Ana Rita Colaço Morais

Mestre em Engenharia Alimentar

High-Pressure CO_2-H_2O Mixture and Ammonia Technologies for Lignocellulosic Biomass Processing within Biorefinery Concept

Dissertação para obtenção do Grau de Doutor em Química Sustentável

Orientador: Dr. Rafał Marcin Łukasik, Investigador Principal, Laboratório Nacional de Energia e Geologia, I.P.

Co-orientador: Prof. Dr. Manuel Nunes da Ponte, Professor Catedrático, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa

Júri:

Presidente: Prof. Doutor José Legatheaux Martins

Arguentes: Doutor Alexandre Babo de Almeida Paiva
Doutor José Carlos Pereira Roseiro

Vogais: Prof. Doutora Susana Filipe Barreiros
Doutora Susana Santos Moita de Oliveira Marques
Doutor Rafał Marcin Łukasik

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UNIVERSIDADE NOVA DE LISBOA

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Para as pessoas mais importantes da minha vida:
Os meus pais e irmãos

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Resumo

O principal objetivo desta dissertação recai sobre a investigação do potencial de duas tecnologias de alta pressão distintas – mistura CO_2/H_2O e amónia – para o processamento de biomassa lenhocelulósica, seguindo o conceito da biorefinaria.

A mistura CO_2/H_2O a alta pressão tira proveito da formação in-situ de ácido carbónico e envolve a integração de ambos os passos de pré-tratamento e hidrólise. Devido ao seu efeito acídico, esta tecnologia foi capaz de hidrolisar seletivamente a fração hemicelulósica da biomassa e produzir uma solução aquosa rica em açúcares C_5 na forma oligomérica. De seguida, estes açúcares C_5 na forma oligomérica foram convertidos com sucesso em furfural com alto rendimento e seletividade usando o mesmo efeito catalítico da mistura CO_2/H_2O a alta pressão. Adicionalmente, os materiais resultantes enriquecidos em celulose demonstraram ser muito suscetíveis para hidrólise enzimática conduzindo à produção de soluções altamente concentradas em glucose.

O processo com amónia a alta pressão baseou-se no desenvolvimento de uma nova tecnologia chamada "Compacted Biomass with Reduced Ammonia" para pré-tratar biomassa peletizada usando menor carga de amónia. Este processo combinou as vantagens da conversão de celulose cristalina I_β em celulose III altamente digerível e os benefícios das reações de amonólise das ligações éster presentes na parede celular. Adicionalmente, o processo proposto permitiu obter açúcares fermentáveis, quer C_5 e C_6, e rendimentos em etanol comparáveis às tecnologias mais relevantes a nível industrial *i.e.* AFEX™ (Ammonia Fiber Expansion) e explosão de vapor. Além disso, esta tecnologia demonstrou ser independente da matéria-prima e eficiente para pré-tratar diversos tipos de biomassa, independentemente da sua composição macromolecular e estrutura morfológica, para a produção de altos rendimentos de açúcares fermentáveis.

Ambas as tecnologias demonstraram ser altamente efetivas para o processamento de biomassa lenhocelulósica, demonstrando o seu potencial como tecnologias sustentáveis para aplicação no conceito de biorefinaria.

Palavras-chave: Biomassa, biorefinaria, alta-pressão, pré-tratamento, hidrólise

Abstract

The main purpose of this dissertation is to scrutinise the potential of two distinct high-pressure technologies - CO_2/H_2O mixture and ammonia – for lignocellulosic biomass processing following the biorefinery concept.

High-pressure CO_2/H_2O mixture took benefits from the formation of in-situ carbonic acid and involved the integration of both pre-treatment and hydrolysis steps. Due to its acidic effect, this technology was able to selectively hydrolyse the hemicellulosic fraction of biomass producing C_5-oligomeric sugar-rich aqueous stream. Later, the C_5-sugars present in this stream were successfully converted into furfural with high yield and selectivity using the same catalytic effect of high-pressure mixture of CO_2/H_2O. Additionally, the leftover materials enriched in cellulose demonstrated to be highly susceptible for enzymatic hydrolysis leading the production of highly concentrated solution of upgradable glucose.

High-pressure ammonia relied on the development of a new ammonia-based technology called "Compacted Biomass with Reduced Ammonia" to pre-treat pelletised biomass, at reduced ammonia loadings. This process combined the advantages of conversion of native crystalline cellulose I_β into highly digestible cellulose III and the benefits of ammonolysis of cell wall ester cross-links. Besides, the proposed process allowed obtaining fermentable sugars, either C_5 or C_6-sugars, and ethanol yields comparable to industrially relevant technologies *i.e.* AFEX™ (Ammonia Fiber Expansion) and steam explosion. Additionally, the proposed approach demonstrated to be a feedstock-independent technology capable to handle different types of biomasses, regardless of their macromolecular composition and morphological structure, to produce high yields of fermentable sugars.

Both explored technologies demonstrated to be highly effectives for lignocellulosic biomass valorisation, showcasing their potential as sustainable technologies applicable in the biorefinery approach.

Keywords: Biomass, biorefinery, high-pressure, pre-treatment, hydrolysis

List of Publications

The present dissertation is based on the following scientific papers:

CHAPTER III - Sara P. Magalhães da Silva, Ana Rita C. Morais and Rafal M. Lukasik. The CO_2-assisted autohydrolysis of wheat straw. *Green Chem.*, 2014, 16, 238-246

CHAPTER IV - Frederico M. Relvas, Ana Rita C. Morais and Rafal M. Lukasik. Selective hydrolysis of wheat straw hemicellulose using high-pressure CO_2 as catalyst. *RSC Adv.*, 2015, 5, 73935-73944.

CHAPTER V - Ana Rita C. Morais, Ana C. Mata and Rafal M. Lukasik. Integrated conversion of agroindustrial residue with high pressure CO_2 within biorefinery concept. *Green Chem.*, 2014, 16, 4312-4322.

CHAPTER VI - Ana Rita C. Morais and Rafal M. Lukasik. Highly efficient and selective CO_2-adjunctive dehydration of xylose to furfural in aqueous media with THF. *Green Chem.*, 2016, 18, 2331-2334.

CHAPTER VII – Patent application and scientific paper: Ana Rita C. Morais, Thapelo Mokomele, Hui Dang, Venkatesh Balan, Bruce Dale, Rafal M. Lukasik and Leonardo da Costa Sousa. New Approach to Ammonia Pre-treatment Integrates Better Feedstock Logistics with Improved Sugar Conversion. *Energy Environ. Sci.*, 2018. (in preparation)

Additionally, during my PhD studies I have contributed to the following scientific productions, which are not included in the present dissertation:

Sebatian Gillet, Mário Aguedo, Laurene Petitjean, Ana Rita C. Morais, André M. da Costa Lopes, Rafal M. Lukasik and Paul Anastas. Lignin Transformations for High Value Applications: Towards Targeted Modifications Using Green Chemistry. *Green Chem.*, 2017, 19, 4200-4233.

Andréia Toscan, Ana Rita C. Morais, Susana M. Paixão, S. M., Luís Alves, Jürgen Andreaus, Marli Camissola, Aldo P. Dillon and Rafal M. Lukasik. High-pressure carbon dioxide/water pre-treatment of sugarcane bagasse and elephant grass: Assessment of the effect of biomass composition on process efficiency. *Bioresour. Technol.*, 2017. 224, 639-647.

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Ana Rita C. Morais and Rafal M. Lukasik. Hydrothermal pre-treatment using supercritical CO_2 in the biorefinery context. Ruiz, H. A., Trajano, H. Thomsen, M. H. (Eds.), Hydrothermal processing in biorefineries - production of bioethanol and high added-value compounds of second and third generation biomass. Springer, Cham 2017. ISBN: 978-3-319-56456-2.

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Mehrdad Arshadi, Thomas M. Attard, Rafal M. Lukasik, Mladen Brncic, André M. da Costa Lopes, Michael Finell, Paul Geladi, Lia N. Gerschenson, Fahrettin Gogus, Miguel Herrero, Andrew J. Hunt, Elena Ibáñez, Birgit Kamm, Immaculada Mateos-Aparicio, Ana Matias, Nikolaos E. Mavroudis, Enzo Montoneri, Ana Rita C. Morais, Calle Nilsson, Emmanouil H. Papaioannou, Aurore Richel, Pilar Rupérez, Biljana Škrbić, Marija Solarov, Jaroslava Švarc-Gajić, Keith W. Waldron and F. J. Yuste-Córdoba. Pre-treatment and extraction techniques for recovery of added value compounds from wastes throughout the agri-food chain. *Green Chem.*, 2016, 18, 6160-6204.

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Chapter I

Introduction

1.1.Challenges & Solutions

Currently, non-renewable fossil sources, such as crude oil, natural gas and coal have been used worldwide to satisfy the everyday needs for fuels, chemicals and materials. On the other hand the use of these fossil resources causes several concerns that nowadays society must address. One of the major problems is that those energy sources are not renewable because they are not replenished in a short lifetime. Another is that the use of fossil sources has been acknowledged as the major responsible for the environmental damages.[1] Additionally to aforementioned issue, the over-dependence on fossil resources along with a continuous increase of world population has driven to serious concerns about the secure and stable supply of energy.[2] All these issues guided to look for alternatives of fossil feedstock. The solution is the use of natural and renewable resources in an effective and sustainable way that is *sine qua non* to guarantee a transition from fossil to a competitive bio-economy without compromising a level of development.

1.2.Importance of Biorefinery

Biomass is the most abundant and the most required feedstocks in all industry sectors of widely understood bio-economy. A comprehensive use of biomass for the production of a wide variety of commodities has been acknowledged as the most promising strategy to fully exploit the value from such feedstocks. To achieve this, in the late of 20th century a concept called "biorefinery" has been proposed. International Energy Agency Bioenergy (Task 42) defines biorefining as the sustainable processing of biomass into a spectrum of marketable products and energy.[3] A close inspection of this definition shows that in fact, biorefinery concept was made as an image of today's petroleum refinery that produces a broad spectrum of commodities from crude (Figure 1.1). However, contrary to crude refinery, biorefinery is more complex because depends on many variables such as type (*e.g.* lignocellulosic biomass, wastes and/or residues, municipal wastes) and origin (*e.g.* industrial, agricultural) of feedstock, technology used (bio-, chem- or thermoconversion) and commodity(ies) produced.[4]

One of platforms explored in biorefinery is biochemical platform called also sugar platform. It comprises the production of fermentable sugars, often referred as building blocks, and their posterior upgrade to chemicals (*e.g.* furfural, oligosaccharides), materials (*e.g.* natural polymers) and energy resources either gaseous such as methane, or liquid fuels (*e.g.* ethanol, long chain fatty acids and long chain fatty alcohols), or solid fuels (*e.g.* pellets). Alternative or often used as complementary platform to sugar one is thermochemical approach. It consists of processes (gasification, pyrolysis, liquefaction) performed at elevated temperature (far above 250 °C) aiming to produce liquid or gaseous intermediates that later *via* bio- or chemo-conversion are upgraded to other value-added products.[5-7]

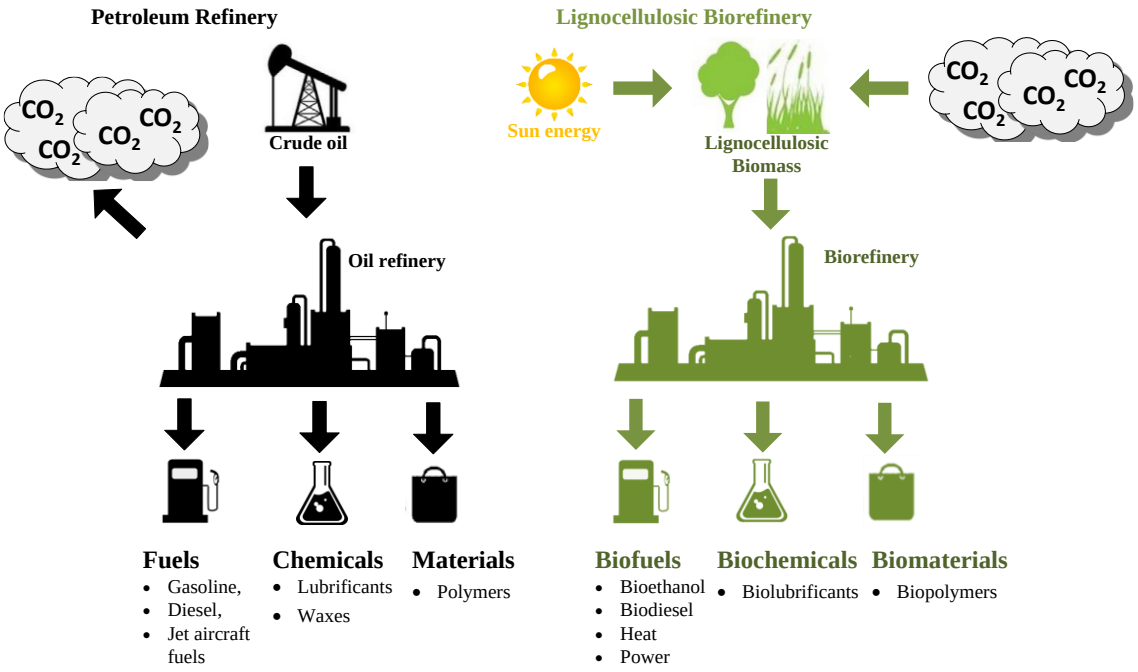

Figure 1.1. Comparative description of today's petroleum refinery and future lignocellulosic biorefinery.

The most recent approach in biorefinery classification is based on the final use of product. Hence, biorefineries can be divided into two categories: i) energy-based and ii) (non-energy) product-based.

The first approach is focused on the production of energy (power, heat, CHP, biogas or advanced liquid biofuels) as main outcome while value-added products are formed only to a lesser extent. This concept was born as a reply of European Union concerns in gaining social development with low-carbon technologies. To address this challenge, as early as in 2006, European Union established the SET-Plan (Strategic Energy Technology Plan).[8] It main aim has been the development and deployment of low-carbon economy that would improve technologies and reduce costs. The SET-Plan has been divided into 10 actions that help to accomplish its objectives. One of SET-Plan actions is action 8, which is dedicated to bioenergy and sustainable biofuels. This action is governed under the umbrella of European Technology and Innovation Platform (ETIP-Bioenergy)[9] with a strong support of European Energy Research Alliance (EERA)[10] Bioenergy Joint Programme.[11]

The second biorefinery approach is directed to obtain a large variety of high value products from each upgradable fraction. Energy-driven products are formed normally from impossible for valorisation remaining.[12] This concept addresses another important issue that is cascade approach in biomass valorisation. The cascade approach of biomass valorisation assumes the stepwise extraction and upgrading of each fractions of biomass (including minor fractions including extractives, proteins, etc.) to value-added products. The aim of cascade approach is at maximising the value of obtained products and reducing the losses of material with a maximal exploitation of the feedstock.

