feedstocks such as sugar crops are suitable to fermentation processes.

Biomass	Cellulose [%wt]	Hemicellulose [%wt]	Lignin [%wt]
Hardwood (poplar)	51-53	26-29	15-16
Softwood (pine)	45-50	25-35	25-35
Wheat straw	35-39	23-30	12-16
Corn cob	34-41	32-36	6-16
Corn stalk	35-40	17-35	7-18
Rice straw	29-35	23-26	17-19
Rice husk	29-36	12-29	15-20
Sugarcane bagasse	25-45	28-32	15-25
Sorghum straw	32-35	24-27	15-21
Barley straw	36-43	24-33	6-10
Grasses	25-40	25-50	10-30
Switchgrass	35-40	25-30	15-20

Herbaceous residues differ in their composition and chemical structure from wood biomass [3.5]. Herbaceous residues have a lower lignin content characterized by a different monomeric composition. Also type of celluloses and hemicelluloses differs. Thus, an individual analysis is required to assess the correct pretreatment conditions to achieve the maximum efficiency of sugar yield.

Proximate analysis give the amount of moisture, ash, volatile matter and fixed carbon of the biomass sample.

Moisture content represents the quantity of water in biomass, expressed as a percentage of the material weight [3.13]. It has a strong influence not only on harvest and preparation , but also on transport, storage, processing and resultant products. The moisture in biomass can remain in external and inherent forms. The external moisture is that above the equilibrium moisture content. It generally resides outside the cell walls. The inherent moisture is absorbed with the cell walls. .

The *ash content* is another parameter to be considered [3.9]: it represent the sure percentage of the feedstock that will not be transformed into valuable fuels. Ashes are the sum of mineral and other inorganic matter (structural or extractable) in the biomass. Structural ash which is inorganic material, is bound in the physical structure of the biomass, while the extractable ash is the inorganic material that can be removed by washing or extracting the material. The primary ingredients of biomass ash is the oxide form of silica, aluminium, iron, calcium, magnesium, titanium, sodium and potassium [3.13]. Knowledge of the chemical and physical properties of biomass ashes is helpful to predict he tendency to form deposits or reactions in the equipment components during conversion.

The volatile matter of biomass is the condensable vapor and permanent gases (exclusive of water vapor) released from biomass when it is heated [3.13]. Its amount depends on the heating conditions, including the heating rate, temperature and residence time. For the determination of the volatile matter content, the biomass sample is heated to a standard temperature, at a standard rate in a controlled environment.

Fixed carbon is the solid combustible residue that remains after biomass is heated and the volatile matter is expelled [3.13]. Fixed carbon is obtained through difference calculation from known values of moisture, ashes and volatile matter.

3.0.1 Importance of water states

Today the most cost effective process in the biomass to fuels industry utilize biological conversion process and indispensable pretreatment where water most certainly takes great effects [3.11]. The vital roles water usually plays in usual common pretreatment are:

- Reactant, constructing mild acid conditions at elevated temperature;
- Solvent or mass transfer medium of catalytic substances, intermediates and end-products;
- Heat transfer medium, determining the heat pattern and efficiency throughout the cellular structure;
- Plasticizer, maintaining a moist and soft texture of fibers by the influence on cell size and fibre strength;
- Explosion medium for analogous explosion pretreatments, tearing materials into small pieces and disrupting micro-structures at sudden decompression.

These indicate that water is directly related to the interaction between substrate and pretreatment and meanwhile highlight the necessity of rehydration operation before pretreatment since most pretreatment objects are dry feedstocks with water content <15% [3.11]. When water is sorbed to lignocellulosic matrix, it is subject to interactions caused by chemical composition and physical structure of lignocellulosic matrix which in turn produces different states and location of water.

Within the matrix, these associating water molecules have properties highly different from properties of bulk water [3.11]. They become localized, more structured and comparatively limited in available degrees of freedom, kinetic motion and ability to exchange with other water molecules compared to water in bulk. Various water states influencing feedstock properties have multiple effects in pretreatment process as summarized above, which could be the key issue that closely related to the extent of pretreatment efficiency. Thus, to unveil the fundament mechanism of water function in pretreatment, it is required to develop an adequate consideration of water states in the architecture of biomass.

In lignocellulosic biorefinering process, engineering-orientated research on the correlation between inner water and mechanical property of feedstock focus on that feedstock swells when exposed to water and dry feedstock is stronger that wet feedstock [3.11]. In typical fiber structure, when a small amount of liquid water was poured onto dry fiber, the water must, by default, be located inside the cell wall matrix as bound water. Hence , the mass of

cell walls increased, as did the volume, which was observed macroscopically as swelling of fiber samples in the work. Concurrently the strength of sample progressively declined as water content increased. With continuous addition of water, the system eventually reached the equilibrium where the liquid water began to accumulate in the voids as free water. When that occurred, the cell wall ceased swelling and the strength of the sample no longer changed with water content.

3.1 Steam explosion

3.1.1 A short introduction

This process was introduced firstly in the twenties of the past century as an efficient method to defibrate wood into fibers [3.1]. Only ten years later steam explosion was patented to produce fermentable sugars and alcohol from wood. Contemporarily it was applied commercially in the Masonite process for the production of fireboards. Recently growing concerns about the depletion of fossil fuels and increasing oil process, as well as environmental issues regarding emission of greenhouse gases, have resulted in increased research on the production on alternative fuels, like ethanol, from various type of biomass. Lignocellulosic biomass is considered as to be the most promising renewable resource for the production of these fuels throughout hydrolysis and fermentation. To enhance these processes pretreatment is necessary and steam explosion attire the attention of researchers also today.

3.1.2 Mechanism

Steam explosion is considered as a physicochemical pretreatment process [3.1]. Biomass materials are put in contact with high temperature (160-280°C) and high pressure (0.7-4.8MPa) saturated steam, for a certain time, called retention time, and then bring back to the ambient pressure in a short time, almost instantaneously, gaining the name of explosion. This process acts on biomass materials in two different way: it causes mechanical shearing and defibrillation of fibers and it starts chemical reactions that leads to acetic acid release and partial hydrolysation of cell wall components.

This last phenomenon is known as *auto-hydrolysis*. Acetic acid is released from acetylated hemicelluloses and it is considered to be the main catalyst for the further hydrolysis of the substrate and, if so the case, for glucose and xylose degradation [3.12]. In addition to acidic acid, formic and levulinic acids, which are formed from the further degradation of furfural and 5-hydroxymethylfurfural (HMF), may contribute to the efficiency of the pretreatment.

During the pretreatment, saturated steam under high pressure penetrates the recalcitrant cell wall by diffusion and the instantaneous decompression of the pretreated biomass causes a release of pressure from the evaporation of condensed moisture and brings to a mechanical separation of fibers [3.1]. Correspondingly, a shear force is generated and acts on the surrounding structure, resulting in the mechanical breakdown of biomass. As the condensed steam penetrating the biomass, reactions proceed and release acetic and uronic acids, which catalytically hydrolyse hemicelluloses into oligosaccharides and monosaccharides. Under severe conditions, amorphous cellulose could be partially depolymerized. In addition, further degradation products (as seen in the previous chapter) could be generated undesirably, which inhibit microbial growth and their fermentation efficiency.

After the pretreatment, condensed water and insoluble solids are recovered in the form of a pretreated material called *slurry* [3.12]. Slurry is composed of a liquid and a solid fraction. The liquid fraction is called prehydrolisate and it contains all the solubilized sugars, mainly hemicellulose sugars and nearly all the degradation products generated during the

pretreatment. In the insoluble solid fraction, the remaining amounts of cellulose, hemicellulose and lignin are recovered.

3.1.3 Process key parameters

The most common theoretical research is based on the optimization of two governing factors: retention time and temperature [3.1]. Overend et al. introduced, in 1987, the *severity factor* that define the severity of steam explosion in terms of the combined effect of temperature and retention time and it is based on the assumption that the process kinetics is first order and obeys Arrhenius' law:

$$R_0 = t \cdot e^{\frac{(T-100°C)}{14,75}}$$

Where R_0 is called *reaction ordinate*, t is the *residence time* [min], T is the *reaction temperature* [°C], 100°C is the base temperature and 14,75 is the conventional energy of activation based on first-order reaction.

Reaction ordinate is not a concept universally valid for the large variety of biomass materials and their chemical and structural differences. However, it remains an useful bookkeeping method for reporting steam explosion conditions.

The *severity factor* (SF) is thus calculated as:

$$SF = \log_{10} R_0$$

To be able to compare results of one-step pretreatment at different consecutive temperatures T_i and reaction times t_i, the severity factor is extended to [3.8]:

$$SF = log_{10}\left(t_1 \cdot e^{\frac{(T_1-100°C)}{14,75}} + t_2 \cdot e^{\frac{(T_2-100°C)}{14,75}} + \cdots + t_i \cdot e^{\frac{(T_i-100°C)}{14,75}}\right)$$

To improve the final sugar yield acids can be added to the raw material [3.1]. It was assessed that application of increasing acid concentration in SE pretreatment results in increased removal of hemicellulose, thus reducing the total glycan content. With the addition of chemicals catalyst, a simple parameter is not suitable for describing the process. Hence, Chum et al. (1990) developed the *combined severity factor* (CSF) to allow comparison of different acid-catalysed process, incorporating an acidity function as the third parameter:

$$CSF = \log_{10}(R_0 - pH)$$

The severity factor ignores the duration of the explosion process [3.3]. The process of SE can be divided into two phases: the steam boiling phase and the explosion phase. The first one acts as a thermochemical reaction, while the second one can be approximated to an adiabatic expansion process and a conversion of thermal energy into mechanical energy. The SF define the first phase. Hence, recently, Zhengdao Yu et al. (2012) introduced a new parameter to define the second phase: the *explosion power density* (EPD). It is founded on the hypothesis of the adiabatic expansion process:

$$P_e = \frac{\Delta H_s + \Delta H_l + \Delta H_m}{t \cdot V} \quad \left[W/m^3\right]$$

Where ΔH_s, ΔH_l and ΔH_m are the enthalpy drop of steam, liquid water and material, t represents the explosion duration and V is the volume of the explosion reactor. Together EPD and SF describe the all SE process.

Assuming equal energy consumption, to maximize the EPD and the conversion from thermal to mechanical energy the deflation time must approach zero. So to achieve the highest efficiency, the process must be carry at low temperature and short durations. It also important to avoid the generation of fermentation inhibitors. The deflation time should be shorter than the pressure balance time between the inside and the outside of the pretreated biomass internal structures, which varies with biomass species. Given a rapid enough decompression, most of the steam and hot liquid water in the biomass will quickly expand and break free of the structure. In a slow decompression, the pressure will have time to equalize across the structure, resulting in a much smaller shearing force.

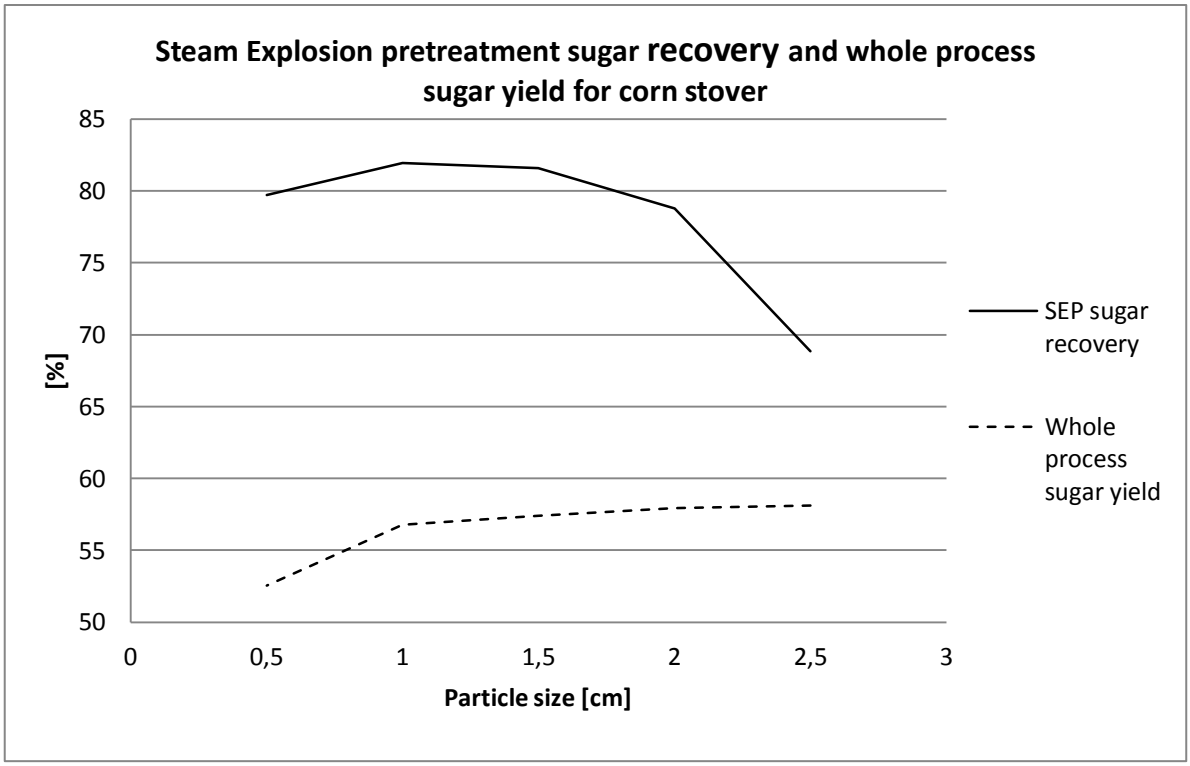
In the formulation of severity factor model, *moisture content* of biomass was not incorporated [3.9]. Moisture content can be used to create a buffer effect and as a tool to dampen the severity of the pretreatment condition, and consequently to moderate the formation of inhibitors. High moisture content could reduce the ability of heat and chemicals to penetrate the chips and then slow down the kinetics, because the voids in the biomass are filled with condensate before the fixed temperature is reached [3.1]. The uneven heating potentially leads to the inhomogeneous degradation of chemical components, clearly being reflected by the variation in the recovery of sugars, lignin and furans in the solid and liquid portions. High feedstock moisture content acts as acid catalyst to hydrolyse biomass during SE. However, the direct contact of biomass with the walls of the reactor will limit and affect the extent of the hydrolysis.

3.1.4 Importance of particle size

During SE pretreatment, heat transfer issue may result in overcooking the surface part of the larger biomass and incomplete pretreatment of the interior part [3.5]. For smaller biomass particles, hemicellulose may be prone to degrade into by-products due to the intense degree of heat. Therefore, optimizing biomass particle size is crucial in term of achieving high sugar conversion and lower production costs.

For example, pre-treating corn stover, the total sugar recovery increases with a reduction of the particle size until 1 cm, but slightly decreases at smaller sizes. These relatively low recoveries might be explained by the hemicellulose solubilisation and degradation to by-products, such as formic acid and furfural, during pretreatment. The literature shows the opposite trend working with softwoods and herbaceous agricultural wastes. This difference might be due to not only the feedstock type but also heat transfer patterns in the biomass pretreatment.

On the contrary, lignin recovery will increase over the 100% with increasing of particle size. This phenomenon had been attributed to the formation of lignin-like compounds from recondensation reactions especially between carbohydrates degradation products and other components from the water extractives which also explained the low sugar recovery.



Liu et al. (2013) [3.5] proposed the mechanistic model for steam for SE pretreatment at different biomass particle sizes and suggest that the small particles could submerge in condensate water and then result in the poor efficiency of explosive depressurization on the biomass solids.

3.1.5 The role of water

The fiber saturated point (FSP) was originally descripted as "the water content which the cell cavities contained no water, but the cell walls were fully saturated with liquid moisture" [3.11]. At FSP, all of the liquid water was bound water, as this represent the maximum amount of water that could be taken up from the vapour phase by unit mass of fiber at a given temperature.

The swelling and strength changes of cellulosic sample when exposed to water are mostly attributed to the increase in the mass of bound water while the contribution of free water is little [3.11]. Especially for SE pretreatment, the softening of chip structure and the enlargement of pores caused by rehydration would allow the entered the end chip and passed rapidly through the chip until it condensed and released its latent heat of vaporisation. If the vessels were initially filled with liquid water or if sufficient condensate accumulated to fill the chip before the steam temperature was reached, further heat transfer was by the slower process of conduction. If all the heat required to heat the moist stalk to steam temperature was supplied by condensation inside the chip, final water content of pretreated samples would rise with increased initial water. The increase of condensed water means the increment of consumed steam or energy. There is a correlation between initial water content of feedstock and energy demand of steam explosion., which is expressed as the steam mass consumed by per unit mass of dry materials. Therefore, much water rehydrated in samples result in a substantial decrease in heat transfer speed and energy utilization efficiency, ne

significant effect of the reduced ability of heat to penetrate the chips is the shortening of actual steaming time at settled temperature. This may cause a deteriorate severity of pretreatment and an uneven treatment of the substrate, which can potentially result in the selective degradation of the outer portion of the chip, while at the same time the interior is less affected by the treatment.

SE is usually carried out at relatively high temperature under mild acid condition which is largely from the decrease of water ionization constant (pKw) at elevated temperature and the release of organic acids from biomass components [3.11]. This high temperature and acidic environment is effective in triggering a series of hydro-thermal reactions. Hemicellulose is thought to be hydrolysed into monomeric, oligomeric sugars and partial soluble sugars are subsequently degraded into small molecular products including furfural by auto-hydrolysis effects with the acidic water, acetic and other acids catalysis derived from acetyl groups. The ester linkages between carbohydrates and lignin are disrupted and hence lignin is melted, solubilized and recondensed. Wet solids showed high glucan and xylan content and low acids soluble lignin (ASL) content after pretreatment compared with the dry ones. This indicated that samples after rehydration with more water were less hydrolysable during pretreatment. While on samples underwent wild hydrolysis of cellulose and hemicellulose, their SE hydrolysates showed an increased amount of soluble sugars (glucose and xylose), which was contrary to expectations. This was due to the weakened degradation reactions as water content increased. It was also supported by decreased content of acetic acids produced from the removal of acetyl groups showed a substantial decrease as water content increased. The observed changes in chemical composition are likely related to a buffering effect of water, whereby water buffers against carbohydrate solubilisation, decomposition and auto-hydrolysis due to the slow rate of heating in the interior of chips. It may result in the predetermined high-temperature and acidic environment being not accessible in a relatively short time. The buffering ability of water thereby weakens SE severity and has a detrimental impact on pretreatment efficacy.

The effect of water states on transfer and reaction behaviours during SE process is inevitably reflected in the pretreatment performance [3.11]. Increasing water content has been shown to have a desirable effect on the recovery of glucose during pretreatment. High water resulted in a decreased yield of enzymatic hydrolysis. This unfavourable hydrolysis could be attributed to the buffering effect of rehydrated water in SE process. On one hand, high water impede effective heat transfer and thus negatively influenced thermochemical reactions during SE process. Since hemicellulose and lignin were not effectively removed from the substrates owing to the deficient solubilisation and degradation, their residues decreased the accessibility of enzymes to cellulosic substrate. On the other hand, the deficient penetration of high temperature steam made it is difficult to achieve the effective pressure difference inside and outside of each cell that drove the evaporation and expansion of water inside the biomass. This was not beneficial to physical tearing action on the rigid and highly ordered fibrils and thus failed to form a loose and porous structure exposed to enzymes. The insufficient chemical and physical changes led to the low efficiency of biomass utilization. It is worth noting that, the cellulose conversion yield went up slightly when the samples were rehydrated from initial water content to FSP, after then experienced a substantial decrease. This tendency is similar to the periodically upward trend of cellulose recovery in pretreated

materials. This indicates the twofold effects of free water on impairing SE efficiency. The first effect is the buffering ability on hindering the normal proceeding of transfer and reaction during SE and the second effect is presented as no obvious contribution on impairing the mechanical strength of rehydrated materials. Finally, as a result of glucose recovery and conversion yield, overall glucose yield of the while process was increased by increasing water content to FSP, and then decrease with the increase of water content from FSP to total saturation. Thus, considering the effects on pretreatment efficacy and energy consumption, the importance of FSP for SE pretreatment is evident. The bound water it determined being the excellent plasticizer that maintains the soft texture of fibers is conductive to SE effect, while the free water it determined presenting the buffering effects on the pretreatment and consuming abundant energy is not conductive . considering pretreatment efficacy and energy consumption, the optimal water states for SE pretreatment is achieved at FSP, specially by spraying water homogeneously to FSP and rehydrating for some hours.

3.1.6 Type of process

There are mainly two type of process: steam explosion in continuous reactors (SEC) and steam explosion in batch reactors (SEB).

The products obtained at the same treatment severity in batch and continuous reactors are different in appearance and chemical components, as well as the enzymatic hydrolysis efficiency [3.1]. These differences are more obvious at higher severities, and the experimental relationship between these two systems was developed to realize the data transformation:

$$\log_{10} R_{0\_batch} = 1{,}50 \cdot (\log_{10} R_{0_{continuous}} - 1)$$

SEB are employed extensively in laboratory scale set ups. First, a certain amount of raw material is weighted for each batch, adjusting moisture and adding chemicals if needed. Then the batch is filled with lignocellulosic biomass through the ball valve on top.

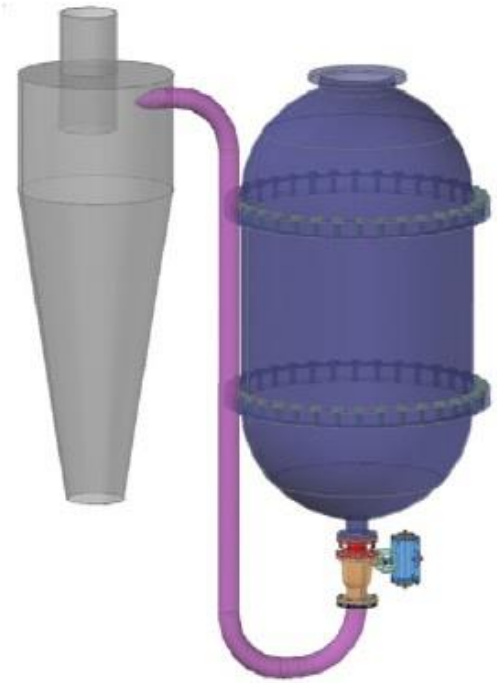


Figura 3.1 - Example of batch steam explosion system [3.3]

Then the ball valve is closed and the saturated steam is inputted into the chamber. Timing begins when the target temperature in the chamber is reached. At the end of the set incubation time, the ball valve at the bottom is opened instantaneously to generate the explosive depressurization and the steam exploded material is shot into the expansion tank. The solid and liquid fractions could be separated easily by a mesh cloth. The collection of gas could be recovered but the qualitative analysis indicated that the gas-phase carbon are maximum a couple of percentage point of feedstock carbon. Mainly gases are composed by CO_2 but some aromatic and furanic compounds are formed and volatilized for steam temperature higher than 200°C.

SEB can be divided into further two models [3.3]: the first model that adopts the classical structure in valve blow mode, which is widely used at present and the second model that adopts a structure in catapult explosion mode that is principally composed of a cylinder and a piston. The second model enables the continuous feeding by a screw conveyer but its energy consumption is too high to apply to the industrial processes, and it is still in the pilot plant scale phase. In the steam boiling phase, the two parts are tightly coupled. During the explosion, the piston, driven by three pneumatic linear actuators, bursts out of the cylinder, a process that is equivalent to a vessel suddenly fracturing into two halves. In the process of the opening piston, the piston rapidly accelerates due to the kinetic energy of the steam and material, as well as the force from the devices driving the process. When the stroke of the piston reaches one fourth of the cylinder diameter, the effective gas deflation passage, the exposed area between the piston and the cylinder, reaches the same area as the cylinder cross section. Hence, the duration of the first period is equivalent to the duration of the piston stroke.

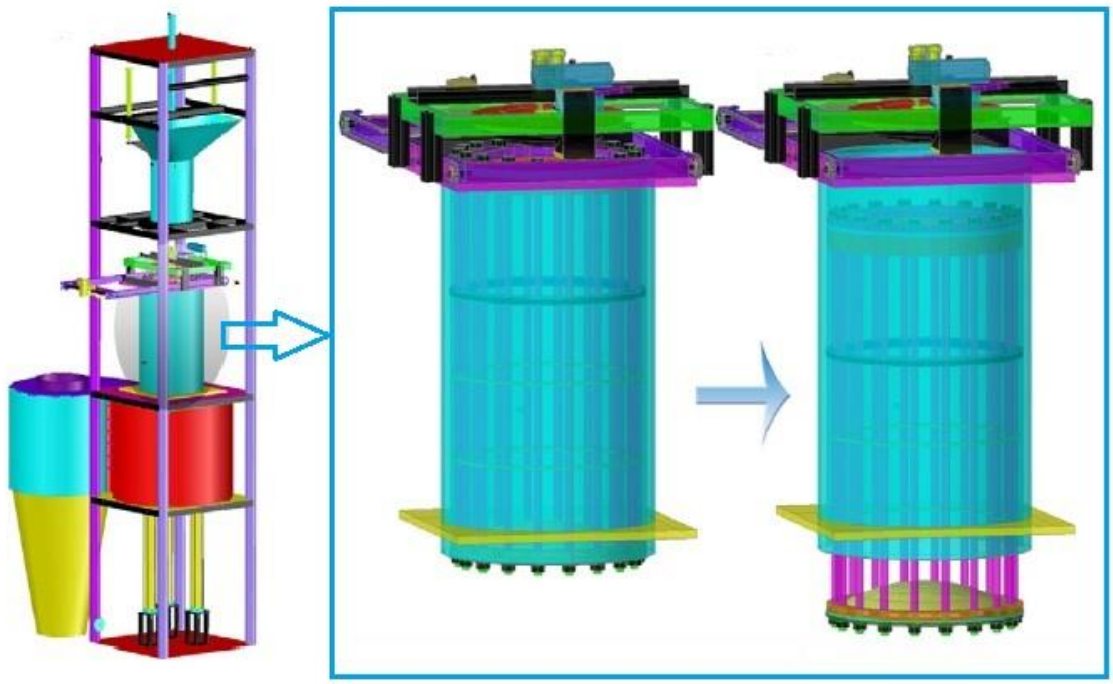


Figure 3.2 - A model of catapult steam explosion. In the zoomed picture, the status of the piston in boiling phase and the status of the piston as the explosion proceeds [3.3]

During steam pretreatment at high temperature it was shown that most of the cellulose and hemicellulose were hydrolysed and became water soluble. As a result, the removal of surface impurities occurred, along with defibrillation of the fiber, due to the removal of cementing materials. Furthermore there is a partial degradation of lignin, which results from the expansion of retained water. In the case of the catapult explosion mode, the kinetic energy of steam explosion fractured the lignin fiber. The fiber diameter and length were also decreased by acid hydrolysis preceding the steam explosion acid hydrolysis led to the formation of hydroxyl, carbonyl and carboxylic groups, which facilitated lignin solubilisation.

Although the catapult mode equipment structure is more complex than the valve blow mode, in scale up application the second results in increasing complexity [3.3]. Non-uniform batch biochemical characteristic, necessity of cooling reactor and heat exchanger to reduce the temperature of the material, charging and operating phases limited to plant project values will increase the complexity of valve blow mode.

The SEC system is employed for large-scale production and arouse more interest for commercial application [3.1]. Typically, SEC systems are acid–catalysed processes and they are composed of an acid supply tanks, a biomass mixer, a high temperature-pressure reactor system and a flash tank. Dried lignocellulosic biomass is dumped into a feed hopper. After being delivered by a belt/screw conveyor and moisture/acid adjusted by a pug mill mixer, the wetted feedstock is screw conveyed to a plug feeder and then force into the pretreatment reactor. Saturated steam at desired temperature and pressure is injected in the reactor and the retention time is fixed by controlling the material level in the reactor, where, on the bottom, a rotating scraper facilitates the movement of the material to the discharge port. Two reciprocating poppet valves operating as a pressure lock direct the pretreatment material into the flash tank, a conical screw mixer. The vent streams are sent to a condenser and the solid

fraction is collected and then pumped to the fermentor. Compared to the batch process, the development of continuous reactors for steam explosion allowed for a better control of the pretreatment variables, critical to achieving optimal processing conditions at high temperatures, as well as higher purity of extracted components because heat transfer limitations are partially overcome, leading to lower accumulation of undesired degradation by-products.

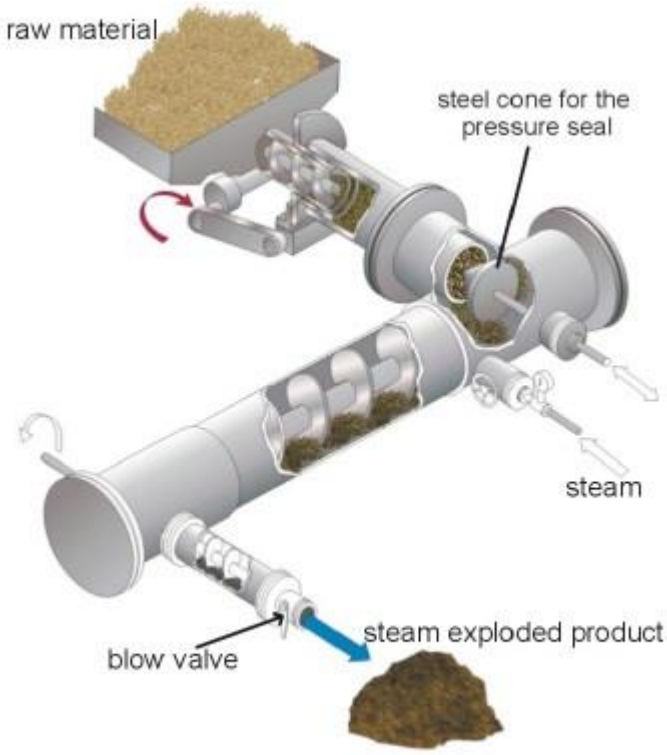


Figure 3.3 - Continuous steam explosion example [3.15]

Studies sustain that with comparable severity, SEB pretreatment removes less hemicelluloses that the continuous system, but, at the same time, enhanced the depolymerisation of the cellulose fraction and resulted in lower lignin content [3.2].

The heat and moisture transfer pattern and efficiency are largely affected by *loading rate* [3.14]. It results in changes of energy consumption of steam explosion process and the final moisture content of steam exploded materials. The former majorly determines the pretreatment economic efficiency, the latter is a crucial regulating parameter related to following drying, enzymatic hydrolysis and other operations. Loading coefficient is also related to explosion effectiveness, since it contributes to the steam deflation speed and influences the physical action of crowded materials during the sudden explosion. The optimization of loading parameters would better be compromised between the economical and processing efficiency of steam explosion to meet the requirements of the economy of the process. Observed variations in the chemical composition of pretreated materials indicate that the pretreatment severity slightly increased with the increase of loading pattern. This is likely because of the strong shearing force among crowded material during the sudden explosion and the rapid deflation speed. In water insoluble solids, increasing the loading pattern which means increasing the volume or compaction degree of loading materials, resulted in a slight decrease of xylan: this reflects the improved solubilisation of

hemicellulose at high loading pattern. As a result, glucan and acid insoluble lignin will increase relatively in the solid fraction. In water soluble fraction, increasing loading pattern prompts the solubilisation and degradation of cellulose, evidenced by the upward trend of glucose and HMF. In relation to enzymatic hydrolysis yield, no clear changes with the increasing of loading pattern appear. Therefore, this little increase of steam explosion severity has almost no effects on the final sugar yield.

3.1.7 Substrate modification

Steam explosion pretreatment produce a slurry material in which is it possible to distinguish the solid (water insoluble solid, WIS) and liquid fractions that can be separated by filter press [3.4]. The WIS fraction consists of cellulose, lignin and the remaining hemicellulose, while the liquid fraction contains the hydrolysed hemicellulose (monomer and oligomer sugars), sugar degradation products and phenolic compounds (released by lignin).

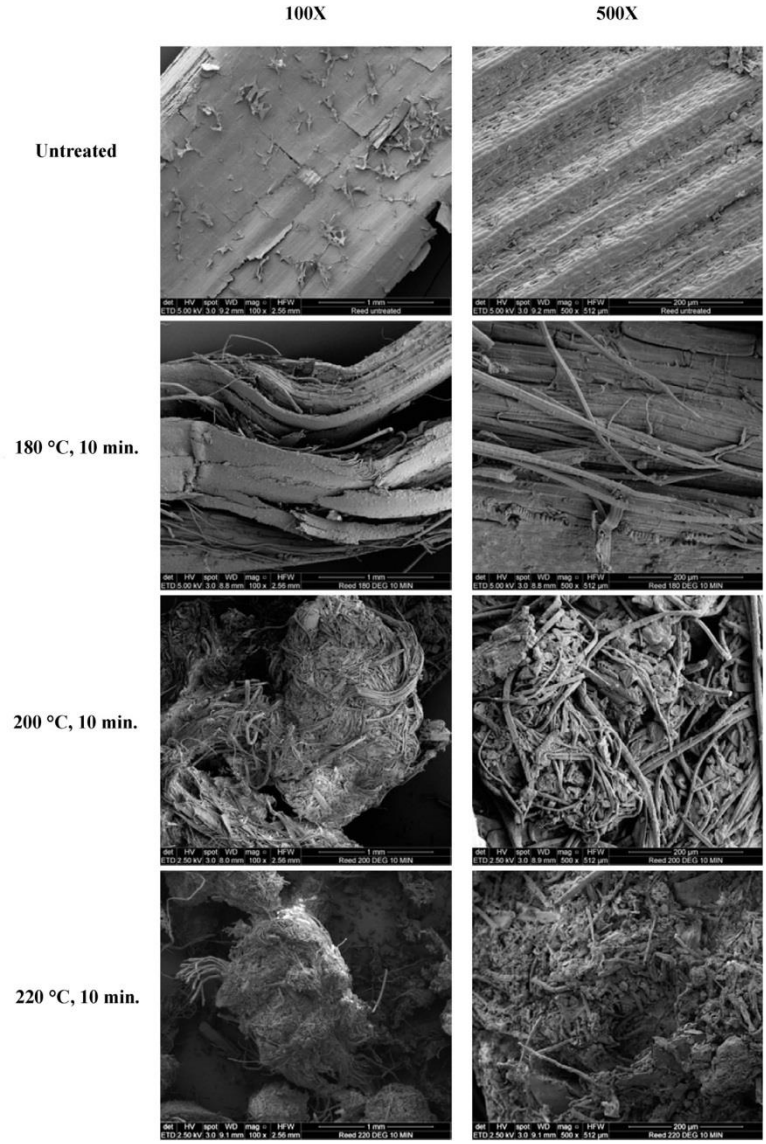


Figure 3.4 - Substrate modification at different severity factor [3.16]

In terms of the morphological property [3.1], extensive defibrillation of fibers was observed after the explosive decompression, mainly ascribing to the mechanical effect from adiabatic

expansion of absorbed water. Compared with compact and regular surface structure of raw lignocellulosic materials, varying degree of destruction of the cell wall are noted after steam explosion pretreatment, depending on severity of the process.

Color characteristics of the SE pretreated samples are a reflection of the severity of the pretreatment conditions [3.9]. This is due primarily to a series of reactions between the chemical constituents of wood cell and extractives under high temperature and humidity conditions. It can be used as an indicator of carbonization during SE pretreatment, as samples tend to darken with increased carbonization.

Figure 3.5 - Example of untreated biomass (left) and SE treated biomass (right) [3.9]

Amorphous cellulose is broken-down with increasing severity of steam explosion, increasing the degree of crystallinity [3.1]. An impregnation under alkaline conditions could lead to a buffer effect in the reactor, which prevented the depolymerisation of cellulose during steam explosion. In literature, different responses have been reported for cellulose content after steam explosion pretreatment under similar conditions [3.16]. While relatively constant concentrations of cellulose were reported for miscanthus and hay, a slight increase in the relative content of cellulose after steam explosion of wheat straw has been reported. It is known that the cellulose fraction of diverse biomass types can vary in configuration (i.e. crystalline and amorphous), leading to different properties and responses to pretreatment.

The hemicellulose fraction begins to solubilize at 150°C [3.16]. The steam provided during the pretreatment, combined with the acetic acid fromed from acetyl groups present in the biomass, catalyzes the hydrolysis of hemicellulose. The hemicellulosic sugars are degraded to oligomers or individual sugars, and are lost under the required conditions for optimum enzymatic hydrolysis [3.1]. Furthermore, hemicelluloses degradation products , such as furfural, 5-hydroxymethyl furfural and aliphatic acids, inhibits subsequent fermentation. Studies about this issue concluded that less severe conditions resulted in better recovery of combined hemicellulosic and cellulosic sugars, although the maximum hydrolysis of cellulose was achieved at more severe conditions. The mild conditions caused less sugar degradation, but the resulting sugar solution was partly fermentable because of the presence

of a high proportion of hemicelluloses derived carbohydrates in an oligomeric form, which strongly inhibit the bioconversion process with enzymes.

Results indicate that the hemicellulose contained in the biomass is highly degraded compared to cellulose [3.9]. The high degradation of hemicellulose is due to its amorphous nature, which degrades easily and evaporates as volatile components during the carbonization process. The crystallinity of cellulose, on the other hand, is responsible for the less degradation. Studies demonstrated that hemicellulose is nearly completely removed at 200°C, while both cellulose and lignin can be dissolve partially.

The content and type of lignin have a significant influence on the enzymatic hydrolysis of cellulose, because lignin acts as both a physical barrier, restricting access of cellulase to cellulose and an attractant to cellulase, resulting in nonproducing and irreversible binding [3.1]. To know the evolution of the lignin content after steam explosion pretreatment is necessary to define the type of biomass [3.16], thus the chemical composition of its lignin. The lignin is fractionated into acid insoluble (AIL) and acid soluble (ASL) lignin [3.9]. The AIL is the residue, as remaining solids, from the hydrolysis suspension. The different type of lignin change radically the behaviour of its evolution. For example, for feedstocks like Miscanthus or Hesperaloe, a notable lignin reduction has been reported, due to its dissolution under harsh steam explosion conditions, while lignin increases pre-treating under similar conditions hay, birch and wheat straw [3.16].

Important and explicit characteristic of steam exploded sample is the agglomeration and redistribution of lignin in depth of the cell wall [3.1]. Because of the low softening point of lignin, it is believed that lignin undergoes melting and condensation to form spherical particles and droplets by the water temperature treatment, which is called coalescence process. The amount and size of lignin droplets are crucially determine by the severity of the steam explosion. The redeposition of lignin on the cellulose surface inhibits cellulases to attack cellulose fibrils and negatively affects the enzymatic hydrolysis. Thereby, an alkaline post-treatment with alkaline solution is necessary to improve bioconversion efficiency of steam-exploded samples, because the lignin is less strongly bound to carbohydrate polymers compared with its native linkages.

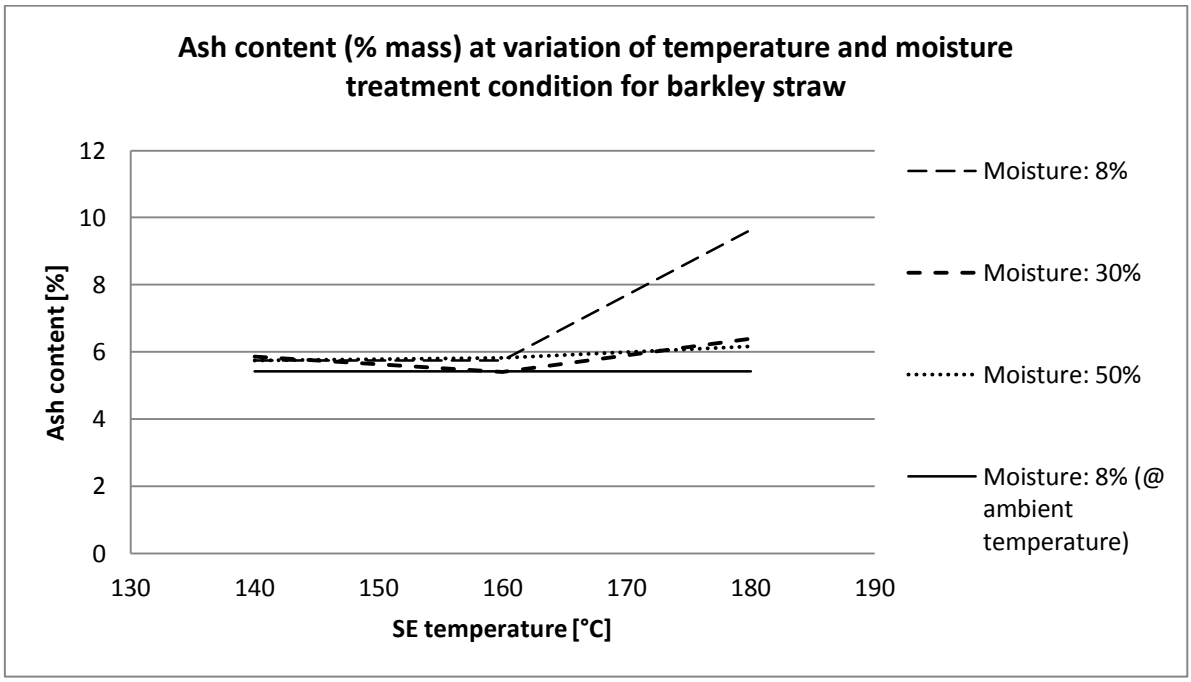
Normally the amount of lignin in the solid residue gradually increases with the severity of steam explosion, either from degradation or solubilisation of carbohydrates or from the generation of pseudo-lignin from sugar degradation products, which could result in artificially high values for lignin using the Klason method [3.1]. In terms of the structural changes of lignin, it was characterized by the simultaneous occurrence of depolymerisation and recondensation reactions between monolignol units.

The increasing of acid insoluble lignin (AIL) with the increasing of temperatures may be also due to the carbonization of the treated biomass from the direct contact with the walls of the reactor [3.9]. Researchers used statistical analysis to show the relation between moisture and AIL. They demonstrated that biomass initial moisture content has a buffer effect by damping the severity of the pretreatment condition; thereby, avoiding the degradation of the amorphous portion of the structural carbohydrates that could be deposited as residue and subsequently increasing the quantity of AIL in the treated biomass. The interaction between temperature, retention time and moisture content have a significantly effect on AIL. It was

observed that the higher are the temperature and the retention time, the higher is the total acid soluble lignin moieties content at higher temperature and retention times the biomass matrix was deconstructed , which enhanced the quantitation of the total acid soluble lignin moieties. It is also verified that the higher is the moisture content, the lower is the total soluble lignin moieties, because of the buffer effect of moisture and it requires higher energy to evaporate the moisture before deconstructing the biomass matrix.

Realizing that it is difficult to reduce the lignin content by varying SE conditions, many researchers have paid attention to the post treatment process for lignin fractionation and utilization.

The ash content in the SE pretreated biomass increases as the temperature and retention time increase [3.9]. Both variables had a significant a significant effect on the ash content of steam exploded biomass. This could also be due to the degradation of the amorphous portion of the hemicellulose and cellulose. High ash content is not a desirable quality of biomass feedstock for thermochemical conversion.



The formation of furfural was also dependent on the severity of the pretreatment conditions [3.9]. The higher are the temperature and the retention time, the higher is the furfural content. This is due to the reactions and degradations of xylose and glucose from hemicellulose and cellulose respectively that takes place at severe conditions. Moisture content varied inversely with furfural content. The higher the moisture content, the lower is the furfural content, because the system was dampened and subsequently reduced the harsh pretreatment environment that would have led to formation of high degree of inhibitors. As seen, at excessive conditions (high pressure and temperature) during Se, degradation of xylose to furfural and glucose to 5-hydroxmethyl furfural occurred. Researchers reported that both temperature and redemption time are variables to optimize SE process, but long retention time increases the production of inhibitory compounds.

The elemental composition of biomass is dependent on the severity of the treatment conditions [3.9]. The carbon content of the steam exploded solid product significantly

increased as a function of temperature and retention time, while hydrogen and oxygen content decrease. Some researches reported that the carbon content of the steam exploded pretreated samples increase because lignin condensed and even carbonizes. This condensation may be accompanied by water loss as evidence by decrease in oxygen content within the pretreated lignin. Loss in hydrogen and oxygen may be due to the formation of water and carbon monoxide and dioxide. The reactions that occur during steam explosion generally cause smaller hydrocarbon molecules (volatiles and gases) with low energy density to volatize or escape, which in turn increase the energy density of the remaining carbon-rich solids. The nitrogen content also increases with increasing process temperature and retention time.

The HHV of the solid produced increase with temperature and decrease with increasing moisture content [3.9]. Increasing temperature caused a reduction in both mass yield and energy recovery. This mass lost may be associated with the decomposition/degradation of some reactive components of the hemicellulose (such as acetic acid) as well as moisture loss. At high residence times, the mass loss can be attributed to the decomposition of the less reactive components of cellulose.

3.2 Chemical hydrolysis

Liquid hot water (LHW) pretreatment is the most simple and common chemical pretreatment for biomass lignocellulosic feedstocks [3.19]. This process is also known as autohydrolysis, because only water is used. The process is done immerging the biomass feedstock in a reactor at high temperature (160°C-240°C) under pressure, to maintain the liquid state of water, for a determined residence time [3.20].

Biomass deconstruction is catalysed by hydronium ions which generated in situ from auto-ionization and acetic acid resulting from acetyl substituents of hemicelluloses, the later having a much higher contribution to the hydrolysis [3.19].

The pH during autohydrolysis treatment indicates treatment severity and the solubility of the hemicelluloses into the liquid phase, which allows for the recovery of carbohydrates as soluble mono and oligosugars [3.18].

The fibers of treated samples became rough and presented a series of irregular holes and folds after pretreatment. The dissolution of hemicellulose and the partial dissolution of lignin enhance the cellulose accessibility to enzyme. A great improvement of surface area, total pores volume and average pore diameter occurs, mainly due to the dissolution of hemicelluloses. The amount of degradation products generated in LHW is largely driven by the severity factor of the process [3.20]. To avoid excessive depolymerization of the cellulose after the extraction of xylose derivatives, the use of relatively mild temperature (between 180°C and 195°C) is suggested.

The solid yield decrease with increasing temperature: this is due to hemicellulose solubilisation and by-products formation, including acetic acid, formic acid, HMF and furfural [3.18].

Auto-hydrolysis is environmental friendly since only water is used as the reaction media [3.18]. It results in very low corrosive process, low undesirable sugar and low costing. In spite of the advantages of auto-hydrolysis, it has not been under consideration as a pretreatment due to the low levels of enzyme recovery. In order to improve the dissolubility of hemicelluloses in the biomass as well as enhance the accessibility of enzymes to the solid residue during the further enzymatic hydrolysis process, many studies related to concentrate acid and diluted acid pretreatment have been carried out.

3.2.1 Acid-catalysed hydrolysis

The use of acid hydrolysis for the conversion of cellulose to glucose is a process that has been widely studied in the last 100 years [3.26]. The commercial use of acid for biomass pretreatment was first reported in 1898 [3.25], when an industrial process was developed in Germany for the production of ethanol from wood.

Acid pretreatment are classified by the acid concentration used. Dilute acid pretreatment (<5% w/v) are usually carried out under moderate conditions of temperature (120-210°C) and pressure (<10bar). Hemicellulose is readily hydrolysed, while low yields of cellulosic sugars are obtained under these conditions. Pretreatment using concentrated acid (>30% w/v) under moderate temperature (<100°C) and atmospheric pressure is able to efficiently

hydrolyse both hemicellulose and cellulose fractions, providing high sugar yields. However, hydrolysis rate is slower for crystalline cellulose than for amorphous hemicellulose, due to their intrinsic properties [3.22]. Consequently, when hydrolysis is performed in one step, hemicellulose-derived pentoses and hexoses are most susceptible for decomposition to furfural and HMF, which are known to have inhibitory effects on subsequent fermentation of sugars. Other disadvantages of concentrated acid include corrosion of the equipment, high consumption of acid, toxicity to the environment and energy demand for acid recovery.

Compared with concentrated acid pretreatment, diluted acid pretreatment is more attractive method as it generates lower amounts of fermentation inhibitors, less issues with corrosion for process equipment and lower emphasis on acid recovery [3.18]. However, high temperature is required and strong conditions should be applied to achieve reasonable yields of sugars, resulting in the degradation of the amorphous hemicelluloses [3.22]. To overcome the drawbacks of concentrated and diluted acids, a two stage process technology has been developed.

Several types of acids, including mineral acids (sulfuric, phosphoric, hydrochloric, hydrofluoric, nitric, and formic acids) and organic acids (maleic, oxalic, acetic, and fumaric acids) can be used for biomass retreatment [3.25]. The most common acid involved in the chemical pretreatment is the sulfuric acid, thanks to its low price and good reactivity with lignocellulosic biomass [3.24]. Phosphoric acid has also been widely studied because it is relatively cheap and able to hydrolyse biomass efficiently [3.25]. Additionally, the neutralization with NaOH of the hydrolysate after phosphoric acid pretreatment results in the formation of sodium phosphate, which is a salt and it is a nutrient for the fermentation microorganism. Thus, its removal it is not necessary. Hydrochloric and nitric acids have similar performance as sulfuric acid or even better but they are relatively more expensive.

More recently, organic acids were suggested as alternatives to inorganic ones in order to avoid machine corrosion, to have lower energy demand for acid recovery [3.22] and lower formation of degradation products [3.25]. However, they are more expensive than mineral acids. Studies revealed that organic acids are better for biomass with high cellulose and lower hemicellulose contents, such as aquatic plants [3.22]. Acid-catalysed hydrolysis with acetic acid results in the highest acidity compared with the other acids [3.18], thus to higher severity. In fact, the highest amounts of total by-products and furfural are observed with acetic acid addition.

3.2.2 Parameters of acid hydrolysis

The *solid loading* (also referred to as solid/liquid ratio) is one of the most important variables affecting the efficiency of the acid pretreatment process [3.25]. High solid loadings, so low volume of liquid per gram of biomass) is very attractive for the economy of the process because it obtains higher concentrations of sugars in the resulting hydrolysate liquor, avoiding or minimizing the concentration step before the fermentation. However, there is an optimum value established to avoid mass transfer limitations. In fact, water is essential in acid pretreatment to facilitate the acid diffusion and also for sugar solubilisation.

As steam explosion pretreatment, acid pretreatment efficiency depends on two variables, temperature and reaction time, that can be correlated in the definition of *combined severity factor* (CSF) of the process, to facilitate a comparison among different processes and conditions [3.25]. Obviously, it has to take into account the pH at which the reaction occurs.

$$CSF = log_{10}\left(t \cdot e^{\frac{(T-100°C)}{14,75}} - pH\right)$$

Usually, the concentration of the degradation products increases with increasing the severity of the process [3.18].

Another useful parameter is the *hydrolysis severity* (HS) [3.23]. It is defined as the ratio between the total sugar concentration and the total inhibitor concentration in the hydrolysate.

$$HS = \frac{\sum S}{\sum I}$$

3.2.3 Dilute acid pretreatment

Dilute acid pretreatment (<5% w/v) are usually carried out under moderate conditions of temperature (120-210°C) and pressure (<10bar) [3.25]. Under these conditions, around 80-90% of hemicellulose sugars are usually recovered, while cellulose and lignin fractions remain almost intact in the remaining solid material after pretreatment. The hemicellulose removal increases the material porosity and enhance the cellulose accessibility to enzymes in the residual solid. After pretreatment with dilute acid, the material structure presents some ruptures due to the hemicellulose removal. Additionally, although only a little lignin is dissolved during this process, the lignin structure is disrupted, favouring the enzymatic digestibility of cellulose. A visible alteration in the colour of the biomass to dark brown is also observed as a consequence of the relative increase of the lignin content in the residual solid material [3.25].

Due to its great ability to selectively remove hemicellulose, dilute acid pretreatment can be used as a first step in the overall process for lignocellulosic biomass fractionation into its three main components [3.25].

The sugar released from the hemicellulose structure are the main components in the hydrolysate obtained after dilute acid pretreatment [3.25]. Usually xylose, arabinose, and glucose are the main monosaccharides present in the hydrolysate, but the type of sugars and their concentrations varies according to the biomass treated and pretreatment conditions. Oligomers from the polymers can also be present in the hydrolysate: this usually happens when the conditions employed for biomass pretreatment are not properly balanced to promote a full conversion of hemicelluloses to monosaccharides. It has to be taken into account to achieve a high yield of conversion to convertible sugars.

Besides sugars, the hydrolysates obtained by diluted acid pretreatment may also contain other components, such as acetic acid (derived from the acetyl groups of the hemicellulose structure), furfural and 5-hydroxymethyl furfural (derived from the degradation of pentoses and hexoses, respectively) [3.25]. These substances are toxic for microorganism and therefore their concentration in the hydrolysate should be as low as possible.

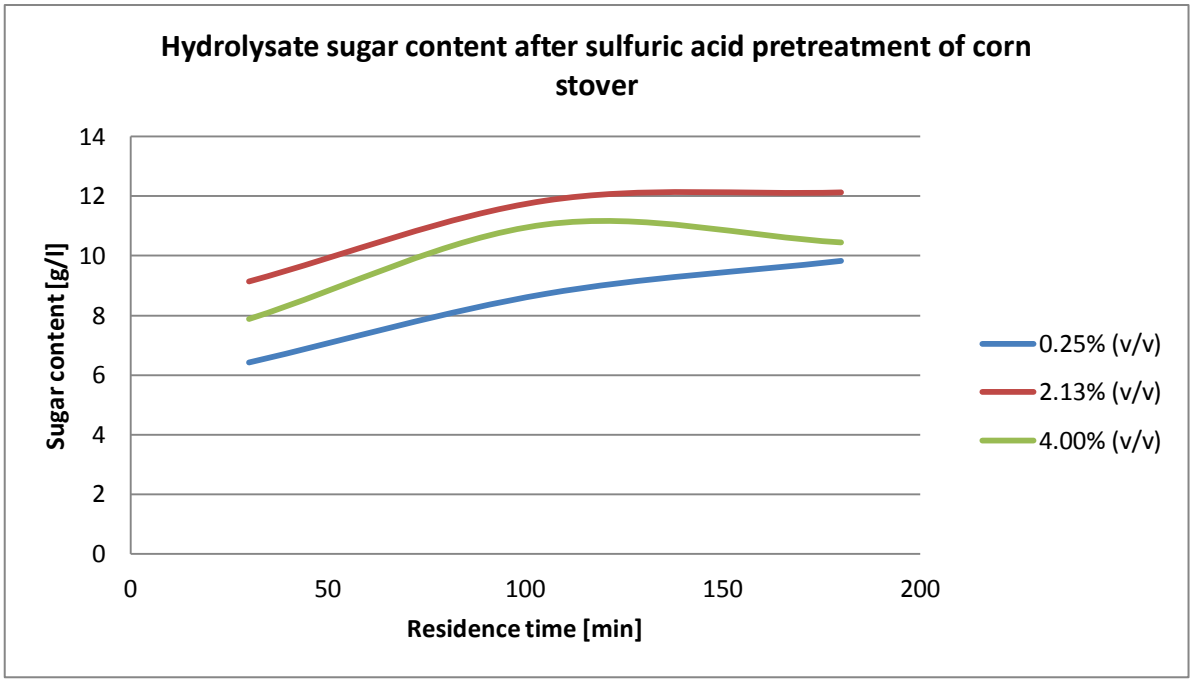
In addition to the main advantages explained before, dilute acid pretreatment is characterized by low consumption of acid, an important advantage in terms of overall cost [3.25]. Furthermore, dilute acids are able to extract nutrients from biomass to the hydrolysate, which can be consumed by the microorganism, reducing the necessity of adding nutrients for fermentation.

The main disadvantage of this method is that the use of acid (even in low concentrations) generates problem related to corrosion of the equipment, requiring expensive materials for the construction of the reactors, increasing the costs [3.25]. The use of high temperature, the overall energy consumption and the necessity of using small particle sizes (a few millimetres) also have an important drawback in the overall cost of the process.

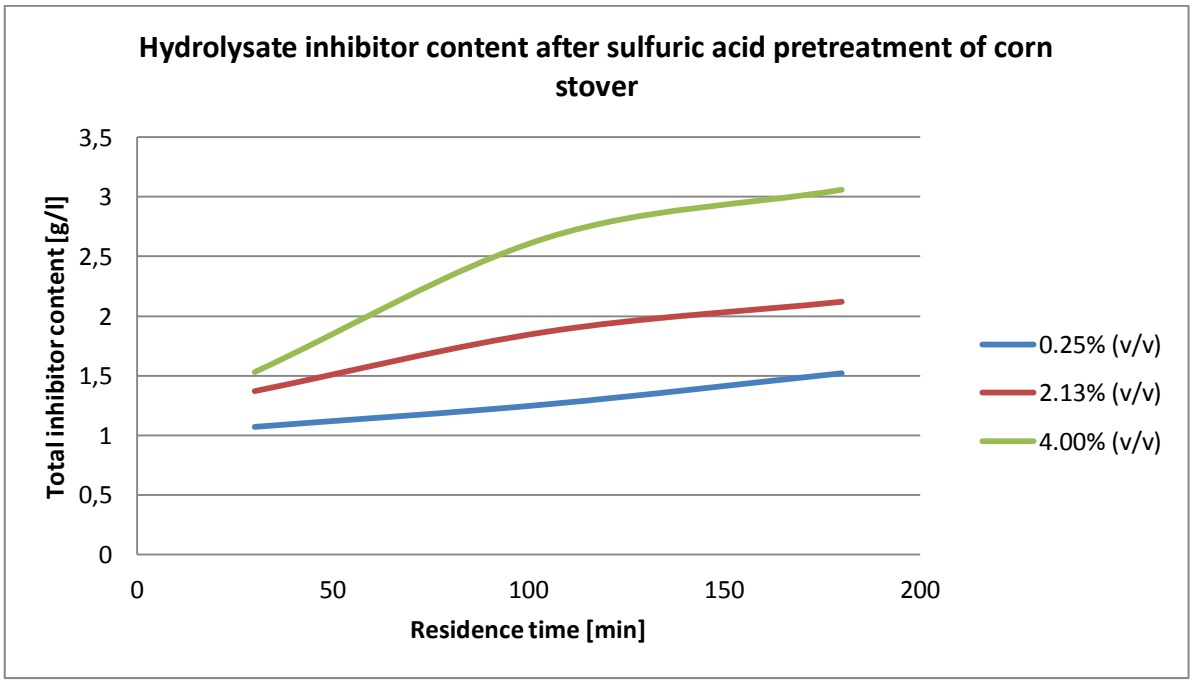
Other disadvantages are related to the nature of the hydrolysate [3.25]. The hydrolysate has a low pH and needs to be neutralized before use as fermentation medium. The salts resulted from the neutralization step need usually to be separated and disposed of. Formation of toxic compounds occurs inevitably during the pretreatment process: a removal is needed if their concentrations will affect the subsequent step. Metal ions resulting from the corrosion of the equipment may also act as inhibitors for the microbial metabolism.

The literature results clearly show that different types of biomass require different conditions of pretreatment to be efficiently converted into sugars [3.25]. Establishing the optimal pretreatment conditions is very important to maximize the sugar yield as well as to minimize the formation of toxic compounds. Nevertheless, the optimal conditions depend on if the goal is to maximize the sugar yield from hemicellulose or from cellulose after enzymatic hydrolysis of pretreated solids or even if it is desired to obtain maximum yield after both steps.

One of the most common treated lignocellulosic biomass is corn stover [3.23]. Analysing the compositions of the hydrolysate at fixed temperature (121°C) but different sulfuric acid concentrations and reaction times, the sugar concentrations increase with acid concentration and reaction time, but further increase in acid percentage and/or in reaction time reduces the sugar concentration. This indicates the dependence of the sugar production on the operational conditions.



In fact, the quantities of inhibitors increase with increasing acid concentration and hydrolysis time [3.23]. Thus an opportune balance is needed between hydrolysis time and acid concentration, in way to optimize the sugar production.



In this respect, response surface methodology (RSM) with central composite design has been considered as a proper tool for determining suitable condition to achieve this optimization purpose [3.24] (see the subsequent chapter).

It can occurs that surface area decreases, although it typically increases after thermal treatment with an acid catalyst [3.24]. This is because the cell wall structure can be damaged through carbohydrate degradation and then the uniformity of the cell wall can deteriorate and morphological irregularity can increase during the diluted acid pretreatment. Conversely, in some studies, the SSA decreases: pores in the cell wall could become

enlarged and they blend together. Thus, mean pore diameter will increase at the expenses of the SSA.

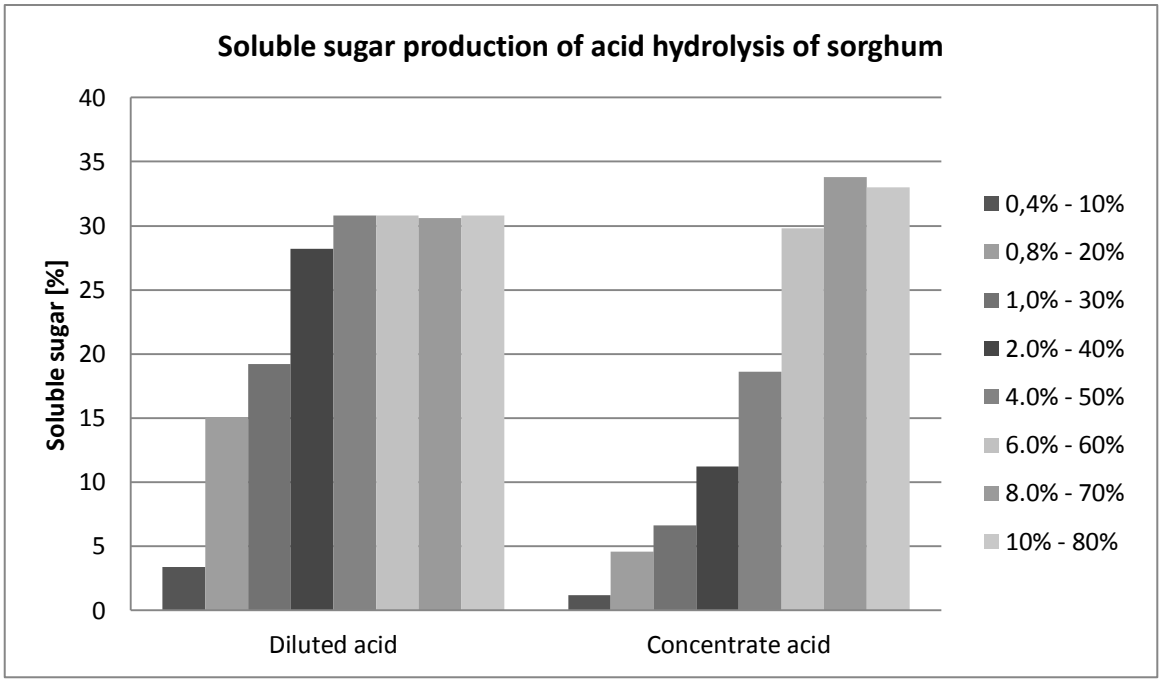
3.2.4 Concentrate acid pretreatment

Biomass pretreatment with concentrated acids is commonly performed using a high acid concentrations (>30% w/v), at ambient to moderate temperature (<100°C) and atmospheric pressure [3.25]. Under these conditions, the acid attacks the lignocellulosic structure and it is able to release sugars from both hemicellulose and cellulose fractions, providing high sugar yields. Concentrated solutions of sulfuric, nitric, hydrochloric, phosphoric and trifluoroacetic acids can be used to treat lignocellulosic materials. However, sulfuric acid is the most commonly used.

The biomass pretreatment with concentrated sulfuric acid is a process usually performed in two sequential steps: an initial stage of solubilisation responsible for decrystallizing the lignocellulose structure and a second stage of hydrolysation that hydrolyse the fragments of cellulose and hemicellulose to monosaccharides [3.25]. The efficiency of sugar recovery by this pretreatment process is affected by operational variables, including acid concentration, solid loading, temperature and reaction time.

Due to its ability to disrupt the structure and release sugars from lignocellulosic biomass, a standard method using concentrated acid was established and is widely used to determine the chemical composition of lignocellulosic materials [3.10] [3.25]. This method is based on a two stage acid hydrolysis using concentrated sulfuric acid (72%) at 30°C for 1 h, followed by a dilution with water to 4% w/w and incubation at 121°C for 1 h. The initial stage using concentrated acid break the lignocellulosic structure, converting polysaccharides mainly into oligosaccharides, which are then converted to monosaccharide in the second stage. The monosaccharide in the resulting hydrolysate are quantified and used for the determination of hemicellulose and cellulose content.

Koradiya et al. [3.38] showed the differences between diluted and concentrate acid hydrolysis on sorghum straw. Hydrolysis process was done with different concentration (%v/v) of sulfuric acid at 225°C for diluted acid, while 75°C for concentrate acid. The soluble sugar yields are shown in the graph below.



It can be observed from the obtained data that the optimum points are reached with 4% v/v of sulfuric acid for diluted acid, while for concentrate acid with 70% v/v of acid. Higher concentration of acid bring to the denaturation of sugars and then the resulted yield will decrease. Diluted acid hydrolysis leads to acceptable soluble sugar level in comparison with concentrate acid hydrolysis. However, it requires higher temperature costs in term of energy demand. On the other hand, high use of acid is characterized of high equipment costs and environmental issues. A specific economical study has to be done to individuate the best pretreatment option.

3.2.5 Layout of acid pretreatment process

Reactors layout are designed to carry out the pretreatment in continuous or discontinuous mode. Co-current rectors, also called extruder reactors, are continuous reactors typical of diluted acid pretreatment [3.26]. Biomass liquid slurry passes through heat exchanger, is heated to the desired temperature and held at the project temperature for the time requested.

Discontinuous reactors, such as batch, percolation and flow-through, works with a defined volume of biomass. In the batch reactors, biomass and reactants are put together and subsequently heated at desired temperature. After completing the reaction, the reactors is cooled and the products filtered. In percolation configuration, the liquid reactants is forced through a packed biomass bed. It may enhance hemicellulose and lignin removal rates.

These reactors, operating in flow through configuration, are characterized by a higher water usage compared to non-flow configuration, which implies dilution of sugar stream and high energy consumption [3.32]. Several configurations have been proposed and investigated to address these concerns, including recirculation flow, partial flow and counter-current flow.

A well-known process for biomass pretreatment using concentrated acid is the Arkenol process, which is based on the use of concentrated acid (especially sulfuric acid 70-80%) in an acid/biomass ratio of 1.25 at low temperature (<50°C) [3.25]. This process is the

conclusion of the researches started in the 1989 by the American Arkenol Company [3.27]. In the first step of the Arkenol process, concentrated sulfuric acid is used to attack the long chain cellulose and hemicellulose molecules, breaking the links of the chain into their simple sugar components. This decrystallization and hydrolysis occurs at relatively low temperatures and pressures. The resulting solution now contains sugar, sulfuric acid, water, and lignin. The lignin is separated from this acidic solution using a filter press. The acidic sugar solution then continues on to a chromotographic separation unit, which effectively separates the acid and sugar solution into a pure sugar stream and a pure acid stream. The acid stream, now diluted, is recycled and reconcentrated for further use. The now slightly acidic sugar stream (the separation of the streams approaches 100%) is neutralized with lime, forming gypsum. The gypsum is filtered from the neutralized sugar solution that is sent on for fermentation to the myriad of possible products.

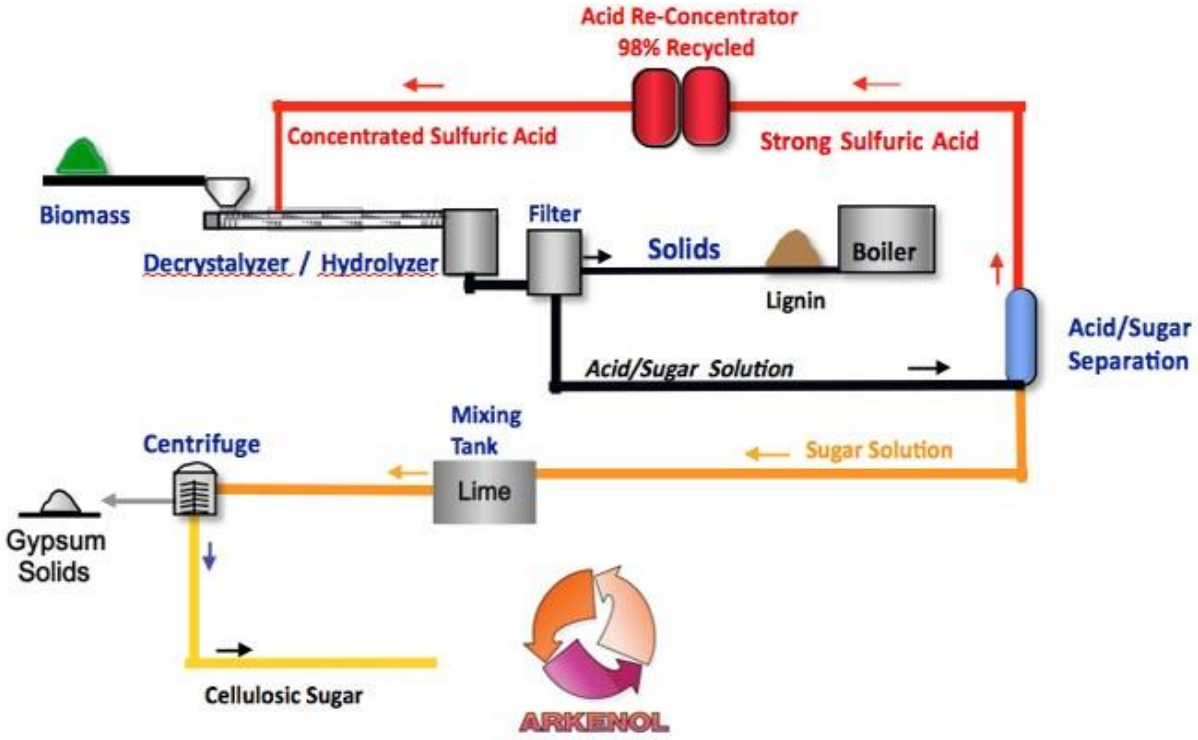


Figure 3.6 - Arkenol process flow diagram [3.27]

Another well-known method for biomass pretreatment using concentrated sulfuric acid solution is the Biosulfurol process for the production of bioethanol [3.28]. This process developed by the Biosulfurol Energy, a Dutch company now dismissed, uses a sulfuric acid solution (70%) in presence of carbon dioxide at ambient temperature. The slurry produced passes an anion-selective diffusion membrane, which separates sulphuric acid from the biomass. About a half of sulfuric acid is transferred to a water phase at the other side of the membrane. That weak acid is to the impregnation reaction after being mixed with SO_3. By adding water recycled from the wastewater treatment, the hemicellulose and cellulose are further hydrolysed and the water neutralised to pH of 4.5. A part of the neutralization take place by exchange of a part of the sulphuric acid via an anion selective membrane. In this

stage the lignin is separated from the dissolved compounds, and dried. Then the hydrolysate is converted to bioethanol via fermentation.

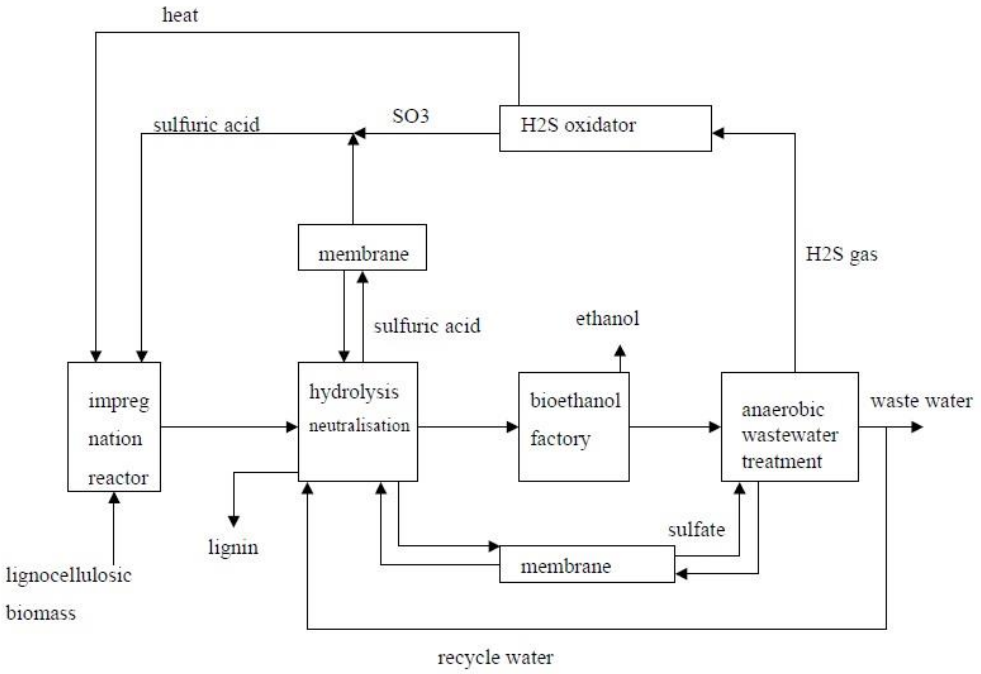


Figure 3.7 - Biosulfurol process flow diagram [3.28]

The main advantages are the no enzyme requirement, the wide range of biomass type utilizable, the possibility to treat relatively large and hard pieces of biomass, very low temperature that allows the use of plastic materials, the low production of inhibitory compounds due to the low temperature, and low overall costs [3.28].

3.2.6 Alkaline pretreatment

The most famous alkaline pretreatment method is the Kraft process (also known as Kraft pulping process): it is a process for conversion of wood into wood pulp, which consists of almost pure cellulose fibers, for the production of paper. The Kraft process utilize a mixture of hot water, sodium hydroxide (NaOH) and sodium sulphide (Na_2S), known as white liquor. The invention of this process is date back in 1879, in Germany, by Carl F. Dahl.

Sodium hydroxide pretreatment for improving in vitro digestibility of straws by ruminants has been found since at least 1919 [3.29]. Thus, the alkaline treatment of cellulosic materials is an old process, which was the focus of the pulp and paper industries for years.

Nowadays, alkaline pretreatment has emerged as one of the favourable pretreatment methods because it has a number of desirable features [3.30]:

- use of mostly non-polluting and non-corrosive chemicals such as ammonia (both aqueous, liquid and gaseous state), sodium hydroxide, sodium carbonate, and calcium hydroxide (lime);
- milder conditions than those needed for acid pretreatment;
- efficient lignin removal.

Alkaline pretreatment reagents are less caustic than acidic reagents and the processes are carried out under milder conditions, some of them even at ambient temperature. Such methods can eliminate the need for expensive materials and special designs to cope with corrosion and severe reaction conditions. It also possible to recover and reuse chemical reagents is some of the alkaline pretreatment methods. The main drawback of this process is time [3.29]: it might take hours or even days to complete the reaction but the reaction time could be saved through the increment of temperature.

The most common alkaline pretreatment methods include lime ($Ca(OH)_2$), and calcium, sodium, barium, lithium and ammonium hydroxides as reactants [3.29]. Among these reactants, sodium hydroxide receives more attention, followed by lime. Lime is largely used thanks to its relatively low costs and its security to use as well as its easy recovery from water as insoluble calcium carbonate by reaction with carbon dioxide.

Sodium hydroxide effectively attacks the linkage between lignin and hemicellulose [3.30]. During the NaOH pretreatment reaction, sodium hydroxide is dissociated into hydroxide ion (OH^-) and sodium ion (Na^+), and, as the hydroxide ion concentration increases, the rate of the hydrolysis reaction increases accordingly.

The major reactions during alkaline pretreatment include dissolution of lignin and hemicellulose, and de-esterification (saponification) of the intermolecular ester bonds [3.30]. These reactions also alter the degree of polymerization (DP) of each component, and bring about changes in the physical properties of treated solids. Such changes might involve surface area, porosity and crystallinity. The change in the crystallinity index is primarily due to the removal of amorphous regions (lignin and hemicellulose) of the biomass, rather than to structural change in the cellulose fibers. This is the reason that the crystallinity index of treated biomass often increases after pretreatment.

During alkaline pretreatment the first reactions are solvation and saponification, that leads to a swollen state of the biomass and makes it more accessible for enzymes and bacteria [3.29]. The saponification of intermolecular ester bonds cross-linking xylan hemicellulose and other components, and the porosity of the lignocellulosic biomass increases with the disintegration of the cross-links. The efficiency of alkaline hydrolysis depends on the substrates and treatment conditions. It is strongly related to the lignin type and content in the biomass. Studies reported that the activation energy for delignification of herbaceous species by alkaline pretreatment was much lower than that required for delignification of wood [3.30]. Consequently, it appears that alkaline pretreatment is much more effective for delignification of grass species than of woody species.

The delignification process of the feedstocks can be enhanced supplying surplus air or oxygen [3.29]. The lignin removal is the cause of the increase of swelling capacity of the lignocellulosic biomass. Furthermore, alkaline treatment enhance the polyionic character of the pretreated lignocelluloses which promotes swelling. Polyionic character is related to the diffusion of basic ions into the lignocelluloses. The ions remain in the lignocelluloses and act as a countercharge to carboxylate ions.

The changes in the cellulose structure by alkaline treatment are mainly involves with changes in crystallinity by mercerization, the distribution of crystalline and amorphous

region as well as partial depolymerisation [3.29]. The effect of cold and hot alkaline pretreatments on the crystallinity index depends on the substrate. When cellulose is treated with alkali solutions, the cellulose swells to various extents, depending on the treatment temperature, alkali concentrations and retention time. A portion of cellulose is degraded during alkali pretreatments: this degradation could be stopped by chemical and physical effects. Chemical stopping is the formation of a non-reactive metasaccharinic end-group by reactive interim products. On the other hand, when the degradation reaches the crystalline regions of the polysaccharide chains, the peeling reaction cannot proceed further for steric reasons, referred to as physical stopping.