Summarising, it shall be stated that the use of biomass in the biorefinery concept has been gaining a strong either political or technological impact being recognised as one of important pathways contributing to achieve more sustainable future.

1.3.Lignocellulosic biomass as renewable feedstock

Biomass has been and continues to be acknowledged as the only renewable carbon natural feedstock capable delivering biofuels, biochemicals and biomaterials on a feasible scale.[13,14] However, the use of 1st generation of biomass, *i.e.* conventional agricultural resources, such as edible crops *e.g.* corn or edible oil seeds, for the production of biofuels is highly questionable because of the "*fuel vs. food*" dilemma.[15] Mitigation of this problem by the increased productivity of edible crops is not the solution too. Joint Research Centre of European Commission showed that that extensive farming, increase of fertilisers' use to accomplish the needs of increase of food crops productivity contribute to increase of emission of greenhouse gasses (GHG). This, overpasses the GHG emission savings from the use of biofuels produced from these edible crops.[16] Hence, in this context, 2nd generation of biomass, such as lignocellulosic biomass (*e.g.* forestry wastes, agro-industrial residues and municipal wastes) has been developed to overcome the drawbacks found for 1st generation ones.[17] Several literature reports have demonstrated that lignocellulosic biomass is a promising and more sustainable alternative to the crude for the production of liquid fuels and chemicals. Firstly, it is a carbon-neutral renewable resource with a potential to reduce the CO_2 emissions and is highly abundant and easily accessible.[18,19] The worldwide production of lignocellulosic biomass is estimated to approach 10 – 50 billion dry tonnes.[20,21] Secondly, lignocellulosic biomass produced from woodworking, forestry and agricultural activities are accumulated in the land in large amounts representing a huge environmental and economic problem.[22] Lastly, lignocellulosic biomass constitutes the non-edible part of the plant whereby does not compete with food production.

1.4.Chemical composition of lignocellulosic biomass

Terms "lignocellulosic biomass" and "lignocellulose" are often used to describe a heterogeneous complex material that is made up of carbohydrate polymers (cellulose and hemicelluloses) and an aromatic polymer (lignin) together with small amounts (up to 10 % dry weight) of acetyl groups, proteins, ash, waxes and phenolic compounds. The ratio between these fractions varies depending on, among other factors, the origin, maturity of the plant cell wall, stage of grow and location.[18,23-26] Table 1.1 present the chemical macromolecular composition of selected types of lignocellulosic feedstocks.[27,28] Depending on the relative abundance of these components within plant cell wall, and *inter alia*, they form a complex inter-linked three-dimensional structure with different degree of organisation.[28] Highly ordered and compacted crystalline cellulose, hydrophobicity of lignin, protection of cellulose by the hemicellulose-lignin matrix and low accessible surface area are the main factors providing resistance to lignocellulosic biomass against to microbial attack.[29,30] These molecular

interactions afford a very complex and recalcitrant structure of biomass making it very difficult to be directly disrupted to sugars. The chemical structure of these polymers and respective role in plant cell wall is discussed below.

Table 1.1. Chemical composition (on dry basis) of selected lignocellulosic biomasses.[24,27]

Biomass type	Feedstock	Cellulose (%)	Hemicelluloses (%)	Lignin (%)
Hardwood	Poplar	45 – 51	25 – 28	10 – 21
	Eucalyptus	45 – 51	11 – 18	22 – 29
Softwood	Spruce	46	23	28
	Pine	42 – 49	13 – 25	23 – 29
Agricultural residues	Wheat straw	35 – 40	22 – 30	12 – 21
	Rice straw	29 – 35	18 – 26	11 – 19
	Corn stover	35 – 40	21 – 25	11 – 19
	Sugarcane bagasse	25 – 45	28 – 32	15 – 25
	Sorghum straw	32 – 35	24 – 27	15 – 21
Grasses	Switchgrass	35 – 40	26 – 30	15 – 20

1.4.1. Cellulose

Cellulose is the main polysaccharide present in the lignocellulosic biomass. Cellulose is a linear syndiotactic (alternating spatial arrangement of the side chains) homopolymer of D-anhydroglucopyranose units linked by β-(l, 4)-glycosidic bonds forming cellobiose units (Figure 1.2.).[31,32]

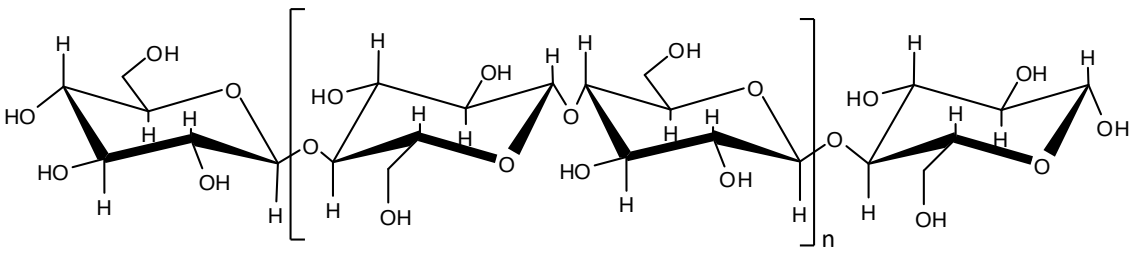

Figure 1.2. Chemical structure of cellulose. The dimeric repeating unit represents cellobiose.

The cellulose chains are assembled in parallel structure of microfibrils which are stabilised *via* inter- and intra-molecular hydrogen bonds and van der Waals interactions which in turns determines the straightness of the cellulose chain.[29] These microfibrils are consisted by highly ordered regions (crystalline cellulose) and small amounts of less ordered structure of amorphous cellulose.[31] Native cellulose is generally found in two distinct polymorphs (crystalline) forms call as cellulose I_α and I_β.[33] The ratio of these two polymorphs varies depending on the cellulose origin. It is believed that wood plants have higher content of cellulose I_β, whereas cellulose I_α has been found in cell wall of some algae and bacteria.[34] The main difference between cellulose I_α and I_β is the relative displacement of cellulose chains resulting in triclinic and monoclinic one-chain unit cells, respectively.[35] In an effort to accomplish the various demands for cellulose industrial applications it is crucial to convert cellulose I to less ordered forms of cellulose II, III, and the most preferably, to cellulose IV (amorphous). Cellulose II can be obtained through the use of alkali treatment, whereas cellulose III is normally the effect of cellulose I treatment in liquid ammonia or by amines.[36,37] In general, cellulose IV cannot be produced directly from cellulose I because firstly, cellulose I needs to be converted into cellulose II or III and then, into cellulose IV.[38] Other structural characteristic of cellulose is the chain length of cellulose fibres. The chain length is expressed as the degree of polymerisation (DP) that is a number of repeating D-anhydroglucopyranose units and varies with the origin of feedstock. The measurements of molecular weight of cellulose have demonstrated that DP of wood cellulose *e.g.* Aspen wood and Douglas Fir, is about 2500, while cotton fibres usually show a DP in the range of 300 –7000.[39]

1.4.2. Hemicelluloses

Hemicelluloses are a heterogeneous class of polymers being, in general, the second most abundant fraction of lignocellulosic biomass (15 – 35 % of dry weight). It is made up of aldopentoses (β-D-xylose and α-L-arabinose), aldohexoses (β-D-mannose, β-D-glucose, α-D-galactose) and/or uronic acids (α-D-4-O-methylgalacturonic, α-D-glucuronic and α-D-galacturonic acids). Small amounts of other sugars, such as α-L-fucose and α-L-rhamnose, and acetyl groups attached to the polysaccharide chain can be also found in hemicellulose structure.[40] In general, hemicelluloses are classifies into four main classes of structurally different polysaccharide types: xylan (xyloglycan), mannan (mannoglycan), xyloglucan and mixed-linkage β-glucans.[41] They differ in terms of side chain types, distribution, localisation and types of glycoside linkages.[41] Hemicelluloses in wood, herbaceous plants, forestry and agro-industrial residues consist mainly of xylan-type, *i.e.* arabinoxylans, whereas hemicelluloses present in softwoods are composed predominantly of mannan-type, such as galactoglucomannans.[42] Arabinoxylans are constituted by β-(1,4)-D-xylanpyranan backbone, branched with arabinose residues attached at positions 2 or 3 and/or at both positions 2 and 3 xylopyranose monomers (Figure 1.3.). Particular structural characteristic of arabinoxylans is the presence of glucuronic and ferulic branches esterified to O-5 of some α-L-arabinofuranosyl residues.[41]

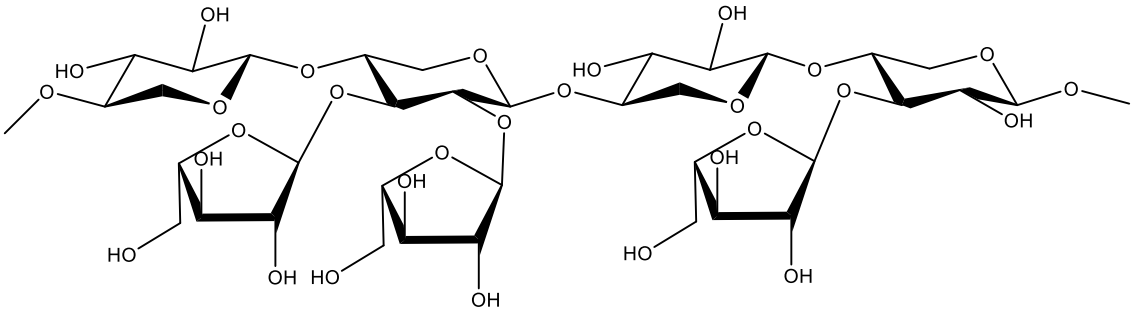

Figure 1.3. Representation of the main structure of arabinoxylan.

Galactoglucomannans (O-acetyl-galactoglucomannans) are composed of a linear backbone of β-(1,4)-linked D-mannopyranose and D-glucopyranose units decorated with D-galactopyranosyl units linked to glucose and mannose by α-(1,6) bonds.[40] The hydroxyl groups of the backbone residues are partially acetylated at C2 or C3 positions. The main structure of galactoglucomannans present in softwoods is depicted in Figure 1.4.

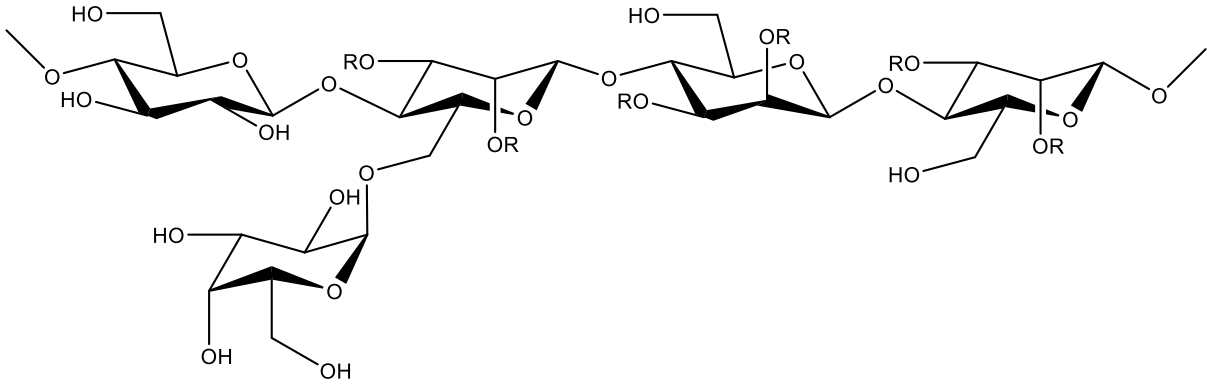

Figure 1.4. Representation of the main structure of galactoglucomannans.

In hardwood and grasses, xyloglucans are the main hemicellulosic polymers found in primary cell walls.[43] This polymer possesses β-(1, 4)-linked D-glucose backbone being most of these residues substituted at O-6 with D-xylose. Other sugar residues, such as D-galactose and L-arabinose have been found attached to xylose residues forming di-, or triglycosyl side chains.[40,44]Additionally, it has been reported that xyloglucan binds to adjacent cellulose microfibrils, keeping their link, or bind covalently to pectins.[45]

1.4.3. Lignin

Lignin is the key fraction in plant cell wall and the most abundant aromatic polymer existing in Nature. Although the exact structure of lignin is not well understood, lignin is regarded as an amorphous material consisting of a three dimensional arrangement of methoxylated phenylpropane units derived from the oxidative polymerisation of one or more of the three types of hydroxycinnamyl alcohol precursors.[46,47] These alcohols originate three different monolignol units, namely *p*-coumaryl, coniferyl and sinapyl alcohols. These monolignols are incorporated into lignin polymer producing *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively, which proportions are dependent

on the specie (*i.e.* softwood, hardwood, grasses). For instance, softwoods are composed mainly by coniferyl units whereas hardwoods consist mostly of syringol units.[46,48] Additionally, a wide-range of ether and carbon-carbon cross-linkages, (C–O–C = β–O–4, α–O–4, 4–O–5) and C–C interunit linkages (β–1, β–5, β–β, 5–5), are formed allowing the connection of *p*-coumaryl, coniferyl and sinapyl units. Figure 1.5. demonstrates an example structure of a lignin fragment with its building blocks highlighted and the most frequent linkages marked.

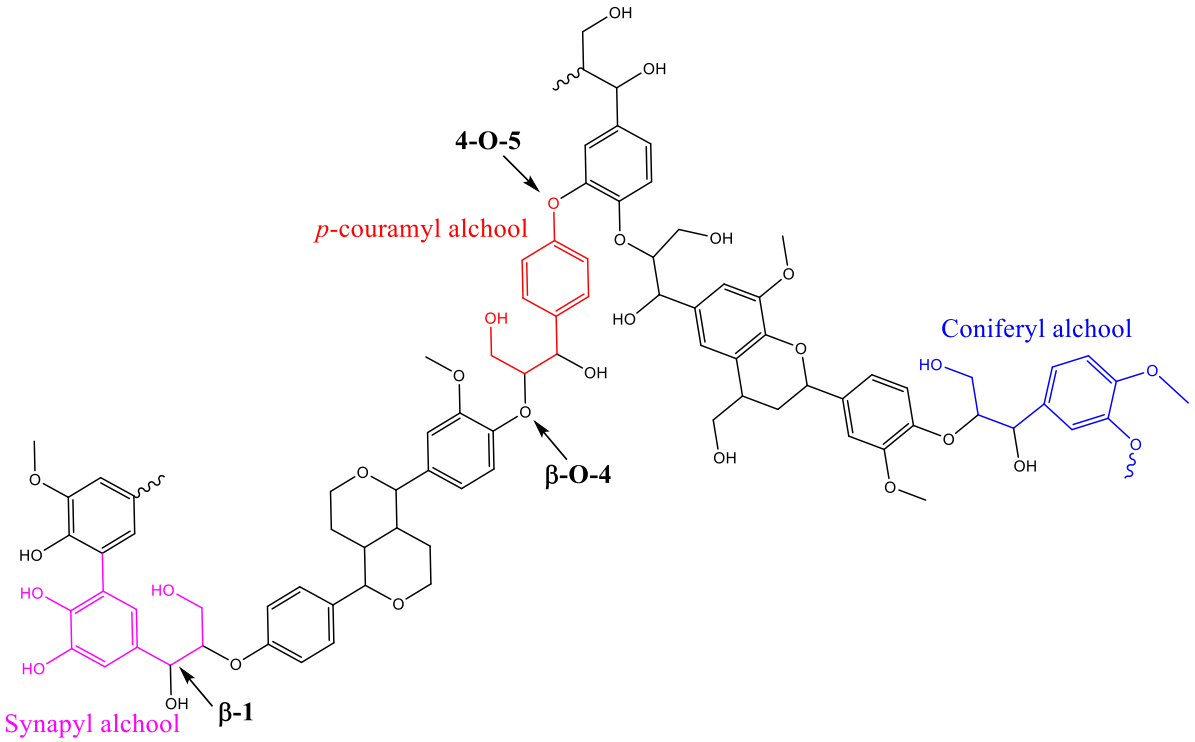

Figure 1.5. Schematic representation of lignin fragment demonstrating its high complexity.

1.5.Importance of Pre-treatment in Lignocellulosic Biorefinery

Lignocellulosic biomass is naturally recalcitrant, having evolved in a physical protection against pathogen penetration. Additionally, inherent factors associated to cellulose crystallinity, degree of polymerisation, low accessible area of cellulose, sheathing of cellulose by hemicellulose-lignin matrix, lignin content and fibre strength makes the upgrading of these materials a challenging task to accomplish.[29,30] Mussato et al. and Yang et al. showed that the enzymatic conversion yield of cellulose of biomass was less than 20 %.[49,50] This means that to increase the conversion yield, higher enzyme loadings would be required. However, it represents significant OPEX (operating expenditure) which on the other hand may hinder the commercialisation. The alternative way to obtain an efficient conversion of cellulose into fermentable sugars is introduction of a pre-treatment step. It aims at changing both physical and chemical properties of the plant cell wall. More specifically, the aim of the pre-treatment step is to disrupt the hemicellulose-lignin matrix and to change the crystalline structure of cellulose, so that catalysts (either biological *e.g.* enzymes or chemical) can easily depolymerise

cellulose into glucose.[30] Figure 1.6. depicts the role of the pre-treatment in partial deconstruction of hemicellulose-lignin matrix of lignocellulosic biomass.

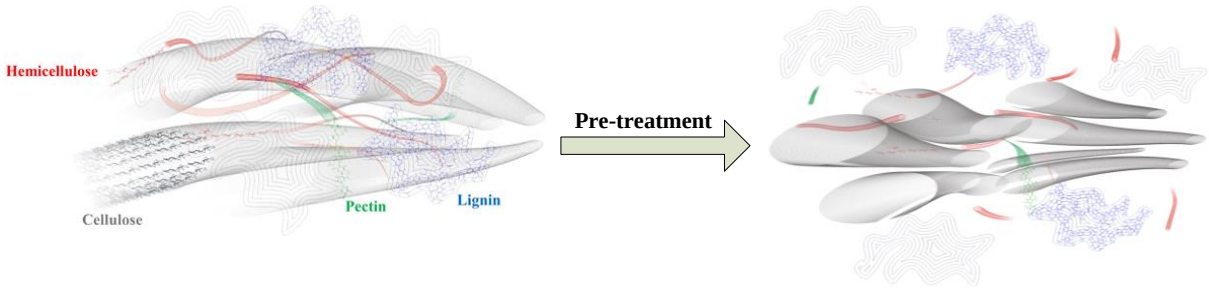

Figure 1.6. Schematic representation of pre-treatment role on disruption of lignocellulosic biomass. Adapted from M. H. L. Silveira, A. R. C. Morais, A. M. da Costa Lopes, D. N. Olekszyszen, R. Bogel-Łukasik, J. Andreaus, L. Pereira Ramos, ChemSusChem **2015**, 8, 3366. Copyright (2015) with permission from Wiley.

Many pre-treatment processes, with varying chemical ways of interaction with the plant cell wall, have been developed to accomplish the aforementioned aim. It should be also highlighted that there is a wide-range of lignocellulosic biomass, *e.g.* hardwoods, softwoods and grasses. Thus, the selection of a given pre-treatment is highly dependent on physical nature, textural properties and chemical macromolecular composition of the feedstock, as well as the extent of modifications desired for.[40,51]

These properties affect deeply downstream processes such as selection of enzyme and microorganism, enzyme and solid loading, conditioning, by-product usage, waste production, and final product purification and recovery.[52] Furthermore, the ideal pre-treatment process should guarantee the valorisation of each polymeric fraction of lignocellulosic biomass in subsequent steps with minimal degradation and biomass losses.[53] In addition to all these requirements, the ideal pre-treatment should be "feedstock agnostic", *i.e.* effective to different biomasses, robust and additionally, economically and environmentally acceptable.[54]

1.6.An Overview of the Current Pre-treatments

Several pre-treatment technologies are available nowadays. They can be classified into the five main categories: (i) physical pre-treatment (milling,[55] extrusion[56]); (ii) chemical pre-treatment under acidic (dilute and concentrated-acid[57,58]), neutral (liquid hot water[59]) and alkaline conditions (ammonia fibre expansion (AFEX),[60] ammonia percolation process (ARP),[61] lime,[62] sodium hydroxide (NaOH)[63]); (iii) solvent fractionation (ionic liquids,[64-66] organosolv;[67,68] (iv) physico-chemical pre-treatment (steam explosion,[69,70] exogenous acid catalysed steam-explosion (CO_2,[71] SO_2,[72,73]); and (v) biological.[74]

In the past five years, several comprehensive reviews have been published demonstrating the various pre-treatment options available today and those that have potential for commercialisation.[52,75-77] On the

basis of these works, an overview of the advantages and disadvantages of the most examined technologies for the pre-treatment of lignocellulosic biomass are depicted in Table 1.2.

Table 1.2. An overview of the advantages and disadvantages of the most commonly studied pre-treatment of lignocellulosic biomass technologies (selected data). Adapted from.[40,75]

Pre-treatment	Advantages		Disadvantages	
Physical				
Mechanical comminution	I.	Reduces cellulose crystallinity	I.	Zero to low upgradable sugar release
	II.	Easily scalable	II.	Energy demanding
Chemical				
Dilute acid	I.	Hemicellulose solubilisation	I.	Formation of fermentation inhibitors
	II.	Production of hemicellulosic monomers	II.	Usage of corrosive chemicals
	III.	Increased cellulose digestibility	III.	No recovery of catalyst(gypsum production)
	IV.	Low CAPEX		
Liquid hot water (hydrothermal)	I.	Hemicellulose solubilisation	I.	Post-hydrolysis step required to obtain monomers
	II.	Production of hemicellulosic oligomers	II.	Low cellulose digestibility
	III.	High cellulose recovery	III.	Formation of fermentation inhibitors
	IV.	No size reduction and additives (catalysts) required	IV.	High temperatures and long reaction times required
	V.	No wastes production	V.	High energy and water input
AFEX	I.	High cellulose digestibility	I.	Safety issues because of handling with NH_3
	II.	Efficient for low lignin content feedstocks	II.	Expensive solvent
	III.	Low formation of fermentation inhibitors	III.	Costly recovery of NH3 (>97 % required)
	IV.	Dry to dry process	IV.	High OPEX (high pressure)
			V.	Less efficient for hardwood biomass

ARP	I.	High lignin removal	I.	High energy and water inputs
	II.	Effective for grasses	II.	Less efficient for hardwood biomass
Lime	I.	Low CAPEX	I.	Long reaction times
			II.	Costly recovery of catalyst
			III.	Scale-up issues
NaOH	I.	High cellulose digestibility	I.	Long reaction times
	II.	Solubilisation of lignin	II.	High water input;
			III.	Costly recovery of catalyst
			IV.	Scale-up issues
Physico-chemical				
Steam-explosion (non-catalysed)	I.	Hemicellulose solubilisation	I.	Post-hydrolysis step required to obtain monomers
	II.	Production of hemicellulosic oligomers	II.	Low cellulose digestibility
	III.	Short reaction times	III.	Low lignin quality
	IV.	Robust for variety of biomass at high-solid loadings	IV.	High OPEX and CAPEX
			V.	High energy input
	V.	No wastes production		
CO_2 explosion (absence of H2O)	I.	High accessible surface area	I.	Hemicelluloses and lignin unaffected
	II.	Cost efficient	II.	High CAPEX
	III.	Low or no formation of degradation products		
Solvent fractionation				
ILs	I.	High cellulose digestibility	I.	Long reaction times
			II.	High CAPEX (expensive solvent)
	II.	High recovery of carbohydrate fraction	III.	Need of solvent recycling and reuse (>99 %)
	III.	Low degradation products formation	IV.	Unknown toxicity of many ILs
	IV.	Effective for a variety of biomasses	V.	Scale-up issues
	V.	Mild operational		

		conditions		
Organosolv	I.	Hydrolysis of hemicelluloses and lignin	I.	Need of solvent recycling and reuse
			II.	High energy input
Biological				
Microorganisms (fungi and bacteria)	I.	Depolymerisation of hemicelluloses and lignin	I.	Long reaction times
	II.	Low energy input	II.	Low sugar yields
	III.	Eco-friendly	III.	On-line monitoring required

AFEX – Ammonia Fibre Expansion; ARP – Ammonia Recycling Percolation; CAPEX - Capital Expenditure; ILs – Ionic liquids

1.6.1. Physical Pre-treatments

The main aim of physical pre-treatments, such as extrusion, micronisation, torrefaction and irradiation (microwaves, electron beam, ultrasound, and gamma ray), is the reduction of particle size often leading to decrease of cellulose crystallinity and its respective DP. This, in turn results in an increase in the surface area and better accessibility of enzymes to subtract.[29,78] Nevertheless, still most of these pre-treatments are characterised by low saccharification yields whereby they are still mainly used as preliminary step for actual pre-treatment steps. Despite the intensive development and advance of physical pre-treatment, they are still energy-demanding and by this a development of feasible biorefineries on the basis of this pre-treatment is still a challenge.[75]

1.6.2. Chemical Pre-treatments

Most of the currently developed pre-treatment technologies methods belong to this category. The main purpose of chemical pre-treatments is the disruption of recalcitrant structure of lignocellulose and hydrolysis (partial or complete) of carbohydrates and/or lignin resulting in higher enzyme accessibility and improved saccharification yields. Chemical pre-treatments can be distinguished according to the chemical character of the occurring process. Hence, alkaline, neutral and acidic pre-treatment can be categorised as following:

1.6.2.1.Acidic Pre-treatments

There are several acid-based pre-treatment processes, such as dilute and concentrated acid pre-treatments. Generally, in this technology, mineral acids such as H_2SO_4, H_3PO_4 and HNO_3 or organic acids *e.g.* maleic and fumaric acids act as catalysts causing the disruption of inter- and intramolecular bonds present in the polysaccharide-lignin matrix provoking the selective hydrolysis of plant cell wall polysaccharides, mostly hemicelluloses.[30] The most commonly used acid in such pre-treatments is H_2SO_4. It has been used worldwide to pre-treat a variety of lignocellulose feedstocks in a range of

temperatures between 160 and 220 ºC with an acid concentration range from 0.2% to 5 % in processes running for a few minutes.[79] The concentration of acid, temperature and reaction time determine the formation of degradation products such as furans (furfural, 5-hydroxymethylfurfural (5-HMF)), aliphatic acids (formic and levulinic acid), aldehydes and a wide variety of phenolic acids which are inhibitory for enzymatic and fermentative purposes.[80-82] To minimise these undesired effects, dilute-acid hydrolyses as alternatives to concentrated-acid pre-treatments were proposed. Dilute-acid pre-treatment promotes the hydrolysis of hemicelluloses and amorphous cellulose consequently more structurally organised (crystalline cellulose) is more accessible for enzymatic attack. Therefore, these pre-treatments aim at minimising the sugar degradation and improvement of the overall sugar yields. . One of the drawbacks is that lignin is also affected and can undergo the depolymerisation.

1.6.2.2.Neutral Pre-treatments

Neutral Pre-treatments are processes normally occurring under acidic conditions, which are formed *in-situ i.e.* without the addition of external acid catalyst. The pre-treatment of lignocelluloses at neutral conditions, *e.g.* liquid hot water, has a similar mechanism to that found for dilute-acid hydrolysis. The only difference is, in the case of neutral pre-treatment, generation of hydronium ions occurs *in-situ via* auto-ionisation of water promoting liberation of short aliphatic acids *e.g.* acetic acid from O-acetyl groups of hemicelluloses.[40,83]

Liquid hot water, also known as hydrothermolysis, hydrothermal pre-treatment, aqueous fractionation, solvolysis, autohydrolysis, or aquasolv, operates at relatively high temperatures (160 to 260 ºC) and pressures (above the saturation point). Reaction times may range from few minutes up to hours depending on the temperature of the process.[40,84] A relatively high hemicellulose hydrolysis yield coupled to low levels of degradation product formation has been obtained in liquid hot water pre-treatment.[85] As a result, most of the hemicelluloses are removed leaving a processed material enriched in cellulose and lignin. Hemicelluloses are only partially depolymerised (staying mainly in oligomeric form in the solution) therefore, an additional post-hydrolysis step (carried by acids or enzymes) is required to obtain monomeric sugars.

1.6.2.3.Alkaline Pre-treatments

These kind of pre-treatments use alkali catalysts, such as ammonia,[86] lime,[62] or NaOH,[63] to soak the feedstock. In general, the mechanism of the process comprises the cleavage of aryl–ether linkages of lignin-carbohydrate matrix and disrupts the lignin structure.[87] The operational conditions used in alkaline pre-treatment are milder when compared to for the acid hydrolysis methods. It results in lower carbohydrate losses. The alkaline pre-treatments have been reported to be little effective when used to treat lignin-rich subtracts, such as softwood biomasses.[88] Among alkaline pre-treatments are also ammonia fibre explosion (AFEX™) and ammonia recycle percolation (ARP).