A large number of reactions may take place at elevated temperatures: the most important reactions are concerned with the dissolution of non-degraded polysaccharides, the formation of alkali-stable end-groups referred to as peeling reactions of end-groups, the hydrolysis of glycosidic bonds and acetyl groups, and the decomposition of dissolved polysaccharides [3.29]. Similar to acid pretreatment, hydrolysis and degradation of hemicelluloses are much faster than cellulose in alkali pretreatment. The endwise peeling continues to degradation until a competing reaction, the so called stopping reaction, takes place, which determinates the degradations. Without the stopping reaction the hemicellulosic components may be completely decomposed by peeling. Since the alkali treatment can liberate mainly acetic acid and some small amounts of other dicarboxylic acids, the degradation under severe conditions can result in the formation of formic acid as an end-product.

It was found that some additives, such as polysulfides, sodium borohydride, hydrogen sulphide, and others, could stabilize polysaccharides against alkaline peeling [3.29]. The effects of these additives, however, have not yet been investigated in lignocellulosic pretreatment.

The hemicellulose extractability through alkaline treatment can usually be easily performed ad has a considerable cost advantage [3.29]. The yield of hemicelluloses is related to the accessibility of aqueous alkalis to the hemicellulosic surface. In addition, different alkaline solutions have diverse disruption and dissolution abilities as they penetrate the wall cell. By exposing more hemicelluloses under the dual treatment of the disruption and dissolution of alkalis, most of the hemicelluloses and lignin dissolves in the alkaline solution; however, there is still a portion of hemicelluloses and lignin that remains as an insoluble residue. This also indicated that a part of the ground substance of the cell wall is still tightly linked together even after treatment with alkali, due to its limited penetration and dissolution of the cell wall.

Generally the hemicelluloses released with weak alkaline solution have much lower molecular weights than those isolated with strong alkalis [3.29]. In contrast to strong aqueous alkalis, weak aqueous alkalis (such as ammonia), have less ability to cleave the hydrogen bonds between the hemicelluloses and cellulose as well as the ester bonds between the hemicelluloses and lignin. Consequently, they can dissociate the hemicellulosic segments with a homogeneous molecular weight but do not easily give rise to the dissociation of large molecular weight hemicelluloses. The differential abilities to isolate hemicelluloses from the cell walls of plants can be defined as “selective extraction process” [3.29].

Simultaneously, lignin has been found to undergo depolymerisation and condensation reactions [3.29]. The effects and reactions in alkali treatment on lignin are complicated because of the different stability and behaviour of different type of linkages and structural elements in this heterogeneous polymer. A small portion of lignin may degrade by cleavage of less frequent linkages, leading to a reduction or total elimination of side chains.

3.2.7 Ammonia Pretreatment

The application of ammonia as a catalyst in alkaline pretreatment of lignocellulose biomass has also been tested and estimated to be a promising alternative [3.31]. Initially ammonia pretreatment has been successfully used to increase the digestibility of low quality forages and crop residues in animal feeding. In contrast to other alkaline catalyst, ammonia can be easily recycled by evaporation, and the residual ammonia provides a potential nitrogen source for biotechnological processes. Different technologies have been developed for ammonia pretreatment processes, such as soaking in aqueous ammonia (SAA), ammonia recycle percolation (ARP) and ammonia fiber explosion (AFEX). These vary in treatment conditions like ammonia concentration, liquid content, process temperature, and pressure.

Ammonia treatment results in the cleavage of complex linkages of lignin-carbohydrate between lignin phenolic and hemicellulose side-chains that allow the removal of lignin and partially hemicellulose [3.31]. Another effect is the influence on crystallization of cellulose, which depends on temperature and treatment duration. Cellulose samples with different degrees of polymerization and crystallinity indexes were subjected to anhydrous liquid ammonia treatments. The treatment resulted in allomorph cellulose of different crystallinity degrees depending on the treatment conditions. Trea'tment at a low temperature (25°C) resulted in a less crystalline product of mainly amorphous structure, whereas treatment at elevated temperatures (130-140°C) gave a more crystalline product.

The effects of ammonia pretreatment are different, depending on the kind of treatment process [3.31]. SAA at a low temperature primarily results in lignin removal and has a little effect on carbohydrates. Raising the treatment temperature leads to an increased solubilisation of hemicellulose with effective removal at temperature above 130°C. Treatment with anhydrous ammonia at a higher temperature and pressure (AFEX) results in lignin and hemicellulose dissolution, which remains in the biomass. After the evaporation of ammonia, the cell wall decomposition products are redeposited onto the surface of the outer cell walls. As a consequence of these chemical changes, the embedded cellulose micro-fibrils are exposed and available for enzyme saccharification processes.

Another aspect of ammonia pretreatment, which is also of economic importance, is the production of high-quality lignin as a secondary product [3.31]. The avoidance of lignin condensation during ammonia pretreatment allows for the conversion to aromatic fine chemicals by controlled depolymerisation.

Lignocellulose treatment by ammonia soaking is usually performed via aqueous ammonia with concentrations between 15-30% [3.31]. High liquid/solid ratio (6-12:1), moderate temperature (30-75°C) and incubation periods of 12-48h up to several days characterize this process. Within the SAA pretreatment process all types of biomass show significant lignin

removal and high glucan retention. Lignin removal increases with higher ammonia concentrations: more than 70% lignin removal has been observed at 30% ammonia concentrations. Apart from treatment conditions, the efficiency of SAA depends on the lignin content of biomass.

Another pretreatment process for biomass using liquid ammonia, ammonia recycle percolation (ARP), uses aqueous ammonia as a pretreatment reagent in recirculation mode and continuous recycling in a packed bed flow through-type reactor (also called percolation reactor) [3.31]. The treatment conditions are temperature of 170-180°C and pressure of about 22-28bar, that prevent ammonia loss by vaporization. The biomass has usually been pre-soaked in an ammonia solution overnight before the percolation process starts. ARP is highly effective in the fractionation of biomass into its main components. Lignin removal is higher in biomass of a low lignin content, such as herbaceous biomass, as compared to woody biomass. The glucan fraction has been retained with high rates (>90%) in all kind of biomass. In most cases water is pumped into the reactor after the ARP process to remove residual sugars and solubilized fractions.

The ammonia fiber explosion (AFEX) is a physicochemical pretreatment in which anhydrous or concentrate (>70%) aqueous ammonia is used to treat biomass at a certain incubation time for a given temperature, moisture content and ammonia loading [3.31]. After a brief incubation, the pressure is released rapidly, causing the biomass to expand. The ammonia is removed from the pretreated biomass by evaporation and can be recycled. After that, the AFEX-pretreated biomass is ready for enzymatic hydrolysis or other biotechnological processes. AFEX is a dry-to-dry process and the entire fraction content remains the same even after pretreatment unless the sample is washed before further use. In contrast to the ammonia pretreatment processes described above, the fractions of lignin and hemicellulose are not removed from the biomass.

Typical AFEX treatment conditions include a temperature about 100°C, 60-80% moisture content, ammonia/dry mass ratio of 1:1, and an incubation time of 5-30min [3.31]. The main effect of AFEX pretreatment is a high solubilisation and conversion of glucan and hemicellulose after enzymatic hydrolysis. The process offers both an effective and economically attractive means for increasing yields of fermentable sugars from lignocellulosic biomass. AFEX has been shown to decrease cellulose crystallinity and particle size, whereas increasing the surface area improves the enzymatic attack.

Ammonia penetrates the cell walls and in the presence of water, ammonolytic and hydrolytic reactions cleave various ester linkages, resulting in the formation of corresponding amides and acids [3.31]. The cleavage of lignin-hemicellulose ester linkages results in solubilisation and the removal of hemicellulose oligomers and other extractable compounds to the outer cell wall parts. At the end of pretreatment, the rapid pressure release results in the formation of large pores in the cell walls.

3.3 Enzymatic hydrolysis

Enzymatic hydrolysis is a relative newcomer with respect to acid-alkaline hydrolysis [3.34]. While the chemistry of sugar production from wood has almost 100 years of process development, enzymes for biomass hydrolysis only have 50 years of serious efforts.

The enzymatic saccharification is a green process for converting the lignocellulose sugars into mono-sugars solution [3.36]. This process is characterized mainly by a long reaction time. To overcome this disadvantage, physical and chemical pretreatment processes, seen in the previous paragraphs, are widely used, enhancing the accessibility to the three main lignocellulose compounds: cellulose, lignocellulose and lignin. Individual pretreatments should be optimized to fit the chemical compositions and internal structure of the pretreated biomass.

In nature, lignocellulose is degraded by a series of hydrolytic and oxidative enzymes, produced by a variety of fungi and bacteria that are able to synergistically degrade cellulose, hemicellulose and lignin [3.34]. Aerobic cellulose degraders, both bacterial and fungal, utilize cellulose through the production of substantial amounts of extracellular enzymes that are freely recoverable from culture supernatants. Anaerobic bacteria degrade cellulose primarily via complexed enzymes systems, called cellulosome, that consists of multiple subunits that interact with each other synergistically and degrade cellulosic substrate.

Lignocellulosic enzymes can be grouped into three main families, in relation to the lignocellulose fraction that they are able to attack [3.36]:

- cellulases, which are multienzyme complexes that consist majorly of endo-glucanases, exoglucanases or cellobiohydrolase, and glucosidases;
- hemicellulases, including mainly arabinanases, galactanases, mannanases, and xylanases;
- ligninases, or lignin-modifying enzymes (LMEs), including mainly peroxidsase and laccase.

Endoglucanases cleave glycosidic bonds within cellulose microfibrils, acting preferentially at amorphous cellulose regions [3.34]. This enzymes fragment cellulose chains to generate attractive ends for exoglucanases, which act subsequently to degrade cellulose, including crystalline cellulose, from either the reducing or non–reducing ends, to generate mainly cellobiose. Thus, glucosidases converts cellobiose into glucose. These enzymes work synergistically, and some prevalent reactions could cause inhibition, such as a high concentration of cellobiose that slows exoglucanases activity.

For maximizing the hydrolysis of lignocellulosic feedstock by cellulase enzymes, the synergic action of hemicellulases is required [3.33]. The cellulose fibers are held together by lignin and hemicellulose. Xylanases cleave internal linkages of the xylan backbone, while xylosidase hydrolyses xylobiose and small xyloligosaccharides to xylose. Often xylans are in partially acetylated form, which are cleaved by acetyxylan esterases. Other debranching enzymes work together to break down the hemicellulose to primarily pentose sugars while improving the accessibility of cellulases to hydrolase cellulose fibers.

In the process of enzymatic hydrolysis of lignocellulosic materials, some proteins have been identified that are capable of nonhydrolytically loosening the packaging of cellulose fibril network, a process calling amorphogenesis [3.39]. These proteins act synergistically along with cellulases, thereby increasing the accessibility of cellulose to the enzymes. Hence, these helper proteins are called amorphogenesis-inducing agents. They promote the dispersion of cellulose aggregations and expose individual chains to the enzyme. This ability makes it an important component in the enzyme mixture use for the hydrolysis of lignocellulosic biomass.

In nature, the lignocelluloses degradation is realized usually by fungi, most of them are of the "white rot" family [3.35]. They use lignin as source of carbon and energy, being the responsible of many diseases of wood and plant species. Lignin modifying enzymes are produced not only by white rot fungi, but also by brown rot fungi. Some bacteria also produce lignin modifying enzymes, although studies showed that fungal enzymes have higher oxidizing power than bacterial enzymes and are able to degrade more complex lignin structures. The main enzyme produced are peroxidases, which depolymerize lignin by oxidation using hydrogen peroxide. Laccases are another class of enzymes found in both bacteria and fungi which have significant lignin-degrading properties. Laccases degrade lignin by oxidation using oxygen.

The main drawbacks for using fungus to delignification the lignocellulosic biomass matrix included long pretreatment times, excess consumption of produced sugars by the fungi, and microorganism contamination problems [3.36].

Although a wide variety of bacteria and fungi produce cellulotyc enzymes able to hydrolyse cellulose, relatively few species produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively [3.34]. Cellulases used for current industrial applications are mainly fungal, primarily due to the efficiencies in fungal enzyme secretion. The hydrolytic enzymes should be of desirable characteristics for their application in the hydrolysis of lignocelluloses [3.33]. These characteristics are catalytic efficiency, thermal stability, adsorption, end-production inhibition resistance, and shear inactivation.

3.3.1 Stabilization of lignocellulolytic enzymes

Enzymes as biocatalysts have found their importance in industrial processes due to their operational ease, environmentally friendly catalysis, and the incorporation of advanced technique such as immobilization and stabilization, which greatly influences the processes with respect to the ease of recovery and reusability of enzymes and in turn contributes to the reduction of costs [3.33]. Enzyme immobilization is a confinement of enzyme to a phase (matrix/support) different from the one for substrates and products [3.37]. Inert polymers and inorganic materials are usually applied as carrier matrices. Apart from being affordable, an ideal matrix must encompass characteristics like inertness, physical strength, stability, regenerability, ability to increase enzyme specificity/activity and reduce product inhibition, nonspecific adsorption and microbial contamination..

Materials used for fabrication of immobilisation supports are various [3.37]. In the family of natural polymers supports we can find alginate (derived from cell wall of brown algae),

collagen (a structural protein of the animal connective tissues), carrageenan (a sulfate polysaccharide extracted from red seaweeds), gelatin (a derived product of collagen), cellulose, starch, pectin. For inorganic supports are mentionable zeolites, ceramics, silica, glass, activated carbon and charcoal. Several methods are used for immobilization and various factors influence the performance of immobilized enzymes

Adsorption/carrier-binding method uses water-insoluble carriers such as polysaccharide derivatives, synthetic polymers and glass [3.37]. Enzyme adsorption results from hydrophobic interactions and salt linkages where either the support is bathed in enzyme for physical adsorption or the enzyme is dried on electrode surface. Adsorbed enzymes are shielded from aggregation, proteolysis (the breakdown of protein into small polypeptides or amino acids) and interaction with hydrophobic interfaces. Researchers have developed several eco-friendly supports, like coconut fibers, which not only prevent cropping up of ethical issues, but also cut the production costs.

In cross-linking/covalent method, bi/multifunctional reagents are used [3.37]. Covalent association of enzymes to supports occurs owing to their side chain amino acids and degree of reactivity based in different functional groups. Highly stable and hyperactive biocatalyst have been reported by covalent binding of enzymes to silica gel carriers modified by silanization with elimination of unreacted aldehyde groups. Increase in half-life and thermal stability of enzymes has been achieved by covalent coupling with different supports like mesoporous silica. Maintaining the structural and functional property of enzymes during immobilization is one of the major roles played by a cross-linking agent

Affinity immobilization exploits specificity of enzyme to its support under different physiological conditions [3.37]. It is achieved by two ways: either the matrix is pre-coupled to an affinity ligand for target enzyme or the enzyme is conjugated to an entity that develops affinity toward the matrix. Affinity adsorbents have also been used for simultaneous purification of enzymes.

Entrapment is caging of enzymes by covalent or non-covalent bonds within gels or fibers [3.37]. Efficient encapsulation has been achieved with alginate-gelatine-calcium hybrid carriers that prevented enzymes leakage and provided increased mechanical stability. Entrapment by nanostructured supports have revolutionized the world of enzyme immobilization with their wide ranging applications in the field of fine chemistry, biomedicine biosensors and biofuels.

A key factor that prevents the commercialization of the enzymatic hydrolysis is the high costs of cellulase enzymes [3.39]. For example, enzyme cost is expected to account for more than 20% of ethanol production. As much of it remain active after hydrolysis, recycling of cellulases makes the overall conversion process more economically feasible. Various methods have been used for recycling enzymes, which include sedimentation followed by ultra-filtration or micro centrifugation, cation exchange chromatography, re-adsorption, and immobilization.

The ultra-filtration method for enzyme recovery has proved to be an efficient way to recover cellulases as well to continuously remove end products that ae generated during hydrolysis that could potentially inhibit hydrolysis reactions [3.39]. Another process is a two-step

system: sedimentation step is combined with membrane filtration step. The sedimentation step is done to remove the largest particles that could block the tubing and the membrane filter. After sedimentation the suspension is clarified using microfiltration, through membranes. Retained cellulases can be reused for hydrolysis (in this way up to 75% can be recycled).

Another method for recycling is using amphiphilic lignin derivatives [3.39]. An amphiphile is a chemical compound possessing both hydrophilic and lipophilic properties. Its effect on cellulase was investigated in a continuous multistage saccharification process, in which amphiphilic lignin is showed as an excellent water-soluble polymeric carrier for immobilization of cellulase to preserve the hydrolytic activity for a long period. The potential economic benefits of the use of these surfactants are demonstrated by different studies, showing a reduction in the total enzyme cost up to 65%.

3.3.3 Parameters influencing enzymatic hydrolysis

It has been observe that the presence of hemicellulose in the pretreated lignocllulose greatly affects enzymatic cellulose degradation [3.33]. While the removal of hemicellulose requires harsh chemical treatment, it becomes an impractical solution as the aim is to reduce the capital cost and minimize the generation of waste by incorporating less severe pretreatment methods. Also, it is known that oligosaccharides released by hemicellulase have inhibitory effects on cellulase. The addition of hemicellulases during the hydrolysis of cellulose increase the rate and also avoids non-productive binding of endoglucanase to the lignocellulose because cellulase and hemicellulase act in synergy to degrade lignocellulose. Biomass contain also a significant percentage of lignin, which has been demonstrated to be the most important limiting factor for the hydrolysis by cellulytic and hemicellulytic enzymes. Several reasons have been put forward as to why the presence of lignin reduces hydrolysis:

- lignin provides a physical barrier, which limits the accessibility of cellulase or hemicellulase to its substrate;
- cellulase becomes non-specifically adsorbed to lignin, which reduces the productive hydrolysis of the substrate
- lignin may also directly inhibit the hydrolytic enzymes.

Researchers indicate that it is not just the presence of lignin but also the type and distribution that have impacts on enzymatic hydrolysis.

In an approach to avoid non-productive bindings and/or the inhibition of lignocellulolytic enzymes by the lignin component of the biomass, several researchers have used certain chemical groups to reduce the non-specific binding of enzyme to lignin [3.33].

Specific surface area, lignin content and biomass crystallinity are the three major factors that have frequently investigated and correlated with enzymatic hydrolysis of biomass after pretreatment [3.6].

The efficiency of enzyme-substrate interactions was strongly influenced by specific surface area (SSA [m^2/g]) [3.5]. The SSA of pretreated biomass increase with increasing biomass particle size, which matches the trend of sugar conversion during enzymatic hydrolysis.

Biomass crystallinity was also believed as an important factor affecting enzymatic saccharification [3.6]. Biomass crystallinity index (CrI [%]) is defined as the mass fraction of crystalline cellulose in the biomass. There are several methods for estimation of cellulose crystallinity index from the powder of X-Ray Diffraction (XRD) data of pure cellulose or biomass samples. In general, it is difficult to extract the absolute value of CrI of biomass samples from the XDR 1-D powder data without additional aid from computer simulation. Many studies analysed biomass CrI from the XRD data and found it increased after steam explosion pretreatment due to removal of amorphous hemicellulose. However, cellulose CrI, obtained by dividing biomass crystallinity with cellulose content in the biomass, reflects real changes in cellulose crystalline structure during pretreatment. Cellulose CrI decreases after pretreatment compared with that of untreated biomass samples. This indicates that the pretreatment disrupts native cellulose crystalline structure.

It has been shown that highly crystalline cellulose is less accessible to cellulase attack than amorphous cellulose and that cellulose accessibility to cellulase is one of the most limiting parameters in enzymatic hydrolysis when the effect of lignin is minimized.

Biomass pretreatment apparently decrease the biomass crystallinity index and hence improved biomass digestibility [3.5]. The crystallinity index of the pretreated biomass decreased with increasing biomass particle size, which also explains why larger biomass particle size had a higher sugar conversion. Lignin and lignin-like compounds have been considered as another major impeding factor in enzymatic hydrolysis due to mostly non-productive binding to cellulase.

Sugar conversion follows the typical hydrolysis pattern, with a rapid rate at the beginning of enzymatic hydrolysis, and then the rate slows down [3.5]. It is hypothesized that the initial enzymatic hydrolysis rate is a function of enzyme accessible surface area, while the slowdown of hydrolysis in the later stages is due to the difficulty of hydrolysing highly crystalline part of cellulose. The relation between biomass particle size and hydrolysis rate differs according to pretreatment process, feedstock preparation and style.

Identification of the relative importance of these factors to enzymatic hydrolysis is complicated by coupling effects between them [3.6]. For example, lignin removal has been shown to facilitate cellulose crystalline structure transformation during pretreatment; both of them contribute to enhanced enzymatic digestibility.

Cellulose CrI does not show apparent correlation with sugar conversion while biomass CrI is negatively correlated with sugar conversion with a linear regression coefficient of about 0.5 [3.6]. Lignin content is negatively correlated with sugar conversion, with a similar linear regression coefficient. Furthermore, it is found that the product of biomass CrI and lignin content correlates negatively with sugar conversion even better than that of crystallinity or lignin content alone. This indicate that there is a relation between these two factors, as studies showed that removing lignin from the plant cell walls facilitated transformation of cellulose crystalline structure.

Pretreatment deconstructs the plant cell walls and causes dissociation of the macromolecules [3.6]. Removal of hemicellulose and relocation of lignin during steam explosion pretreatment increases specific surface area, however, most of the lignin still remains in the pretreated solid residues that can result in non-productive enzyme adsorption. Therefore, the final sugar conversion is determined by a balance between these two factors. It is generally believed that increased SSA leads to improve enzyme accessibility. It should be mentioned that higher affinity of the biomass for cellulases does not necessarily result in increased saccharification due to the changes in chemical structures of lignin or cellulose crystalline structures. Variations of lignin content or biomass CrI disrupt plant cell walls, also leading to changes in SSA.

3.3.4 Time course of enzymatic hydrolysis

During cellulose hydrolysis, the combined action of endoglucanases and cellobiohydrolases produce changes in the solid substrate features and in cellulose accessibility that results in rapid changes in hydrolysis rates [3.34]. The rate and extent of the enzymatic hydrolysis of lignocellulosic substrates are highly influences by enzyme loadings, hydrolysis time and physical characteristics of pretreated substrate.

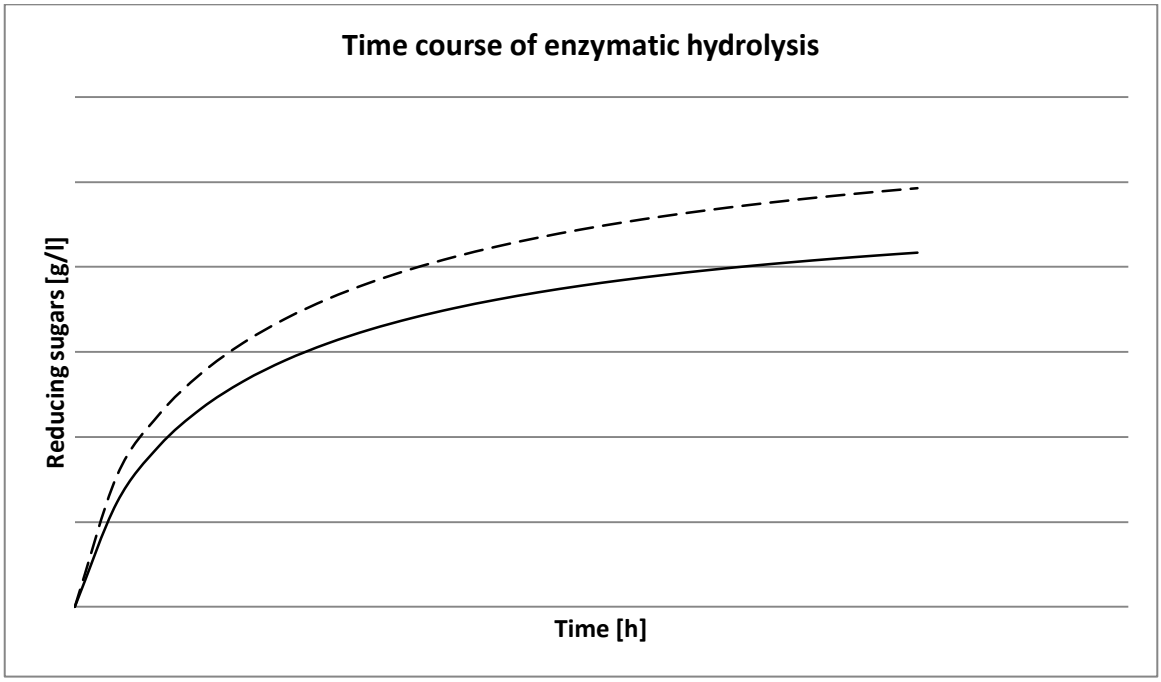
Typically, the rate of cellulose hydrolysis by enzymes decreases rapidly with conversion, leading to decreased yields, long processing times, and high enzyme usage. During the course of enzymatic hydrolysis of a pretreated lignocellulosic substrate, after an initial rapid phase, the hydrolysis rate decrease rapidly. The reaction rate continuously declines as the conversion percentage of cellulose increases and, in most cases, as a result of an incomplete hydrolysis, a recalcitrant cellulosic residue remains. Possible causes may be enzyme inactivation due to thermal effects, inhibition by hydrolysis products formation of an inactive enzyme substrate complex cellulase, substrate transformation into a less digestible form, and/or the heterogeneous structure of the substrate.

The specific rate of lignocelluloses hydrolysis, at a given percentage of conversion, also decreases with increasing lignocelluloses concentration [3.34]. However, the economy of biorefinery based on lignocellulosic materials is improved at higher substrate loadings due to reduced capital cost for smaller reaction vessels, decreased water usage and the higher sugar concentration after saccharification. Performing the saccharification reaction at high levels of insoluble solids creates a number of process-related problems associated with enzymes-substrate mixing and cellulase effectiveness such as sugar inhibition. At increased levels of solids, the ability of the enzyme to reach the reaction site is reduced and, at the same time, sugar inhibition is higher due to the increasing difficulty in diffusion of sugars away from catalytic site.

Experimental enzymatic hydrolysis time courses can be fitted with the double quadratic hyperbola [3.2]:

$$reducing\ sugar\ concentration\ [\frac{g}{l}] = \frac{a \cdot time\ [h]}{b + time\ [h]} + \frac{c \cdot time\ [h]}{d + time\ [h]}$$

The graph below represent the behaviour of the reducing sugars concentrations of two pretreated substrates. The straight line represents the steam explosion pretreated substrate enzymatic hydrolysis without acid addition, while the dotted line is represents the SE pretreated substrate with acid addition.



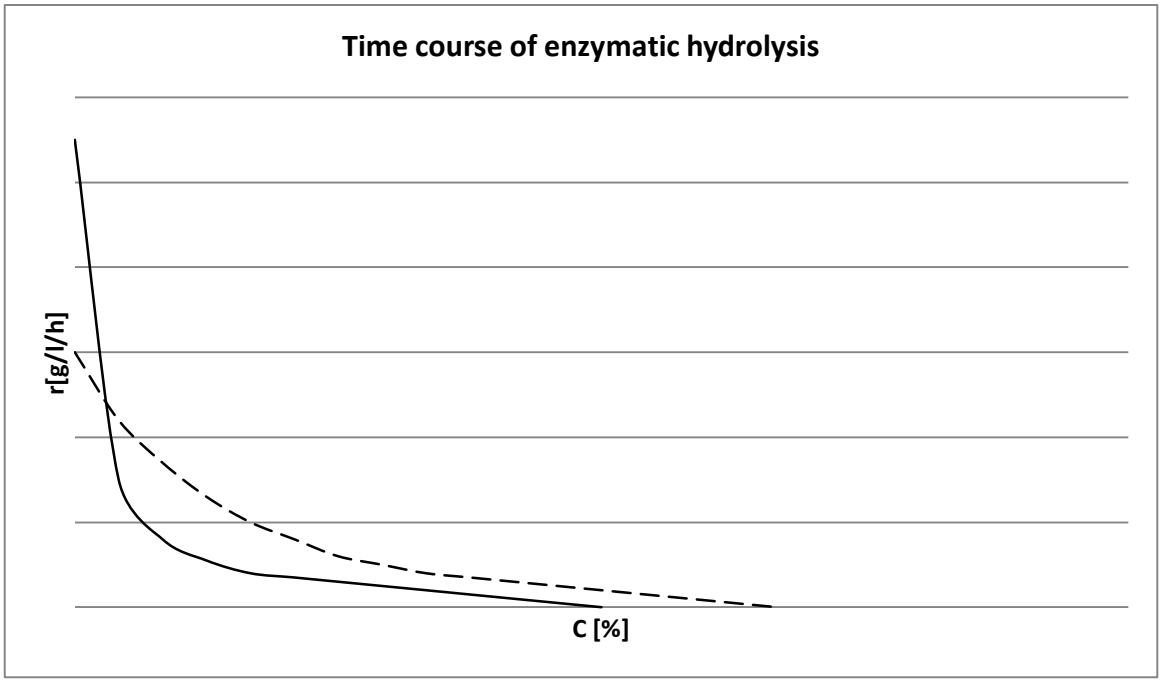
The volumetric hydrolysis rate r [g/l/h] is the behaviour of the conversion process in time. The time span can be chosen in an opportune way, but usually it is in the order of 10^{-1}h. The formula that describes this parameter is:

$$r = \frac{\Delta reducing\ sugar\ concenration\ [\frac{g}{l}]}{\Delta time\ [h]}$$

The reducing sugar concentration at each time step defined the saccharification. It is expressed by S and it is measured in moles [M]. This parameter is used to introduce another key factor of the process:

$$\mathrm{C}\ [\%] = \frac{\mathrm{S}\ [\mathrm{M}]}{\mathrm{sugar}_{\mathrm{substrate}}[\mathrm{M}]} \cdot 100\%$$

It is the conversion of the sugars portion present in the starting lignocellulosic substrate. The relation between C and r can be described roughly by a third-order polynomial curve, showed in the graph below:



The straight line represents an enzymatic hydrolysis of a steam explosion pretreated substrate without acid addition, while the dotted line is represents an enzymatic hydrolysis of a steam explosion pretreated substrate with acid addition.

Auto-catalysed SE pretreatment results in lower R-values. High concentration of acid during pretreatment has as consequence a high removal of hemicellulose, that is crucial to have a high initial rate of conversion. At the same time this is not beneficial to the continuity in time of the conversion rate, that decrease rapidly. Low acidic conditions have r-values much more constant.

Thus there is an optimal condition that has to be defined with reference to substrate composition, processing time and space.

High saccharification within short hydrolysis time is crucial for industrial exploitation. High concentrations of formic and acetic acid during hydrolysis step could be connected to the rapid slow-down of the hydrolysis rate. Acetic and formic acids are generated during pretreatment by degradation of furfural or acetyl groups. Both compounds were shown to inhibit the hydrolysis rate of lignocellulose. It can be assumed that high concentrations of carboxylic acids alter the pH value of the reaction mixture, thereby inactivating cellulases.

3.4 A comparison of different physicochemical pretreatment methods

Pretreatment method	Operating temperature	Operating pressure	Major effects	Advantages	Disadvantages
Steam explosion	160-280°C	7-48bar	- Remove of hemicellulose - Alteration of lignin structures	- Cost effective - High yield of accessible carbohydrates	- Partial hemicellulose degradation - Acid catalyst needed to make the process efficient - High energy consumption - Toxic compounds generation
Liquid hot water	160-240°C	>1bar	- Remove of hemicellulose - Partial remove of lignin	- No catalyst needed - Low costing	- High energy/water input - Corrosion (even mild) - Toxic compounds generation
Dilute acid hydrolysis (<5% w/v)	120-210°C	~1bar	- Remove of hemicellulose	- Selectively remove of hemicellulose - Low acid consumption	- High energy consumption - High cost for equipment - Toxic compounds generation
Concentrated acid hydrolysis (<30% w/v)	<100°C	~10bar	- Remove of hemicellulose - Partial solubilisation of cellulose - Alteration of lignin structure	- High sugar yield	- High costs for acids - High cost for equipment - Toxic compounds generation
Alkaline hydrolysis	20°C-150°C	1-30bar	- Partial remove of hemicellulose - Decrystallization of cellulose - Remove of lignin	- Selectively remove of lignin - Low formation of toxic compounds - Low costs of catalyst - Low energy demand	- Long retention time (for low temperature)

4. Optimization methods

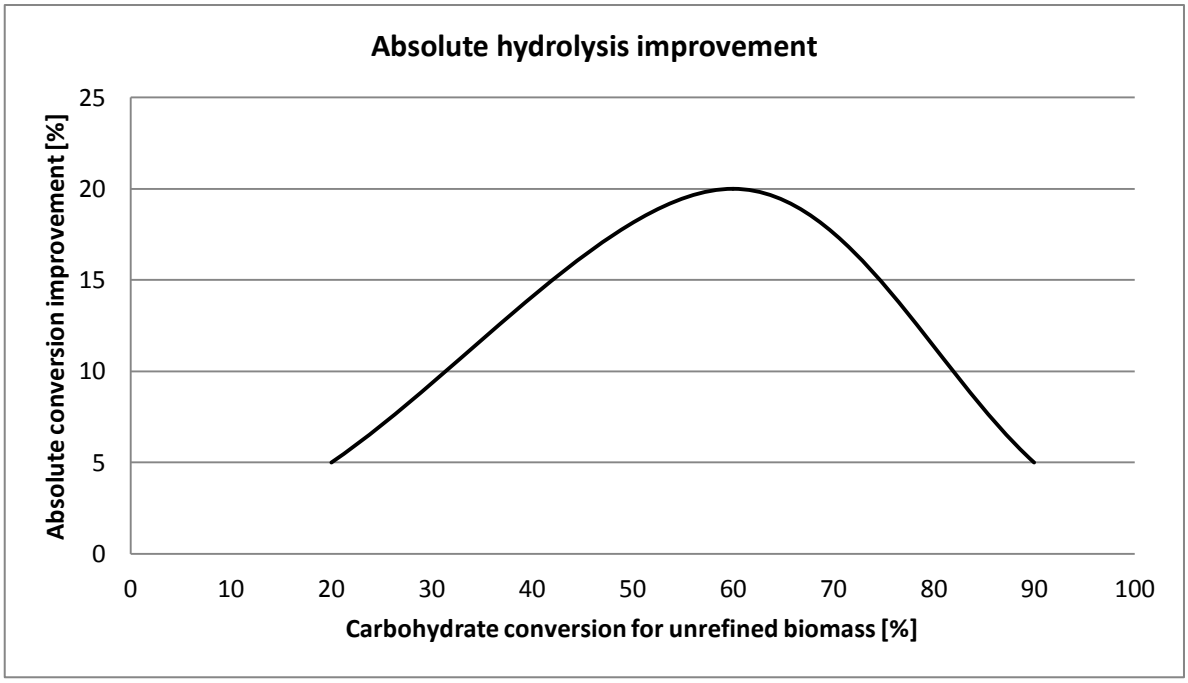
The optimization of the processes, to ensure the maximum substrate utilization, is based on four parameters [4.2]: the recovery of cellulose in the water insoluble fraction, the susceptibility of cellulose to enzymatic hydrolysis, the recovery of hemicellulosic sugars in the prehydrolisate and the low formation of degradation products. The operating conditions that maximize each of these parameters are usually different and, even, opposite. Therefore a compromise among them should be reached. At temperature below 150°C, there is no release of organic acids from the lignocellulosic biomass and the autohydrolysis is not able to significantly modify the cellulose and lignin matrix, although a large fraction of hemicellulose can still be converted in oligosaccharides. On the other hand, the exothermic degradation of cellulosic and hemicellulosic sugars, due to the xylose ring of the hemicellulose, begins at temperatures above 195°C. Thus the effective hydrolysis of carbohydrates is in conflict with their excessive degradation.

4.1 Mechanical refining

Recently, mechanical refining has been proposed as viable process that can overcome the biomass recalcitrance [4.1]. However, this kind of mechanical treatment requires high energy consumption, but still relatively low with respect to the entire biorefinery process. Refining is an established technology in the pulp and paper industry, and its aim is to separate fiber bundles and to create high surface area by fibrillation of cell wall structures. This structural and morphological alteration of fibers can also be beneficial for biomass conversion processes. There were several types of refiners utilized in the history of the pulp and paper industry, but disc refiners are the most modern. Disc refiners are composed of three components: a rotating disc plate that is connected to an electric motor, a stationary disc plate and a housing that enclose these disc plates. It can operate at ambient pressure or in overpressure.

Mechanical stress on cellulose fibers during the refining process can induce alterations to the fiber structure and morphology. Mechanical refining systems have been widely used in the past to increase the specific area of wood fibers through the combination of targeted fibrillation and delamination of cellulose fibers. The same technology can be directly applied to biomass deconstruction to increase enzyme accessibility and the subsequent carbohydrate conversion efficiency. As consequence to the application of this first step, pretreatment severity can be reduced, resulting in minor costs of chemicals and their recovery. Lower severity means also lower inhibitor compounds concentration that allows fermentation to be more effective. Thus, enzymes utilization can be reduced since substrates are more readily digestible, with benefits on economy of the process.

Analysing the hydrolysis data, there is a maximum in the relative yield improvement of carbohydrate conversion after refining.



The maximum point and the width of the curve are highly dependent on the type of biomass, the pretreatment conditions and the enzymatic hydrolysis conditions. Hence to evaluate the effectiveness of the mechanical refining, a specific refining reactivity study is needed.

Furthermore, mechanical refining technology is versatile, it can be installed in conjunction with any type of process scheme and it is a commercially proven technology for high process flows of biomass. Despite being identified for its potential in combination with chemical pretreatment, the use of mechanical refining in biochemical conversion processes is still in initial stage.

Recently, the use of refining to fibrillate the fibers following autohydrolysis has shown to improve sugar recovery considerably and make the overall process more viable [3.18].

4.2 Steam explosion optimization

4.2.1 Two stage steam explosion pretreatment

It is demonstrated that the higher the operating conditions (temperature and time) the higher was the severity factor, resulting in a higher recovery of cellulose and lignin, which enhanced the accessibility and digestibility of cellulose [3.9]. However, this recovery has an expense of increasing the destruction and degradation of hemicellulose. The optimum treatment severity of each of the polymer fractions involve a two stage treatment cycle, with the removal of the bulk of the hemicellulose and lignin at low severity and a post-treatment to obtain the cellulose in high purity though at a low degree of polymerization.

Some tissues or cells, such as thin-walled cells that easily hydrolyse, will be excessively degraded because of the structural homogeneity of lignocellulose, and some inhibitors will be generated under same pretreatment conditions [4.5]. Results showed, compared to one-step steam explosion, two-step SE with an intermediate separation of fiber cells (ISFC) can

increase enzymatic hydrolysis by more than 10%, reduce inhibitor conversion by 30% and increase fermentation products.

Lignocellulosic material possesses the characteristics of a complex, heterogeneous and multi-level structure. From the cell composition perspective, it includes fiber cells (FCs) and parenchyma cells (PCs) (including catheters, thin-walled cells, and epidermal cells) [4.5]. Different pretreatment conditions are required due to the differences in structure and in morphology between the two cell types. FCs, with high degree of lignification and compact structure, have high heat and mass transfer resistance and are hard to break. On the other hand, thin walled cells are sensitive to heat and mass transfer and are easily torn physically. Pretreatment conditions for the different tissues and cells should be optimized accordingly to achieve the best hydrolysis effect and to minimize simultaneously side reactions, this suggests two-step SE pretreatment, with the first step performed under mild conditions to hydrolyse PCs and PCs, and the second step, in which just difficult-to-hydrolyse FCs from the first step is pretreated again under normal conditions to increase the enzymatic hydrolysation and reduce the generation and concentration of inhibitors.

Zhang et. al (2012) [4.5] proposed a two-step SE combined with an intermediate separation of fier cells to optimize fermentation of corn straw hydrolysate. The conditions in the first step were chosen to give a high recovery of hemicellulose-derived fermentable sugars and a low concentration of inhibitors in the liquid. In the second step, FCs separated from the first step were pretreated again under normal conditions to enhance enzymatic digestibility. The two-step SE combined with ISFC process was optimized with respect to the total inhibitor conversion, enzymatic hydrolysation of materials and conversion ratio.

4.2.2 Chemical addition to feedstock materials

It was assessed that application of increasing acid concentration in SE pretreatment results in increased removal of hemicellulose. In addition is detected an increase in concentration of acid insoluble lignin. The formation of extraneous polymeric materials by condensation reactions can cause an apparent increase in the overall recovery yield of lignin, sometimes beyond the theoretical calculation based on the lignin content of starting material. Pretreatment caused a degradation of particles into smaller fragments [3.2]. It is found that increasing acid concentrations gradually reduced particles sizes. Is known that a reduction of particle size in pretreatment is associated with an increase in specific and accessible surface area.

H_2SO_4 and SO_2 have been largely employed to enhance enzymatic hydrolysis, decreasing the production of inhibitory compounds, leading to more completely removal of hemicellulose [3.1]. SO_2 is used in gaseous phase, because it is more effective than H_2SO_4 in terms of diffusion in lignocellulosic materials and it has a better recyclability. SO_2 is not the actual catalyst: it is transformed in sulfuric acid trough oxidation or disproportionation reactions. SO_2 acts also as a weak acid by dissolving in the inherent water in cell wall to form sulphurous acid and then attack lignin. Nevertheless, H_2SO_4 impregnation results in higher sugar yields that SO_2 impregnation. Therefore higher concentrations of inhibitor compounds were observed in the H_2SO_4-impregnated hydrolysate. This is caused by the

higher lignin removal acted by sulfuric acid. Using herbaceous biomass, sugar yields are more similar.

Substrates pretreated by steam explosion without acid or at low acid concentrations, remained in a fibrous shape, while high acid concentrations resulted in a granulate-like morphology [4.2]. Increasing pretreatment severity causes a separation of cell types and individual fibers, a fragmentation of biomass particles and an accumulation of droplet-like structures on the particle surface. These structures most likely represent lignin droplets formed by condensation reactions during pretreatment.

Application of increasing acid concentrations in pretreatment resulted in a darkening of the substrates [4.2]. It has been related to a break-down of lignin and extractives or sugars caused by high temperatures and to the relocation of lignin onto the particle surface. The colour change is also accompanied by a partial defibration or separation of individual fibers and cell types of the substrate.

4.3 Lignin removal

Unlike the other two major components in lignocelluloses which are released as monomeric carbohydrates, lignin is composed of three different phenylpropane monomers [4.7]. It contributes as much as 30% of the weight and 40% of the energy content of lignocellulosic biomass. As seen up here, lignin limits the conversion of biomass into bio fuels, but its native structure allows its versatility of applications and other value-added applications are also being developed. Since the bioconversion of fractionated cellulose to biofuels is not economically competitive due to the little use of other biomass components, the effective utilization of lignin offers a significant opportunity for the commercial operation of a lignocellulosic bio refinery. Extraction and separation of lignin is proved to be positive to the conversion of hemicellulose and cellulose to bio fuels.

Alkaline extraction is a relatively easy and efficient process to obtain lignin from lignocellulosic biomass compared with other solvent extraction due to the high solubility of the polymer in alkali [4.7]. Generally lignin is depolymerized in a heated alkaline medium and dissolved in the liquid.

Lignin alkaline extraction is a process that includes the NaOH transfer from the solution to the solid surface, heterogeneous chemical reaction and the product transfer from the solid raw material into the solutions [4.7]. Alkaline extraction is a first order reaction and the change rate of lignin removed per unit mass of raw materials is related to the amount of lignin remaining in the solids and the concentration of sodium hydroxide. Thus, the following apparent kinetics mechanism for dissolved lignin is proposed:

$$\frac{dL}{dt} = k \cdot (L_e - L) \cdot S$$

Where the term on the left is the lignin extraction rate, k is the chemical reaction rate constant, Le is the equilibrium concentration of total lignin in the liquid extract [g/L], L is

the dissolved lignin concentration at a given extraction time t [g/L], and S is the concentration of NaOH at the same time t [g/L].

The product of k and S can be replaced by a new constant k1 and the model is rewritten as:

$$\frac{dL}{dt} = k_1 \cdot (L_e - L)$$

Carrying out the integral, with time from 0 to t, the equation becomes:

$$L = L_e[1 - exp(-k_1 \cdot t)]$$

the relationship between k1 and extraction temperature T can be numerically characterized by the activation energy for linearized Arrhenius equation:

$$\ln(k_1) = ln(A) - (1/T) \cdot (E_a/R)$$

Where A is a pre-exponential factor; Ea is the activation energy for the lignin extraction [kJ/mol], R is the gas constant whose value is 8.314 J/(mol K), and T is the absolute temperature [K]. The slope of the straight line (-Ea/R) is obtained by plotting ln(k1) versus 1/T and the activation energy is calculating using the slope.

For untreated biomass, these equations descript in a good way the phenomenon of lignin extraction. In the case of steam exploded pretreatment, this alkaline extraction kinetics model is not suitable for the simulation of lignin extractions [4.7]. Obviously the physicochemical changes of the biomass during the steam explosion lead to the variation of lignin extraction process. As described, steam explosion includes two effects: high temperature cooking and instantaneous expansion force on raw materials. Firstly, the instantaneously expansion force causes the enhancement of specific surface area of the fibrous materials which increases the exchange area between the solid and the alkali liquor. Secondly, the reaction of lignin during the high-temperature cooking may also change its structure and facilitates its dissolution into liquid which is similar to the process in alkaline extraction. In this case, the extraction of lignin is mainly simple dissolution of lignin from the solid to the liquid phase. This process can be described by the second-order rate law based on the extraction of solvent-soluble compounds from ground plant tissues:

$$\frac{dL}{dt} = k_2 \cdot (L_e - L)^2$$

Where k2 is the second-order extraction rate constant [L/(g min)] and the meaning of the other letters are the same seen before. After the integration, the second-order extraction kinetics can be present as a linearized equation:

$$L = \frac{k_2 \cdot L_e^2}{t \cdot (1 + t \cdot k_2 \cdot L_e)}$$

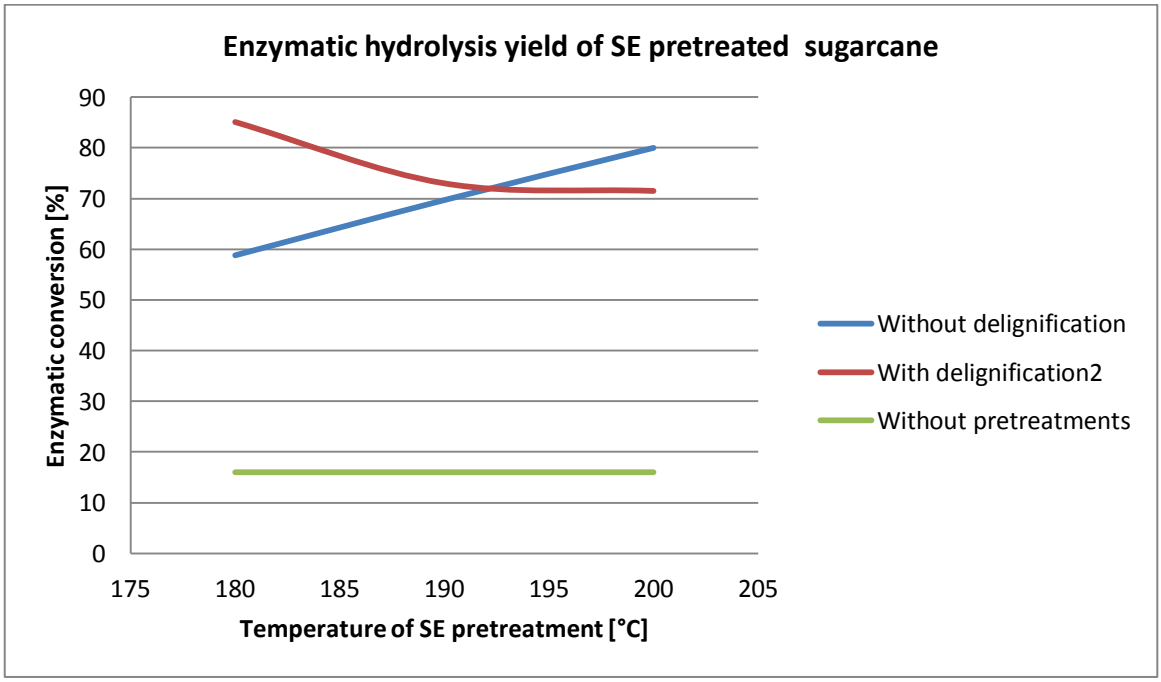
The increase of k2 with increasing temperature also could be described by the Arrhenius equation. The good linear relationship of ln(k2) versus inverse of absolute temperature (1/T) demonstrates that the second-order model can satisfactorily simulate the extraction kinetics of lignin from steam-exploded biomass. The activation energy of lignin extraction from steam exploded biomass is estimated from the slope of the plot lnk2 versus 1/T, which

decreases by several percent compared with that of lignin extraction without steam explosion.

The experimental studies of Wang et. al (2016) demonstrates that a first order model could readily simulate the lignin extraction of untreated biomass, but for steam exploded biomass, a second order model is necessary [4.7]. Their results shows that milder extraction conditions were available to extract lignin from steam-exploded biomass.

The addition of an oxidant agent to alkaline pretreatment, can improve the performance by favouring lignin removal [3.29]. Hydrogen peroxide is commonly used in papermaking. Alkaline peroxide method provides an effective for delignification and it is considered to be a promising approach for the low-energy pretreatment of lignocelluloses under mild conditions. The principle of this method relies the formation of hydroperoxide anion in alkaline media. The oxidative action of the hydrogen peroxide derived radicals is thought to contribute to the depolymerisation of lignin by attacking lignin side chains and fragmenting the lignin macrostructure into a number of low molecular weight compounds. However, in this process pH is one of the most important parameters for the efficient application of peroxide. In fact, depending on the pH adopted during lignin oxidation, no significant changes in the chemical structure might be observed.

Hydro peroxide (H_2O_2) use has been studied by several researchers [3.4]. The purpose of the pretreatment with HP is the delignification: HP is a chemical oxidizing which is common used as disinfectant. Its oxidizing ability allows to detach and solubilize lignin, loosening the lignocellulose matrix, increasing the amount of cellulose available for the hydrolysis by enzymes. Hydroperoxyl and hydroxyl radicals which are generated by decomposition of HP initiate delignification. The use of HP is considered "environmental friendly" and the chemicals costs, compared to the use of other effective pretreatment chemicals, are lower. The main disadvantage is that the high oxidative degradation of lignin by HP can lead to the accumulation of aliphatic, aldehydes and phenolic inhibitory products.



Oliveira et al. (2013) showed the benefit of a combination between SE and alkaline delignification before the enzymatic hydrolysis [4.3]. They run the hydrolysis in three ways: an enzymatic hydrolysis without any type of pretreatment, an enzymatic hydrolysis after a SE pretreatment and an enzymatic hydrolysis after a SE pretreatment and an alkaline delignification.

Lignin solubilisation reach the maximum at the minimal temperature (18=°C) of steam explosion. Increases pretreatment temperature doesn't lead to further delignification. Probably, this phenomenon might be associated with the impossibility of the alkaline treatment to remove pseudo lignin formed at temperatures. This indicates that an efficient alkaline delignification can be achieved after steam explosion at moderate temperature, and extremely severe pretreatment conditions can be avoided without affecting delignification degree. This high solubilisation degree is a consequence of the increased susceptibility towards alkaline delignification exhibited by materials pretreated by hydrothermal methods.

In parallel with delignification, the alkaline treatment also led to a significant solubilisation of hemicelluloses. Considering the amount of hemicelluloses that had already been solubilized in the pretreatment, hemicellulose solubilisation during delignification was directly proportional to the residual amount of that component in the cellulignin.

Cellulose was also partially solubilised during delignification, although at a lower extent than during pretreatment.

The enzymatic conversion after the SE pretreatment rise proportionally with the increase of pretreatment temperature. The delignification step lead to a significant enhancement of the enzymatic hydrolysis. However the effect of delignification is not similar for all the pretreatment conditions. The improvement of enzymatic hydrolysis decrease from modest to worse than the enzymatic hydrolysis without delignification step. A hypothesis for explaining this unexpected fact could be related to an increase of recalcitrance of the material as consequence of the drastic conditions of pretreatment and delignification. It could also be hypothesized that the pseudo-lignin formed by pretreatment at severe conditions, and apparently not removed by the alkaline treatment, interferes with the enzymatic hydrolysis.

Thus, the best options to applying SE to sugarcane straw are a SE pretreatment at low temperature with subsequent delignification (saving the energy the required by higher temperature) or a SE pretreatment at high temperature without delignification (saving all costs of the whw3ole delignification stage). Additionally, at high temperatures, hemicelluloses will completely be solubilized, and the sugars can be recovered in the pre-hydrolysate.

4.4 Ozone pretreatment

Ozone is the strongest commonly available oxidant: it oxidation potential is higher than hydrogen peroxide and oxygen [4.8]. It is capable of reacting with many inorganic and organic substances. Ozone has several usage in the daily world. Furthermore it can be used a biomass pretreatment method thanks to its property of carbon-carbon double and triple bonds cleavage.

Among the chemical oxidants, ozone is highly reactive towards compounds with functional groups with high electron densities, such as those present in lignin [4.8]. The degradation of lignin increases, as seen, the accessibility to the cellulose fraction. Moreover, ozone is a very selective oxidant, and no significant carbohydrate losses occur. Another advantage is that ozonolysis is carried out at room temperature and pressure. On the contrary, high dosage of ozone can be necessary, so operating conditions have to be optimized to be cost-effective.

Ozone reacts mainly due to two mechanisms [4.8]: direct reactions with molecular ozone and indirect reactions with the radical species that are formed when ozone decomposes in water. Direct reactions are selective and limited to unsaturated aromatic and aliphatic compounds as well as specific functional groups. Due to its dipolar structure, the ozone molecule may lead to 1-3 dipolar cycloaddition on unsaturated bonds, with the formation of ozonides according to the Criegee mechanism. Ozonides further decompose into carbonyl compounds (aldehyde or ketone) and hydrogen peroxide. The electrophilic nature of ozone also provokes its reaction with molecular sites with a strong electronic density, such as aromatic compounds. Aromatics substituted by electron donor groups (hydroxyl or amine groups) are highly reactive with ozone. On the contrary, aromatics substituted by electron withdrawing groups (-CCOH, -CN, -NO_2) are weakly ozone reactive. The attack of ozone leads to the formation of quinoids that further react according to the Crieege mechanism, resulting in the opening of the aromatic cycle and the formation of aliphatic products with carbonyl and carboxyl groups. Direct reactions with ozone are dominant under acidic conditions and in the presence of radical scavengers. The more common inhibitors of the free-radical reactions are bicarbonate and carbonate ions, phosphate, alkyl groups, and tertiary alcohols. At high pH values and in the presence of hydrogen peroxide, UV radiation, or other compounds (metals, metal oxides, formic acid, aryl groups), ozone decomposes, giving radical species, which are highly unstable molecules with an unpaired electron. Ozone decomposition in water involves a series of single electron and atom transfer processes and the intermediacy of hydroxyl radicals. Under these conditions, indirect and nonselective radical reactions prevail. The very high reactive hydroxyl radical (OH) is the common species in these systems together with hydrogen peroxyl radicals (HO2). The mechanism of the reaction of ozone with a substance may involve both direct reactions with ozone and indirect reactions with OH radicals. This is because promoters of free-radical reactions such as hydrogen peroxide are formed in the course of direct ozonation processes.

Ozone is a promising reagent for the oxidation of lignin due to its reactivity with aromatic and unsaturated compounds [4.8]. The most substantial effect of ozone is on lignin degradation. Hemicellulose can also be slightly degraded through nonselective ozone reactions. As the lignin content decreases, the solubilisation of hemicellulose can increase because both compounds are solubilized together in a lignin-hemicellulose complex. Although glycoside linkages in cellulose can also be cleaved by ozone, cellulose is hardly affected. The removal of lignin from fiber surfaces by ozone pretreatment is expected to increase the reactivity of enzymes with carbohydrates in the fiber walls. Ozone pretreatment can also increase total pore volume as well as specific surface area, and this could improve the enzyme's access to the fiber walls. The first stage of the reaction of ozone with the aromatic ring of the lignin structure involves the electrophilic addition mechanism with a subsequent aromatic ring opening and formation of o-quinones and dicarboxylic acid

derivatives. Other reaction are also possible, such as the hydroxylation of aromatic rings of the coniferylic or p-coumarylic structures of lignin or the oxidative cleavage of the methoxyl groups. The reactions with carbon double bonds and the β-O-4 moieties are believed to be fast. The subsequent reaction of ozone with intermediate products occurred with a much lower rate.

As ozone degrades lignin, it weakens the adhesive strength between the cellulose microfibrils, which can improve the accessibility of the enzyme to cellulose and hemicellulose [4.8]. Lignin is degraded easily at the beginning of the reaction. However, there is a fraction of lignin that is difficult to remove. This could be explained because the remaining fraction of lignin could be located in positions that are less accessible to ozone. On the other hand, it could be a nonreactive lignin fraction that is difficult to remove under usual reaction conditions. Part of the lignin has a chemical structure condensed by the formation of the new carbon-carbon bonds as a result of the autohydrolytic process suffered, making it more resistant to ozone attack.

When most lignin has been already removed, the oxidation of holocellulose can start [4.8]. Ozone can cleave glycoside bonds, leading to the oxidation of functional groups to carbonyl and carboxyl compounds, lactones, and hydroperoxides. The degradation of carbohydrates can also be due to the formation of nonselective hydroxyl radicals. The main paths for hydroxyl radical formation are ozone decomposition reactions catalysed by the presence of metal ions and the direct reactions between ozone and lignin in the Crieege mechanism. The ozone reaction should be carried out under acidic conditions to reduce the formation of non-selective hydroxyl radicals. In any case, the ozone decomposition rate is usually low compared to the direct reaction of ozone with organic compounds when the pH<12.

In the lignocellulosic biomass pretreatment, ozone is usually used as a single oxidant [4.8]. However there are organic compounds that can act as protectors of cellulose. The mechanism can be explained as follows. First, transition metal ions on lignocellulosic materials promote radical reactions. The radical species, mainly hydroxyl radicals, can react very quickly with carbohydrates through nonselective reactions. These organic compounds act as scavengers of hydroxyl radicals, favouring selective ozone reactions with lignin. On the other hand, it the presence of these additives, cellulose swells less than when it is only impregnated with pure water. Therefore cellulose accessibility to the oxidizing agents decreases. Finally, additives improve ozone mass transfer by reducing the interfacial tension of the liquid phase and the size of the bubbles. These organic compounds are xalic aacid, tert-butyl alcohol, 1-butyl alcohol, diethyl ether, ethyl acetate, and acetic acid. The use of these additives on an industrial scale is limited because of their ccost and potential impact in further steps (enzymathic hydrolysis and fermentation) and process effluent.

The effect of ozone as a delignifying agent has the following characteristics over other chemical treatments [4.8]:

1. Degradation is essentially limited to lignin, although hemicellulose can be slightly attacked, and cellulose is hardly affected. The specificity of ozone toward lignin, in comparison with other delignifying processes, is a clear advantage, because it produces lower weight losses during pretreatment.

2. Ozone can be generated on-site as needed, thereby avoiding chemical supply problems in isolated areas, storage, transport costs, and the safety problems associated with shipping and handling.
3. Ozone generation does not require a large-scale application to be economically feasible. Its use is very attractive for local use with various types of biomass available in steady supply.
4. Reactions are carried out at room temperature and pressure, which reduces the capital and energy costs.
5. The atmospheric pollution impact of the process is minimal, because residual ozone can be easily decomposed to oxygen using a catalytic bed. In any case, the process has to be designed to optimize ozone consumption, so ozone leftover should be minimal.
6. Ozone does not leave strongly acidic, basic, or toxic residues in the treated material. Therefore, ozonated lignocellulosic materials can be used as animal feed.

On the other hand, ozone treatment led to the production of inhibition compounds for the enzymatic hydrolysis [4.8]; the inhibiting compounds can be divided into three groups: weak acids furan derivate, and phenolic compounds. Non-dissociated weak organic acids can penetrate microbial cells and decrease the intracellular pH. Lignin degradation products, such as phenolic compounds, can damage the microbial cellular membranes. Inhibitors can interact antagonistically on microbial growth. Cellulose conversion in enzymatic hydrolysis can also be affected by the presence of inhibitory compounds. Phenolic compounds can cause the deactivation of the enzyme and reduce the hydrolysis rate. Formic acid is known to inhibit saccharification.

Various researches had been carried out with the aim of optimizing the factors affecting the ozonation of lignocellulosic materials, such as moisture content, particle size, ozone concentration in the gas phase, reaction pH, and ozone dose (ozone applied per dried mass) [4.8]. The moisture content and ozone concentration were shown to be the most significant factors. As seen up to here, the optimal parameters are specific for the type of raw material. Ad hoc studies must be carried out to optimize the process.

4.5 Response Surface Methodology

Common in all pretreatment processes, severe pretreatment conditions can cause further degradation of cellulose and force it into the liquid fraction, which can reduce the glucose yield of the hydrolysis [3.24]. Therefore, suitable condition that can prevent excessive isolation of the cellulose fraction and lead to high elimination of the hemicellulose fraction should be investigated. In this respect, response surface methodology (RSM) with central composite design (CCD) has been considered as a proper tool for determining suitable condition to achieve the purpose.

In statistics, the experimental design is a methodology to collect the results of the experiment and to build up a model that relates several independent variables (x_i) with one or more dependent variables (y_i). It is relevant in those cases in which theory are not able to

explain the relation between the variables. This relation can be written as a function, with a noise, or error, ε in the response y [4.6]:

$$y = f(x_i) + \varepsilon$$

In case of two independent variables, x_1 and x_2, the response y is a surface, called response surface.

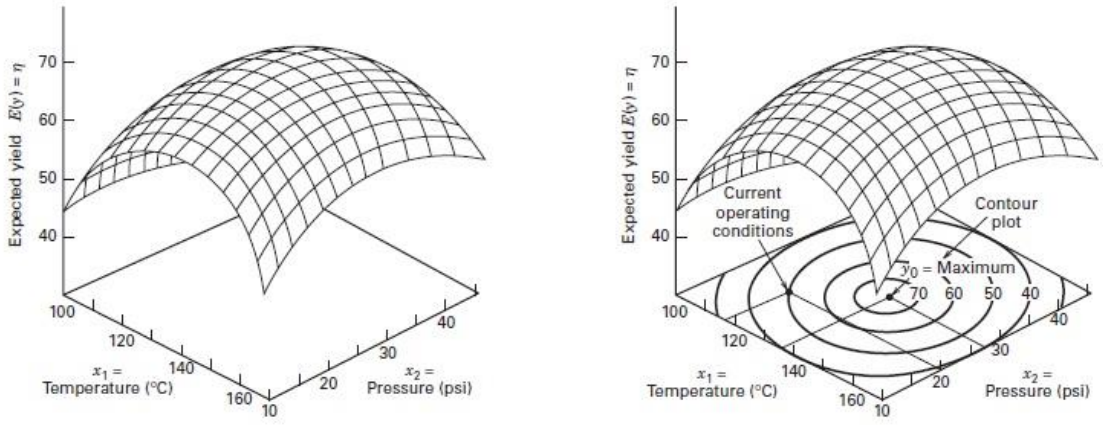


Figure 4.1 - Typical response surface graph [4.6]

To help visualize the shape of a response surface, it is useful to plot the contours of the response surface. In the contour plot lines of constant response are drawn in the x_1, x_2 plane. Each contour corresponds to a particular height of the response surface.

Response surface methodology is largely used to optimize pretreatment processes. Variables like temperature and reaction time, temperature and chemicals concentration, temperature and pressure, are usually used to establish the optimal conditions of the pretreatment methods.

If the response is well modelled by a linear function of the independent variables, the approximating function is the first-order model, in the form:

$$y = b_0 + b_1 x_1 + b_2 x_2 + \varepsilon$$

The definition of the coefficient can be done with the regression models.

If a quadratic polynomial is needed to represent the function, a second-order model is used, in the form:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2 + \varepsilon$$

It is unlikely that a polynomial model will be a reasonable approximation of the true functional relationship over the entire space of the independent variables, but for a relatively small region they usually work quite well.

To solve these equations, several methods are used and several software are been developed, such as Design Expert.

The method of least squares is used to estimate the parameters in the approximating polynomials. The response surface analysis is then performed using the fitted surface. If the fitted surface is an adequate approximation of the true response function, then analysis of the

fitted surface will be approximately equivalent to analysis of the actual system. The model parameters can be estimated most effectively if proper experimental designs are used to collect the data. Designs for fitting response surfaces are called response surface designs.

Frequently, the initial estimate of the optimum operating conditions for the system will be far from the actual optimum. In such circumstances, the objective of the experimenter is to move rapidly to the general vicinity of the optimum. When we are remote from the optimum, we usually assume that a first-order model is an adequate approximation to the true surface in a small region of the independent variable. Methods, such as steepest ascent/descent, are used to move in the direction of the optimum.

When the experimenter is relatively close to the optimum, a model that incorporates curvature is usually required to approximate the response. The central composite design (CCD) is a method usually used for fitting the second order models.

Then, an analysis of variance (ANOVA) of the model is carried out to evaluate the statistical significance of the model.

4.6 Improving enzymatic hydrolysis

The cost of currently available cellulase preparations is a major obstacle in the enzymatic biomass hydrolysis route to valuable products [4.9]. Many researchers have realized that advancements are possible in multiple directions, such like optimizing the hydrolysis process, enhancing the cellulase activities, optimizing the reaction conditions, enzyme and substrate cocktail composition, enzyme recycling and recovery strategies.

One interesting approach that has been recently developed is the optimization using enzymes from different sources and mixing in an appropriate proportion using the statistical approach of factorial design. Another researchers branch is directed to the development of enzymes that can tolerate both acid and heat. These enzymes are produced naturally by extremely thermophilic microbes or so called extremophiles.

The isolation of microbes producing stable hydrolytic enzymes which can work at higher temperature/pH is of great value at an industrial scale [4.10]. Also, the enzymes which can withstand organic solvents, metal ions, and high levels of radiation or high salt environment are very useful for industrial applications. The usefulness of these enzymes lies in range of industries ranging from pharmaceutical, food, textile, feed, starch, etc..

A largely used strategy to improve the efficiency of enzymatic hydrolysis is to improve the specific activities of cellulases by genetic engineering [3.39]. Non-productive bindings and inactivation of enzymes by lignin component are the most important limiting factors for lignocellulosic substrates. Mainly two strategies are used to improve the properties of individual cellulase components: rational design and direct evolution.

Rational design is the earliest approach to protein engineering. This strategy requires a detailed knowledge of protein structure. The first step in rational design involves the selection of a suitable enzyme. In the next step, the amino acid site to be changed will be identified on the basis of a high-resolution crystallographic structure. Finally, the resultant mutant will be characterized. The success is very difficult because the information of

structures and mechanism of the target enzyme is not available for a vast majority of enzymes. Even if the structure and catalysis mechanism of the target enzyme are well characterized, the molecular mutation basis for the desired function may not be achieved.

The second method, direct evolution, has the advantage to be independent of enzyme structure and of the interactions between enzyme and the substrate. The most important challenge of this method is developing tools to correctly evaluate the performance of mutants generated by recombinant DNA techniques.

While improvements in enzymes and process development are expensive and long term solutions, optimising the process operations to improve economic feasibility can develop a short term solution [4.10]. Considering the complexity and non-linearity of the process, model based optimization using systematic models is more appropriate than through heuristic strategies.

A part from non-productive adsorption due to lignin and inhibition by products of hydrolysis and degradation products of pretreatment, the amount of enzyme also reduces due to deactivation by temperature. The deactivation temperature for a given enzyme is dependent on the source of the enzyme. Therefore, only the enzyme available after deactivation takes part in adsorption as well as hydrolysis reaction. Representing enzymatic hydrolysis of cellulose by a kinetic model is essential to study the performance of the hydrolysis process and improving the same.

One of the major limiting features of the enzymatic hydrolysis is the slow rate of hydrolysis and hence longer retention times. Therefore, minimisation of the retention time was considered as the second objective of the optimal control problem.

In spite of intensive research efforts over the past decades, the enzyme hydrolysis step remains a major techno-economic bottle-neck in the lignocellulosic biomass conversion process.

5. Corn stover experimental conversions

One of more studied biomass is corn stover, due to its large diffusion in the world. The subsequent chapter shows the optimum of main parameters that characterize the conversion of corn stover into valuable sugars for the production of biofuels. To have a real sum-up of the behaviour of the yields and efficiencies is important to refer to a standard process. In absence of availability to test directly these behaviours, a collection of optimized parameters related to studies available in literature is shown.

These values are not absolute optimal values! The complexity of the processes shift the optimum point in relation to different variables, such as chemical composition of raw material, that as you can see in the next paragraph is not a constant, size of the raw material, and every feature that characterize the equipment, e.g. dimensions.

Enzymatic hydrolysis will be not treated: the variables are mainly two: the enzymes and the time of reaction. There are other two variables: temperature and agitation, but they are set to standard values (50°C and 150rpm) . Several types of enzymes, more or less effective, are commercialised. Usually the enzyme used in a study or in another study is due to availability, also economical. Time of reaction can be prolonged to infinity. It is clear that the more the reaction go on, the more it is complete. It will be a choose about cost, both of energy and of time, to interrupt the reaction at a given time. In laboratory scale, the economy of the process is set aside.

Starting from the data collected in the studies present in literature, some empirical formulas had been carried out with the aid of Microsoft Excel tools. To verify the validity of the model proposed, the R-squared index was used.

The coefficient of determination, also called R-squared (R^2), is a statistical measure that represents the proportion of the variance for a dependent variable that's explained by an independent variable or variables in a regression model.

$$R^2 = \frac{Explained\ Sum\ of\ Squares\ (ESS)}{Total\ Sum\ of\ Squares\ (TSS)} = 1 - \frac{Explained\ Sum\ of\ Squares\ (ESS)}{Residual\ Sum\ of\ Squares\ (RSS)}$$

Where, in the case of n values:

$$ESS = \sum_{i=1}^{n} (y_{pi} - y_m)^2$$

$$TTS = \sum_{i=1}^{n} (y_i - y_m)^2$$

$$RSS = \sum_{i=1}^{n} (y_i - y_{pi})^2$$

The observed data are y_i, their mean value is y_m and the predicted value, according to the defined model, in the i-th position is y_{pi}. R-squared ranges from 0 to 1: the more R^2 is close to 1, the more the proposed model follows the observed values.

5.1 Corn stover chemical composition

The first contradictory data is the chemical composition of corn stover. It differs for different varieties of corn, different climate and land characteristics, etc. The table shows the difference between several studies took into account for the subsequent paragraphs. A mean value is calculated in the last column.

	3.5	3.14	4.12	4.14	4.15	4.16	4.17	4.18	4.19	4.20	4.21	4.22	4.23	4.24	**Mean values**
Glucan	31.7	31.7	31.6	31.6	30.7	36.1	35.6	35.1	35.6	33.9	32.6	36.1	34.4	N.A.	**33.6**
Xylan	17.1	12.0	16.8	20.5	15.0	20.7	19.7	21.9	21.0	21.4	22.5	21.4	22.4	N.A.	**19.4**
Lignin	12.6	11.8	16.0	22.8	14.2	18.6	18.0	20.6	22.6	12.4	20.7	N.A.	N.A.	N.A.	**17.3**
Ashes	N.A.	0.9	3.9	8.7	8.8	N.A.	7.9	N.A.	8.7	1.7	4.1	N.A.	N.A.	N.A.	**N.A.**

5.2 Steam explosion optimization

5.2.1 Size optimization of steam exploded corn stover

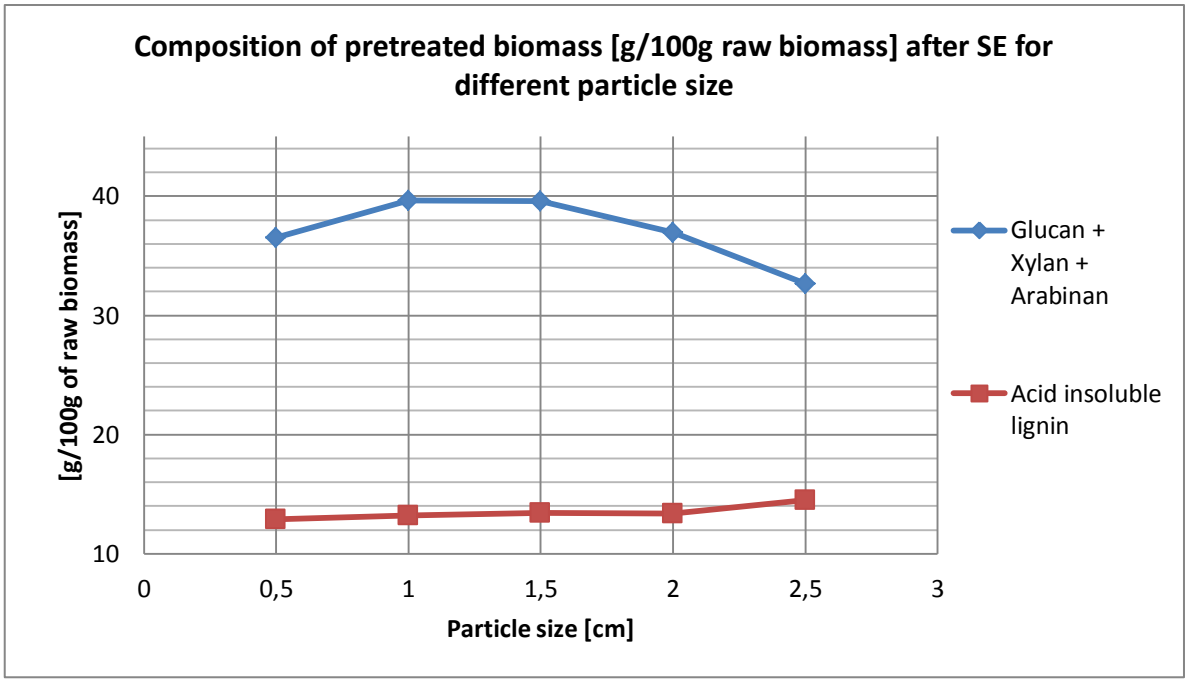
Liu et al. [3.5] carried out a study on the influence of chip size on the steam explosion performance. Corn stover, prior to be pretreated, was manually cut into different particle size: 2.5cm, 2.0cm, 1.5cm, 1.0cm and 0.5cm. Samples were sprayed with demi water to the moisture content of 30%.

The pretreated reactor system consists of a reactor chamber (15L working volume) and a reaction chamber (150L of working volume). During pretreatment, 150g of corn stover (dry basis) was top-loaded into the reactor chamber. High pressure steam was then filled into the reactor until the temperature reached 200°C (1.6MPa). After 5 minutes of exposure to the saturated steam, corn stover was exploded into the reception chamber by the ball-valve.

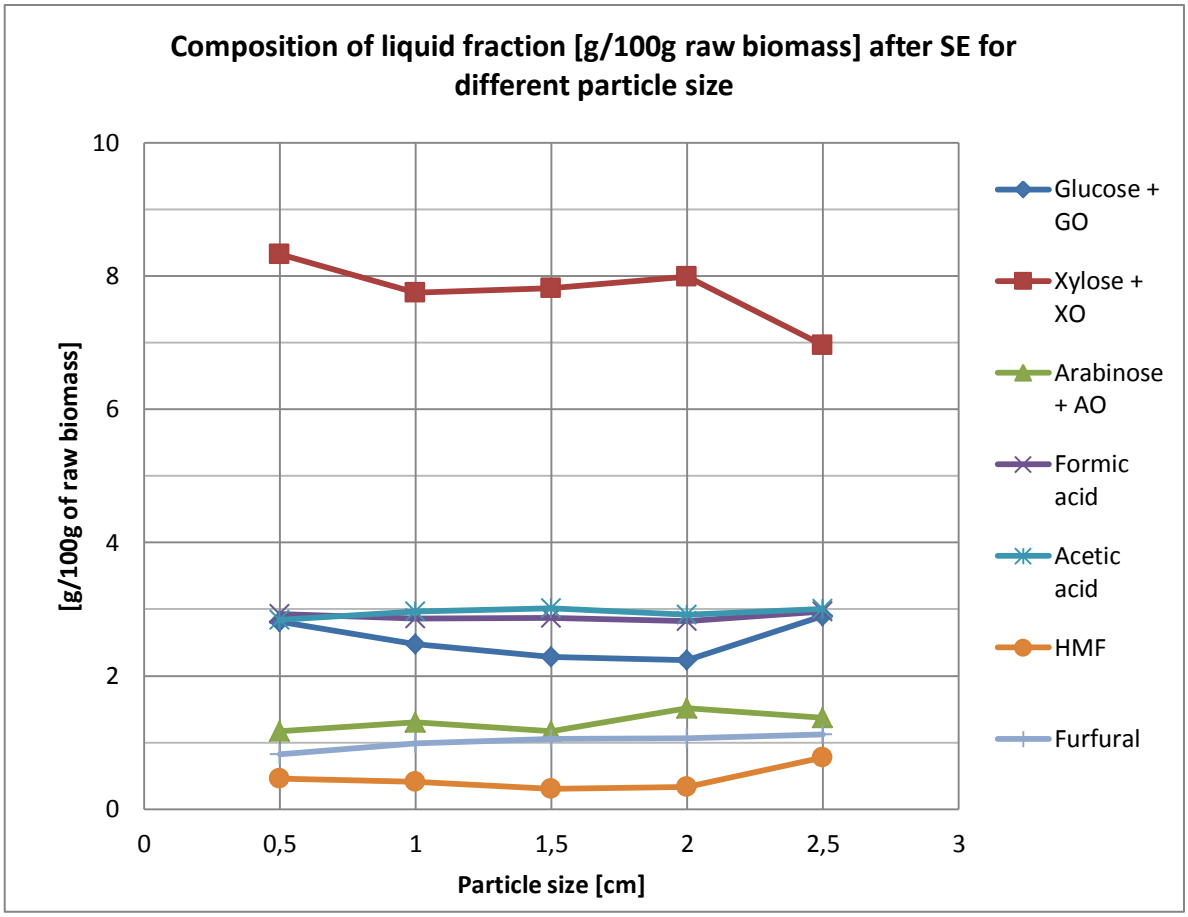
The enzymatic hydrolysis was carried out following the NREL standard protocol LAP-009. Commercial cellulase (Accellerase 1500) was used. The pretreated biomass was hydrolysed at a glucan loading of 1% or 6% (w/v) in a 0.05M citrate buffer solution (pH 4.8) with an Accellerase 1500 loading of 15 or 60 FPU/g glucan and a β-glucosidase loading of 64pNPGU/g glucan. Samples were hydrolysed at 50°C at 200rpm for 168h.