Ammonia Fibre Expansion (AFEX^{TM}) process uses high concentrations of anhydrous ammonia (0.3-2 kg NH_3 / kg of dry biomass) under high pressures but milder temperatures (160-180 ºC) and is followed by a quick release of pressure.[89,90] This method results in a swelling and physical deconstruction of biomass fibres that promotes a cellulose decrystallisation. An interesting feature of this technology is its ability to cause some chemical modifications of lignin, mainly due to the cleavage of aryl-ether bonds, producing intermediate molecular weight extractable lignin fragments.[91] Differently from chemical and neutral pre-treatments, in AFEX process, hemicelluloses remains practically intact (only deacetylation of hemicelluloses can lead to some losses) resulting in high recovery yields with very low moisture content.[92] Consequently, to obtain superior yields of fermentable sugars, the AFEX-treated materials besides of cellulases require also the hemicellulose hydrolytic enzymes. The main advantage of AFEX is the low formation of inhibitors for subsequent enzymatic and fermentative steps.[93]

Ammonia Recycle Percolation (ARP) is performed in a flow-through a packed bed column reactor in recycle mode with percolating aqueous ammonia (10-15 wt.%) at milder temperatures (150-170 ºC) and pressures.[94,95] This process results in the extraction of hemicelluloses and lignin to the liquid stream. Similar to AFEX^{TM} pre-treatment, ARP has been reported to be effective for low lignin containing biomasses.[96]

1.6.2.4.Oxidative Pre-treatments

This type of pre-treatments involve the use of oxidising agents *e.g.* ozone[97] or hydrogen peroxide[98,99] with the aim to remove lignin and hemicellulose from biomass. These oxidising agents selectively hydrolyse lignin aromatic and alkyl/aryl ether linkages leading to formation of less polymerised chemical molecules.[96] On the other hand, lignin and hemicellulose degradation products, *e.g.* aliphatic acids and aldehydes, can be formed that may have a negative impact on the performance of enzymatic hydrolysis.

Wet oxidation is usually carried out in the presence of pressurised oxygen/air or oxygen peroxide along with water at temperatures between 150 and 350 ºC and pressures from 50 up to 200 bar.[100] A disadvantage of this pre-treatment is a possibility of partial lignin and hemicellulose degradation.

1.6.3. Fractionation with solvents

Solvent fractionation based pre-treatment applies the principle of differential solubilisation and partitioning of various constituents of lignocellulosic biomass.[101] There are numerous literature reviews on solvent fractionation-based pre-treatments; however, among the diverse solvents explored, the ones that have attracted a main interest are the organosolv processes and in last two decades, ionic liquids.

Organosolv processes comprise the use of organic or aqueous/organic mixtures, usually alcohols (*e.g.* ethanol, methanol) or aliphatic acids (*e.g.* acetic, formic and oxalic) in the presence (or absence) of an acid catalyst (*e.g.* H_2SO_4, HCl) in order to disrupt inter- and intramolecular bonds between the biomass carbohydrates and lignin.[102] Organosolv processes are reported to be carried out in the large range of operational temperatures *e.g.* 90-120 ºC for grasses and 150-220 ºC for woods with a reaction time between 25 and 120 min. During the process, lignin is extensively removed and occurs the hydrolysis of hemicelluloses. It results in cellulose-rich pulp, which is highly susceptible to enzymatic processes.[96] The solvent is recovered to make the process economic and environmentally-friendly whereas high quality lignin can be used as an additive binder for other valuable end-uses.[88]

Ionic liquids (ILs) are molecular solvents with melting point below 100 ºC composed solely of cations, normally large organic, and ions, generally smaller and inorganic.[96] ILs demonstrate several important properties, such as low vapour pressure and consequently low volatility, high thermal and chemical stability and posse a great solvent power. Especially the last property makes them an interesting alternative to traditional solvents for lignocellulosic biomass. Pre-treatment with ILs can be executed in several ways however the most common is the complete dissolution of biomass followed by the regeneration of each fraction using anti-solvents.[64] IL pre-treatments are carried out at temperatures ranging from 90 to 140 ºC in reactions lasting from single hour to several hours. ILs have a great potential for lignocellulosic biomass pre-treatment, producing an highly susceptible material to enzymatic hydrolysis. One of the main and commonly recognised limitations is the cost of these solvent. The recent achievements in this field confirm that some ILs can be as cheap as classical organic solvents e.g. acetone.[103] Nevertheless, ILs must be appropriately recovery and a recycling strategy must be always employed in an effort to guarantee the economic feasibility of the process.

1.6.4. Biological Pre-treatments

Biological pre-treatments involve the use of microorganisms, *e.g.* brown, white, and soft-rot fungi, that secret extracellular enzymes *e.g.* laccases and lignin peroxidases able to remove substantial amount of lignin from the plant cell wall.[20] The major advantages of these pre-treatments are low energy inputs, mild operational conditions and avoidance of hazardous chemicals; however, very slow conversion rates constitute a serious drawback for their application in large-scale biorefinery installations.[104] Additionally, most of microorganisms convert part of available carbohydrates for cellular growth affecting negatively the final sugar yields.

1.7. Considerations for an ideal pre-treatment

A variety of pre-treatment technologies is constantly investigated to improve their technological factors, *i.e.* modification of physical and chemical structure of lignocellulosic biomass and also to advance economic and reduce the environmental effect of these processes. Due to the diverse reasons discussed below universal pre-treatment does not exist, whereby each technology differs from their

mode of action affecting differently the lignocellulosic biomass. Still, all pre-treatments should meet certain primary requirements, such as disruption of lignocellulose structure aiming to modify its polymerisation degree and cellulose surface area, decrease of cellulose crystallinity and lignin content without their significant losses and no formation of fermentable inhibitors.[75,105] The choice of a pre-treatment technology depends on many factors that must be taken into account when selecting the most adequate one for particular scenario. Among them are:

1. Feedstock: The macromolecular, chemical and morphological composition of the feedstock implies particular types of pre-treatment. This in turn, determines the entire value chain from the field side to the product formulation. Normally, as transport of the feedstock is cost consuming the selection of the feedstock is made on the basis of endogenous resources (either natural or industrial).
2. End-product: The production of desired commodity has an important aspect on the selection either the feedstock or pre-treatment.
3. The industrial symbiosis: The possibility to share either industrial streams *e.g.* energy, steam or intermediates is highly desired and may determine the type of pre-treatment deployed.
4. Environmental aspects: Use of more sustainable methodologies with less hazardous (non-polluting, non-corrosive, inflammable, etc.) chemicals and processes performed under milder conditions are more adequate.
5. Local conditions: Legal aspects as well as presence of adequate infrastructure and qualified human resources are crucial to determine the adequate technologies of biomass pre-treatment.

At least these five factors must be taken into account because they help to take a decision regarding the adequate pre-treatment steps that is always interlinked within the larger value chain of the biorefinery.

1.8.High-pressure CO_2-H_2O mixture Pre-treatment

1.8.1. Fundamentals

Following the statement of Professor Roger Sheldon "(…) *the use of water and supercritical CO_2 as reaction media is also consistent with current trend towards the use of renewable, biomass-based raw materials (*…)" is clear that water along CO_2 are going to play an important role in the development of innovative and environmentally-friendly technologies for the processing/pre-treatment of lignocellulosic biomass.[106] For reaction mixture of CO_2 + H_2O, at temperatures above 160 and up to 250 ºC and pressures above 200 bar, the reaction medium (water-rich phase) contains up to 98 mol% of water, whereas the gas phase (CO_2-rich phase) has a density comparable to those found for liquids

containing at least 30 mol% of water.[107] In the liquid phase occurs the dissolution of CO_2 (in molecular form) in water leading to the formation of unstable carbonic acid (H_2CO_3).

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$

As H_2CO_3, even being in presence of CO_2 dissolved in water, is unstable acid it undergoes dissociation to form hydronium ion (H_3O^+) and hydrogen carbonate ion (HCO_3^-).

$$CO_2 + 2H_2O \leftrightarrow HCO_3^-$$

The subsequent reaction of further dissociation is less symptomatic as pKa_2 is only 10.32[108] hence in the aqueous solution it can be stated that exist equilibrium between CO_2 dissolved in water and hydrogen carbonate ion from carbonic acid dissociation. This equilibrium depends on the conditions of the reaction, namely on temperature and pressure.

To estimate the amount of CO_2 dissolved in water, Henry's law can be used, as long as the temperature is not close to critical temperature.[109] In generally, higher concentration of hydronium ion, due to the formation of carbonic acid, causes acidification of the medium to a relatively low pH value (2.8 - 3.0) under temperatures in the range of 25-70 ºC and pressures of 70-200 bar.[110]

1.8.2. Mechanism of Action

The dissolution of CO_2 in water promotes the hydrolysis of biomass polymers in a mechanism similar to that found for acid-based pre-treatments. During this process, the formation of hydronium cation act as a protonation actor of either glycosidic oxygen or oxygen in the carbohydrate ring and form a carbocation as an intermediate. Next hydronium cation promotes the cleavage of acetyl groups from hemicelluloses increasing the acidity of the medium causing the classical acid hydrolysis and formation of saccharides, either in monomeric or in oligomeric form. The removal of hemicellulose increases the accessibility of cellulose polymer subjected to improved enzymatic saccharification process.[111] Clearly, CO_2-based processes offer similar advantages to those found for both liquid hot water and dilute-acid hydrolysis without the typical bottlenecks found especially for mineral acid-based technologies, such as need of acid neutralisation and waste disposal.[112]

1.8.3. Reaction Severity

The severity factor is a tool to assess and compare the performance of the pre-treatment under various operational conditions such as temperature, pressure of CO_2 and reaction time. To evaluate the effect of CO_2 in the severity of the reaction, where the determination of the pH value is essential, van Walsum proposed the combined severity factor ($CS_{P_{CO_2}}$)[113] according to the following formula: $CS_{P_{CO_2}} = \log(R_O) - pH$, where R_O is the severity factor, T is temperature (ºC) and pCO_2 is partial pressure of CO_2 (atmospheres).[114] Considering the influence of temperature, partial pressure and solubility of CO_2 in water, the same authors proposed an equation to estimate the pH value in high-

pressure CO_2-H_2O mixture. They suggested the following formula: $pH = (8.00 \times 10^{-6})T^2 + 0.00209 \times T - 0.216 \times \ln(p_{CO_2}) + 3.92$, where the temperature is in the range of 100 and 250 ºC and the partial pressure of CO_2 is up to 151.9 bar.

1.9.High-pressure Ammonia Pre-treatment

At the beginning of the 20th century, Fritz Haber and Carl Bosch developed the process for ammonia synthesis from atmospheric nitrogen and hydrogen (typically obtained from natural gas reforming, but it could also be derived from green resources).[115,116] Ammonia is a colourless alkaline gas with a characteristic sharp odour, highly recoverable, non-corrosive and easily handled.[117] In comparison to other chemicals used in biomass processing *e.g.* ionic liquids, ammonia is considered an inexpensive chemical. It can be easily liquefied due to strong hydrogen bonding between NH_3 molecules; the liquid ammonia boils at -33.3 ºC and has a vapour pressure of 10 bar at 25 ºC.[118] Ammonia-based technologies for biomass pre-treatment have been investigated extensively and used for over 80 years as a swelling agent of cellulose and for changing the cellulose fibre morphology.[119] Ammonia has been used in both aqueous and anhydrous forms to promote a range of physicochemical modifications in plant cell walls. While aqueous ammonia systems typically explore hydroxyl ions and ammonia reactions with lignocellulosic biomass components, anhydrous ammonia systems only use ammonia as the major pre-treatment agent. Though both systems are effective in pre-treating lignocellulosic biomass, their interaction with cell wall components is slightly different. By exploring those differences, a range of ammonia pre-treatment methods *e.g.* ARP, AFEX™, ammonium hydroxide and extractive ammonia have been evaluated and reported, showing significantly different results.

1.9.1. Fundamentals of Alkali-based Pre-treatments

1.9.1.1.Polysaccharides Reactions

Understanding the interaction of hydroxyl ions with biomass polysaccharides is essential for the tailor-made development of an effective alkali pre-treatment with high overall sugar yields. Firstly, the solvation of hydroxyl groups by hydroxyl ions causes a swollen effect in biomass.[120] At elevated temperatures, a number of reactions targeting the polysaccharides take place. The most important ones are: dissolution of undegraded polysaccharides; peeling of end groups with formation of alkali stable end groups; alkaline hydrolysis of glycosidic bonds and acetyl groups and degradation, and decomposition of dissolved polysaccharides and peeled monosaccharides.[111] Peeling and hydrolytic reactions under strong alkaline conditions are largely responsible for the loss of fermentable sugars and reduction of the degree of cellulose polymerisation.

The *peeling reactions* start at temperatures *ca.* 100 ºC, leading to the reduction of polysaccharide chains from the existing reducing end groups. The mechanism for endwise peeling starts with Lobry de Bruyn Alberda van Ekenstein rearrangement, which is the isomerisation of the reducing end group

to a ketose in equilibrium with the corresponding 2,3-enediol. The C-4-substituent is disrupted, due to its alkali-labile property, leading to a new reducing end in the polysaccharide chain. The generated monomeric sugar is tautomerised to a dicabonyl compound that is rearranged to produce isosaccharinic acids. Other possible degradation compounds that can be found are lactic acid, 2-hydroxybutanoic acid and 2,5-dihydroxypentanoic acid. In general, hemicelluloses are more susceptible to peeling reactions than cellulose; however, these reactions occur differently depending on the hemicellulose type. For instance, xylans are less susceptible and more stable than arabinans and glucomanans. The easily breakable arabinose side groups present in xylans of softwoods have a stabilising effect against alkaline peeling, since an alkali-stable metasaccharinic acid end group is produced after the loss of the arabinose side group. Then, the reaction occurring during the endwise peeling stops when competing reactions, also known as stopping reactions, take place in order to prevent the disruption of polysaccharide fibers and, consequently their degradation.[111,121,122]

The *hydrolytic reactions* of polysaccharide chains occur at temperature *ca.* 150 ºC, leading to the formation of new reducing ends, which are subjected to endwise reactions (secondary peeling). A β-hydroxy elimination at the C2 position in cellulose kicks-off the hydrolytic reactions forming a tautomeric intermediate that is further converted into an alkali stable metasaccharinic acid group or C2-methylglyceric acid. Other low molecular weight aliphatic acids, such as acetic and formic acids, are also produced; acetic acid is formed *via* cleavage of the acetyl side chain groups present in hardwood and grass xylans, whereas formic acid is produced from the peeling reactions of polysaccharides. Additionally, glucuronic acid side groups of xylan are also hydrolysed under strong alkaline conditions. Thus, the avoidance of hydrolytic reactions would prevent the formation of undesired aliphatic acids.[111]

1.9.1.2.Lignin Reactions

The most important aspects in lignin degradation are the varied reactivity of lignin subunits and the stability of bonds existing in lignin polymers.[123] The reactivity of such lignin subunits is highly dependent upon whether the phenolic group is etherified or not. In alkaline conditions, the dissolution of lignin is mainly achieved by the cleavage of the most labile bonds *i.e.* ester and aryl-ether bonds. Aryl-alkyl or alkyl-alkyl bonds are also cleaved, but to a lesser extent than aryl-ether bonds, whereas diaryl ether and C-C bonds are usually the most stable ones and remain unchangeable during alkali reactions.