The composition of the pretreated biomass and liquid fraction with different particle sizes are showed in the graphs below. The mass recovery of pretreated biomass and their compositions were different depending on biomass particle size. The mass recovery of glucan and xylan increased with biomass particle size decreased from 2.5cm to 1cm but then slightly decreased for both glucan and xylan at particle size of 0.5cm.

Most of the arabinan was solubilized in liquid under all tested conditions. The lignin composition increased with increasing biomass particle size.



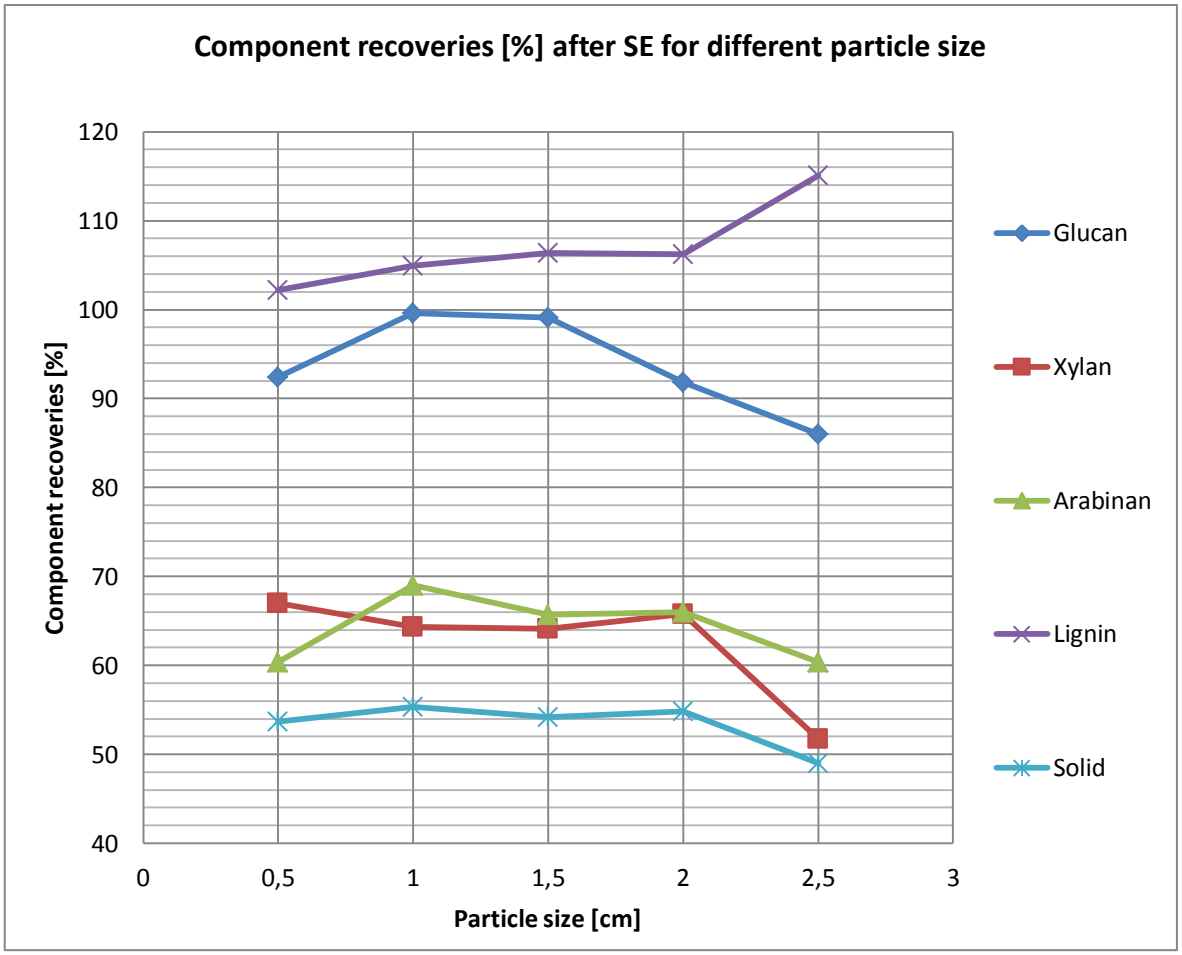
In liquid fractions, the concentration of soluble glucose (monomer plus oligomers) was found increasing with reduced biomass particle size except for the particle size of 2.5cm which gave the highest value of this concentration. Both xylose and furfural amounts decreased with decreasing biomass particle size, but xylose oligomer amount increased with decreasing biomass particle size. It was interesting to note that the conditions generating high amount of glucose and xylose in the liquid fraction typically also generated high amount of HMF and furfural. No significant differences of formic acid and acetic acid amount were observed among the feedstock with different biomass particle size.



As a result, the feedstock with particle size of 2.5cm lead to higher cellulose and hemicellulose solubilisation and degradation compared with the smaller particles at the same pretreatment conditions.

The graph below shows the component recoveries during pretreatment as a function of the biomass particle size. The recoveries of glucan and solid biomass increased with particle size reduced from 2.5cm to 1.0cm, but slightly decreased at 0.5cm. The xylan recovery increase of more than 15% with the decreasing of particle size, while the arabinan recovery decreased of almost 5%. These relatively low recoveries of xylan and arabinan might be explained by the hemicellulose solubilisation and degradation to by-products (e.g. formic acid and furfural) during pretreatment.

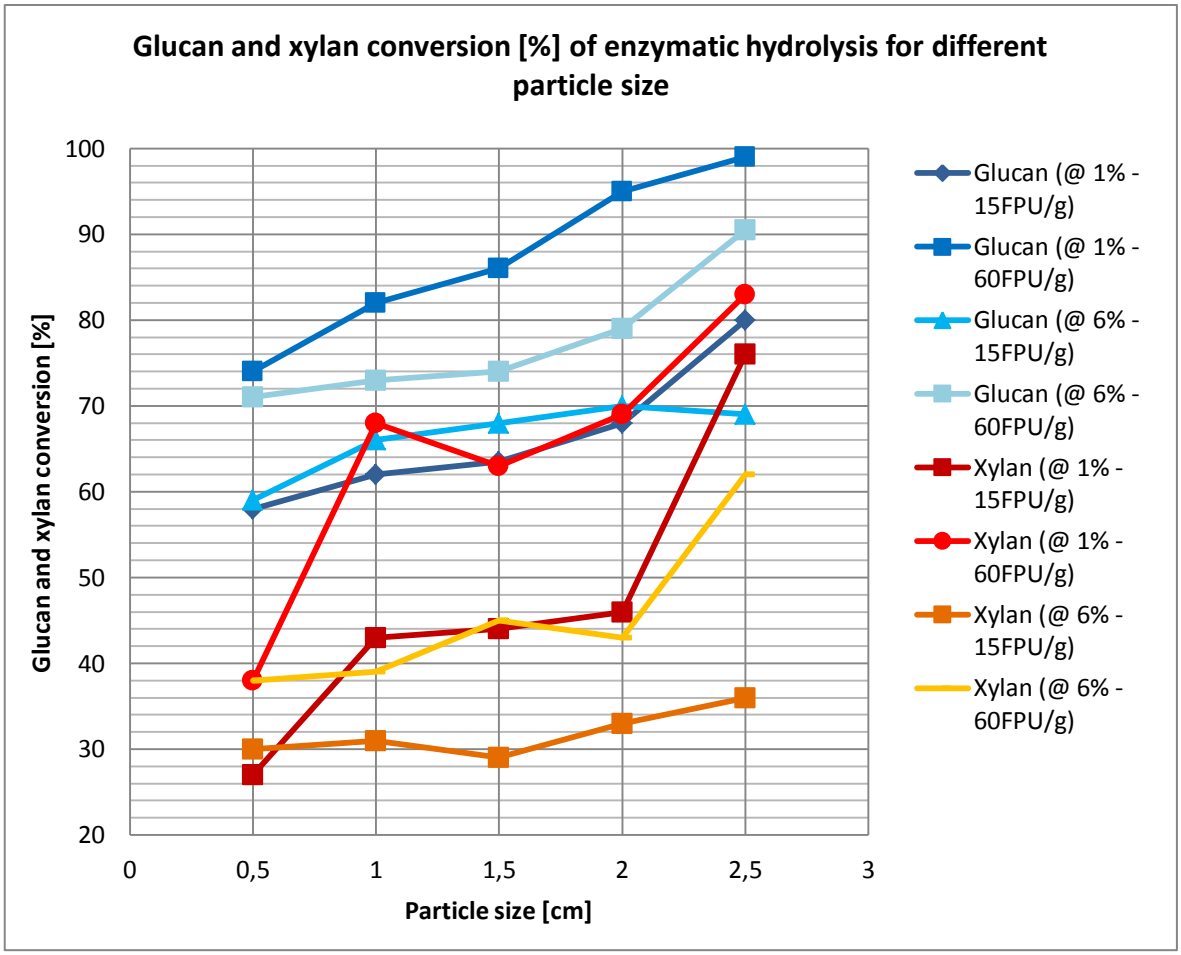
Solubilisation or degradation of lignin during pretreatment was not found. On the contrary, lignin recovery increased from 102% to 115% with increasing biomass particle size. This phenomenon had been attributed to the formation of lignin-like compounds ("pseudo-lignin") from recondensation reactions especially between carbohydrates or carbohydrates degradation products and other components from the water extractives, which also explained the low xylan recovery. These above results infer that the corn stover with larger biomass particle size is subjected to more sufficient pretreatment reaction than smaller biomass particle size in the scope from 2.5 to 0.5cm, due to higher pretreatment severity leading to degradation products of smaller molecules in the glucan and xylan degradation pathways.



The corn stover with different biomass particle sizes was steam exploded and then hydrolysed using commercial enzymes at two glucan loadings (1 and 6%) and two enzymes loading (15 and 60 FPU/g glucan). The conversions of glucan and xylan, which reflect the digestibility of the pretreated biomass, obviously increased with increasing biomass particle size at almost all tested conditions (different glucan loading and different enzyme loadings). Enzymatic hydrolysis at 1% glucan loading with the enzyme loading of 60FPU/g glucan achieved a glucan conversion and a xylan conversion as high as 99% and 83% at the particle size of 2.5cm. However, these two values for the particle size of 0.5 were low as 73% and 37%.

Considering the economics, high sugar concentration is required for industrial process. The maximum glucose and xylose concentrations reached 60 and 6 g/l, respectively, at particle size of 2.5cm by enzymatic hydrolysis at 6% glucan loading and 60FPU/g glucan.

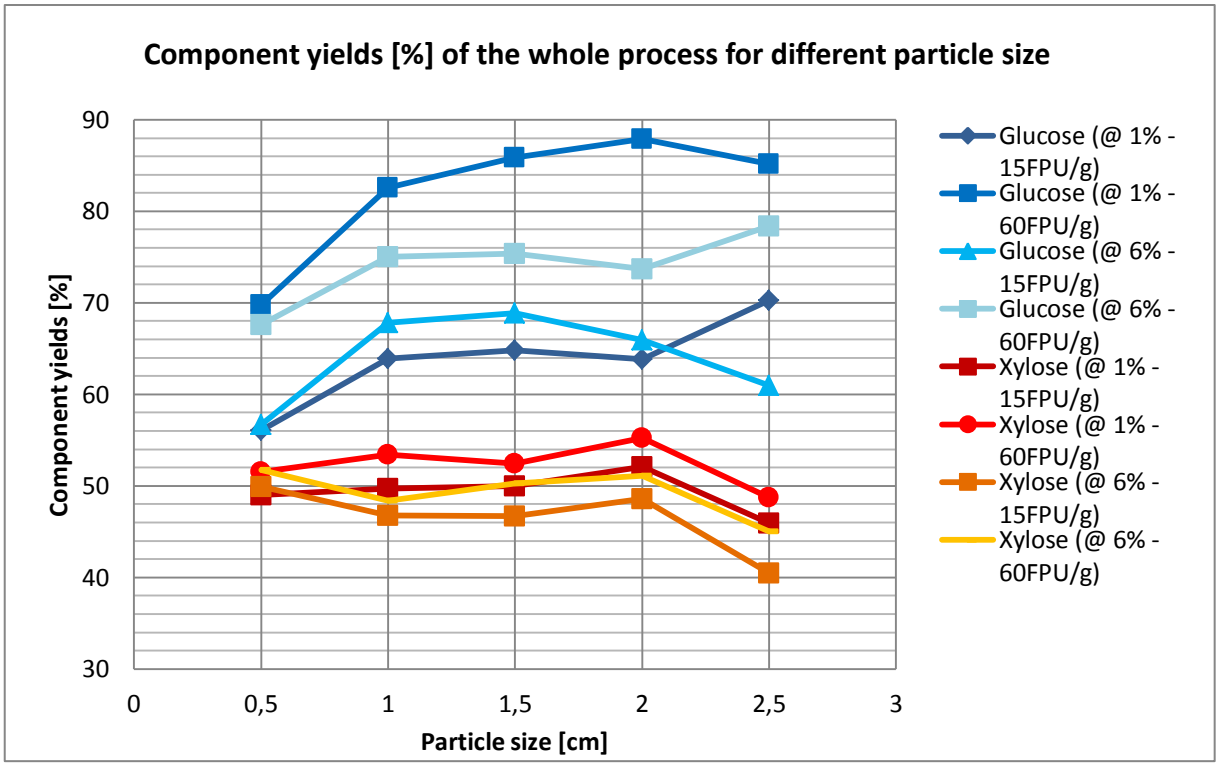
Thus, corn stover with larger biomass particle size achieved the better enzymatic hydrolysis performance, which reflected the higher pretreatment efficiency. From the biorefinery and economical point of view, the larger biomass particles producing high sugar conversions and reducing the electric power consumption during biomass size reduction process can be considered as the best ones for biomass conversion process.



Many techno-economic analyses of lignocellulose pretreatment had emphasised the importance of glucose and xylose yields on the optimization of the process and ensuring maximum biomass utilization. Glucose and xylose yields from pretreatment and enzymatic hydrolysis are showed in the graph below. The glucose yield at 1% glucan loading with an enzyme loading of 15 FPU/g glucan and at 6% glucan loading with an enzyme loading of 60FPU/g glucan increased with increasing biomass particle size. However, the maximum glucose yield at 1% glucan loading with an enzyme loading of 60FPU/g glucan and at 6% glucan loading with an enzyme loading of 15FPU/g glucan was obtained at particle size of 2.0 and 1.5cm respectively.

As for xylose yields, the maximum value at 1% glucan loading were obtained at particle size of 2.0cm. thus, the relatively high glucose and xylose was obtained at the particle size of 2.5 and 2.0cm, which also implied that the utilization of larger biomass particles resulted in the higher pretreatment and enzymatic hydrolysis efficiency.

There is no evident mass loss for glucan, but most of xylan and arabinan was solubilized and degraded during pretreatment. Approximately 88% and 55% of glucan and xylan was hydrolysed to glucose and xylose, respectively, through pretreatment and enzymatic hydrolysis at particle size of 2.0cm by 1% glucan loading with an enzyme loading of 60FPU/g glucan.



5.2.2 Moisture optimization

Sui et al. [3.14] studied the effect of moisture content of the corn stalk chips on SE pretreatment. The feedstock was air-dried to the moisture content of about 10%. Then samples are sprayed with distilled water to 20, 40, 60, 80 and 100% moisture content. The steam explosion was carried out at 198°C in a batch reactor (20 litres) for 5 minutes; then, corn stalk was exploded into the reception chamber by the ball valve. The size of corn stalk chips was in the range of 5-8cm. Enzymatic hydrolysis was carried out at 50°C in a rotary shaker at 150rpm for 48h.

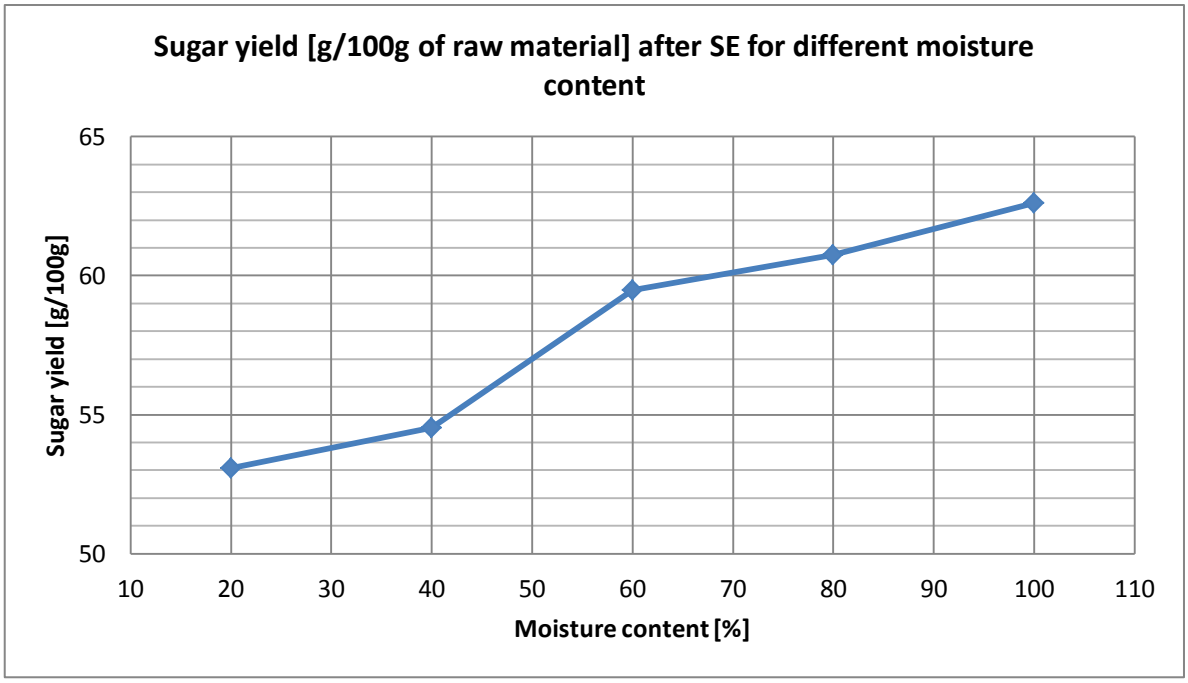
The percentage of glucan and xylan in the solid fraction increased as moisture content increased, inferring the reduction of sugar solubilisation. In liquid fraction, high amount of glucose and xylose obtained at high moisture content with few degraded product HMF also support above observation. These indicate that increased moisture content restricted the quantity of carbohydrates that are liberated into the liquid portion and subsequently degraded. Therefore, the recovery of glucose and xylose increased with the raise of moisture content. The relation between sugar yield and moisture can be grossly approximated (R^2=0.951) with the formula obtained, a 1st grade polynomial, based on the collected data:

$$\text{Sugar yield } [\%] = 0.126 \cdot x[\%] + 50.51$$

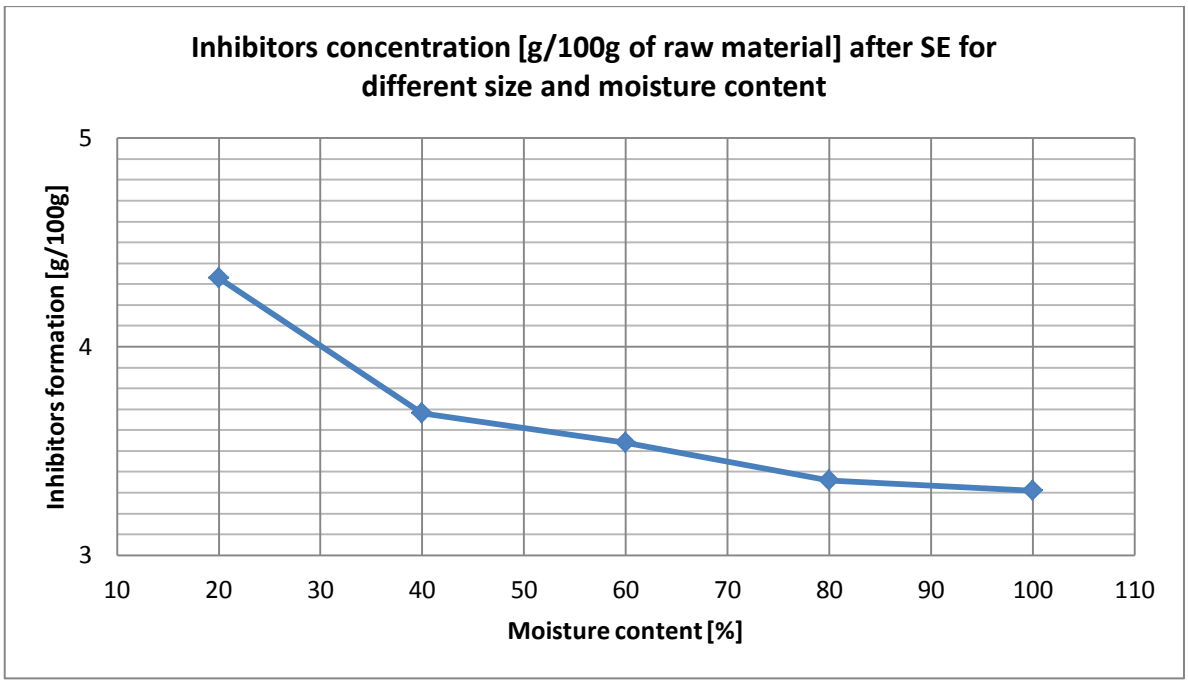
While the inhibitors content can be described by a power trend line (R^2=0.968):

$$Inhibitors\ content \left[\frac{g}{100 g_{raw\ material}}\right] = 7.01 \cdot x[\%]^{-0.167}$$

Where x is the moisture content.

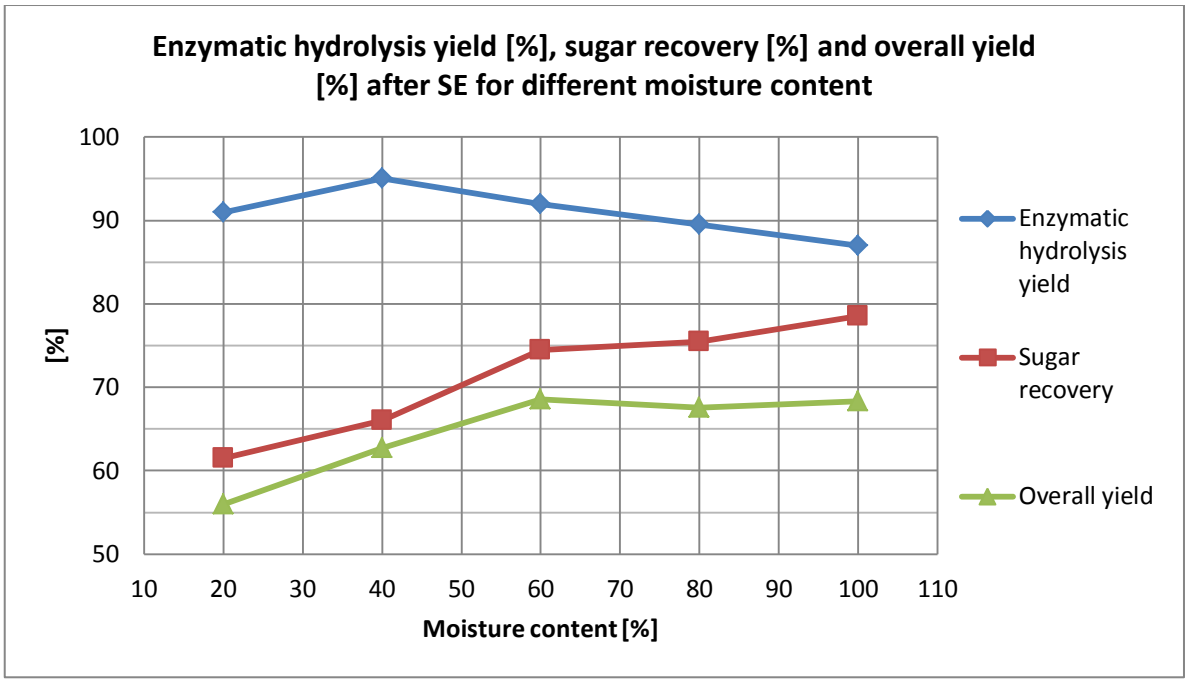


This buffer effect of water presents hurdles for carbohydrate solubilisation, decomposition and efficient auto-hydrolysis due to high heat capacity of water and slow heating rate in the interior of the chips. Thus it is suggested that high-moisture materials require longer steaming time for development of the maximum digestibility.



As exhibited in the graph below, the enzymatic hydrolysis yield of glucose first reached a peak of almost 90% at 40% moisture content and then decreased as moisture content increased. The enzymatic hydrolysis yield of xylose showed no difference among different moisture content, and it is almost 100%. As the combined results of sugar recovery and enzymatic hydrolysis, the overall glucose yield decreased with increased moisture content due to the reduction of hydrolysis yield while the overall xylose yield increased resulting from the increase of xylose recovery. The maximum yield of total sugars was still achieved

at 60% moisture content. Therefore, low moisture content leads to high pretreatment severity and enzymatic hydrolysis yield, although these improvements are at the expense of slight drop of recovery.



The buffer effect caused by extra water provided mild pretreatment to substrate and was responsible for the poor hydrolysis performance. Therefore, high moisture content should not be suitable for pretreatment of corn stalk, due to the waste of energy and low enzymatic digestibility. The study of [3.14] show that corn stalk with 60% moisture content generated the maximum enzymatic hydrolysis yield under relatively low water and energy consumption, thereby is possibly the optimum subject.

To approximate the behaviour of the enzymatic hydrolysis and overall yield, a second order polynomial is not enough: the value of R^2 is no more than 0.835 and 0.969 respectively. With a third order polynomial they become 0.974 and 0.975 respectively, even if the maximum of overall yield curve has been shifted to the right.

$$EH\ Yield\ [\%] = 7.29 \cdot 10^{-5} \cdot x^3[\%] - 1.54 \cdot 10^{-2} \cdot x^2[\%] + 0.889 \cdot x[\%] + 78.9$$

$$Overall\ Yield\ [\%] = 2.69 \cdot 10^{-5} \cdot x^3[\%] - 8.21 \cdot 10^{-3} \cdot x^2[\%] + 0.805 \cdot x[\%] + 42.7$$

5.2.3 Temperature, steam pressure and residence time

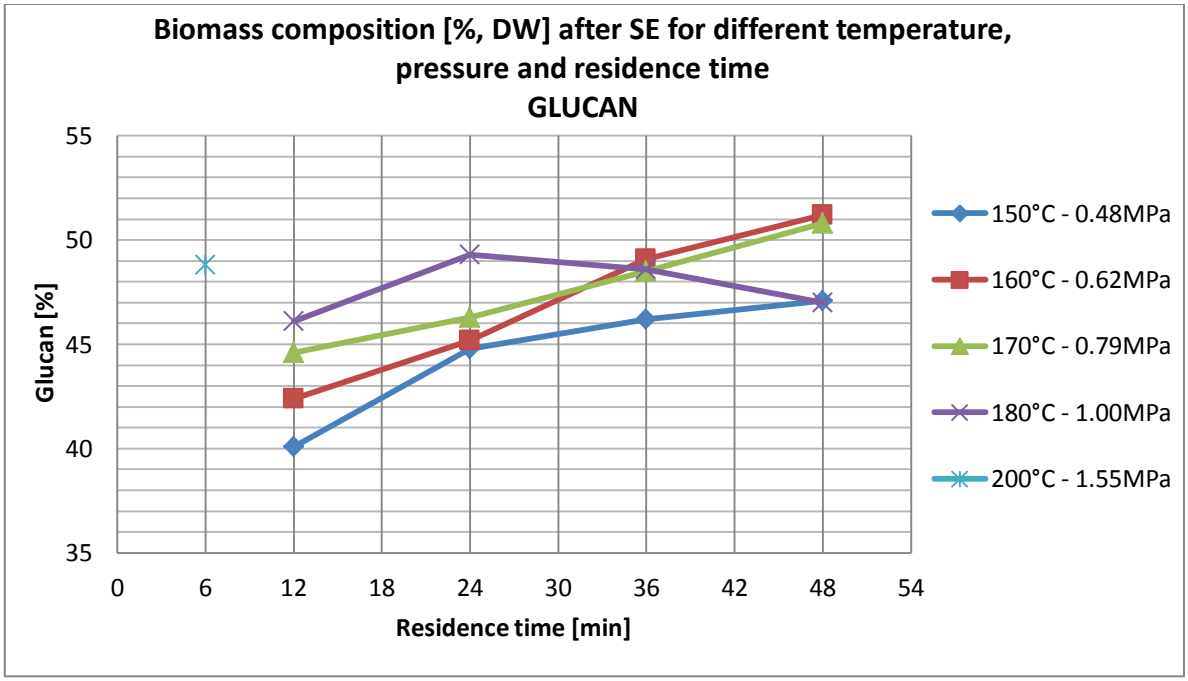
Liu et al. [4.12] analysed in their study the relation between glucose and xylose production with temperature, steam pressure and residence time of steam explosion on corn stover. The corn stover was in 2.0cm size and adjusted to 35% (w/w) moisture content. The SE was carried out in a 20L reactor with 1kg of corn stover on dry basis each time. High temperature steam was injected into the reactor until holding the temperature reached to the desired values (150-160-170-180°C). The reaction system was then maintained for a certain residence time (12, 24, 36 and 48 min). As the desired residence time was reached, the pressure of reaction system was rapidly increased to 1.5MPa, and the corn stover biomass was then exploded into a reception tank. Time used for the explosion step before the corn

stover biomass was exposed to atmospheric pressure was less than 2s. The control experiment of SE was conducted at 200°C for 6min.

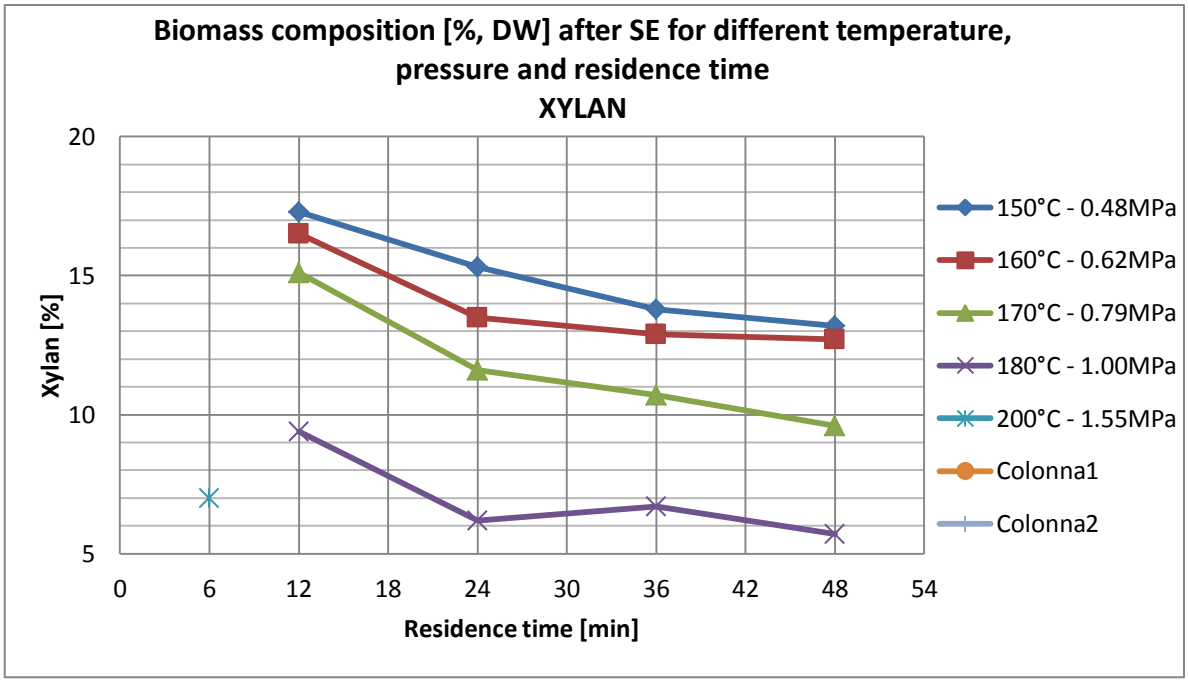
The enzymatic hydrolysis was carried out at different solid loading and enzyme loading. For the experiments of solid loading the hydrolysis was carried out at a certain solid loading of 1%, 6%, 12% and 18% in a citrate buffer solution (50mM, pH 4.8) with an enzyme loading of 10FPU/g solid. For the enzyme loading, the digestibility was conducted at 12% solid loading with an enzyme loading of 5, 10, 15 and 20FPU/g solid. The above experiments were carried out in a water baths shaker at 50°C with 200rpm for 120h.

The total sugar content of untreated corn stover was about 50%, while the lignin content was 16%. Acetyl content that is side chains to the xylan backbone was 3,7%. High sugar content and low lignin content was beneficial to the lignocellulosic biomass conversion. High acetyl content should facilitate the auto-hydrolysis effect in SE.

The data collected after the SE treatment are shown in the graphs below. They have been related to the residence time in the SE reactor and divided by temperature at which the SE have been hold.

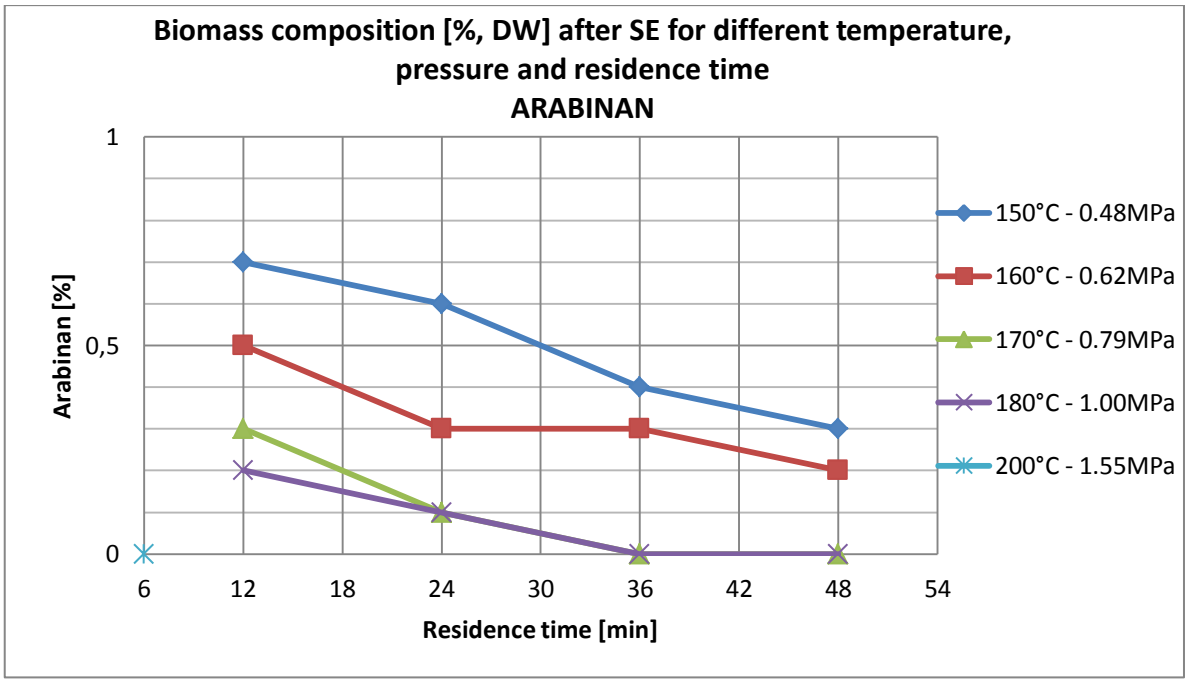


The glucan content of treated corn stover increased with holding temperature increasing from 150°C to 170°C or with residence time increasing from 12min to 48min, respectively. As for 180°C, glucan content reached the maximum value at 24min and then decreased with the increase of residence time. Results indicated that glucan may degraded at high pretreatment severity. It is interesting to note that the glucan content at 150, 160 and 170°C for 36 and 48min were approximate to or even higher that that at 180°C and at 200°C for 6min, respectively. The highest glucan content (51.2%) was obtained at 160°C and 48min. These results suggested that SE with low holding temperature and long residence time increased glucan content and avoided its excessive degradation compared with that at 200°C for 6min.

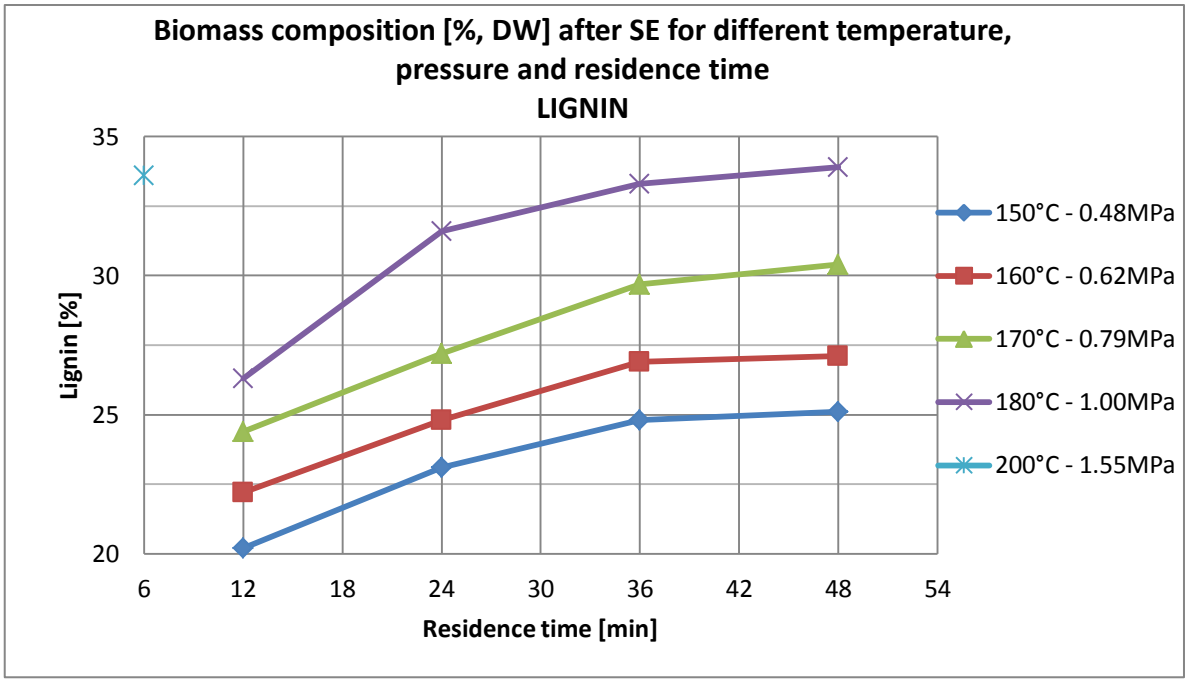


The xylan content of steam exploded corn stover at all SE conditions, except at 150°C for 12min, was lower than that of untreated corn stover. Results indicated that xylan was dissolved and/or degraded in SE. The xylan content decreased with increase of holding temperature and residence time.