The *cleavage of α-aryl and α-alkyl ether linkages* comprises an alkali-assisted structural rearrangement of the phenolates, which promotes the cleavage of linkages between α-C in phenylpropane units and the O of the aryl ethers or the alkyl ethers, to the corresponding quinone methide (Figure 1.7). Then, several reactions, such as elimination and addition reactions may take

place. In hardwoods and softwoods, α-aryl ether linkage is one of the most common bonds playing a great role on the delignification rate.[123]

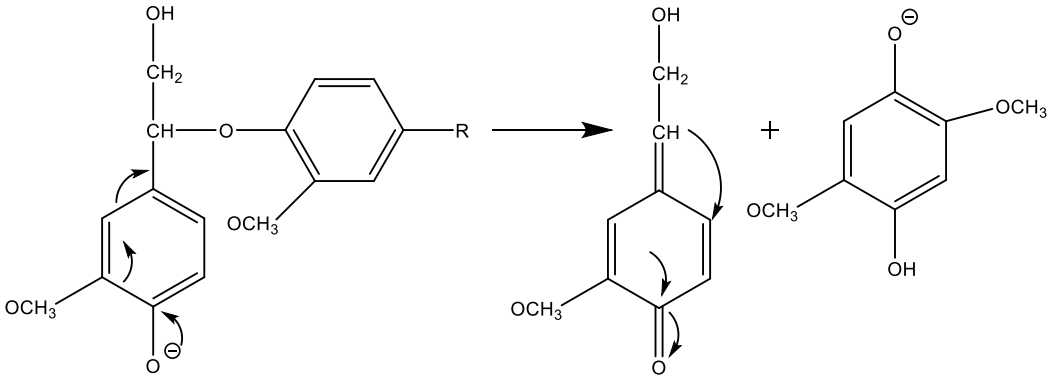

Figure 1.7. Representation of an α-aryl ether linkage cleavage.

The *cleavage of phenol β-aryl ether linkages* involves the nucleophilic attack of hydroxyl on the α-carbon leading to the formation of epoxides. These compounds promote the subsequent cleavage of β-aryl ethers. These phenol type linkages play an important role on the diversity of connections in lignin structure, particularly in softwoods.[123]

The *cleavage of non-phenol β-aryl ether linkages* only occurs in the presence of a hydroxyl group on α- carbon. This group can be easily ionised under extreme alkaline conditions, and the formed oxygen ion targets the β- carbon generating an epoxy compound.[123]

1.9.2. Reactions involving Ammonia

1.9.2.1.Ammonolysis

Ammonolysis are reactions that occur between ammonia and ester bonds to form alcohols and amides. They can occur in the absence or presence of water, in liquid and vapour phases. The cleavage of ester linkages, particularly found in lignin-carbohydrate complexes, such as di-ferulate (which cross-link polysaccharides), lignin–ferulate and lignin–diferulate linkages (cross-link polysaccharides to lignin), are expected to facilitate the removal of lignins from the cell wall, thus increasing the biomass susceptibility to enzymatic attack.[37,124] Chundawat et al. reported the formation of acetamide and various phenolic amides during the AFEX™ pre-treatment of corn stover, which are a result of ammonolysis reactions between ammonia and those ester linkages present in lignocellulosic biomass (Figure 1.8).[124] In aqueous medium, ammonia dissociates into ammonium and hydroxyl ions. Those hydroxyl ions can compete with ammonia for cleaving ester linkages via hydrolysis reactions, generating carboxylic acids instead of amides. French et al. reported, for the first time, the effect of aqueous ammonia in a wide range of esters composed of different leaving groups with different molecular weights.[125] The authors found that different esters with decreasing molecular weights lead to an increase of the ratio of ammonolysis to hydrolysis and reactivity as well.

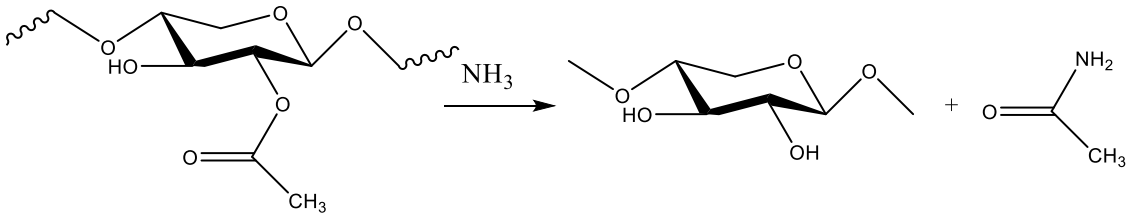

Figure 1.8. Schematic representation of the ammonolysis reaction targeting ester linkages present in acetylated arabinoxylans, resulting in formation of acetamide and deacetylated arabinoxylan.

1.9.2.2.Maillard Reactions

Maillard-type reactions occur when ammonia reacts with polysaccharides. Maillard reaction chemistry is characteristically composed of a complex network of reactions. In order to have an idea how complex are these reactions, Shibamoto identified more than fifty by-products, including imidazoles, pyrazines, ketones, aldehydes and amides, when D-glucose was subjected to ammonia treatment.[126] Chundawat et al. has also observed the presence of this family of compounds in AFEX pre-treated corn stover, which have been produced by subjecting the biomass at high temperatures (> 120 °C) in the presence of ammonia. Minimizing the formation of Maillard-type reactions during pre-treatment is not only important to preserve carbohydrates for subsequent fermentation, but also to avoid the formation of fermentation inhibitors, which reduce final product yields.[127]

1.9.2.3.Conversion of crystalline cellulose I_β to cellulose III_I

Cellulose I_β (monoclinic) is the most common allomorphic form of cellulose in higher plants[128]. Several pre-treatments have been acknowledged to affect the cellulose crystallinity at different levels. For instance, native cellulose I_β can be converted into cellulose II *via* ILs, or into cellulose III_I through treatment with anhydrous liquid ammonia and certain amines, such as 1,2-diaminoethane (Figure 1.9). During the anhydrous liquid ammonia treatment, ammonia penetrates into cellulose lattice leading to the formation of intermediate cellulose-ammonia complex by breaking hydrogen bonds and interacting with the cellulose hydroxyl groups.[129,130] In particular, when native cellulose is subjected to anhydrous liquid ammonia, it undergoes into changes in interplanar distances of the 101 planes leading to an increase in the volume of unit cell from 671 (native cellulose) to 801 cubic Å found in cellulose-ammonia crystal complex. Once the ammonia is removed from the intermediate complex, the volume of unit cell is reduced to 702 cubic Å, due to establishment of a new hydrogen bond network and adjustments on the packing of cellulose chains.[131] This leads to the formation of cellulose III_I allomorph. Chundawat et al. reported that the conversion of native cellulose into cellulose III_I resulted in an improvement of enzymatic digestibility of approximately 5-fold in glucose yield in comparison to the starting cellulose I, and 80 % from corn stover;[132] however, it is important to point out that the degree of conversion of cellulose I into cellulose III_I is highly reliant over various operating

conditions, such as temperature at both pre-treatment and ammonia extraction, residence time and moisture content.[133]

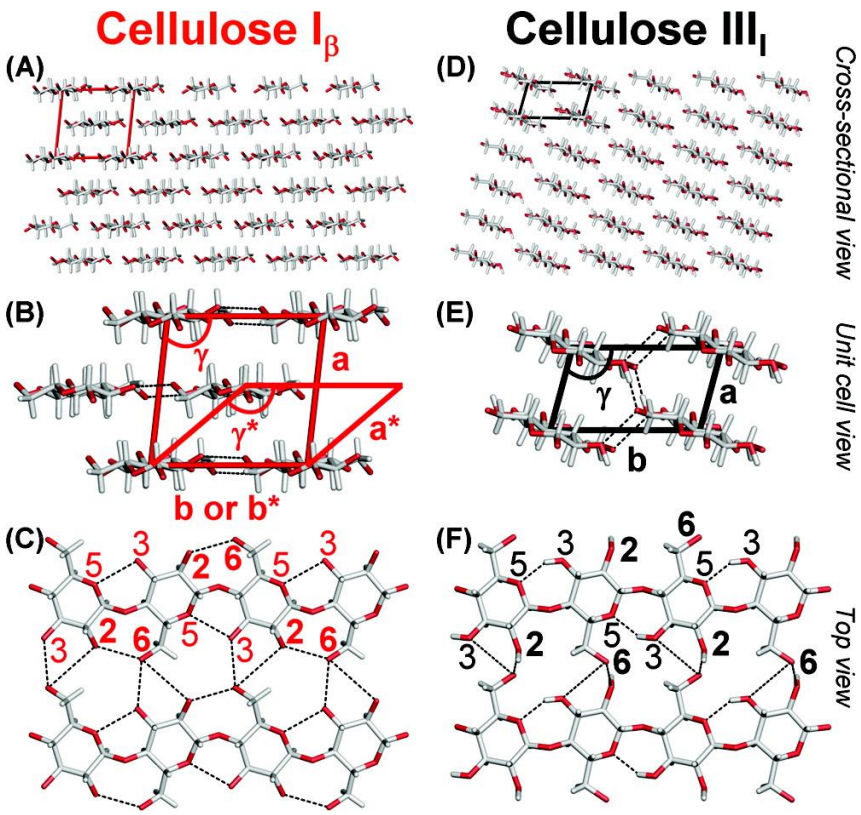

Figure 1.9. Schematic representation of cellulose I_β (A-C) and III_I (D-F) in cross-sectional (A-D), unit cell (B-E), and top (C-F) views. Glucan chain organisation and hydrogen bond network are represented by dotted lines. Oxygen atoms are depicted in red and numbered considering the adjoining carbon atoms (C-F). Reprinted from S. P. S. Chundawat; G. Bellesia; N. Uppugundla; L. C Sousa; D. Gao; A. M. Cheh; U. P. Agarwal; C. M. Bianchetti; G. N. Phillips Jr.; P. Langan; V. Balan; S. Gnanakaran; B. E. Dale; *J. Am. Chem. Soc.* **2011,** 133, 11163-11174. Copyright (2011) with permission from American Chemical Society.

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Chapter II

Scope and aims of the thesis

2.1.Scope and aims of the thesis

This dissertation is focused on the development of high-pressure technologies showing the full exploitation of polysaccharides (cellulose and hemicelluloses) present in lignocellulosic biomass. The valorisation of various lignocellulosic biomasses was investigated using two distinct technologies based on the chemical character of studied fluids, namely:

1. Acidity of CO_2/H_2O mixture through the catalytic effect of carbonic acid formed *in-situ*;
2. Alkaline character of high-pressure ammonia.

The very distinct chemical character of the studied technologies greatly influenced the efficiency of lignocellulosic biomass processing, namely performance of pre-treatment, hydrolysis, dehydration, enzymatic conversion and fermentation.

A thoughtful assessment of the selective hydrolysis of hemicellulose fraction of wheat straw into C_5-aqueous stream and of the improvement of cellulose digestibility for the enzymatic hydrolysis was performed to understand the potential benefits of new approach involving high-pressure CO_2/H_2O mixture. The same phenomenon has been employed for the selective dehydration of C_5-sugars into furfural. In this concept the use of high-pressure CO_2 as an effective and more sustainable catalyst in an H_2O/THF system was applied.

Furthermore, a new approach of high-pressure ammonia-based technology has been established. It aimed decreasing the ammonia loading by the use of densified biomass. The performance of this new ammonia treatment has been assessed through the production of fermentable C_5 and C_6 sugars *via* enzymatic hydrolysis and their biological conversion to ethanol. In addition, this thesis aims verifing the lignocellulosic feedstock versatility of the new ammonia-based concept. For this purpose, hardwoods, herbaceous dicots and monocots, for the production of fermentable sugars were used.

2.1.1. High-pressure CO_2/H_2O mixture

The efficiency of high-pressure CO_2/H_2O mixture to selectively hydrolyse the hemicellulose fraction from wheat straw and to simultaneously improve the enzymatic hydrolysis of cellulose-rich fraction is presented in **CHAPTERS III**, **IV** and **V**. The application of the same catalytic approach for the furfural production has been scrutinised in **CHAPTER VI**.

The work presented in **CHAPTER III** aims to demonstrate the advantages from the addition of high-pressure CO_2 to liquid hot water system in the biomass pre-treatment. For this, high-pressure CO_2/H_2O pre-treatment of wheat straw was examined under various operational conditions. The formed *in-situ* carbonic acid was found to result in higher production of pentose oligomers in comparison to CO_2-free pre-treatment (*i.e.* liquid hot water). Moreover, the effect of reaction time and pressure of CO_2 applied on the performance of hemicellulose hydrolysis into C_5 sugars has been studied in **CHAPTER IV** as well.

In addition, the influence of the pre-treatment methodology on the performance of enzymatic hydrolysis of cellulose-rich leftover solids has been scrutinised in **CHAPTER V**. Both chemical and physical effects of high-pressure CO_2 addition to the pre-treatment was found to improve the enzymatic hydrolysis yields when compared to those obtained for liquid hot water.

Besides the biomass pre-treatment, high-pressure CO_2 can act as catalyst in further conversion of pentose sugar stream to furfural. Additional effect of high-pressure CO_2 as a furfural-stripping agent, in a presence of an organic solvent, allowing the improvement of final furfural yields is discussed in **CHAPTER VI**.

2.1.2. High-pressure ammonia

CHAPTER VII describes a new high-pressure ammonia technology called Compacted Biomass with Reduced Ammonia (COBRA). This technology aims to increase the pre-treatment solid loading by the use of pelletised biomass, which in turns has a significant influence on the ammonia loading. The alkaline character of the COBRA pre-treatment of densified sugarcane bagasse allowed achieving conversion of cellulose I_β to cellulose III, ammonolysis of cell wall ester cross-links and removal of lignin from the biomass plant cell wall. The performance of COBRA under industrially-relevant hydrolysis conditions, high-solid loading enzymatic hydrolysis followed by fermentation step were carried out. The obtained results, in terms of fermentable sugar and ethanol yields, were compared to those achieved by mature technologies. Lastly, the robustness and efficiency of COBRA to handle different sources of biomass, including hardwoods, herbaceous dicots and monocots, for the production of fermentable sugars was also addressed.

2.2.Author contribution

The chapters presented in this thesis are based on information and data acquired and published in scientific papers with contributions of several co-authors. Thus, my direct contribution to each chapter is listed below:

CHAPTER III

1. Set-up and development of high-pressure CO_2/H_2O mixture process for the wheat straw processing.
2. Chemical characterisation of liquid and solid streams from biomass pre-treatment.
3. Discussion of the obtained results and manuscript drafting.