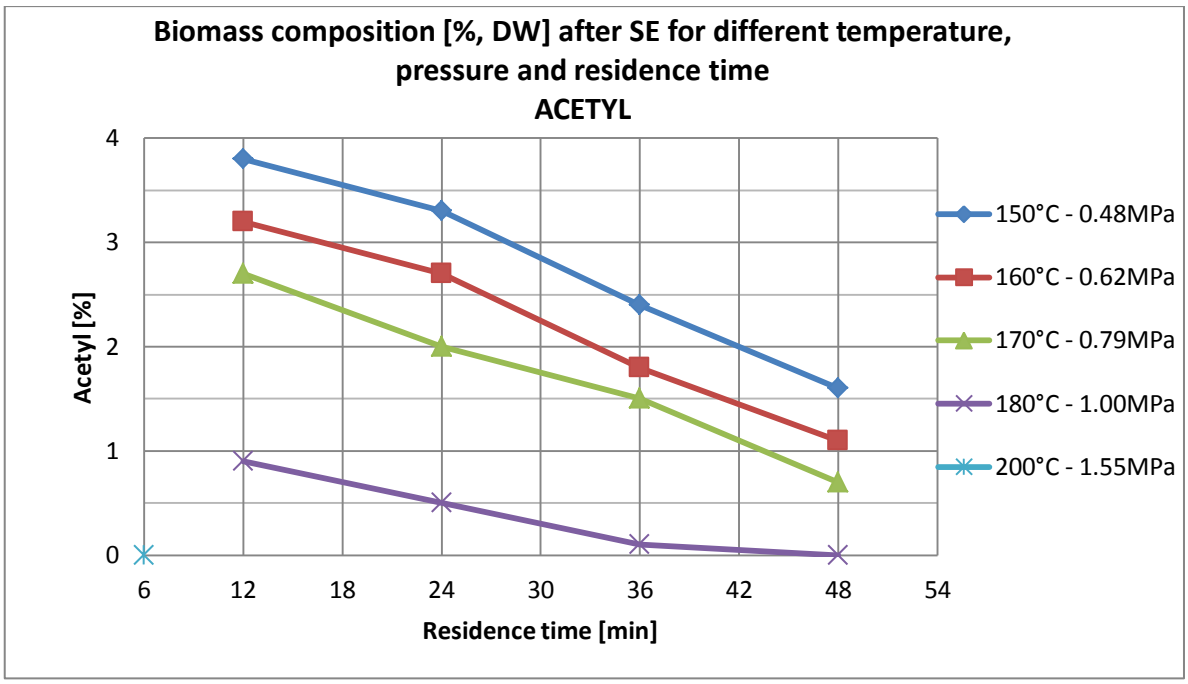
The result indicated that most of xylan in corn stover biomass was removed at high pretreatment severity. Results suggested that SE with low holding temperature and short residence time should protect xylan from excessive degradation.



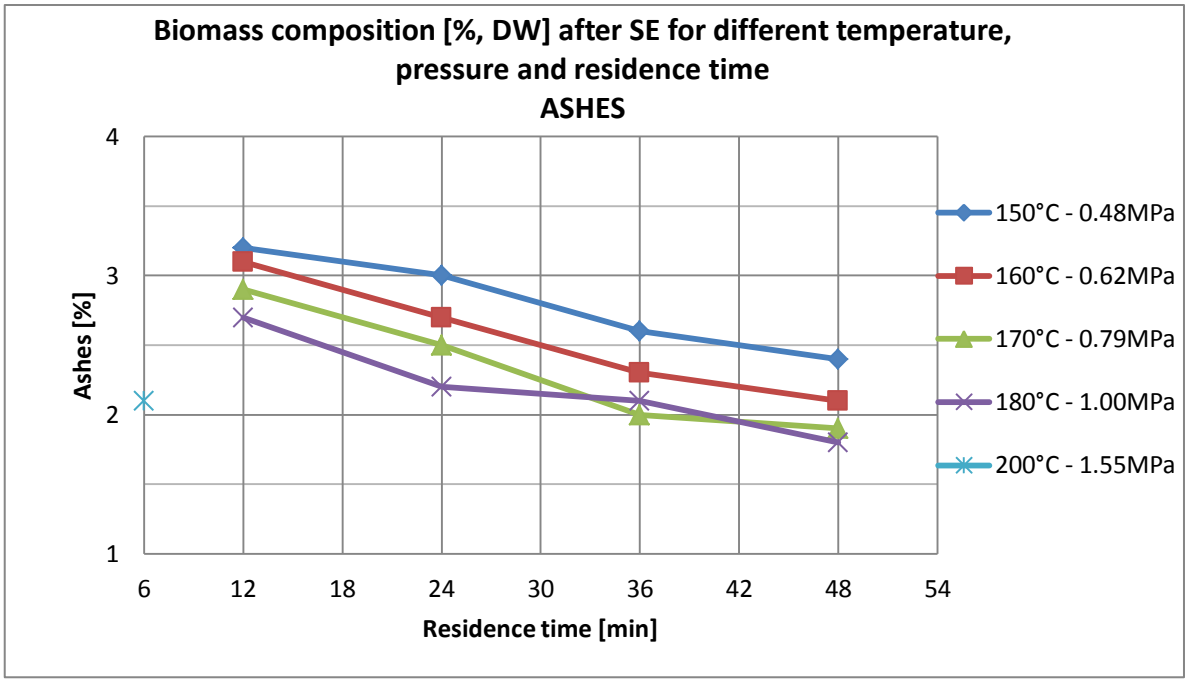
The arabinan content, which is a part of hemicellulose, follows a similar trend to the xylan content.



The lignin content of steam exploded corn stover increased with respect to the untreated biomass as discussed in the previous paragraphs. However, the lignin content at 150, 160 and 170°C were 3.2-13.4% less than that at 200°C for 6min, which may be beneficial to the subsequent digestibility.

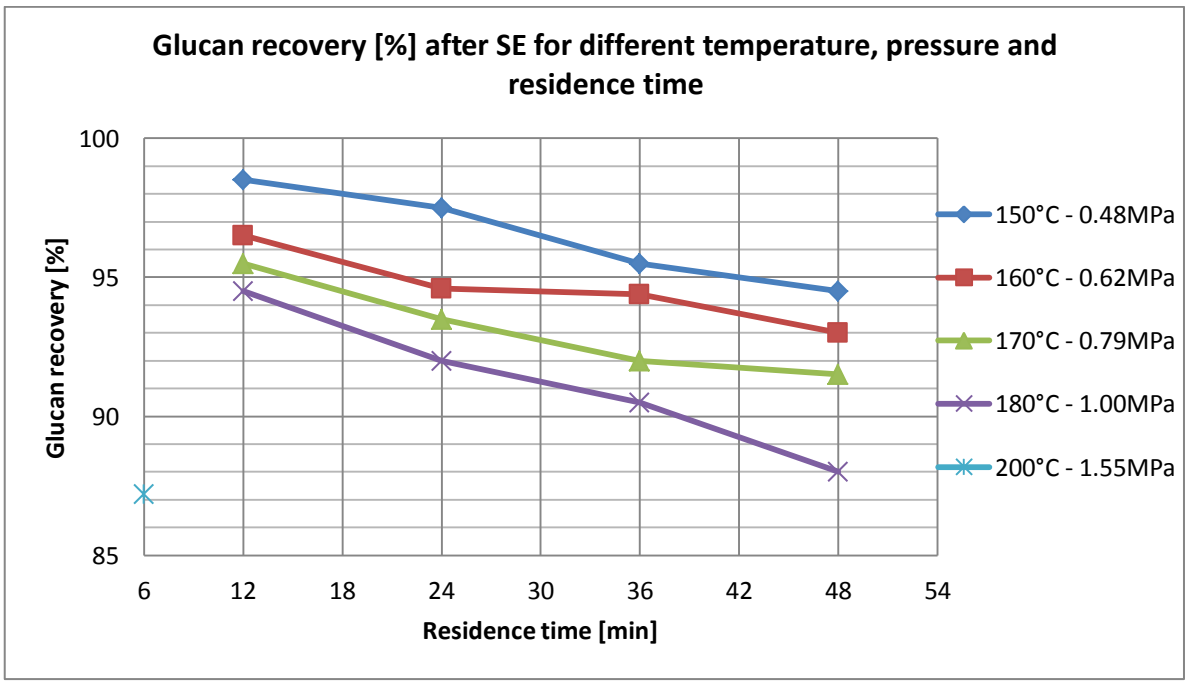


The acetyl content was lower than that of untreated corn stover. It decreased with the increase of holding temperature and residence time. The removal of acetyl suggested high steam explosion efficiency and should facilitate the subsequent digestibility.



Ash content showed a similar trend to acetyl content.

The next data collection shows how each chemical component evolves in quantity in respect with the untreated condition.



It is interesting to note that glucan recovery decreased by less than 3.0% at 150°C and 160°C with residence time increasing from 12 to 48min., while it decreased by more than 5.0% at 170°C and 180°C. Glucan recovery at 150°C and 160°C was more than 93.5%, which was 7.2–12.5% higher than that at 200°C for 6 min (87.2%). Results implied that higher pretreatment severity lead to more glucan degradation, which should be avoided in SE.

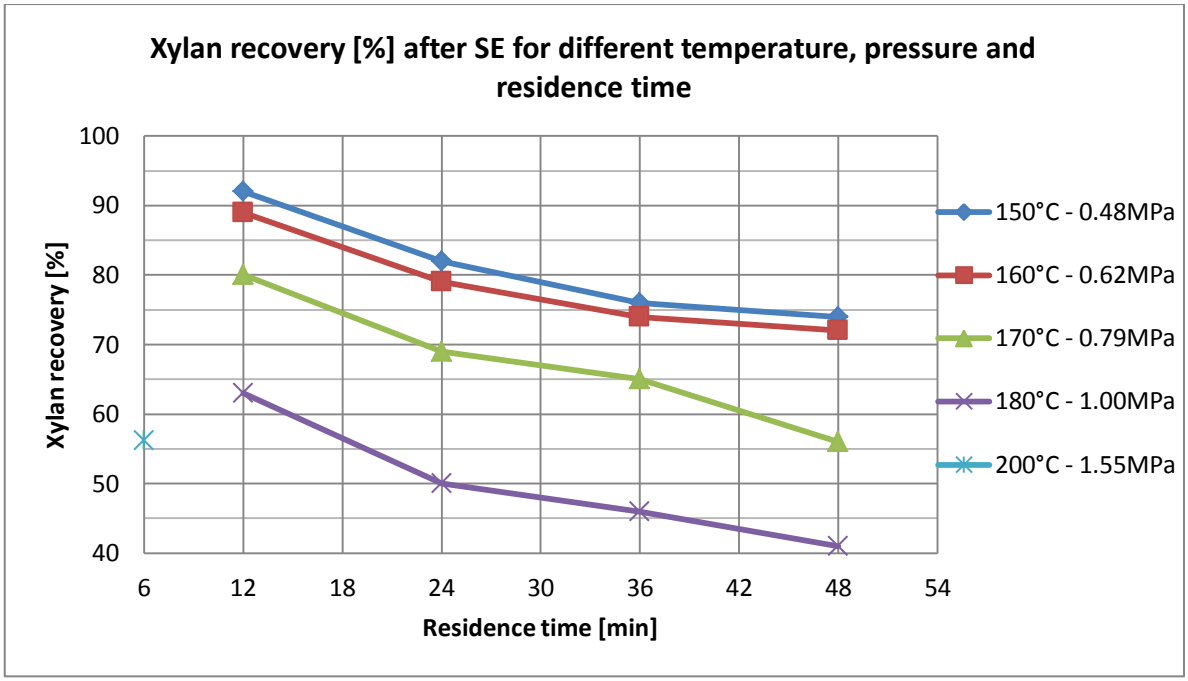
Component recovery can be represented with an exponential square-root model of decay. The initial quantity, at time zero, is 100%. So the formula is written as:

$$Glucan\ rec.[\%] = Glucan\ rec._{t=0} \cdot e^{-\lambda(T)\sqrt{t}}$$

Where the λ is the decay rate dependant on the temperature. In these, the relation between the decay rate and the temperature was fitted with a second order polynomial:

$$\lambda(T) = 2.20 \cdot 10^{-6} \cdot T^2 - 3.77 \cdot 10^{-4} \cdot T + 1.41 \cdot 10^{-2}$$

With this model, the value of r-squared was 0.884. This is because of the behaviour of glucan recovery at 150°C, that is more similar to a linear relation than to an exponential. If we remove it from the calculation, r-squared reached 0.989.



Xylan recovery also decreased with the increase of holding temperature and residence time. It decreased by only 2.2-3.0% with holding temperature increasing from 150°C to 160°C, and then decrease by 27.0-31.4% with holding temperature increasing from 160°C to 180°C.

Like glucan recovery, it can be represented by a decay expression:

$$Xylan\ rec. = Xylan\ rec._{t=0} \cdot e^{-\lambda(T)\sqrt{t}}$$

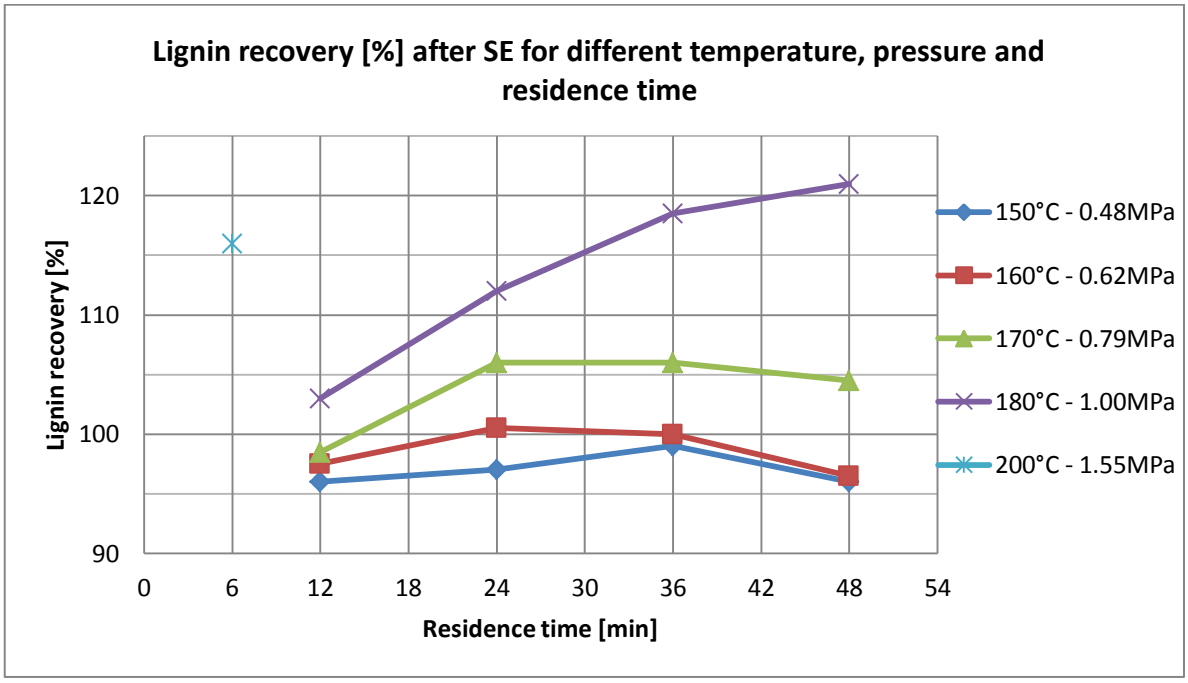
With these data, the relation between the decay rate and the temperature was fitted better with a second order polynomial:

$$\lambda(T) = 1.29 \cdot 10^{-4} \cdot T^2 - 3.97 \cdot 10^{-2} \cdot T + 3.08$$

With this model, the r-squared value is 0.930. It could be better, as previously, not considering the curve at 150°C. In fact, without those data, the model reaches a r-square value of 0.971.

It is interesting to note that xylan recovery decreased by about 17% at 150°C and 160°C and about 23% at 170°C and 180°C with residence time increasing from 12min to 48min. These results implied that high holding temperature and long residence time within the scope of this study led to the excessive degradation of xylan. However, it should be noticed that xylan recovery increased by 15.8–34.2% at 150°C and 160°C compared with that at 200°C for

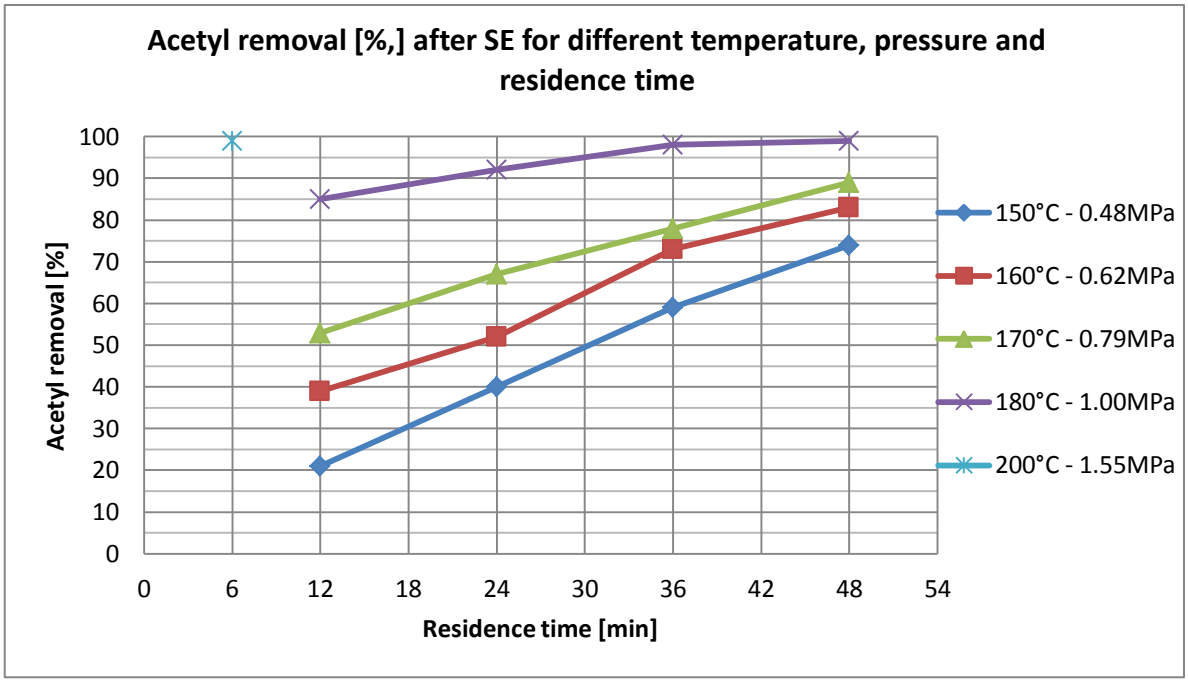
6min (56.2%), indicating that low temperature SE prevented xylan from degrading excessively.



Lignin recovery hardly changed with holding temperature increasing from 150°C to 160°C. However it rapidly increased with holding temperature increasing from 160°C to 180°C. It should be noticed that lignin recovery was more than 100% at 170°C and 180°C for all residence times, while it was 116% at 200°C for 6 min. The reason was that the lignin-like compounds were generated by recondensation reactions among carbohydrates, DPs and water extractives.

The behaviour of the lignin recovery is less immediate to simplify. It could be approximated to a third order polynomial, with coefficients dependent on the temperature.

Lignin content in pretreated solid is a main factor affecting the enzyme activity and hence the digestibility efficiency, implying that the holding temperature of 170, 180 and 200°C may be adverse for the conversion of corn stover biomass.



The generation of acetic acid enhanced the auto-hydrolysis effect in SE, and hence improved the SE efficiency. Acetyl removal rate, corresponding to acetic acid yield, can be considered as a key metric for the assessment of the SE performance. Acetyl removal rate increased with the increase of holding temperature and residence time, respectively. Results indicated that higher pretreatment severity should lead to more removal of acetyl groups. Acetyl removal rate was more than 80% at 180°C for all residence times or at 48min for all holding temperatures except 150°C, respectively. Almost 100% of acetyl removal rate was obtained at 180°C for 36 and 48 min and at 200°C for 6min. Previous study confirmed that more removal of acetyl groups resulted in higher digestibility efficiency. Therefore, the efficiency of SE should be the balance of sugar recovery, lignin recovery and acetyl removal rate.

Acetyl removal rate can be expressed with an exponential behaviour:

$$Acetyl\ removal\ [\%] = 100\% \cdot (1 - e^{-\lambda(T)t})$$

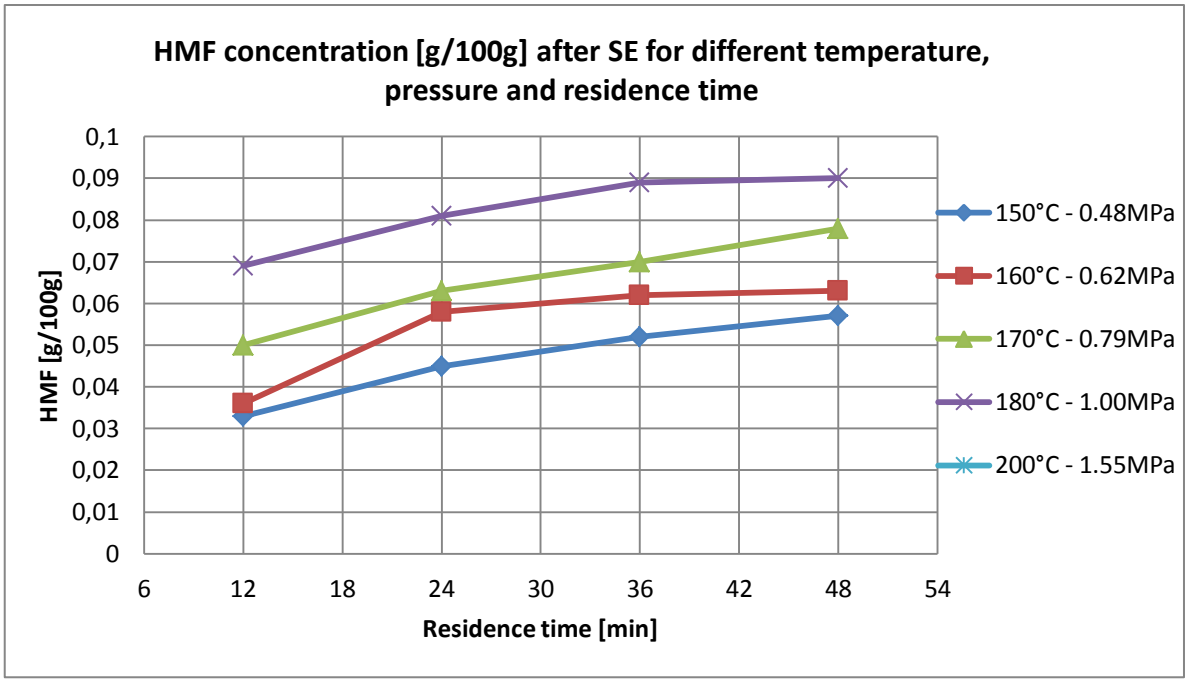
With these data, the relation between the decay rate and the temperature was fitted better with a third order polynomial:

$$\lambda(T) = 1.38 \cdot 10^{-5} \cdot T^3 - 6.60 \cdot 10^{-3} \cdot T^2 + 1.05 \cdot T - 56.0$$

With this model, the value of r-squared is 0.982.

Degradation products (DPs) can be used as the main factor for evaluating the pretreatment efficiency and they obviously affected the subsequent conversion process. The data shown below represent the concentration in grams per 100 grams of feedstock.

HMF concentration, which is a degradation product of glucose, slightly increased with holding temperature increasing from 150°C to 160°C at a certain residence time and then sharply increased with the increase of holding temperature. HMF concentration also increased with the increase of residence time at a certain holding temperature.



Results suggested that higher pretreatment severity led to more degradation of glucan, and hence higher HMF concentration, which was consistent with the results of glucan recovery. However, HMF concentration in low temperature SE was less than 0.10g/100g feedstock, which was lower than that at 200°C for 6min (0.31g/100g feedstock) and as reported in previous studies.

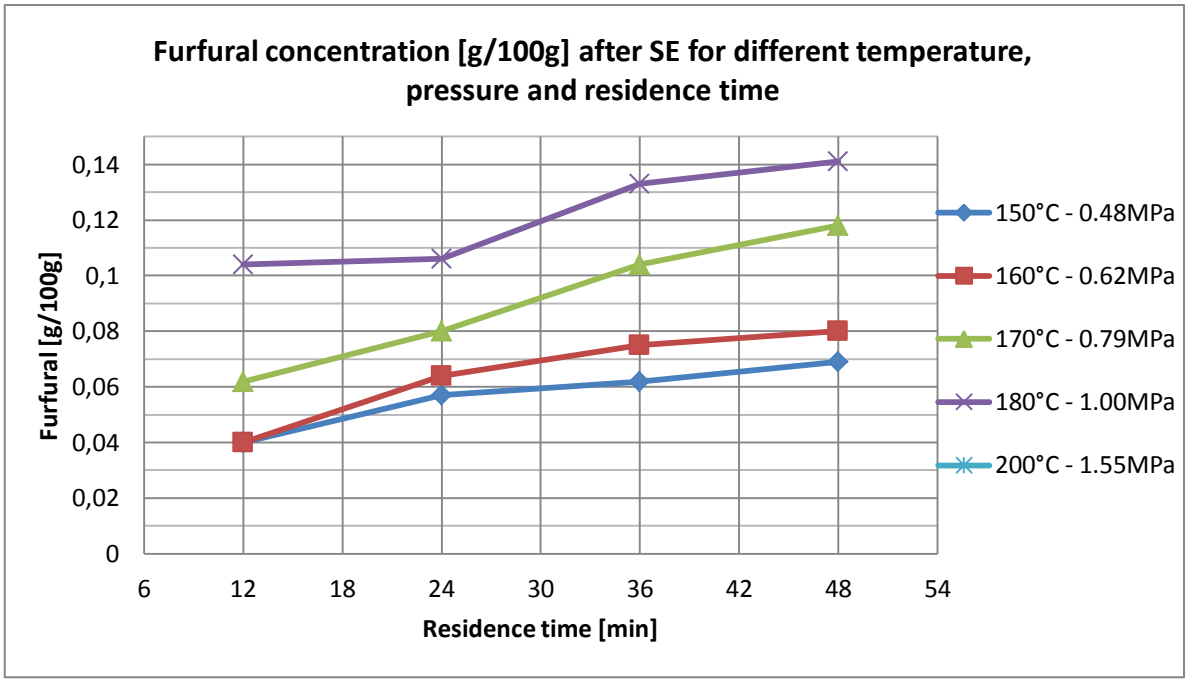
HMF concentration can be expressed with a power behaviour:

$$HMF\ conc.[g/100g] = A(T[°C]) \cdot time[min]^{B(T[°C])}$$

$$A(T[°C]) = 4.51 \cdot 10^{-5} \cdot T^2 - 0.139 \cdot 10^{-2} \cdot T + 1.08$$

$$B(T[°C]) = -3.26 \cdot 10^{-4} \cdot T^2 - 0.101 \cdot T - 7.38$$

With this model, the value of r-squared is 0.875. The behaviour of the data at 160°C doesn't follow the model, if we remove this curve, the model reached a value of 0.971.



Furfural concentration, which is a degradation product of xylose, hardly changed with holding temperature increasing from 150°C to 160°C at a certain residence time. But it then rapidly increased with holding temperature increasing from 160°C to 180°C. It is interesting to note that furfural concentration increased with residence time increasing from 12min to 36min, but it then decreased at 48min at a certain holding temperature. The reason was that long residence time resulted in further degradation and/or polymerization of furfural with other DPs. Although high holding temperature and long residence time led to more degradation of xylan, furfural yield in low temperature SE was less than 0.15g/100g feedstock, which was lower than that at 200°C for 6min (0.92g/100g feedstock) and previous studies.

Furfural concentration can be expressed with a power behaviour:

$$Furfural\ conc.[g/100g] = A(T[°C]) \cdot time[min]^{B(T[°C])}$$

$$A(T[°C]) = 1.02 \cdot 10^{-4} \cdot T^2 - 3.25 \cdot 10^{-2} \cdot T + 2.59$$

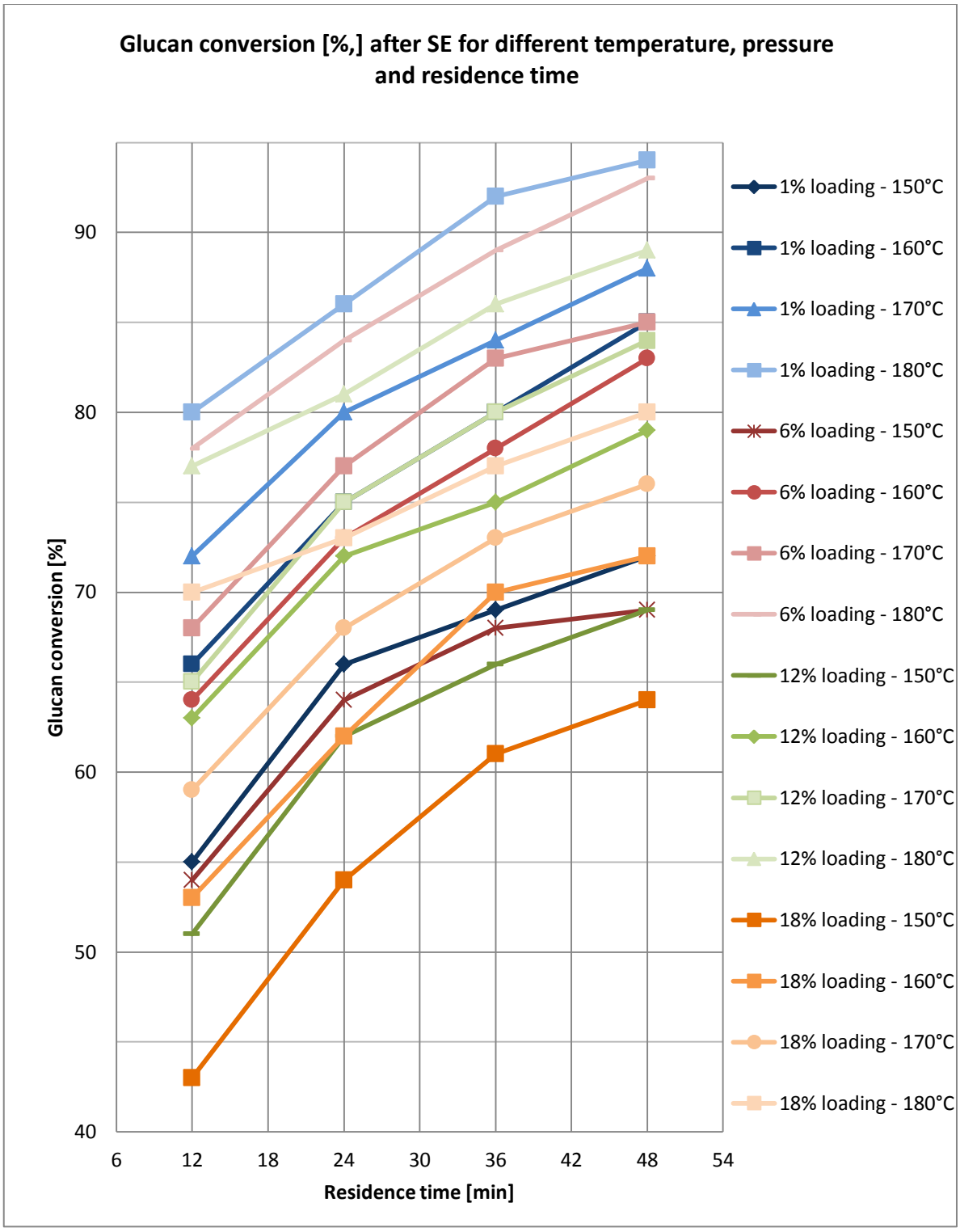
$$B(T[°C]) = -9.06 \cdot 10^{-4} \cdot T^2 - 0.294 \cdot T - 23.33$$

With this model, the value of r-squared is 0.803. The behaviour of the data at 180°C doesn't follow the model (R^2=0.815), if we remove this curve, the model reached a value of 0.962.

Regarding the overall process economy, it is important to get a sugar conversion and concentration as high as possible in digestibility. Thus, enzymatic digestibility was performed at different solid loadings and at different enzyme loading for each previous experiment. The large number of data collected are shown, as clearly as possible, in the graphs below.

For the experiments of solid loading, the digestibility was carried out at a certain solid loading of 1%, 6%, 12% and 18%, respectively, in citrate buffer solution (50mM, pH 4.8) with an enzyme loading of 10 FPU/g solid. For the experiments of enzyme loading, the

digestibility was conducted at 12% solid loading with an enzyme loading of 5, 10, 15 and 20FPU/g solid, respectively. The above experiments of digestibility were carried out in an water baths shaker at 50°C with 200rpm for 120h.



Glucan conversion under different solid loadings increased with increase of residence time at a certain holding temperature. Results implied that high holding temperature and long residence time resulted in high digestibility performance.

As for different solid loadings, glucan conversion decreased with solid loading increasing. The reason was that high solid loading led to high viscosity of digestibility mixture and poor mass transfer efficiency, and thus low glucan conversion.

The behaviour of glucan conversion can be approximated, with a coefficient of determination (R^2) comprised between 0.991 and 1.000, with a logarithmic function, in the form:

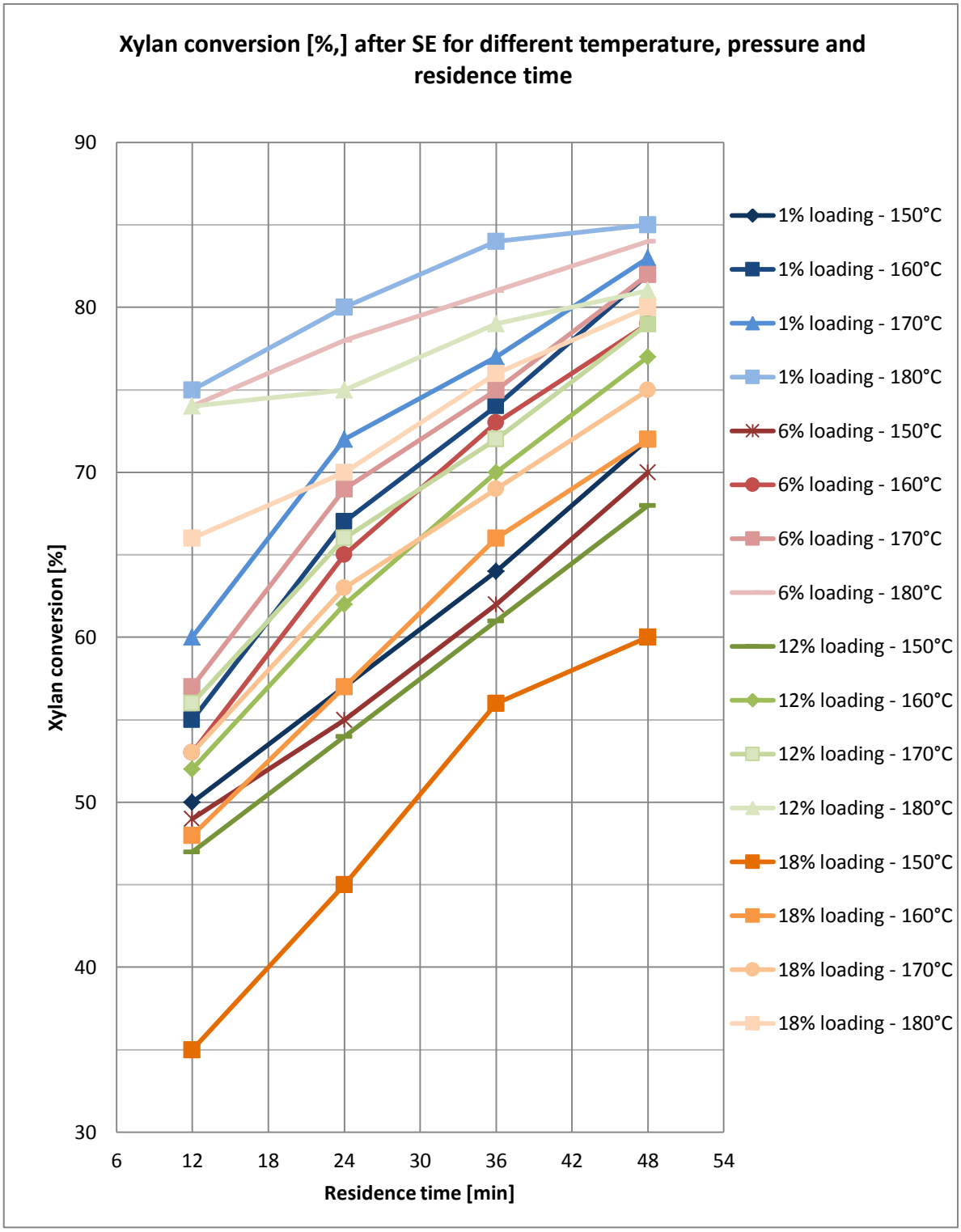
$$glucan\ conversion[\%] = A(T, solid\ loading) \cdot \ln(time[min]) + B(T, solid\ loading)$$

Where A and B are coefficients dependent on temperature and solid loading. At the same time, the behaviour of these coefficients can be represent by a relation with temperature and loading rate, with a mean coefficient of determination of 0.895 and 0.924 respectively. The behaviour of A and B in function of temperature, at a constant solid loading can be represent by a linear function, while the relation with solid loading can be fitted better with a second order polynomial equation:

$$A(T, solid\ loading) = -1.08 \cdot 10^{-4} \cdot l[\%]^2 \cdot (T[°C] - 107.4) + 7.40 \cdot 10^{-4} \cdot l[\%] \cdot (T[°C] - 158.1) + 0.118 \cdot T[°C] - 5.67$$

$$B(T, solid\ loading) = 6.26 \cdot 10^{-5} \cdot l[\%]^2 \cdot (T[°C] - 436.1) - 2.19 \cdot 10^{-3} \cdot l[\%] \cdot (T[°C] - 179.9) + 0.319 \cdot T[°C] - 20.15$$

It is interesting to note that xylan conversion rapidly increased with holding temperature increasing from 150°C to 160°C at a certain residence time under different solid loadings and then slightly increase with holding temperature. Xylan conversion also increased with the increase of residence time increasing at a certain holding temperature. As for different solid loadings, xylan conversion decreased with solid loading increasing.



Thus, these results indicated that the high sugar conversion was obtained below 12% solid loading with SE conditions of 160-180°C at 48min, with xylan conversion between 80% and 85% while glucan conversion was between 85% and 93%.