CHAPTER IV

1. Designing of the experiments to be executed.
2. Chemical characterisation of either liquid or solid streams.
3. Discussion of the obtained results and the manuscript writing.

CHAPTER V

1. Planning and execution of the enzymatic hydrolysis assays.

2. Chemical characterisation of the produced streams.
3. Analysis of morphological changes (SEM and FTIR) in the obtained leftover solids.
4. Designing and execution of experiments for the determination of chemical and physical effect of high-pressure CO_2 addition to the pre-treatment.
5. Discussion of the obtained results and the manuscript preparation.

CHAPTER VI

1. Designing and execution of dehydration experiments to produce furfural.
2. Chemical characterisation of obtained streams.
3. Discussion of the obtained results and the manuscript writing.

CHAPTER VII

1. Optimisation and execution of experiments related to ammonia pre-treatment, enzymatic hydrolysis and fermentation trials.
2. Chemical and morphological characterisation of samples obtained from all aforementioned experiments.
3. Discussion of the obtained results and the manuscript preparation.

Chapter III

The CO_2-assisted autohydrolysis of wheat straw

This chapter is based on the published manuscript: Sara P. Magalhães da Silva, Ana Rita C. Morais and Rafal Bogel-Lukasik. *Green Chem*. 2014, 16, 238.

3.1.Introduction

Lignocellulosic feedstock is mainly composed of cellulose (35-50 %), hemicellulose (20-35 %) and lignin (5-30 %),[1] wherein the composition is dependent upon several parameters. Due to the complex macroscopic structure of lignocellulosic materials, several pre-treatment technologies are currently employed to overcome this recalcitrance against chemical and microbial attacks. The pre-treatment methods can be categorised according to various criteria.[2,3] Pretreatments can be segregated between conventional (dilute acid hydrolysis, alkali),[4] hydrothermal (steam-explosion,[5] wet oxidation,[6] microwave treatment,[7] and autohydrolysis)[8] and alternative methods (ionic liquids,[9,10] sub- and supercritical fluids, mostly water and CO_2).[11-13] The autohydrolysis process uses compressed hot water (pressure above saturation point) with a general range of temperature between 150 and 230 °C and various reaction times from seconds to hours according to the operation mode applied.[4] Hydronium ions generated *in-situ* by water auto-ionization and acetic acid from dissolution of acetyl substituents of hemicelluloses have the capability to act as catalysts formed *in-situ* in the autohydrolysis processes. A high recovery of hemicellulose in the liquid-fraction (mainly in oligomeric form) and of cellulose and lignin in the solid fraction with negligible losses are generally reported. The hemicellulose-rich liquor can be a source of value-added products. Xylooligosaccharides (XOS) are one of these products and can be obtained directly from autohydrolysis pre-treatment.[8] Xylitol, important due to its application in the food, pharmaceutical and cosmetic industries, can be produced after the bioconversion of hemicellulose liquor as well.[14] Moreover, production of reducing sugars, organic acids (such as acetic acid or propionic acid), bio-composites, furfural and other miscellaneous compounds can be developed too.[15] On the other hand, the solid fraction obtained is mainly converted to sugar monomers and further to bioethanol along with a possibility to produce value-added commodities.

Supercritical CO_2 ($scCO_2$) is a non-toxic, non-flammable and inexpensive reagent[16] and its employment generally lowers the temperature of the process leading to minor generation of degradation products and a higher yield of the reaction.[4,17] Technologies involving the use of sub- (near its critical conditions) and supercritical treatments have been investigated for lignocellulosic material pre-treatments. In these processes sub- or supercritical water and/or supercritical CO_2 were commonly used. Considering economic efficiency supercritical water or liquid hot water (LHW) treatments seem to be superior due to the water facilitated feasibility of hydrolysis that provides an acidic environment at high temperatures.[18] Particularly in the subcritical range of temperature and pressure ($P < 210$ bar, $T < 380$ °C), the ion product and the hydrolysis capacity of H_2O increases due to the increased temperature. At 250 °C the ion product for water, K_w, reaches a maximum of 6.34×10^{-12}, resulting in a 5.5 pH for water at 220°C.[18] Thus hemicellulose could be completely separated from the lignocellulose and enzymatic digestibility of cellulose can be significantly enhanced by treating the lignocellulosic material under ascribed conditions.[19,20] Recent studies on the sub-pressurised water effect of $scCO_2$ in biomass pre-treatment[13,18] showed that the presence of water

favours the separations in a manner that in the presence of CO_2, the mixture becomes more acidic due to the *in-situ* formation and dissociation of carbonic acid according to the following equation: $CO_2 + 2H_2O \leftrightarrow HCO_3^- + H_3O^+; HCO_3^- + H_2O \leftrightarrow CO_3^{2-} + H_3O^+$. By the dissolution of CO_2, the pH of the water–CO_2 mixture decreases to approximately 3.0 (depending on pressure and temperature) making the environment strongly acidic, thus facilitating the biomass hydrolysis.[21]

Compared to the conventional hydrothermal treatments, the combined sub- or supercritical processes demonstrate a much higher reaction rate; thus, neither the use of an additional catalyst nor inhibition of the reaction towards intermediates is required.[22,23] The use of scCO_2 can overcome the drawbacks resulting from the conventional pre-treatments with organic acids due to the CO_2 practical neutralisation caused by a pressure reduction. The use of scCO_2 was reported to not cause a significant change in the microscopic morphology of wood.[24] Studies aimed at checking the scCO_2 effect on raw lignocellulosic materials with different moisture contents under various pre-treatment conditions (temperature, time and pressure)[11]demonstrated that an increase in moisture content to 73% (w/w) at 214 bar and 165 °C resulted in a significant increase of the final sugar yields from the enzymatic hydrolysis. An important conclusion on a pronounced effect of the moisture content in pre-treatments with scCO_2 was drawn. Addition of CO_2 to the autohydrolysis pre-treatment of beech wood showed an increase of xylan hydrolysis.[25] On the other hand, CO_2 applied in the autohydrolysis process at 100 bar did not enhance the degree of biomass dissolution[12] due to the unfavourable acidic water/CO_2 system.

The presented work was devoted to examine effect of CO_2 on the autohydrolysis pre-treatment of wheat straw, in order to enhance the selectivity of the dissolved hemicellulose fraction. Moreover, this work aimed at evaluation of the temperature and non-isothermal operational mode effects on the composition of both liquid and solid fractions as a function of the severity factor (Log R_0)[26] (according to the following equation: $R_0 = \int_0^t e^{\left(\frac{T(t)-100}{14.75}\right)} dt$, where t is time expressed in minutes, T abbreviates temperature (°C) and 14.75 is an empirical parameter related with temperature and activation energy).

Additionally, the influence of CO_2 density was also studied using the Peng-Robinson equation of state.[27]

3.2.Materials and methods

3.2.1. Raw material

Wheat straw was kindly supplied by INIAV, I.P. – Estação Nacional de Melhoramento de Plantas (Elvas, Portugal). The material was ground using a knife mill (IKA® WERKE, MF 10 basic, Germany) to particles smaller than 1.5 mm, and stowed at room temperature. The wheat straw moisture was determined to be 8 %. CO_2 with a purity ≥99.99 % bought from Air Liquide, AlphaGaz™ gamma, Paris, France was used. For post-processing filtrations, paper filters (Ø=150 mm, nº 1238) from Filter-Lab, Microchip Technology Inc., Arizona, USA were used. For all experiments the following reagents were used: distilled water (17 MΩ/cm)

produced by the PURELAB Classic Elga system, 72 % (w/w) H_2SO_4 aqueous solution was prepared from concentrated H_2SO_4 solution (96 % purity) supplied by Panreac Química, Barcelona, Spain. In addition, ethanol at 96 % purity (v/v) for gas phase capturing was acquired from Carlo Erba Group - Arese, Italy.

3.2.2. CO_2-assisted autohydrolysis of wheat straw

The CO_2-assisted autohydrolysis treatments of wheat straw were performed in a stainless steel 600 mL reactor (series 4560, Parr Instruments Company, Moline, Illinois, USA). The reactor was equipped with two four-blade turbine impellers, and the temperature and pressure were controlled using a Parr PID controller, model 4842. An external fabric mantle was used to heat the reactor, while an internal stainless steel loop was used to cool the system with cold water. Figure 3.1 illustrates a scheme of the apparatus used.

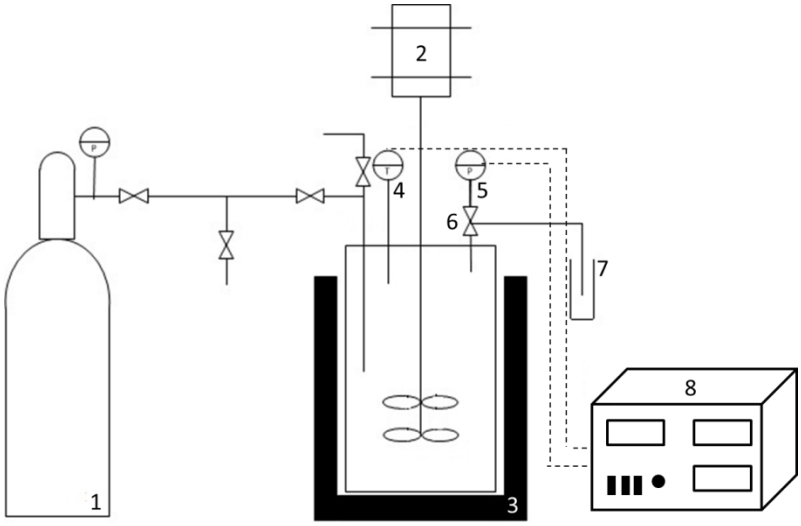

Figure 3.1. Scheme of the CO_2-assisted autohydrolysis pre-treatment apparatus. 1 – CO_2 cylinder; 2 – magnetic drive; 3 – heating mantle; 4 – thermo par; 5 – pressure transducer; 6 – depressurisation valve; 7 – vial filled with ethanol; 8 – pressure and temperature PID controller.

The CO_2-assisted autohydrolyses of wheat straw were carried out at three temperatures, namely 180, 200 and 210°C, selected based on the literature data.[8] An initial pressure of 60 bar at room temperature and an agitation speed of 70 rpm were maintained constant in all experiments. Different mixture loadings were used: 250 g of H_2O/25 g of wheat straw; 150 g of H_2O/15 g of wheat straw and 75 g of H_2O/7.5 g of wheat straw. When the final desired temperature was attained, the reactor was rapidly cooled down to quench the reaction. A slow depressurization (2 bar ·minute^{-1}) of the reaction mixture was executed when the temperature was lower than 20 °C to minimise the presence of volatile compounds in the vapour phase. The depressurised gas phase passed through a vial placed in the ice at temperature 0 ºC filled with a known amount of ethanol. This procedure allows for dissolution of volatile compounds for posterior qualitative and quantitative analyses. The liquid (liquor) and solid

fractions were recovered through by vacuum filtration. The qualitative and quantitative analyses of all fractions were performed using the procedures presented below.

3.2.3. Chemical Analyses

3.2.3.1.Characterisation of the feedstock material composition

The feedstock material was ground in a knife mill to a particle size <0.5 mm and the moisture was determined by drying at 105 °C for at least 16 h to obtain constant weight. The biomass was characterised analysing glucan, xylan, arabinan and acetyl group content after treatment with 72 % (w/w) H_2SO_4 according to standard methods.[28] Syringe filters (0.2 μm) from Whatman, GE Healthcare Life Generations, Buckinghamshire, United Kingdom were used to filter all samples before running on High-Performance Liquid Chromatography (HPLC). Monosaccharides (glucose, xylose and arabinose) and acetic acid were investigated using an Agilent 1100 series HPLC system, Santa Clara, CA, USA equipped with a Bio-Rad Aminex HPX-87H column (Hercules, CA, USA). The set conditions of the column were: 50 °C, 0.4 mL/min flow rate with 5 mM H_2SO_4. A refractive index (RI) detector was employed to examine sugars and acetic acid content. The acid insoluble residue was considered as a Klason lignin after correction for the acid insoluble ash (determined by igniting the content at 550 °C for 5 h). Protein quantification was performed by the Kjeldahl method using the Nx6.25 conversion factor.[29]

3.2.3.2.Characterisation of the processed solids

The solid fractions were washed with distilled water at room temperature, and oven-dried at 40 °C for at least 48 h. The processed solids recovered were subjected to the same chemical characterisation of the feedstock except for the determination of protein and ash.[28]

3.2.3.3.Liquor and post-hydrolysate characterisation

The concentration of reducing sugars (glucose, xylose and arabinose), as well as acetic acid, furfural and 5-hydroxymethylfurfural (HMF) present in the liquor recovered from the CO_2 pretreatment were determined by HPLC. In this case, a flow rate of 0.6 $mL \cdot min^{-1}$ and furfural and HMF analyses occurred with a UV/Vis detector at 280 nm. The liquor sample was subjected to hydrolysis with 4 % (w/w) H_2SO_4 at 121 °C for 1 h in an autoclave (Uniclave, Portugal) to convert soluble oligosaccharides from hemicellulose into its constituent sugar monomers.[30] After post-hydrolysis, the concentration of oligosaccharides was expressed as an increase in sugar monomers determined by HPLC. After post-hydrolysis, oligosaccharides concentrations were expressed as an increase in sugar monomers analysed by HPLC.

3.2.3.4.Gas phase

To examine the presence of volatile degradation products, namely furfural and acetic acid, the gas phase recovered during slow depressurisation was analysed by HPLC.

3.3.Results

3.3.1. Feedstock composition

The chemical composition of the wheat straw used in the CO_2-assisted autohydrolysis pre-treatment was characterised. These data are compiled in Table 3.1.

Table 3.1. Macromolecular composition of wheat straw (% of dried weight).

Component	This work[a]	Carvalheiro et al.[8]
Cellulose[b]	38.5 ± 0.1	38.9 ± 0.2
Hemicellulose	24.9	23.5
Xylan	19.1 ± 0.6	18.1 ± 0.3
Arabinan	3.0 ± 0.1	3.0 ± 0.2
Acetyl groups	2.7 ± 0.2	2.5 ± 0.1
Klason lignin	17.7 ± 0.1	18.0 ± 0.5
Ash	10.7 ± 0.1	9.70 ± 0.03
Protein	4.7 ± 0.1	4.5 ± 0.5
Others[c]	3.5	5.5

[a] Average of two replicates; [b] Determined as glucan; [C] Determined by difference.