In the same way, the behaviour of xylan conversion can be approximated, with a value of coefficient of determination (R^2) between 0.953 and 0.997, with a logarithmic function, in the form:

$$xylan\ conversion[\%] = C(T, solid\ loading) \cdot \ln(time[min]) + D(T, solid\ loading)$$

Where C and D are coefficients dependent on temperature and solid loading. The behaviour of these coefficients can be represent by a relation with temperature and loading rate. The relation of C and D in function of temperature, at a constant solid loading can be represent by a linear function, while the relation with solid loading can be fitted better with a second order polynomial equation, with a mean coefficient of determination of 0.837 and 0.914 for C and D respectively:

$$C(T, solid\ loading) = 1.95 \cdot 10^{-4} \cdot l[\%]^2 \cdot (T[°C] - 181.0) - 2.75 \cdot 10^{-3} \cdot l[\%] \cdot (T[°C] - 155.3) + 0.103 \cdot T[°C] - 4.26$$

$$D(T, solid\ loading) = 4.74 \cdot 10^{-4} \cdot l[\%]^2 \cdot (T[°C] - 188.2) - 6.17 \cdot 10^{-3} \cdot l[\%] \cdot (T[°C] - 162.1) + 0.311 \cdot T[°C] - 22.02$$

Since an improvement in enzyme loading is of a great importance for the capital cost of digestibility, the solid loading was fixed to 12% and the influence of enzyme loading on sugar conversion was investigated.

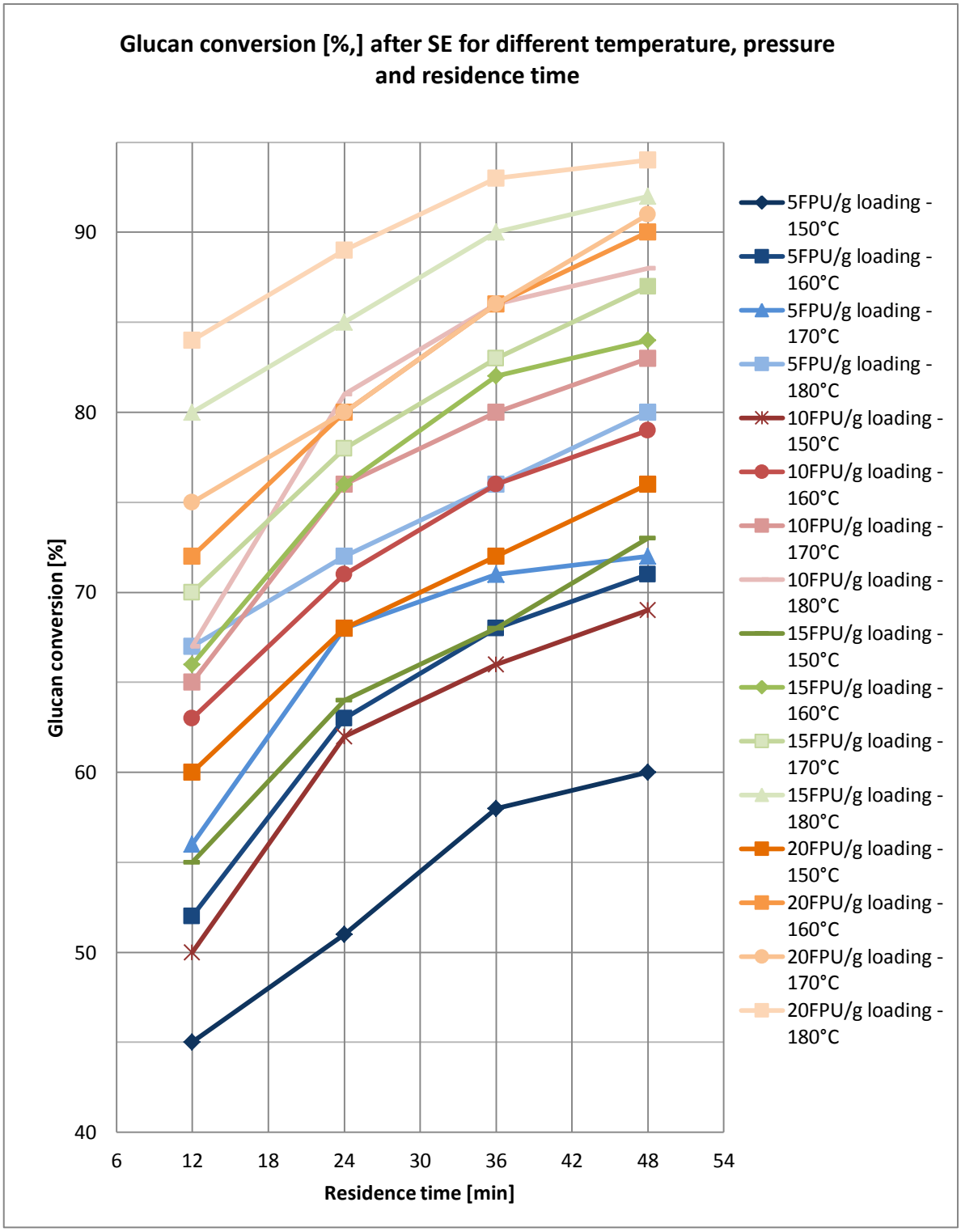
As previously, glucan conversion can be approximated, with a mean coefficient of determination (R^2) between 0.988 and 1.000, with a logarithmic function, in the form:

$$glucan\ conversion[\%] = A(T, enzyme\ loading) \cdot \ln(time[min]) + B(T, enzyme\ loading)$$

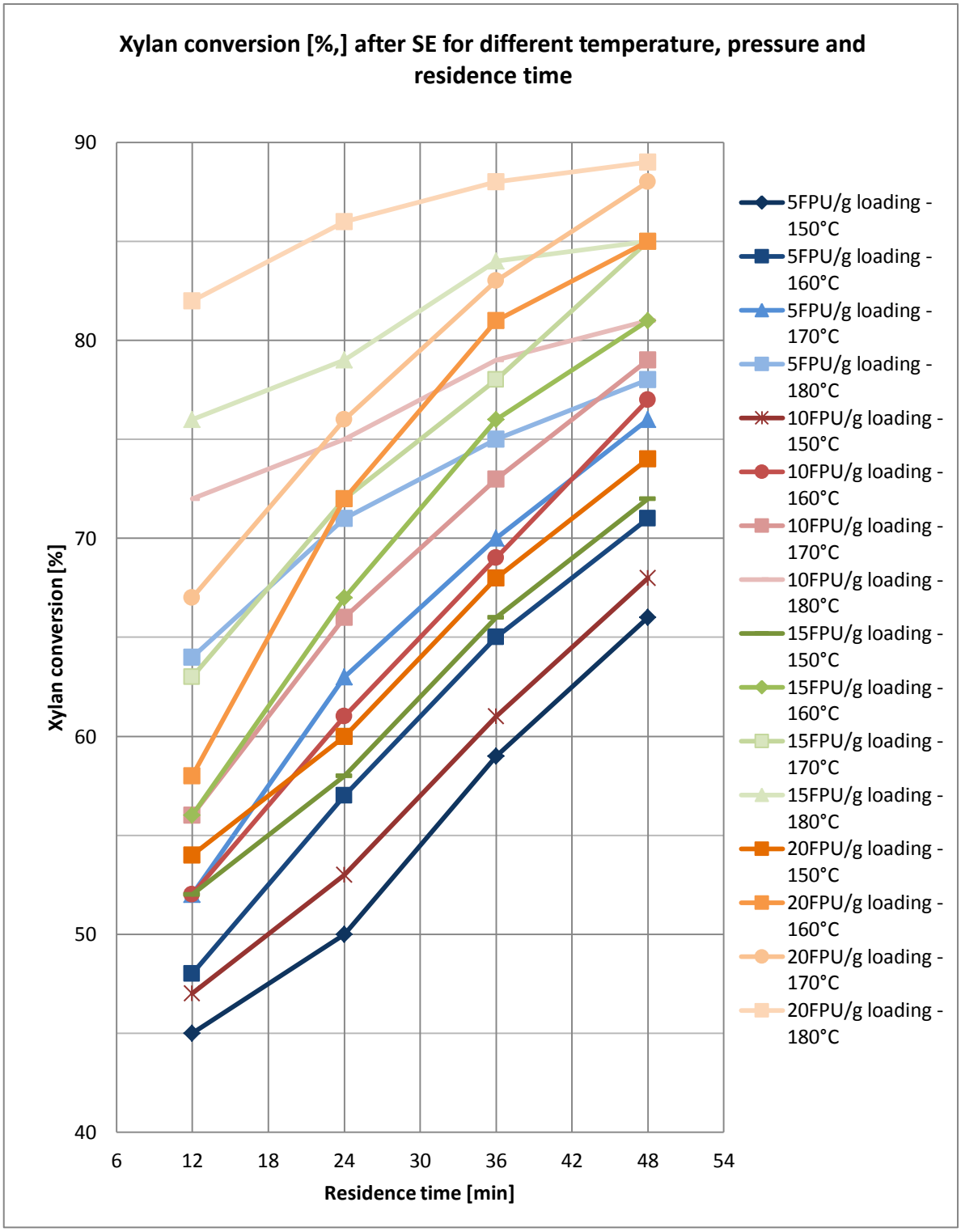
Where A and B are coefficients dependent on temperature and solid loading. At the same time, the behaviour of these coefficients can be represent by a relation with temperature and loading rate, with a mean coefficient of determination of 0.830 and 0.933 respectively. The behaviour of A and B in function of temperature, at a constant solid loading can be represent by a linear function, while the relation with solid loading can be fitted better with a second order polynomial equation:

$$A(T, enzyme\ loading) = -9.60 \cdot 10^{-5} \cdot l[FPU/g]^2 \cdot (T[°C] - 72.4) + 2.31 \cdot 10^{-3} \cdot l[FPU/g] \cdot (T[°C] + 9.48) + 9.59 \cdot 10^{-2} \cdot T[°C] - 6.12$$

$$B(T, enzyme\ loading) = -3.76 \cdot 10^{-4} \cdot l[FPU/g]^2 \cdot (T[°C] - 110.6) + 9.64 \cdot 10^{-3} \cdot l[FPU/g] \cdot (T[°C] - 63.4) + 2.47 \cdot 10^{-1} \cdot T[°C] - 18.2$$



Glucan and xylan conversion increased with the increase of holding temperature and residence time, with an enzyme lading of 5FPU and 10FPU/g solid. However, glucan and xylan conversion with an enzyme loading of 15 FPU and 20FPU/g solid slightly increased with holding temperature increase from 160°C to 180°C at 24, 36, and 48 min, respectively. As for different enzyme loadings, glucan and xylan conversion increased with the increase of enzyme loading at all SE conditions.



In the same way, the behaviour of xylan conversion can be approximated, with a value of coefficient of determination (R^2) between 0.987 and 0.999, with a logarithmic function, in the form:

$$xylan\ conversion[\%] = C(T, enzyme\ loading) \cdot \ln(time[min]) + D(T, enzyme\ loading)$$

Where C and D are coefficients dependent on temperature and solid loading. The behaviour of these coefficients can be represent by a relation with temperature and loading rate. The relation of C and D in function of temperature, at a constant solid loading can be represent

by a linear function, while the relation with solid loading can be fitted better with a second order polynomial equation, with a mean coefficient of determination of 0.387 and 0.618 for C and D respectively:

$$C(T, loading) = 7.70 \cdot 10^{-5} \cdot l[FPU/g]^2 \cdot (T[°C] - 158.4) - 8.35 \cdot 10^{-4} \cdot l[FPU/g] \cdot (T[°C] - 391.8) + 9.24 \cdot 10^{-2} \cdot T[°C] - 4.55$$

$$D(T, loading) = 1.05 \cdot 10^{-4} \cdot l[FPU/g]^2 \cdot (T[°C] - 165.7) + 2.30 \cdot 10^{-3} \cdot l[FPU/g] \cdot (T[°C] - 0.173) + 0.246 \cdot T[°C] - 16.57$$

These low values of R^2 for C and D mean that the model is not so close to the beginning data: the logarithmic function approximated well the results, but the dependence of temperature and enzyme loading may be approximated better with a further polynomial order.

The increased glucan and xylan conversion decreased with the increase of enzyme loading, indicating that the increase of enzyme loading beyond 10 FPU/g solid should be no helpful to improve the digestibility efficiency significantly. Thus, considering the sugar conversion and the cost of enzyme, an enzyme loading of 10FPU/g solid was considered as the optimal condition.

The digestibility efficiency is a function of time and sugar conversion rate (SCR). The trends of glucan and xylan conversion at the same sampling points were consistent with those of above digestibility experiments. During a specific time interval of digestibility, glucan and xylan conversion were between 40% and 70% at 6h under all SE conditions. However, it should be noticed that the increased glucan and xylan conversion decreased with increase of digestibility time.

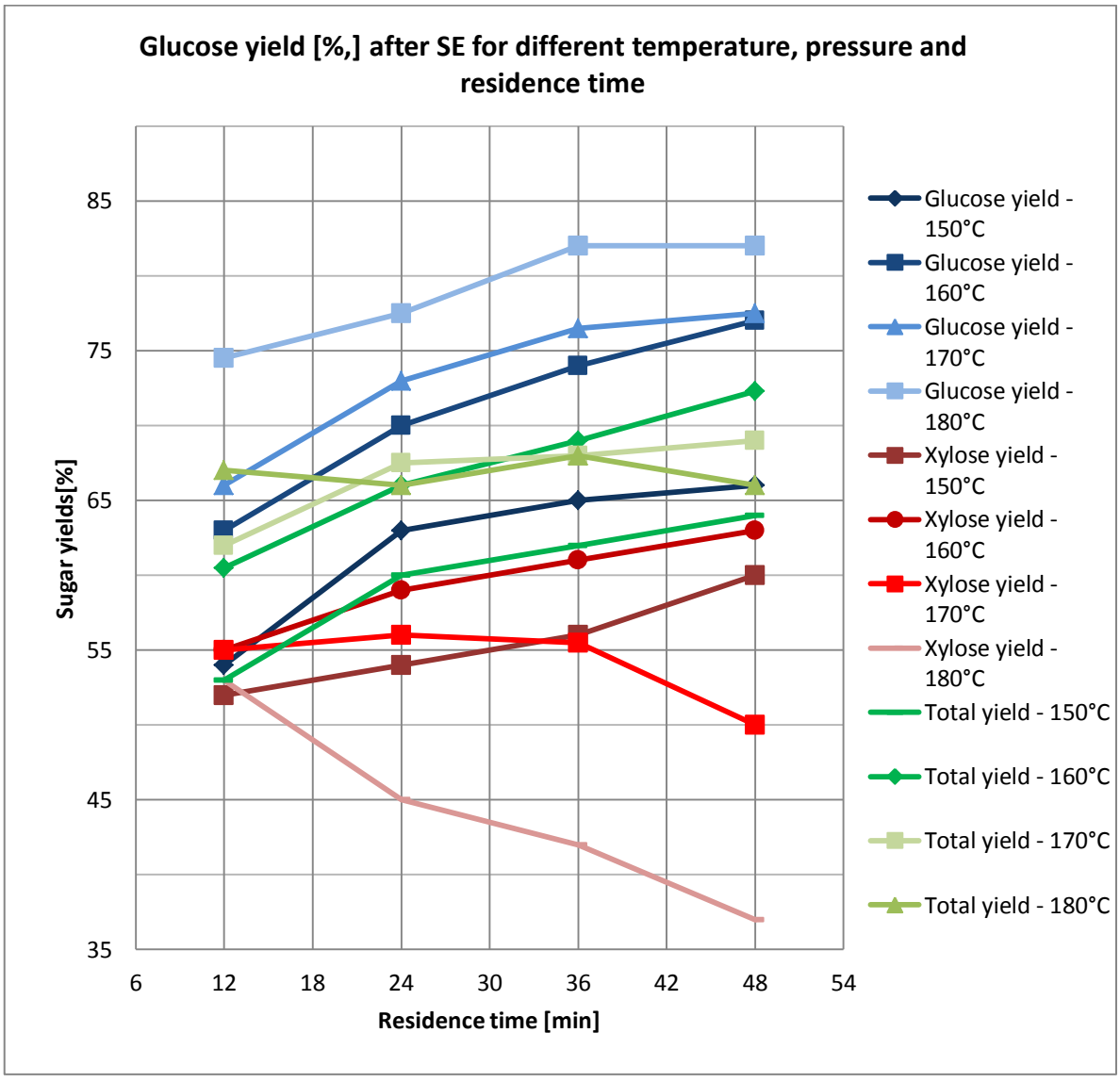
Pretreatment results must be balanced against their impact in xylan recovery, DPs formation and digestibility efficiency. The pretreatment severity below 170°C was lower than that at 200°C for 6min. Xylan recovery below 160°C was more than 70% which was higher than that at 200°C for 6min (56.2%). Xylan removal should affect the digestibility performance. The optimal solubilisation and digestibility of xylan can be obtained by either high holding temperature with short residence time (200°C, 6min) or low holding temperature with long residence time (160°c, 48min). Results implied that SE with low holding temperature and long residence time was more favourable.

DPs formed from sugars and lignin may have a potential inhibitor's effect in digestibility and fermentation. Low temperature SE resulted in lower yield of HMF and furfural compared with 200°C and 6min and previous studies, which corresponded to less degradation of sugars.

The digestibility efficiency is a key metric to evaluate the pretreatment performance. Furthermore, the high digestibility efficiency is also required in the lignocellulosic biomass conversion process. Glucan and xylan conversion at 6% solid loading with an enzyme loading of 10FPU/g solid loading was more than 82.3% and 79.6% beyond 160°C at 48min. results suggested that low temperature SE improved the digestibility efficiency. Water holding capacity and specific surface area of SE corn stover increased with the increase of

holding temperature and residence time, respectively, which was higher than that for untreated corn stover. the increased of water holding capacity implied that the highly rigid and ordered structure of untreated corn stover was disrupted and the hydrophilic groups of cellulose and hemicellulose were exposed after SE. the increase of specific surface area indicated that the particle size of untreated corn stover was reduced and the pore structure of corn stover was expanded. SE increased the water holding capacity and specific surface area of SE and consequently improved the digestibility performance.

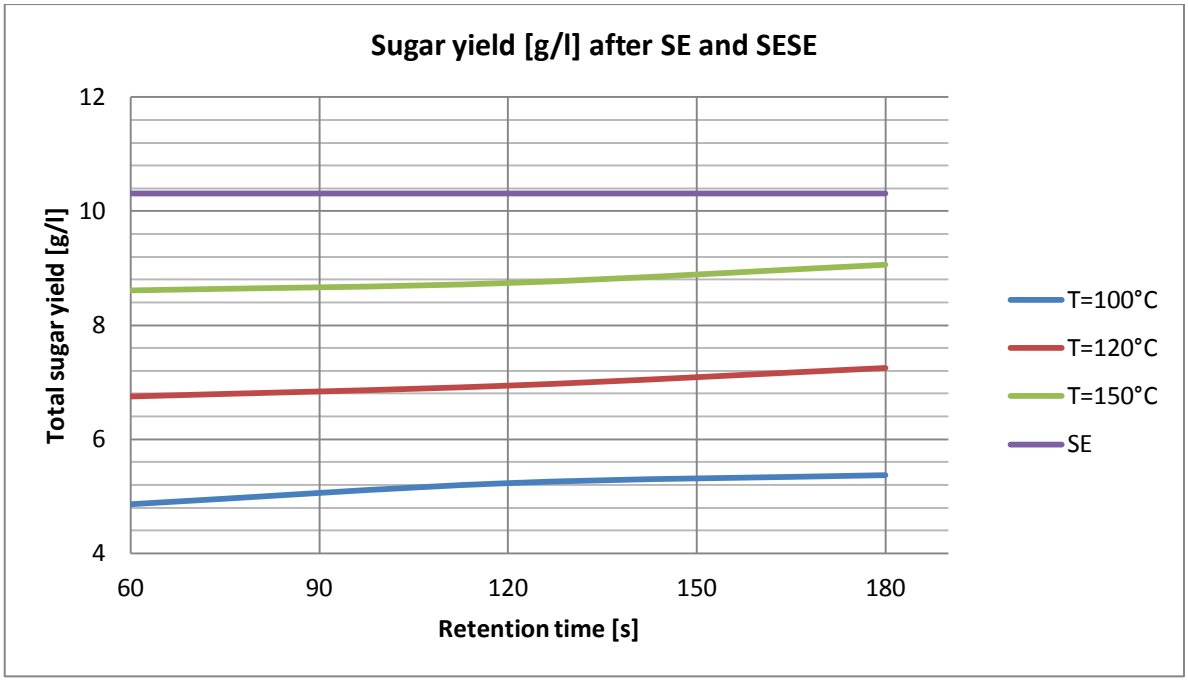
Glucose, xylose and total sugar yield in the whole process are show in the graphs below:



The optimal conditions of low temperature SE were 160°C and 48min, under which glucan and xylan recovery was 93.4% and 71.6% respectively. Glucan and xylan conversion was 82.3% and 79.6% at 6% solid loading at 10FPU/g, respectively. In the whole process, glucose, xylose and total sugar yields reached 77.3%, 62.8% and 72.3% respectively. Low holding temperature and high exploding pressure steam explosion combined with digestibility should be an effective process to improve sugar production.

5.3 Screw extruder steam explosion

Chen et al. [4.11] analysed a screw extruder steam explosion (SESE), in which a screw extrusion is combined with steam explosion to feed and explode the corn stover continuously. SE is considering as an high energy consuming batch process, with high operating temperature and high pressure. After the SE pretreatment, high enzymatic hydrolysis yield is obtained (>88 %) but various fermentation inhibitors are also generated at these conditions. In the this study, corn stover was submitted to SESE at the temperature ranging from 100°C to 150°C, without previous cut up. Corn stover was previously dried at room temperature to equilibrium moisture content of 8.0% without chipping. Raw materials are heated up to determined temperature in the feed zone and then, together with hot steam, enter into the compression zone of SESE device, heated and compressed by the energy provided by the friction and compression among the screw, the inter wall of the device and the material. Right after the material is extruded out of the metering zone. Researchers investigate the process with temperature of 100°C, 120°C and 150°C and residence time of 1min, 2min and 3min. After the explosion, the material was recovered in a cyclone. The solid was stored at 4°C for subsequent characterization and enzymatic hydrolysis. The liquid after pretreatment was collected by filtration from washing the treated corn stover. The enzymatic hydrolysis was carried out at 50°C, 4.8 pH, 150rpm in a shake flask. The solid-to-liquid ration of 1:10. Enzyme loading of 60 FPU/g of glucan cellulase, with 15 CBU of β-glucosidase.



During pretreatment, lignocellulose degrades into sugars and other chemicals. Therefore, pretreatment influence can be partly evaluated by composition of the liquid samples, mainly composed by monosugars and inhibitors. As showed in the graph, with the increasing of treating temperature, more monosugars, mainly xylose, were detected.

Banally, the total sugar yield behaviour can be approximated with a linear function, with a R-squared value between 0.937 and 0.981, of retention time (t) and process temperature (T):

$$Total\ sugar\ yield\ \left[\frac{g}{l}\right] = A(T) \cdot t + B(T)$$

Where A and B are function of T. They can be written as a second order polynomial with an R-squared value of 1.000:

$$A(T) = a \cdot T^2 + b \cdot T + c = -1.95 \cdot 10^{-7} \cdot T^2 + 3.88 \cdot 10^{-5} \cdot T + 2.33 \cdot 10^{-3}$$

$$B(T) = a \cdot T^2 + b \cdot T + c = -5.88 \cdot 10^{-4} \cdot T^2 + 0.221 \cdot T - 11.59$$

SE showed the multiply content (60g/l) of fermentation inhibitors compared with SESE process, due to the higher temperature applied.

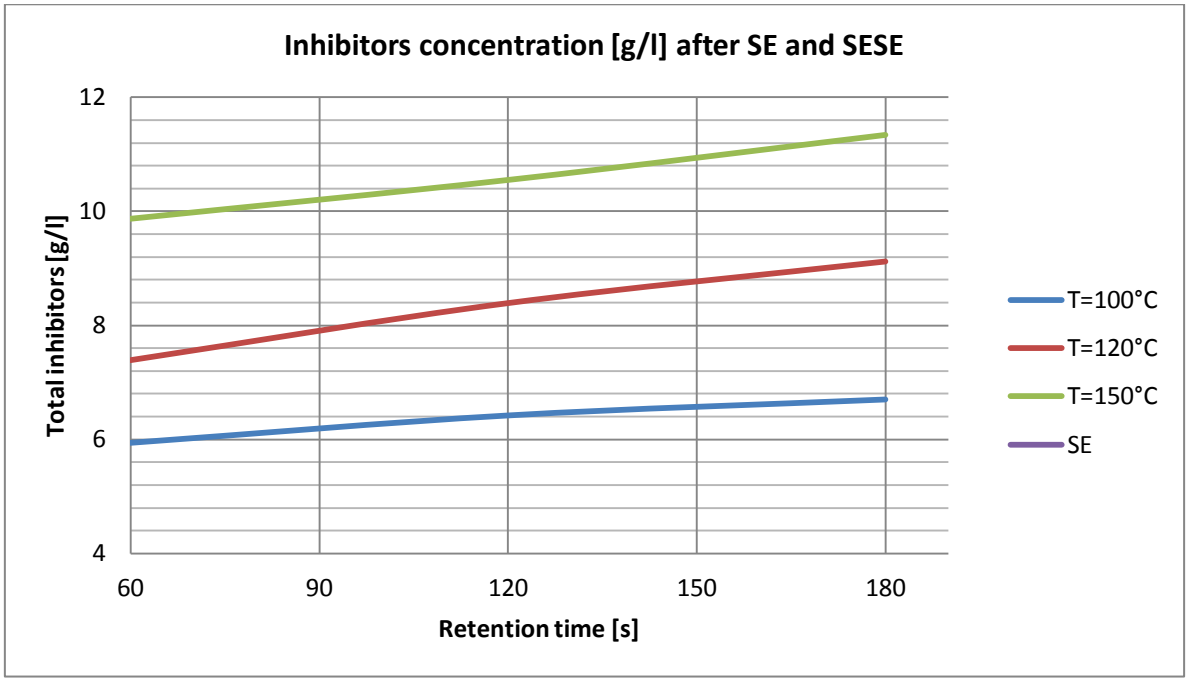
As for sugar yield, also inhibitors concentration can be approximated with a linear function of retention time (t) and process temperature (T):

$$Inhibitors\ concentration\ \left[\frac{g}{l}\right] = a(T) \cdot t + b(T)$$

Where A and B are function of T. They can be written as a second order polynomial:

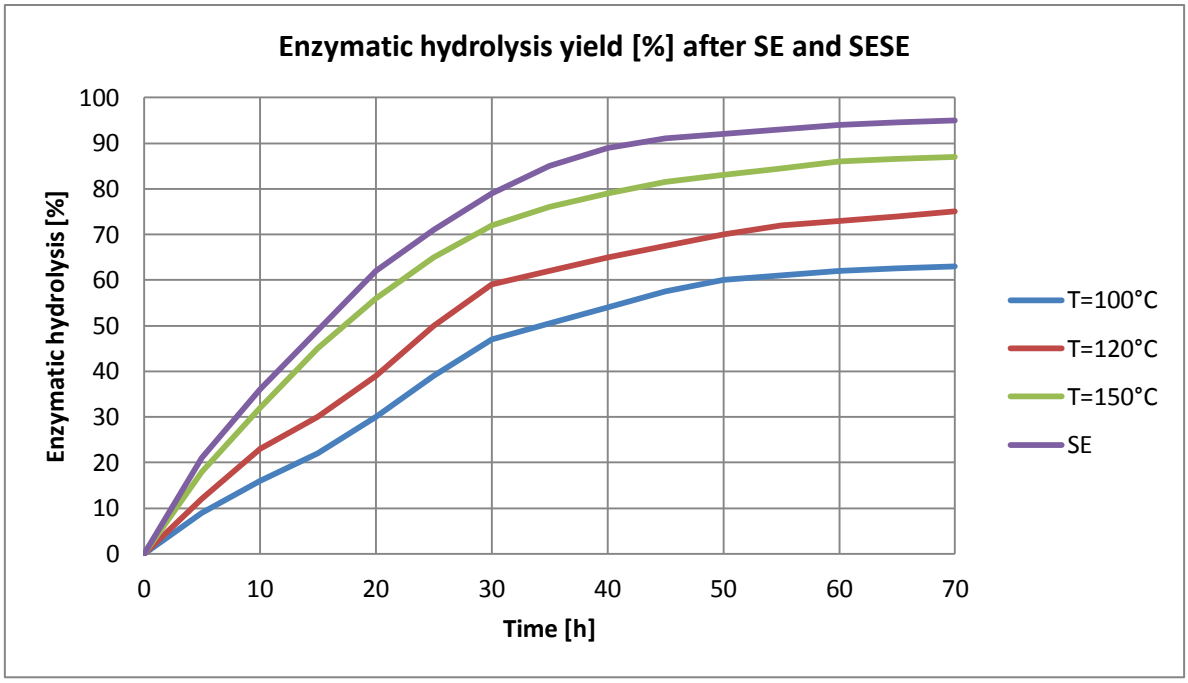
$$A(T) = a \cdot T^2 + b \cdot T + c = -1 \cdot 10^{-5} \cdot T^2 + 2{,}5 \cdot 10^{-3} \cdot T - 0{,}148$$

$$B(T) = a \cdot T^2 + b \cdot T + c = 7 \cdot 10^{-4} \cdot T^2 - 0{,}110 \cdot T + 9{,}363$$



The slope of these curves increases with the temperature.

The graph below shows that enzymatic hydrolysis sugar yields were improved by SE and SESE pretreatments. The lowest sugar yield of the treated solid sample is SESE (100°C - 2min) with the sugar yield of 63%, while the sugar yield of untreated corn stover at the same enzymatic hydrolysis conditions was only 32%. The maximum yield at 70h (89%) was obtained from SESE (150°C – 2min), which is competitive compare to the enzymatic hydrolysis yields of SE (95%).



The SESE pretreatment achieved 89% enzymatic hydrolysis yield at the optimum conditions (150°C – 2min). Although the yield of enzymatic hydrolysis is not very high compare to SE process, but the amounts of fermentation inhibitors were lower and the operation fees were much cheaper.

SESE process is aim to be a competitive alternative to SE, especially in large scale continuous bio-refineries: continuity, low energy demand, and discrete conversion yield are features that characterized SESE pretreatment.

Experimental enzymatic hydrolysis time courses can be fitted with the double quadratic hyperbola [3.2]:

$$EH\ [\%] = \frac{a(T,t)\cdot time\ [h]}{b(T,t) + time\ [h]} + \frac{c(T,t)\cdot time\ [h]}{d(T,t) + time\ [h]}$$

Where the coefficient a, b, c, and d will be functions of process temperature and retention time.

5.4 Chemical hydrolysis

5.4.1 Sulfuric acid

Lloyd et al. [4.22] tried to identifying conditions to realize the highest yields glucose and xylose from dilute acid pretreatment and from subsequent digestion.

A sample was pre-soaked at room temperature in the appropriate concentration (0.22-0.98% w/w) of dilute sulfuric acid solution at 5% solids (w/w) for at least 4h. Then, the pre-soaked slurry was transferred to the reactor and it is bring up to the desired temperature: the range 140-200°C was investigated.

After filtering and washing the contents, the wet solid residue (about 1g of dry matter) was transferred to a 100ml shake flask, and 30ml of 0.05M acetate buffer was added. The flask was put in a shaking water bath set at 50°C and 150rpm and allowed to equilibrate for several hours. The appropriate amounts (from 3-60 FPU/g and 6-120 CBU/g) of cellulase (Genencor, Spezyme CP) and cellobiase (Novozyme, Novozym 188) enzyme were then added. At 24, 48, and 72h, 0.5ml aliquots were removed and filtered, and the filtrates were analysed for glucose and xylose.

Overall, up to 93.0% of the total sugars originally available in the corn stover used could be recovered for coupled dilute acid pretreatment and enzymatic hydrolysis at 140°C for 40min and a H_2SO_4 concentration of 0.98% and at 60 FPU/g glucan, respectively. Xylose yield and glucose yield in the hydrolysate of the first step are 32.4% and 4.3%, respectively, while their values in the enzymatic hydrolysis step are 4.5% and 51.8%, respectively.

5.4.2 Nitric acid

Zhang et al. [4.17] investigated the optimization of diluted nitric acid pretreatment of corn stover. The size of corn stover chips are very small, lower than 0.5mm. The reaction temperature was set to 150, 165 and 180°C and the nitric acid concentration to 0.2, 0.4 and 0.6% w/w. All pretreatment experiments are carried out in a laboratory scale stainless steel vessel with a total volume of 0.5L. the stainless steel was equipped with a heat exchanger, an impeller. type mixer and a thermocouple. An electrical heated jacket with temperature controller was used to control the set temperature.

The highest xylose concentration (22.03g/L) was reached at 2min in the experiment performed with 0.6% HNO_3 at 150°C. At this condition, the conversion of xylan into xylose was 96.1%. It was also observed that xylose concentration reached a maximum value and then decreased with reaction time, and the decreasing rate increased with increasing reaction temperature and HNO_3 concentration. The conversion of xylan into xylose also decreased rapidly and the lowest conversion was 5.7% under the harshest conditions (180°C, 0.6% HNO_3, 60min). These results confirmed that undesirable decomposition reactions occurred, which were more obvious under the severest conditions.

Arabinose was released more quickly than glucose and xylose. The highest concentration (2.95g/L) of arabinose was reached after 5min in the experiment performed with 0.4% HNO3 at 150°C. Under this condition, about 100% of the initial arabinan was converted into arabinose, demonstrating that arabinosyl substituents were susceptible to hydrolysis. It was

observed that arabinose concentration reached a maximum value and then decreased with reaction time, indicating that degradation to furfural occurred.

The acetic acid concentration increased quickly with time and nitric acid concentration during the first 10min. After that, the rate of release was very low but the acetic acid concentration was not decreased, which indicated that no decomposition reaction took place. To date, the effect of acetic acid on the growth of microorganisms is not clear. In this study, the highest value was 5.86 g/L, obtained in the experiment performed using 0.6% HNO_3 at 180 ◦C with a reaction time of 10 min.

Furfural was generated as a degradation product from pentoses. The concentration of furfural increased with temperature and the concentration of nitric acid. The maximum concentration was 14.23g/L, obtained in the experiment performed with 0.6% HNO_3 at 180°C with a reaction time of 60min, which was 79.9% of the potential concentration of furfural. This result implied that most of the pentoses could be decomposed to furfural even under the harshest conditions.

It is important to obtain hydrolysates with high sugar concentrations (carbon source for microorganism growth) and low concentrations of growth inhibitors (acetic acid and furfural) if they are to be used as fermentation media. Based on this consideration, the operational conditions were optimized using the kinetic modelling of each concentration.

The condition of 0.6% HNO_3 at 150°C for 1min is proposed as the overall optimum condition. This condition gives a high concentration of fermentable sugars but also saves time, thus reducing energy consumption and therefore the cost of the hydrolysis process.

Under this optimum condition, the conversions of glucan, xylan, arabinan into respective monosaccharides were 4.7%, 95.9%, 99.2%. This result was comparable with the data reported by other researchers.

5.4.3 Combined diluted acid and alkaline oxidation

Lee et al. [4.15] explored the feasibility of applying sequential dilute acid and alkali pretreatment into the hydrolysis of corn stover.

The raw material was milled to small particles in a blender, and then screened through sieves to obtain the -20/+80 mesh fraction (relevant to the particle sizes of 180–850μm). These corn stover particles were air-dried at 50°C and stored in a sealed container at room temperature until use.

All pretreatment tests were conducted in a stainless steel reactor with a working volume of 100ml. The reactor was equipped with a temperature control unit consisting of thermo-couple, modular controller and electric heat wire. In each test, 8g of the corn stover particles and 80ml of dilute H_2SO_4 solution with varying concentrations were mixed together and introduced to the reactor. The reaction started when the reactor temperature reached a desired operating temperature. The applied reaction temperature ranged from 140 to 180°C, the reaction time from 1 to 90min and the H_2SO_4 concentration from 0.25% to 1.00% (w/v).

When the pretreatment reaction was finished, the reactor was immediately cooled to room temperature by using tap water. The pretreated solution was obtained by filtration and the filtered solids were washed with deionized water until a neutral pH was reached. The washed corn stover residue was dried at 50°C for more than 3 days.

Then, 5g (in dry weight) of the pretreated corn stover was mixed with 100ml 2% (w/v) NaOH solution and loaded into the same reactor. The alkaline pretreatment reaction was conducted at 80°C for 1h. After completion of the second pretreatment, only the solid residue was separated using a sieve. The obtained corn stover residue was extensively washed with deionized water and dried in an oven at 50°C, then kept under anhydrous condition.

EH of the pretreated corn stover residue was carried out in a digestion solution containing sodium citrate buffer (pH 4.8) and sodium azide, which was prepared in accordance with NREL standard procedures. An amount of pretreated corn stover residue equivalent to 1g of glucan was loaded in 250ml Erlenmeyer flask and an enzyme mixture of cellulase (Celluclast 1.5L) and b-glucosidase (Novozyme 188) was added together with the digestion solution, so that the final volume of the reaction mixture could be 100ml. The enzyme loadings were 15 or 60 FPU/g-glucan for cellulase and 30 CBU/g-glucan for b-glucosidase, respectively. The reaction mixture was digested in a rotary shaker at 150rpm and 50°C for 72h. Hydrolysates were sampled periodically for sugar analysis. The raw corn stover and a-cellulose were also subjected to the same EH test as a control and as a reference, respectively.

Diluted sulfuric acid pretreatment reaches is optimum at 180°C for 1min with a concentration of H_2SO_4 of 0.5% (w/v). Solid recovery is of 49.4% while glucan recovery and xylan recovery are 51.8% and 6.5%, respectively. In the hydrolysate, the glucose and xylose yields are 14.2% and 71.0% with a generation of inhibitors of 3.75g/L of hydrolysate. Enzymatic hydrolysis yield is only 72.1% and the overall glucose yield of 75.8%.

Coupling the dilute acid pretreatment with the alkaline pretreatment, the enzymatic hydrolysis reached the 100% of digestibility with an overall glucose yield of 97.9%.

H_2SO_4 used in the first step selectively hydrolysed 74.6–77.3% of xylan and NaOH used in the second step removed 85.9–89.4% of lignin, from the raw corn stover. Compared to single dilute acid pretreatment, the proposed combined pretreatment minimized the generation of by-products such as acetic acid, furfural and hydroxyl-methyl-furfural in the hydrolysates, and enhanced the enzymatic hydrolysis of the solid residue. The changes in the structural features (porosity, morphology, and crystallinity) of the solid residue were strongly correlated with the enhancement of enzymatic digestibility. The overall glucose and xylose yields finally obtained after enzymatic hydrolysis reached 89.1–97.9% and 71.0–75.9%, respectively.

An et al. [4.21] analysed a two-stage pretreatment method developed to improve sugar recovery. Firstly, the corn stover was pretreated with acidic dioxane to remove lignin, then the residue was subjected to dilute hydrochloric acid to eliminate the negative effects of hemicelluloses on enzymatic hydrolysis as well as increasing xylose yield.