The level of the wheat straw moisture was determined to be 8 %. A total of 63 % of wheat straw biomass were polysaccharides among which 38.5 % was cellulose (estimated as glucan). Wheat straw hemicellulose was composed of β-D-(1,4)-linked xylopyranosyl backbone, substituted with arabinofuranose, 4-O-methylglucuronic acid, acetyl groups, xylose and phenolic acids.[8] The total hemicellulose, 24.9 %, was measured as the sum of xylose, arabinose and acetyl group content. In relation to the Klason lignin content, the obtained value was corrected for the ash content of acid insoluble residue and it was determined to be 17.7 %. The obtained data are in good agreement with those reported by Carvalheiro et al.[8]

3.3.2. Composition of the liquors

The composition of the liquors was one of the parameters examined in this study. The wheat straw CO_2-assisted autohydrolyses resulted in liquors containing a mixture of sugar oligomers (mainly XOS), monosaccharides (glucose, xylose and arabinose), acetic acid (from acetyl groups present in hemicellulose) and sugar decomposition products, namely HMF and furfural. According to the literature reports, the formation of these chemicals depends on the severity of pre-treatment

conditions, namely temperature. The composition of liquors obtained from the CO_2-assisted autohydrolysis under various conditions is depicted in Table 3.2. Xylooligosaccharides (XOS) were found to be major components present in liquors in all reactions. Considering all the biomass loading studied, the highest amount of XOS produced was determined to be at the lowest studied biomass loading (75/7.5) under the most severe conditions ($Log\ R_0$ = 3.48). On the other hand, at the highest biomass loading (250/25) under similar severity condition ($Log\ R_0$ = 3.54), the concentration of XOS was as much as 36 % lower than presented before. The obtained concentration was comparable to the concentration (10.64 g/L) with the lowest biomass loading (75/7.5), but under less severe conditions ($Log\ R_0$ = 2.60). The remaining oligosaccharides (GlcOS and AcO) exhibited a significant concentration in the liquid fraction, which decreased with an increase of the severity of the reaction conditions. During the pre-treatment of wheat straw, pentoses are also co-produced from xylan and arabinan with xylose being the main monosaccharide present in all assays followed by arabinose revealing that pentose concentration enhances steadily with the conditions' severity. Under the conditions leading to the maximal XOS recovery (biomass loading of 75/7.5 and $Log\ R_0$ = 3.48) the maximal arabinose concentration was obtained while the highest concentration of xylose (4.03 g/L) was achieved at the biomass loading of 150/15 and under the severity conditions of $Log\ R_0$ = 3.44. The same trend was observed for monomers of glucose, acetic acid and for the degradation products. The sugar degradation products, HMF and furfural, were detected in low amounts in almost all reactions. The exceptions were reactions at the harshest conditions, for which an increase was more pronounced as the furfural concentration increased by 9 and 4.5 times in the case of the transition from 200 to 210 °C for 150/15 and 75/7.5 biomass loading, respectively.

The pH values of liquors from CO_2-assisted autohydrolysis pre-treatments are also presented in Table 3.2 The pH of liquors decreases from 4.38 to 3.93 and from 4.55 to 4.03 with the increase of temperature for biomass loadings of 150/15 and 75/7.5, respectively.

The analysis of the influence of the CO_2 amount shows that a larger amount of CO_2 obtained by the relative reduction of the biomass amount by half in the reactor leads to an increase of XOS recovered by 1/3 (at 210 °C) and is counterbalanced by a reduction of xylose and furfural concentrations by 17 % and 30 %, correspondingly.

Table 3.2. Composition of the liquors (g/L) from CO_2-free autohydrolysis of wheat straw[8] and composition (g/L) and yields of each product present in the liquors (g/ 100 g of initial polymer present in the feedstock) from the CO_2-assisted autohydrolysis of wheat straw with an initial CO_2 pressure equal to 60 bar.

Reaction	CO_2-free[a]	CO_2-assisted													
Biomass loading[b]	250/25	250/25				150/15						75/7.5			
T (°C)	210	210		180		200		210		180		200		210	
Log R_0	3.83	3.54		2.58		3.16		3.44		2.60		3.08		3.48	
pH	4.32	3.85		4.38		4.13		3.93		4.55		4.39		4.03	
Composition/Yields	g/L	g/L	g/100 g	g/L	g/100 g	g/L	g/100 g	g/L	g/100 g	g/L	g/100 g	g/L	g/100 g	g/L	g/100 g
XOS	9.5	10.0	55.1	5.5	29.6	11.4	62.6	11.8	51.2	10.6	57.7	12.9	70.3	15.7	70.6
GlcOS	0.5	4.3	11.8	3.5	9.6	3.4	11.2	3.2	8.8	5.2	12.6	5.1	12.6	4.1	9.8
AcO	0.2	0.7	-	1.5	-	1.2	-	1.1	-	1.8	-	1.3	-	1.2	-
Xylose	1.7	3.4	16.4	2.0	9.7	2.4	11.8	4.0	19.6	0.5	2.3	0.5	2.4	3.3	16.1
Arabinose	1.2	0.9	17.8	1.3	13.2	1.3	15.4	2.0	25.5	0.4	3.7	0.5	4.2	2.1	20.7
Glucose	1.0	1.2	2.8	1.1	2.8	1.2	3.0	1.8	4.4	0.4	0.9	0.4	1.0	2.0	5.0
Acetic Acid	2.1	2.4	-	0.6	-	1.0	-	3.0	-	1.1	-	1.6	-	2.7	-
HMF	0.0	0.1	0.49	0.0	0.0	0.1	0.3	0.2	0.7	0.0	0.1	0.0	0.1	0.1	0.5
Furfural	0.1	5.4	40.6	0.1	0.5	0.5	3.9	4.6	35.0	0.3	2.4	0.7	5.4	3.2	24.0

[a] Data taken from ref.[8] [b] g of water/g of wheat straw. XOS – xylooligosaccharides; GlcOS – gluco-oligosaccharides; AcO – acetyl groups linked to oligosaccharides; n.a. – not available.

3.3.3. Composition of the processed solids

The results of the composition of the processed solids along with the solid yield after CO_2-assisted autohydrolysis treatments for different biomass loadings are shown in Table 3.3.

Table 3.3. The solid phase composition (g/ 100g processed solids) and solid yield (g/ 100 g feedstock) obtained after the CO_2-assisted autohydrolysis of wheat straw for different biomass loading.

Reaction	CO_2-assisted						
Biomass loading[a]	250/25	150/15			75/7.5		
T (°C)	210	180	200	210	180	200	210
Log R_0	3.54	2.58	3.16	3.44	2.60	3.08	3.48
Solid Yield	56.3	77.8	60.5	55.9	70.5	62.9	54.6
Glucan	58.4	45.5	49.9	55.6	54.1	54.4	64.3
Xylan	8.9	15.6	6.7	5.0	9.2	6.0	2.2
Arabinan	0.0	0.5	0.0	0.0	0.1	0.0	0.0
Acetyl groups	1.4	2.6	0.5	0.0	2.7	1.2	0.0
Klason Lignin	28.3	20.0	23.9	28.9	21.0	21.5	26.7

[a] g of water/g of wheat straw.

The hydrolysis condition affects the solid yield recovery. The elevated temperature and thus log R_0 lead to the decrease of the solid yield. Depending on the biomass loading used the solid yield might decrease from approximately 70–78 % to around 55 %. On the other hand, the amount of xylan in the processed solids decreases as the severity of the conditions increases and a complete removal of arabinan from the processed solids is verified except for the reaction at 180 °C. For this reaction a noticeable amount of arabinan (0.53 g/L) was detected in the solid phase. Furthermore, harsher reaction conditions facilitate the complete dissolution of acetyl groups as they are absent in the solid phase. Moreover it can be observed that the applied treatment influences neither cellulose nor Klason lignin since their relative contents increase with the severity factor.

3.3.4. Composition of the recovered gas phase

In the gas phase recovered from the reaction mixture depressurisation procedure, it was sought to detect the presence of a significant amount of volatile compounds. Figure 3.2 depicts the influence of biomass loading and reaction temperature on the amount of furfural (the only volatile product found) recovered from the gas phase. The increase of the severity under the hydrolysis conditions led to an increase of furfural in the gas phase where under the most severe conditions its concentration reached 6 g/L. Furthermore, a lower amount of biomass loaded on the reactor counterbalanced by the larger amount of CO_2 present affects the furfural removal as well.

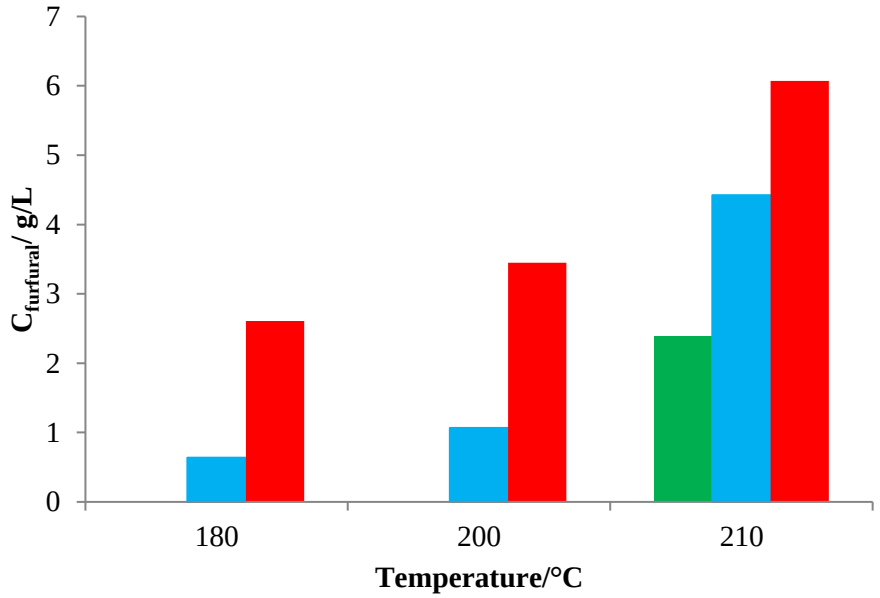

Figure 3.2. Furfural concentration (g/L) in the recovered gas phase from depressurisation for studied temperatures and biomass loadings. Green bar - 250g of water/25g of biomass, blue bar - 150g of water/15g of biomass, red bar - 75g of water/7.5 g of biomass.

3.4.Discussion

3.4.1. Effect of temperature

The CO_2-assisted autohydrolysis of wheat straw was carried out at three temperatures (180, 200 and 210 °C) selected according to the literature reports.[8] Two different biomass loadings were used to study the influence of temperature (Table 3.2). In the case of both the examined ratios (150/15 and 75/7.5), the percentage of components present in liquor depends on the temperature of the process. In fact, the increase of the reaction severity is responsible for the decrease in the density and in the dielectric constant of water allowing the water dissociation which enables the disruption of the recalcitrant structure and, hence, leads to easier hydrolysis of xylan producing XOS-rich liquors.[4]

The water dissociation caused by an increase of the temperature of the reaction can be proven by a decrease of medium pH for the same biomass loading reported elsewhere.[8] The results presented in this work are in good agreement with these results; for higher temperature, decreases in the solubility of the gas is observed; therefore after depressurisation of the reactor, the equilibrium in the system for reactions at high temperatures can be achieved faster and the pH of the liquor is higher as is confirmed in this study.

The amount of solubilised xylan increases with increasing temperature to reach 37 to 85 % of the initial amount at the maximal reaction temperature studied for a biomass loading of 150/15. The same trend was found for the biomass loading of 75/7.5 in which it was noticed that xylan dissolution depends strongly on temperature. The maximal yield of xylan solubilised as XOS was obtained under the severest conditions corresponding to 51 % and 71 % of the initial xylan amount for a biomass loading of 150/15 and 75/7.5,

respectively. The obtained data demonstrate that the CO_2-assisted autohydrolysis pre-treatment results in XOS-rich liquor, which with the increase of severity conditions is randomly hydrolysed leading to smaller oligosaccharides or even to xylose monomers. This fact can be confirmed by the increase of xylose concentration accompanied by the increase of temperature.

Similar to xylose and XOS, the increase of arabinose concentration with an increase in temperature is observed. The arabinose release was achieved at lower temperature compared to xylose as arabinose shows a higher thermal sensitivity.[31]

During the pre-treatment the acetyl groups attached to the xylan backbone are released in the liquor. Thus, the content of acetic acid in liquors increases with temperature reaching a 3-fold higher value at 210 °C than at 200 °C. Yet the acetic acid content was still relatively lower even under maximal XOS concentration conditions. In addition, it is important to highlight that after pre-treatment, some acetyl groups remain bonded to oligosaccharides in the liquor solution, which explains why acetic acid concentration is augmented after post-hydrolysis of the liquors. Furthermore, the achieved results permit us to draw a conclusion that increasing the temperature of the process leads to the formation of more sugar derived degradation products. For a biomass loading of 150/15 at 180 and 200 °C, insignificant concentrations of furfural and HMF were detected but at 210 °C, a furfural concentration equal to 4.60 g/L was observed. This is due to the fact that under the examined conditions, the increase of the severity factor results in the formation of degradation products, namely furfural that is a product derived from the arabinose present as a xylan sidechain. A similar increase of furfural concentration together with an increase of severity conditions correlates well with the results described for CO_2-free autohydrolysis for different biomasses such as eucalyptus wood, wheat straw and brewery's spent grain.[8,32,33] Nevertheless, under the conditions of maximal production of XOS, the furfural amounts were in the range of 0.33–3.19 g/L for 75/7.5 biomass loading.

It is worth to underline that under the highest furfural concentration conditions the concentration of HMF remains very low (0.20 g/L) although 19.3 % cellulose dissolution did occur. Furthermore, the concentration of glucose in the liquor is very low (1.76 g/L) reaching a yield of 4.37 % of glucan in feedstock. The severity of the pre-treatment seems to be insufficient to produce degradation products such as HMF in high concentration although enough to produce a relatively high concentration of gluco-oligosaccharides (3.20 g/L).

The chemical composition of the processed solids (Table 3.3) shows an enrichment of lignin and cellulose contents accompanied by an increase of temperature. It is caused by complete hydrolysis of the hemicellulose fraction which additionally led to a continuous decrease in solid residue yield to reach values below 60 % for the experiment with a final temperature of 210 °C. Furthermore, the increase of lignin content along with the increase of severity of pre-treatment conditions is related to condensation reactions between lignin and sugars and/or degradation products resulting from precipitation of the fibre inducing an

apparent increase in Klason lignin content.[34,35] Under the most severe conditions, a high recovery of lignin (91 %) from the initial amount of lignin present in wheat straw (Table 3.1) was also attained and only 0.22 g of lignin was dissolved and found in the liquor. This strongly indicates that the CO_2 presence does not lead to a significant dissolution of lignin. Regarding cellulose, 19.3 % of glucan present in raw feedstock was dissolved in the liquid fraction. However, its percentage on solid residues increased steadily with the severity of treatments, chiefly due to the resistance of this polymer to hydrothermal treatments.[32]

The amount of xylan present in the processed solid decreases with an increase of temperature, showing a recovery of 14.6 % at 210 °C. Therefore, to achieve complete removal more severe conditions are required although the results obtained in this work and available in the literature[8] show that higher temperature promotes extensive degradation (Table 3.3).