In the first stage, 4g dried corn stover and 40mL different acid concentrations (0.2, 0.4, 0.6, 1.0, 1.2 and 1.5% w/w) in dioxane solution were mixed in 70mL batch reactor with agitation. Different temperature (70, 80 and 90°C), reaction time (10, 20, 30, 40 and 50 min), dioxane–water ratios (1 : 1, 4 : 1, 9 : 1, 12 : 1, v/v) have been investigated. After pretreatment, the reactors was removed from the heating jacket and directly cooled to room temperature with circulating water. Then the obtained hydrolysate was separated from the residues by filtration. The hydrolysate was divided into halves, one half was used to analyse the lignin recovery and the other half was analysed to determine its glucose, xylose and lignin contents. The optimal condition was chosen according to the consumption of energy and acid as well as the recovery of glucose, xylose and lignin. All the reactions were conducted in duplicated. The solid residue was used for the second stage pretreatment. In the second dilute acid stage, the wet residue from the first stage was further pretreated under the optimal conditions (1% w/w HCl, 120°C, 40min) which were investigated in our previous work. After this process, the residual solid was collected by filtration, washed with deionized water until it was free of acid and then stored for enzyme hydrolysis stock. The hydrolysate was collected to determine the glucose and xylose content with the same method in the first stage.

Enzymatic hydrolysis was performed by using a cellulase concentrate (Cellulast 1.5 L) supplemented with b-glucosidase (Novozym 188). The enzyme activities was 67.8 filter paper units (FPU) per mL (expressed as micromoles of glucose produced per minute, with filter paper as a substrate) and 210.5 cellobiose units (CBU) per mL (expressed as micromoles of cellobiose that is converted to glucose per minute, with cellobiose as a substrate) for Celluclast 1.5 L and Novozyme 188, respectively. Enzymes were directly used without further purification. The enzymatic hydrolysis of untreated and wet pretreated samples (0.2g dry substrate) were conducted in a centrifuge tube containing 50mM sodium acetate buffer (pH 4.8). The mass concentration was set at 5% (w/v). Sodium azide (0.3%, w/v) was also added for inhibiting the microbial infection. Cellulase (3, 5 and 10 FPU per gram substrate) and b-glucosidase (20 CBU per gram substrate) were added into the tube as well. The enzyme hydrolysis experiments were performed in a shaking incubator at 50°C, 120 rpm. After 72 h, the hydrolysate was separated from the mixture by centrifugation.

The optimal condition was 90°C, 20min, and 9/1 (v/v) dioxane–water including 1.0 wt% HCl solution in the first stage followed by 120°C and 40min for 1.0 wt% dilute hydrochloric acid in the second stage. The total yields of glucose and xylose were 91.5% and 79.7%, respectively, with a low cellulase dosage of 3 FPU g_1 of substrate. This two-stage pretreatment was effective due to the removal of lignin in the first stage and the hydrolysis of hemicelluloses in the second stage, resulting in a very high sugar recovery with a low enzyme loading.

An et al. [4.18] analysed a two-stage dilute hydrochloric acid (DA)/aqueous ammonia wet oxidation (AWO) pretreatment to recover the sugars of corn stover.

The corn stover was dried and grinded by a cutting mill to pass through a 0.4mm screen. The corn stover was the pretreated using 1% w/w HCl at 120°C for 40min with a solid-to-liquid ration of 1:10 (g/mL) in 70mL batch reactor with mechanical stirring. After the pretreatment, the reactor was moved away from the heating jacket and cooled down to room

temperature with cold water. Then the reaction mixture was separated by filtration and washed with demi water.

The corn stover was pretreated using the combination of aqueous ammonia and oxygen. The effects of different ammonium hydroxide concentrations (6.2, 12.6, 16.0, 19.4, 22.9, 26.5 wt%), reaction temperatures (100, 110, 120, 130, 140, 150°C), reaction times (20, 30, 40, 50, 60min) and oxygen pressures (1.0, 2.0, 2.5, 3.0, 3.5, 4.0MPa) on the glucan yield from enzymatic hydrolysis were investigated. The desired temperature of experiments need 12 min. The reaction time was counted when the desired temperature was reached. The pretreatment was terminated immediately in cold water. After the pretreatment, the residues were separated by filtration, washed by deionized water until the filtrate reached the native pH

The cellulases were loaded at 3 FPU·g−1 glucan and supplemented with 20 CBU of β-glucosidase. 0.03 g/mL sodium azide was added as an antibiotic to inhibit microbial infection during the enzymatic hydrolysis. The hydrolysis process proceeded for 72 h. After enzymatic hydrolysis, the hydrolysate and residues were separated by centrifugation.

The results showed that DA-AWO process demonstrated a positive effect on sugar recovery compared to AWO-DA. 82.8% of xylan was recovered in the first stage of DA-AWO process at 120°C for 40min with 1% w/w HCl. The second stage was performed under relative mild reaction conditions (130°C, 12.6% w/w ammonium hydroxide, 3.0MPa O_2, 40min), and 86.1% lignin could be removed. 71.5% of glucan was achieved with a low enzyme dosage (3FPU/g) in the following enzymatic hydrolysis. DA-AWO pretreatment was effective due to its sufficient hydrolysis of hemicellulose in the first stage and remarkably removal of the lignin in the second stage, resulting in high sugar recovery with a low enzyme dosage.

5.4.4 Sodium hydroxide + sodium sulphite pretreatment

Liu et al [4.24] analysed the optimization of alkaline sulphite pretreatment of corn stover. The influences of pretreatments on solid yield, delignification, and carbohydrate recovery under different pretreatment conditions and subsequent enzymatic hydrolysis were investigated. The effect of pretreatment was evaluated by enzymatic hydrolysis efficiency and the total sugar yield.

The pretreatment of corn stover (50g oven-dried) was carried out in a pot with a total volume of 1.5L. Corn stover was subjected to pretreatment using sodium hydroxide (NaOH)−sodium sulphite (Na_2SO_3) with total titratable alkali charge of 6%, 8%, 10%, 12%, and 14%. The molar ratio of NaOH−Na_2SO_3 was 1:1. The ratio of pretreatment liquor to corn stover (oven-dried) charge was 4:1, 6:1, 8:1, 10:1, and 12:1, respectively. Both solution and corn stover were heated to the desired temperature in 20min from 30°C. The pretreatments were done at five levels in the range of 120−160°C at holding time ranging from 10 to 50min. After pretreatment, the bombs were cooled in water to room temperature. The solids were collected and washed with deionized water to remove residual chemicals and dissolved compounds and then used for determination of total solid, lignin, and polysaccharides prior to enzymatic hydrolysis.

The pulp pretreated with alkali was beaten in a PFI mill. Beating conditions were as follows: pulp consistency 10%, beating gap 0.3mm, revolutions in the range 1500−6000rpm and increments of 1500 revolutions. The refined pulp was collected and stored in the refrigerator.

Enzymatic hydrolysis of the substrates was conducted on a 0.4g (oven-dried) basis in a serum bottle with substrate consistency of 2% (W/V) at 50°C using an incubator shaker at 95rpm for 48h. Sodium citrate buffer (pH = 4.8) was added to maintain the pH at 4.8, while 0.02% sodium azide was used in the mixture to inhibit the microbial infections. An enzyme cocktail of cellulase (20PFU/g of dry biomass) and β-glucosidase (10IU/g of dry biomass) was used for enzymatic hydrolysis.

The optimum pretreatment conditions were obtained, as follows: the total titratable alkali* (TTA) of 12%, liquid/solid ratio of 6:1, temperature of 140°C, and holding time of 20min. Under those conditions, the solid yield was 55.24%, and the removal of lignin was 82.68%. Enzymatic hydrolysis rates of glucan and xylan for pretreated corn stover were 85.38% and 70.36%, and the total sugar yield was 74.73% at cellulase loading of 20FPU/g and β-glucosidase loading of 10IU/g for 48h. Compared with sodium hydroxide pretreatment with the same amount of total titratable alkali, the total sugar yield was raised by about 10.43%. Additionally, the corn stover pretreated under the optimum pretreatment conditions was beaten by PFI at 1500rpm. After beating, enzymatic hydrolysis rates of glucan and xylan were 89.74% and 74.06%, and the total sugar yield was 78.58% at the same enzymatic hydrolysis conditions. Compared with 1500rpm of PFI beating after sodium pretreatment with the same amount of total titratable alkali, the total sugar yield was raised by about 14.05%.

*Titratable alkali (TA) refers to the total concentration of free protons and undissociated alkali in a solution that can react with a strong acid and be neutralized

5.4.5 Sodium hydroxide methanol solution

Yuan et al. [4.19] investigated the sodium hydroxide-methanol solution (SMs) pretreatment of corn stover to overcome biomass recalcitrance for the first time. Effects of sodium hydroxide loading, solid-to-liquid ratio, processing time and temperature were studied in detail.

Corn stover was milled and passed through 1mm size screen. The milled materials were washed with water for three times to remove the field dirt, and then dried at 105 °C to a constant weight.

The general procedure of the SMs pretreatment of corn stover was as follows. Firstly, NaOH and methanol ranging from 0 to 2.5g and 10 to 100mL, respectively, were added into a 100mL Schott red gap bottle to form a homogeneous SMs. Corn stover (5.0g) was then added into the bottle and mixed thoroughly with the SMs. The bottles were then sealed and heated up to the reaction temperature of 20–100°C in a water bath and maintained at the constant temperature for a processing time of 0.5–3.0h. When the SMs pretreatment was finished, the whole slurry was quickly cooled below 50°C by water, filtrated through

Buchner funnel and washed three times by equal volume of methanol or water. Finally, the regenerated samples were dried at 105°C to a constant weight before use.

The regenerated solids after the SMs pretreatment were resuspended in water and adjusted to pH 4.8 with 4M H_2SO_4 to a final solid loading of 5% (w/v). Unless otherwise specified, the enzymatic hydrolysis was conducted at 50°C for 72h in the presence of 15 FPU cellulase, 5 mg β-glucosidase and 5 mg xylanase per gram corn stover in a shaking incubator. Cellic® $CTec_2$ of 15FPU/g corn stover was also investigated for enzymatic saccharification. Sodium azide of 0.03% (w/v) was added to prevent microbial growth.

The SMs pretreatment could significantly enhance the enzyme accessibility of corn stover, minimize the degradation of sugar polymers, and decrease the energy consumption. 97.5% glucan and 83.5% xylan were preserved in the regenerated corn stover under the optimal condition: 1h of pretreatment with alkali loading 0.1g/g, at temperature of 80°C and solid-to-liquid ratio of 50% (w/v). Subsequent enzymatic digestibility of glucan and xylan reached 97.2% and 80.3%, respectively. The enzyme susceptibility of the regenerated samples was explained by their physical and chemical characteristics. This strategy provides a promising alternative for better techno-economic of the lignocelluloses-to-sugars routes.

5.4.6 Alkaline hydrogen peroxide pretreatment

Banerjee et al. [4.23] analysed the effects of biomass loading, hydrogen peroxide loading, residence time, and pH control in combination with subsequent digestion with a commercial enzyme preparation, optimized mixtures of four commercial enzymes, or optimized synthetic mixtures of pure enzymes.

Corn stover was ground to pass a 5 mm screen and stored at room temperature. Before use, it was further ground in a mill to pass a screen size of 0.5 mm.

For AHP, a solution of H_2O_2 (diluted from a commercial 30% stock) was titrated to pH 11.5 (±0.2) with 5M NaOH and mixed with the biomass. When comparing different biomass loadings, a fixed amount of biomass (1g) was added to a fixed amount of H_2O_2 plus NaOH and a variable amount of water to give the desired final biomass loading. Pretreatment biomass loadings are given as nominal % w/v, for example. All pretreatments were performed at 23°C in 125ml flasks with shaking at 90rpm for 24h or 48h. After AHP pretreatment, the biomass suspensions were neutralized to approximately pH 7 with concentrated HCl, treated with catalase to destroy residual H_2O_2, heated at 90°C for 15min to inactivate the catalase, and lyophilized to dryness.

After AHP pretreatment and neutralization, biomass samples were typically lyophilized. The main reason for this was a concern about the stability of wet biomass during storage prior to the enzyme hydrolysis step. However, air drying biomass has been shown to adversely affect subsequent enzymatic digestibility, and therefore the effect of lyophilisation on digestibility was tested. Corn stover (10g) was placed in each of two 500ml glass bottles at a final biomass loading of 10% and subjected to AHP pretreatment for 24h at an H_2O_2 loading of 0.5g/g biomass. One sample was then lyophilized whereas the other sample was subjected directly to enzymatic hydrolysis. The enzymatic hydrolysis conditions for both samples were 0.2% glucan loading, 15mg enzyme/g glucan, pH 4.6, 48h, and 50°C. The enzyme

mixture comprised 64% Accellerase 1000, 9% Multifect Xylanase, and 27% Multifect Pectinase, which proportions were derived from small-scale experiments with the same pretreatment conditions. Digestibility of the two samples were within 3% of each other (95±2.2% for lyophilized vs 92±2.0% for non-lyophilized). We conclude that drying the pretreated biomass by lyophilisation does not reduce subsequent sensitivity to enzymatic

Optimization experiments with four-component commercial enzyme cocktail mixtures used an augmented quadratic experimental design and a fixed enzyme protein loading of 15 mg/g glucan.

At a pretreatment biomass loading of 10% and an H_2O_2 loading of 0.5 g/g biomass, an optimized commercial mixture at total protein loadings of 15 mg/g glucan gave monomeric glucose yield and xylose yield of 95,0% and 75.1%, respectively.

5.4.7 Urea pretreatment

Wang et al. [4.20] investigated the high-solid pretreatment of corn stover using urea. Effects of solid loading (30–70%), temperature (60–80°C), time (2–14d), and ratio of urea to corn stover (1:10–1:1), were studied.

Pretreatment was conducted in 500mL flask sealed by screw cap. 20g of grinded corn stover was loaded and adjusted to desired solid loading with distilled water and then was mixed with urea. When the pretreatment finished, the flasks were unsealed and the ammonia was released in a fume hood for 10min. The pretreated corn stover was washed with distilled water and was filtered by a vacuum pump for removing the remaining ammonium and soluble lignin and other matters. A portion of the solid mass after filtration was used for enzymatic saccharification.

Enzymatic saccharification experiment was performed in a 150mL Erlenmeyer flask containing 50mL sodium acetate buffer (pH 5.00) and 0.02% (w/v) sodium azide to prevent microbial growth at 2% (w/v, grams dry weight per 100mL) solid content. Enzyme loading (Accellerase 1500) was 1mL/g. The flasks were incubated at 50°C and 140rpm for 72h in a shaking incubator and the hydrolysate was withdrawn for sugar analysis at the end of saccharification.

Generally, the glucose and xylose yield increased as the reaction temperature, time, and ratio of urea to corn stover increased. Urea enhanced enzymatic saccharification at a relatively high-solid loading and low temperature. The results showed that 80°C for 10 days with urea to corn stover ratio of 1:1 and 50% solid loading are optimum treatment condition to achieve high glucose recovery and enzymatic saccharification efficiency. Under optimum condition, 97.24% glucan and 61.63% xylan were preserved in the treated corn stover, and the maximum enzymatic saccharification efficiencies of 84.11% and 78.54% were achieved for glucose and xylose, respectively. Therefore, the urea pretreatment was a promising approach featuring with comparable efficiency of enzymatic saccharification as other pretreatment methods, high biomass loading, low temperature, low pressure, low water usage, and ammonia recycle.

5.5 Optimization parameters

	Optimized parameter	Glucan recovery	Xylan recovery	Sugar recovery	Inhibitors concentration	Enzymatic hydrolysis yield	Overall yield
SE+EH (size optimization 2.0cm) [3.5]	SE Temp: 200°C Time: 5min R.H.: 8% EH Temp: 50°C S-L ratio: 1% w/v Enzyme: Accelerase 1500 Conc: 60FPU/g Time: 168h	91.8%	66.0%	78.9%	N.A.	82%	71.5%
SE+EH (moisture optimization 60%) [3.14]	SE Temp: 198°C Time: 5min EH Temp: 50°C Enzyme: Cellic CTEC 2 Time: 48h	N.A.	N.A.	74.5%	3.54g/100g	92.0%	68.5%
SE+EH [4.12]	SE Temp: 160°C Time:48min EH Temp: 50°C Enzyme: Cellic CTEC 2 Conc: 10FPU/g S-L ratio: 6% Time: 120h	93.4%	71.6%	N.A.	0.14g/100g	N.A.	72.5%
SESE+EH [4.11]	SESE Temp: 150°C Time: 2min EH Temp: 50°C Enzyme: Sigma Aldrich Conc: 60FPU/g S-L ratio: 1:10	N.A.	N.A.	N.A.	8.90g/L	89%	63%

	Time: 70h						
DA+EH (Sulfuric acid) [4.22]	Temp: 140°C Conc: 0.98% w/v S-L ratio: 1:20 w/w Time: 40min Enzyme: Spezyme CP + Novozym 188 Time: 72h Temp: 50°C	N.A.	N.A.	N.A.	N.A.	N.A.	93.0%
DA+EH (Sulfuric acid) [4.15]	Temp: 180°C Conc: 0.5% w/v S-L ratio: 1:10 w/v Time: 1min Enzyme: Celluclast 1.5L + Novozym 188 Time: 72h Temp: 50°C	51.8%	6.5%	N.A.	3.75g/L	72.1%	73.4%
DA+ALK+EH (sulfuric acid + sodium hydroxide) [4.15]	DA Temp: 180°C Conc: 0.5% w/v S-L ratio: 1:10 w/v Time: 1min ALK Temp: 80°C Conc: 2% w/v S-L ratio: 1:20 w/v Time: 1h EH Enzyme: Celluclast 1.5L + Novozym 188 Time: 72h Temp: 50°C	51.8%	6.5%	N.A.	3.75g/L	100%	84.5%
DA (Nitric acid) [4.17]	Temp: 150°C Conc: 0.6% w/w HNO_3 Time: 1min	N.A.	N.A.	N.A.	2.66g/100g	N.A.	66.6%
DA+EH (hydrogen peroxide)	Temp: 23°C Conc; 0.5g/g H2O2 Time: 24h	N.A.	N.A.	N.A.	N.A.	N.A.	85%

[4.23]	S/L ratio: 10% EH Temp: 50°C Conc: 15mg/g Time: 48h S/L ratio: 0.2%						
ALK + DA+EH (dioxane-water + HCl) [4.21]	ALK Temp: 90°C Conc: 9/1 v/v dioxane + 1% w/w HCl Time: 20min DA Temp: 120°C Conc: 1% w/w HCl Time: 40min EH Enzyme: Celluclast 1.5L + Novozym 188 Time: 72h Temp: 50°C	N.A.	N.A.	N.A.	N.A.	N.A.	85.6%
DA+ALK+EH (Hydrochloric acid + NH3 H2O) [4.18]	DA Temp: 120°C Conc: 1% w/w S-L ratio: 10% Time: 40min ALK Temp:130°C Conc: 12.6% w/w + 3MPa O_2 S-L ratio: 10% Enzyme: Cellulases Time:72h Temperature: 50°C	83.8%	N.A.	51.9%	2.5g/100g (raw)	85.4%	81.4%
ALK+EH (Sodium hydroxide +Sodium sulphite) [4.24]	ALK Temp: 140°C Conc: 12% Time: 1h S-L ratio: 6:1 EH	N.A.	N.A.	N.A.	N.A.	81.9%	78.6%

	Enzyme: Celluclast 1.5L + Novozyme 188 Time: 48h S-L ratio: 2% w/v Temperature: 50°C						
ALK+EH (Sodium hydroxide methanol) [4.19]	Temp: 80°C Conc: 0.1 g/g Time: 1h Solid loading: 50% Enzyme: Cellic® CTec2 Time: 72h Temperature: 50°C	97.5%	83.5%	90.5%	N.A.	88.8%	63.6%
ALK+EH (Urea) [4.20]	Temp: 80°C Conc: 1g/g Time: 10d Solid loading: 50% Enzyme: Accellerase 1500 Time: 72h	97.2%	61.1%	79.2%	N.A.	81.3%	65.1%

6. Conclusions

Biomass-derived fuels or biofuels are an important contributor in the modern renewables slice of the energy source distribution. In particular, bio-hydrogen is believed to be one of the biofuels of the future, combining its ability to potentially reduce the dependence on foreign oil and contribute to lower the GHG emissions. The future role of hydrogen as a clean fuel for fuel cells producing near zero emissions and as an intermediate energy carrier for storage and transport of renewable energy is increasingly recognized worldwide.

Lignocellulosic biomass is of particular interest as a sustainable source of sugars and platform chemicals for conversion into renewable fuels, fine chemicals, and materials. It is also the only accessible non-fossil source of carbon that can be processed into liquids, which are easily incorporated into the existing transportation fuel infrastructure. However, biomass recalcitrance is the biggest obstacle in the development of large-scale, second-generation cellulosic fuel production and use. Biomass recalcitrance hinders the effectiveness of enzymes during the bioconversion process due to a lack of accessibility and mostly arises from the complex structure and ultrastructure of lignocelluloses.

Hence, biomass must be pretreated to overcome recalcitrance, allow for enzyme accessibility to cellulose, and maximize the recovery of multiple products for improving the economics of second-generation lignocellulosic bio-refineries. Pretreatment has several aims, such as disrupting the physical structure of the biomass by breaking the lignin barriers, disrupting cellulose crystallinity, and removing non-cellulosic components in order to increase the cellulose accessibility.

Pretreatments play a fundamental role in the path of transformation from the raw lignocellulosic biomass to biofuels. Several efforts have to be done yet to reach a sustainable and affordable state of the art of conversion. I hope this work will help someone to have smattering about the argument and create a base to start a serious study on pretreatments of lignocellulosic biomass.

Bibliography

[2.1] *Biomass Resource Basics*, U.S. Office of Energy Efficiency and Renewable Energy (2013)

[2.2] *World Energy Resources – Bioenergy 2016*, World Energy Council (2016)

[2.3] Bharadwaj Kummamuru, *WBA Global Bioenergy Statistics 2017,* World Bioenergy Association (2017)

[2.4] Roger M. Rowell, *Handbook Of Wood Chemistry And Wood Composites, chapter 3: Cell Wall Chemistry*, CRC Press (2005)

[2.5] Zahid Anwar, Muhammad Gulfraz, Muhammad Irshad, *Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review*, Journal of Radiation Research and Applied Sciences (2014)

[2.6] Luiz Pereira Ramos, *The Chemistry Involved In The Stream Treatment Of Lignocellulosic Materials*, Departamento de Química, Universidade Federal do Paraná (2003)

[2.7] Adepu Kiran Kumar, Shaishav Sharma, *Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review*, Bioresources and Bioprocessing (2017)

[2.8] Leif J.Jönsson, Carlos Martín, *Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies to minimize their effects*, Bioresource Technology 199 (2016) 103-122

[2.9] L. Canilha, A.K. Chandel, T.S. Milessi, F. Antunes, W. Freitas, M. Almeida Felipe, S. da Silva, *Bioconversion of Sugarcane Biomass into Ethanol: An Overview about Composition, Pretreatment Methods, Detoxification of Hydrolysates, Enzymatic Saccharification, and Ethanol Fermentation*, Department of Biotechnology, School of Engineering of Lorena, University of Sao Paulo (2012)

[2.10] K. Wilson, A.F. Lee, J.P. Dacquin, *Catalysis for Alternative Energy Generation - Chapter 7: Heterogeneous catalysts for converting renewable feedstock to fuels and chemicals*, School of Chemistry, Cardiff University (2012)

[2.11] S. Devi, A. Dhaka, J. Singh, *Acid and Alkaline Hydrolysis Technologies for Bioethanol Production: an Overview*, International Journal of Advanced Technology in Engineering and Science, vol. 4 (2016)

[2.12] J.B. Kristensen, *Enzymatic hydrolysis of lignocellulose: substrate interactions and high solids loadings*, Forest & Landscape, University of Copenhagen (2009)

[2.13] Valeria Reginatto, Regina Vasconcellos Antônio, *Fermentative hydrogen production from agroindustrial lignocellulosic substrates*, Departamento de Quimica, Universidade de Saõ Paolo (2014)

[2.14] R. Braun, B. Drosg, G. Bochmann, S. Weiß, R. Kirchmayr, *Recent Developments in Bio-Energy Recovery Through Fermentation – Chapter 2*, Department for Agrobiotechnology, IFA-Tulln – University of Natural Resources and Applied Life

Sciences, Tulln, Austria and Department of Environmental Biotechnology, Graz University of Technology, Graz, Austria (2009)

[2.15] J.H. Reith, R.H. Wijffels, H. Barten, *Bio-methane & Bio-hydrogen: Status and Perspectives of Biological Methane and Hydrogen Production*, Dutch Biological Hydrogen Fundations (2003)

[2.16] S.N.A. Rahman, M.S. Masdar, M.I. Rosli, E.H. Majlan, T. Husaini, *Overview of Biohydrogen Production Technologies and Application in Fuel Cell*, Fuel Cell Insistute, University of Malaysia (2015)

[2.17] Peter R. Seidl, Adriana K. Goulart, Pretreatment for lignocellulosic biomass conversion to biofuels and bioproducts, Current Opinion in Green and Suistainable Chemistry 2 (2016) 48-53

[3.1] Kun Wang, Jinghuan Chen, Shao-Ni Sun, Run-Cang Sun, *Pretreatment of Biomass – Chapter 6: Steam explosion*, Elsevier B.V. (2015)

[3.2] Mareike Monschein, Bernd Nidetzky, *Effect of pretreatment severity in continuous steam explosion in enzymatic conversion of wheat straw: Evidence from kinetic analysis of hydrolysis time courses*, Bioresource Technology 200 (2016) 287-296

[3.3] Zhengdao Yu, Bailiang Zhang, Fuqiang Yu, Guinzhuan Xu, Andong Song, *A real explosion: The requirement of steam explosion pretreatment*, Bioresource Technology 121 (2012)

[3.4] A. Verardi, A. Blasi, I. De Bari, V. Calabrò, *Steam pretreatment of Saccharum officinarum L. bagasse by adding impregnating agents for advanced bioethanol production*, Ecotoxicology and Environmental Safety 134 (2016) 293-600

[3.5] Zhi-Hua Liu, Lei Qin, Feng Pang, Ming-Jie Jin, Bing-Zhi Li, Yong Kang, Bruce E. Dale, Ying-Jin Yuan, *Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover*, Industrial Crops and Products 44 (2013) 176-184

[3.6] Xin Zhang, Qipeng Yuan, Gang Cheng, *Deconstruction of corncob by steam explosion pretreatment: Correlations between sugar conversion and recalcitrant structures*, Carbohydrate Polymers 156 (2017) 351-356

[3.7] Roberto A.Agudelo, María P.García-Aparicio, Johann F.Görgens, *Steam explosion pretreatment of triticale (× TriticosecaleWittmack) straw for sugar production*, New Biotechnology – Volume 33 – Issue 1 (2016) 153-163

[3.8] Sanam Monavari, Andrea Bennato, Mats Galbe, Guido Zacchi, *Improved One-Step Steam Pretreatment of SO2-Impregnated Softwood with Time-Dependent Temperature Profile for Ethanol Production*, Wiley Online Library (2010)

[3.9] Kingsley L. Iroba, Lope G. Tabil, Shahab Sokhansanj, Tim Dumonceaux, *Pretreatment and fractionation of barley straw using steam explosion at low severity factor*, Biomass and Bioenergy 66 (2014) 286-300

[3.10] National Renewable Energy Laboratory - Biomass compositional analysis: *https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html*

[3.11] Wenjie Sui, Hongzhang Chen, *Effects of water states on steam explosion of lignocellulosic biomass*, Bioresource Technology 199 (2016) 155-163

[3.12] A. Duque, P. Manzanares, I. Ballesteros, M. Ballesteros, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 15: Steam Explosion as Lignocellulosic Biomass Pretreatment*, Elsevier (2016)

[3.13] Junmeng Cai, Yifeng He, Xi Yu, Scott W. Banks, Yang Yang, xingguang Zhang, Yang Yu, Ronghou Liu, Anthony V. Bridgwater, *Review of physicochemical properties and analytical characterization of lignocellulosic biomass*, Renewable and Sustainable Energy Reviews 76 (2017) 309-322

[3.14] Wenjie Sui, Hongzhang Chen, *Study on loading in steam explosion process of corn stalk*, Bioresource Technology 179 (2015) 534-542

[3.15] http://www.trisaia.enea.it/it/laboratori-e-impianti/impianto-steam-explosion

[3.16] Javier Lizasoain, Marìa Rincòn, Franz Theuretzbacher, Ramòn Enguìdanos, Paal J. Nielsen, Antje Potthast, Thomas Zweckmair, Andreas Gronauer, Alexander Bauer, *Biogas production from reed biomass: effect of pretreatment using different steam explosion conditions*, Biomass and Bioenergy 95 (2016) 84-91

[3.17] Zhi-Hua Liu, Lei Qin, Ming-Jie Jin, Feng Pang, Bing-Zhi li, Yong Kang, Bruce E. Dale, Ying-Jin Yuan, *Evaluation of storage methods for the conversion of corn stover biomass to sugars based on steam explosion pretreatment*, Bioresource Technology 132 (2013) 5-15

[3.18] Murat Ertas, Qiang Han, Hasan Jameel, *Acid-catalysed autohydrolysis of wheat straw to improve sugar recovery*, Bioresource Technology 169 (2014) 1-8

[3.19] Qiang Yu, Jing Liu, Xinshu Zhuang, Zhenhong Yuan, Wen Wang, Wei Qi, Qiong Wang, Xuesong Tan, Xiaoying Kong, *Liquid hot water pretreatment of energy grsses and its influence of physisco-chemical changes on enzymatic digestibility*, Bioresource Technology 199 (2016) 265-270

[3.20] Michele Michelin, José Antonio Teixeira, *Liquid hot water pretreatment of multi feedstocks and enzymatic hydrolysis of solids obtained thereof*, Bioresource Technology 216 (2016) 862-869

[3.21] Marzieh Badiei, Nilofar Asim, Jamilah M. Jahim, Kamaruzzaman Sopian, *Comparison of Chemical Pretreatment Methods for Cellulosic Biomass*, APCBEE Procedia 9 (2014) 170-174

[3.22] Harifara Rabemanolontsoa, Shiro Saka, *Various pretreatment of lignocellulosics*, Bioresource Technology 199 (2016) 83-91

[3.23] Guangli Cao, Nanqi Ren, Aijie Wang, Duu-Jong Lee, Wanqian Guo, Bingfeng Liu, Yujie Feng, Qingliang Zhao, *Acid hydrolysis of corn stover for biohydrogen production using Thermoanaerobacterium thermosaccharolyticum W16*, International Journal of Hydrogen Energy 34 (2009), 7182-7188

[3.24] Soo-Kyeong Jang, Jong-Hwa Kim, Hanseob Jeong, June-Ho Choi, Soo-Min Lee, In-Gyu Choi, *Investigation of conditions for dilute acid pretreatment for improving*

xylose solubilisation and glucose production by supercritical water hydrolysis from Quercus mongolica, Renewable Energy 117 (2018) 150-156

[3.25] S.I. Mussatto, *Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 8: Biomass Pretreatment with Acids*, Elsevier (2016)

[3.26] A. Shahbazi, B. Zhang, *Bioalcohol Production – Biochemical conversion of lignocellulosic biomass – Chapter 5: Dilute and concentrated acid hydrolysis of lignocellulosic biomass*, Woodhead Publishing (2010)

[3.27] http://arkenol.com/

[3.28] J.W. van Groenestijn, J.H.O. Hazewinkel, R.R. Bakker, *Pre-treatment of lingo-cellulose with biological acid recycling (the Biosulfurol process)*, TNO, Techno Invent, Wageningen University & Research Center (2007)

[3.29] J.-K. Xu, R.-C. Sun, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 19: Recent Advances in Alkaline Pretreatment of Lignocellulosic Biomass*, Elsevier (2016)

[3.30] Jun Seok Kim, Y.Y. Lee, Tae Hyun Kim, *A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass*, Bioresource Technology 199 (2016) 42-48

[3.31] U. Merretig-Bruns, B. Sayder, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 20: Pretreatment with Ammonia*, Elsevier (2016)

[3.32] Veronique Archambault-Léger, Lee R. Lynd, *Fluid mechanics relevant to flow through pretreatment of cellulosic biomass*, Bioresource Technology 157 (2014) 278-283

[3.33] Shuddhodana, D. Mohmot, Ranjita Biswas, V.S. Bisaria, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 23: Enzymatic Hydrolysis of Lignocellulosic Residues*, Elsevier (2016)

[3.34] M. Ballesteros, *Bioalcohol Production – Biochemical conversion of lignocellulosic biomass – Chapter 6: Enzymathic hydrolysis of lignocellulosic biomass*, Woodhead Publishing (2010)

[3.35] A. Aquilanti, P. Barghini, S. Silvi, M. Fenice, *Idrolisi di materiale lignocellulosico per l'ottenimento di substrati idonei alla produzione di idrogeno*, Università della Tuscia, ENEA (2012)

[3.36] Hongliang Guo, Yingju Chang, Duu-Jong Lee, *Enzymatic saccharification of lignocellulosic biorefinery: Research focuses*, Bioresource Technology 252 (2018) 198-215

[3.37] Sumitra Datta, L.Rene Christena, Yamuna Rani Sriramulu Rajaram, *Enzyme immobilization: an overview on techniques and support materials*, Biotech (2013) 1-9

[3.38] Manoj Koradiya, Srinivas Duggirala, Devayani Tipre, Shailesh Dave, *Pretreatment optimization of Sorghum pioneer biomass for bioethanol production and its scale-up*, Bioresource Technology 199 (2016) 142-147

[3.39] Parameswaran Binod, K.U. Janu, Raveendran Sindhu, Ashok Pandey, *Biofuels – Alternative Feedstocks and Conversion Processes – Chapter 10: Hydrolysis of Lignocellulosic Biomass for Bioethanol Production,* Academic Press – Elsevier (2011)

[4.1] Junyeong Park, Brandon Jones, Bonwook Koo, Xiaowen Chen, Melvin Tucker, Ju-Hyun Yu, Thomas Pschorn, Richard Venditti, Sunkyu Park, *Use of mechanical refining to improve the production of low-cost sugars from lignocellulosic biomass*, Bioresource Technology 199 (2016) 59-67

[4.2] A. Duque, P. Manzanares, I. Ballesteros, M. Ballesteros, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 15: Steam Explosion as Lignocellulosic Biomass Pretreatment*, Elsevier (2016)

[4.3] Fernando M.V. Oliveira, Irapuan O. Pinheiro, Ana M. Souto-Maior, Carlos Martin, Adilson R. Goncalves, George J.M. Rocha, *Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products*, Bioresource Technology 130 (2013) 168-173

[4.5] Yuzchen Zhang, Xiaoguo Fu, Hongzhang Chen, *Pretreatment based on two-step steam explosion combined with an intermediate separation of fiber cells-Optimization of fermentation of corn straw hydrolysates*, Bioresource Technology 121 (2012) 100-104

[4.6] Montgomery, Douglas C., *Design and Analysis of Experiments (8th Edition) - Chapter 11: Response Surface Methods and Designs*, John Wiley & Sons (2013)

[4.7] Guanhua Wang, Hongzhang Chen, *Enhanced lignin extraction process from steam exploded corn stalk*, Separation and Purification Technology 157 (2016) 93-101

[4.8] M. Coca, G. Gonzalez-Benito, M.T. Garcia-Cubero, *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery – Chapter 18: Chemical Oxidation With Ozone as an Efficient Pretreatment of Lignocellulosic Materials*, Elsevier (2016)

[4.9] Ananda S. Amarasekara, *Handbook of Cellulosic Ethanol – Chapter 6: Enzymatic Hydrolysis of Cellulose and Hemicellulose*, Department of Chemistry – Prairie View A&M University, Scrivener Publishing (2014)

[4.10] Fenila F., Yogendra Shastri, *Optimal control of enzymatic hydrolysis of lignocellulosic biomass*, Resource-Efficient Technologies 2 (2016) S94-S104

[4.11] Jingwen Chen, Wengui Zhang, Hongman Zhang, Qiuxiang Zhang, He Huang, *Screw extrude steam explosion: A promising pretreatment of corn stover to enhance enzymatic hydrolysis*, Bioresource Technology 161 (2014) 230-235

[4.12] Zhi-Hua Liu, Hong-Zhang Chen, *Xylose production from corn stover biomass by steam explosion combined with enzymatic digestibility*, Bioresource Technology 193 (2015) 345-356

[4.13] Sun Min Kim, Bruce S. Dien, M.E. Tumbleson, Kent D. Rausch, Vijay Singh, *Improvement of sugar yields from corn stover using sequential hot water pretreatment and disk milling*, Bioresource Technology 216 (2016) 706-713

[4.14] Qiyu Liu, Wenzhi Li, Qiaozhi Ma, Shengxin An, Mianghao Li, Hasan Jameel, Hou-min Chang, *Pretreatment of corn stover for sugar production using a two-stage dilute acid followed by wet-milling pretreatment process*, Bioresource Technology 211 (2016) 435-442

[4.15] Jae Won Lee, Ji Young Kim, Hyun Min Jang, Min Woo Lee, Jong Moon Park, *Sequential dilute acid and alkali pretreatment of corn stover: Sugar recovery efficiency and structural characterization*, Bioresource Technology 182 (2015) 296-301

[4.16] Lei Qin, Zhi-Hua Liu, Bing-Zhi Li, Bruce E. Dale, Ying-Jin Yuan, *Mass balance and transformation of corn stover by pretreatment with different dilute organic acids*, Bioresource Technology 112 (2012) 319-326

[4.17] Rui Zhang, Xuebin Lu, Youshan Sun, Xinying Wang and Shuting Zhang, *Modelling and optimization of dilute nitric acid hydrolysis on corn stover*, Society of Chemical Industries, 2010

[4.18] Shengxin An, Wenzhi Li, Ying Xia, Tingwei Zhang, Feng Huang, Qizhao Lin, Liang Chen, *Combined dilute hydrochloric acid and alkaline wet oxidation pretreatment to improve sugar recovery of corn stover*, Bioresource Technology 271 (2019) 283-288

[4.19] Wei Yuan, Zhiwei Gong, Guanghui Wang, Wenting Zhou, Yi Lui, Xuemin Wang, Mi Zhao, *Alkaline organosolv pretreatment of corn stover for enhancing the enzymatic digestibility*, Bioresource Technology 265 (2018) 464-470

[4.20] Lili Wang, Ke Zhang, Youjie Xu, Meng Zhang, Donghai Wang, *High-solid pretreatment of corn stover using urea for enzymatic saccharification*, Bioresource Technology 259 (2018) 83-90

[4.21] Shengxin An, Wenzhi Li, Qiyu Lui, Minghao Li, Qiaozhi Ma, Longlong Ma, Hou-min Chang, *A two-stage pretreatment using acidic dioxane followed by dilute hydrochloric acid on sugar production from corn stover*, Royal Society of Chemistry (2017)

[4.22] Todd A. Lloyd, Charles E. Wyman, *Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids*, Thayer School of Engineering, Dartmouth College, Hanover U.S. (2005)

[4.23] Goutami Banerjee, Suzana Car, John S. Scott-Craig, David B. Hodge, Jonathan D. Walton, *Alkaline peroxide pretreatment of corn stover: effect of biomass, peroxide, and enzyme loading anc composition on yields of glucose and xylose*, Biotechnology for Biofuels (2011) 4-16

[4.24] Huan Liu, Bo Pang, Haisong Wang, Haiming Li, Jie Lu, Meihong Niu, *Optimization of Alkaline Sulfite Pretreatment and Comparative Study with Sodium Hydroxide Pretreatment for Improving Enzymatic Digestibility of Corn Stover*, Journal of Agricultural and Food Chemistry 63 (2015) 3229-3234