3.4.2. Effect of CO_2

3.4.2.1.Influence of CO_2 presence

Results obtained in this study provide evidence supporting the assumption that the presence of *in-situ* formed carbonic acid enhances the hydrolysis of hemicellulose fractions. A previous literature result demonstrates that with pure xylan, carbonic acid significantly increases hydrolysis activity compared to the CO_2-free autohydrolysis process.[25] A similar conclusion can be drawn from the results presented in this work as they illustrate that addition of CO_2 leads to an increase of XOS concentration when compared to the CO_2-free autohydrolysis under the same severity conditions reported elsewhere.[8] It is especially evident for the same severity factor (log R_0 = 3.53) and biomass loading (250/25 g). An increase of 65 % and 100 % of the XOS and xylose concentrations, respectively, can be observed. On the other hand, a high conversion of xylan into XOS was achieved (70.6 %) with a joint contribution of XOS and xylose accounting for 86.7 % of the initial xylan.

The presence of GlcOS in the liquor was also found to be higher when CO_2 was used (6-fold higher) in the reaction. The maximal GlcOS production was achieved at 180 °C and 75/7.5 biomass loading corresponding to a yield of GlcOS close to 13 %. Zhao et al. achieved the same maximal yield of GlcOS produced from corn stalks and wheat straw at supercritical water hydrolysis (384 °C).[22] This fact means that the presence of CO_2 allowed obtaining approximately the same yield of GlcOS with a decrease by 204 °C allowing the use of subcritical conditions. Furthermore this also clearly indicates that the presence of CO_2 leads to the minor dissolution of cellulose, even under less severe conditions than without CO_2[8] but further degradation of hexoses to HMF was negligible as HMF was detected in minimal concentration (0.14 g/L).

On the other hand, the presence of CO_2 contributes to the formation of further degradation products from the hemicellulose fraction, *e.g.* furfural. This is caused by the easier degradation of pentoses to furfural while hexoses are less susceptible to the degradation to HMF.

Furthermore, CO_2 plays an important role in the pH of the hydrolysate. It was found that the pH varies from 3.85 to 4.55. The decrease of the hydrolysate's pH can be explained by the fact that carbonic acid is formed *in-situ*, especially that no additional acetic acid compared to the CO_2-free autohydrolysis reaction[8] was produced. Conversely, Walsum et al.[13] revealed that CO_2 addition leads to an increase of the final pH of the hydrolysate in comparison to the autohydrolysis without CO_2. This inconsistency between the results presented by Walsum et al. and those in this work comes from the difference in the reaction conditions. The work of Walsum shows that the CO_2/water ratio is equal to 0.04 while in this work the CO_2/ water ratio is at least 3-fold higher. Therefore, a relatively higher amount of CO_2 leads to a considerably lower pH created in the course of the reaction; thus after the depressurisation, CO_2 dissolved in the liquid phase acts as an acidifier of the medium.

Comparison of these results with those reported in the literature[8] illustrates that the amount of XOS depends on the CO_2 presence, and the same concentration of XOS can be achieved under less severe conditions. To produce 10 g/L of XOS a 5 °C higher temperature is needed that also corresponds to a 11 % higher log R_0.

The effect of CO_2 is also observed in the composition of the processed solids. The main differences between treatments with and without CO_2[8]are perceptible in enrichment of the glucan and the Klason lignin content in the case of CO_2-assisted reactions, as well as in complete removal of arabinan. In addition, lower xylan content is observed in the wheat straw CO_2 pre-treatment. It indicates that CO_2 enhances the dissolution of hemicellulose (xylan, arabinan and acetyl groups) and retains cellulose and lignin in the solid phase.

The results of CO_2-assisted hydrothermal hydrolysis showed that the lignocellulosic materials can be partially solubilised and hydrolysed at temperatures significantly below the supercritical point.

3.4.2.2.Influence of CO_2 concentration

One of the aims of the CO_2-assisted autohydrolysis pre-treatments was to examine the influence of CO_2 on the XOS formed. The CO_2 concentration was calculated using the Peng–Robinson equation of state (PR-EOS) with the initial temperature of 20 °C and a pressure of 60 bar. The CO_2 density was calculated using the following relation:

$$P = \frac{RT\rho}{(1-b\rho)} - \frac{a\rho^2}{1+2b\rho-b^2\rho^2},$$

where $a \equiv a_c\alpha$;

$$a_c \equiv 0.45723553\frac{R^2{T_c}^2}{P_c};$$

$$\alpha \equiv \left[1+\kappa\left(1-\sqrt{T_r}\right)\right]^2;$$

$$\kappa \equiv 0.37464 + 1.54226\omega - 0.26993\omega^2.$$

The constants used are: T_c (CO_2) = 304.2K; P_c (CO_2) = 73.8 bar; ω (acentric factor) = 0.228;[36] R (gas constant) = $8.314 \cdot 10^{-2}$ L·bar/ K/mol.

The increase of CO_2 concentration makes liquors richer in hemicelluloses-derived products. Figure 3.3 shows that at 210 °C, an increase of 17 % and 57 % of the XOS concentration is attained with the reduction of water/wheat straw loading from 250/25 to 150/15 and to 75 g of H_2O/7.5 g of wheat straw, respectively.

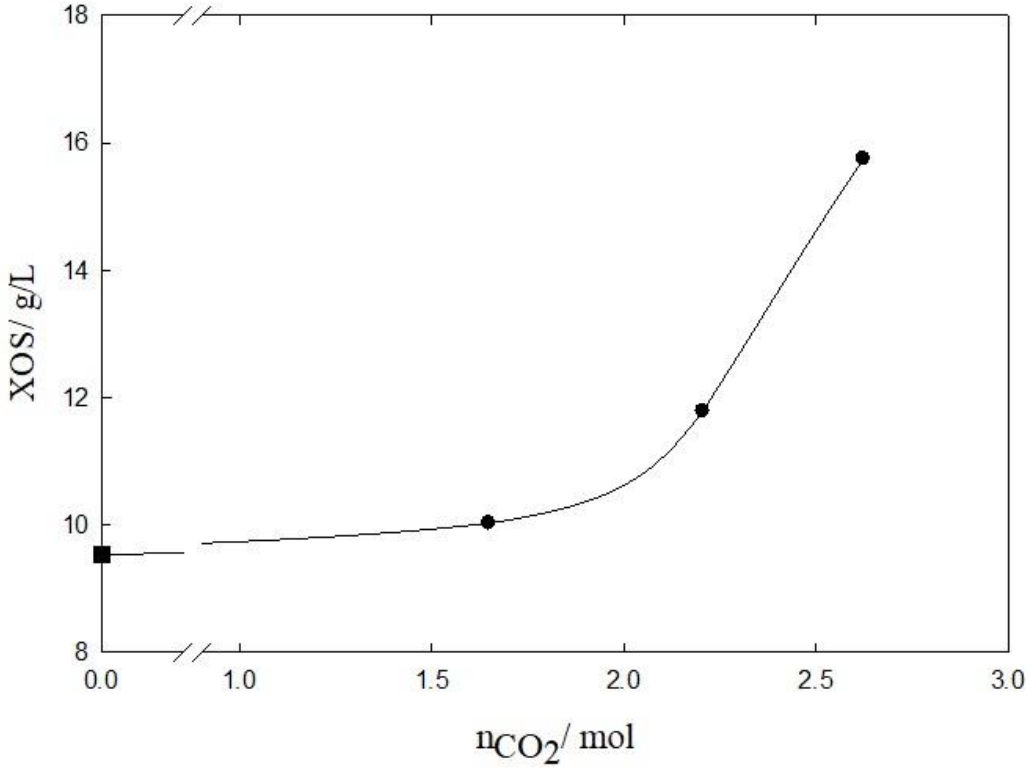

Figure 3.3. The XOS concentration (g/L) as a function of CO_2 number of moles at 210 ^{0}C. The data for the CO_2-free reaction (■) taken from literature.[8]

A biomass loading reduction by half (from 150/15 to 75/7.5) led to an increase of the number of moles of CO_2 by more than 20 % (Table 3.4). Therefore, as is expected the XOS concentration increases with an increase of the CO_2 concentration in the system; however, the pH of the solution became less acidic.

Table 3.4. The CO_2 density determined using the PR-EOS, as well as number of CO_2 moles present in the reactor at the initial reaction conditions.

Biomass loading[a]	250/25	150/15	75/7.5
ρ(CO_2)/mol·dm^{-3}		5.071	
"free volume" [b]/mL	325.0	435.0	517.5
n(CO_2)/mol	1.65	2.21	2.62

[a] g of H_2O/g of wheat straw; [b] "Free volume" was determined by the difference between the reactor volume (600 mL) and the volume occupied by the biomass loaded.

This controversial result can be explained by the fact that after the CO_2 decompression, a prolonged time is needed to achieve the equilibrium in the system. In other words, the pH measurement is done immediately after the experiment is performed under non-equilibrium conditions. Furthermore, the time needed to achieve the equilibrium is strongly dependent on the amount of water present in the system due to the diffusion limitation of CO_2 in the liquid phase. The complementary research on the pH of solutions 20 days after reactions was shown to be very similar to those reported elsewhere.[8] Solutions obtained from the reaction produced at 180 and 210 °C for 75/7.5 biomass loading after 20 days get similar pH, that is, 5.79 and 5.92, respectively. The observed increase of the solutions' pH proves again the fact that CO_2 is released slowly from water and even more the obtained values are very similar to that reported by Carvalheiro et al. for which at the same severity factor ($\log R_0 = 2.60$) the pH of the solution was 5.61.[8]

3.4.3. Volatile products

The volatile compound formed from the hemicellulose fraction was found to be in the gas phase. The obtained data depicted in Figure 3.2 show that the biomass loading and reaction temperature play an important role in the amount of furfural recovered. The increase in furfural formation can be considered to be dependent upon the process temperature and the CO_2 present. The temperature effect on furfural formation has already been discussed in this work. Another important aspect influencing the furfural volatility is the presence of acetic acid. To examine the acid–base interaction between furfural and acetic acid, the effect of different contents of acetic acid on the distribution behaviour of furfural and of the solvent properties of the weak acid on carbon dioxide has to be taken into account. The literature results show that up to a concentration of 5 wt %, acetic acid has modifier properties and enhances furfural extraction.[37] Another interesting aspect is that, besides the acetic acid present in the liquor, acetic acid was not detected in the gas phase entrapped after the reaction. The estimation of the VLE data for the system of CO_2 + water + acetic acid provides information on the negligible solubility of acetic acid under the reaction conditions.[38] Additionally, in the presence of CO_2 there is an equilibrium between CO_2, H_2O and acetic acid; thus acetic acid is dissolved in water not only in molecular but also in ionic form[39] inhibiting its volatility.

3.5.Conclusions

The CO_2-assisted autohydrolysis treatment of wheat straw was investigated, in order to selectively dissolve the hemicellulose fraction. The autohydrolysis with CO_2 allowed producing a liquid fraction rich in hemicellulose (mainly in oligomer form) and a solid containing mainly glucan together with lignin. These results prove the high selectivity of the pre-treatment towards hemicellulose fraction. The *in-situ* formation of carbonic acid resulted in an increase of both xylose monomers and an increase of XOS concentration in comparison to the CO_2-free pre-treatment of the wheat straw under analogous conditions (temperature and LSR). The effect of temperature on pre-treatments with CO_2 addition was also examined. It was noticed that higher temperature (more severe conditions) led to an increase of xylose and XOS concentrations.

Furthermore, a decrease of the final pH of the hydrolysate was observed. Therefore, the acidic character originating from the formation of carbonic acid enables the disruption of the chemical bonds between hemicellulose and lignocellulose. Thus, it can be concluded that carbonic acid contributes to autohydrolysis. Nevertheless, further studies are required in order to determine the optimal conditions under which the consensus between temperature/initial pressure and hemicellulose dissolution is attained without extensive formation of degradation products.

The CO_2-assisted autohydrolysis towards XOS formed at elevated amounts under much less severe conditions compared to the CO_2-free process is proposed. Therefore, the valorisation of agriculture residues towards high value added products is more economically and environmentally favourable, especially when green compounds such as CO_2 and water are used in the proposed processes. The major products of CO_2-assisted autohydrolysis are XOS that can be later obtained in a pure form after the membrane separation process, for direct end-uses as prebiotic ingredients, or, alternatively, can be subjected to a post-hydrolysis followed by biofuel production through C_5 fermentation.[8]

3.6.References

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Chapter IV

Selective hydrolysis of wheat straw hemicellulose using high-pressure CO_2 as catalyst

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4.1.Introduction

Worldwide energy demands coupled with a reduction of readily and economically available fossil feedstock and their environmental impacts have resulted in an extensive need for novel and sustainable sources of energy. Lignocellulosic biomass is the unique economic and environmentally acceptable alternative since it is abundant, renewable and low-cost, and does not compete with food and feed applications.[1,2] Nowadays, lignocellulosic biomass is one of the most important energy sources, having an estimated annual production of 10–50 billion metric tons worldwide.[3] One great example of the importance of lignocellulosic biomass is wheat straw, which is produced throughout the world as a residue of wheat cultivation. Wheat straw has drawn special attention due to its many interesting features that facilitate its valorisation.[4] For instances, it is produced in high amounts and it does not present an excessive commercial value.[5] Presently, it is employed in low added-value applications such as animal-feed and bedding,[6] mulch[7] and pulp production.[8] Furthermore, it is considered the agro-industrial residue that represents the uppermost potential for the production of second generation of bioethanol in Europe since its annual production is around 170 million tons per year.[9,10]

Lignocellulosic biomass has a very heterogeneous composition as it is generally composed of three main fractions: cellulose, hemicelluloses and lignin.[11] Cellulose and hemicelluloses are constituted by polymers of hexosans and pentosans representing 35–50 % and 20–40 % of biomass, respectively. Lignin is a complex polymer matrix of aromatic alcohols constituting between 10 and 25 % of the weight of entire biomass. The aforementioned complex composition and recalcitrant structure of lignocellulosic biomass creates a great challenge for its valorisation in the biorefinery framework. In an effort to obtain all benefits of each biomass component, specific technologies are needed to deconstruct them and to make biomass available for further conversion to value-added products.[12] Various physical, chemical, physico-chemical and biological pretreatment technologies have demonstrated to be efficient in deconstruction of recalcitrant structure of biomass increasing its susceptibility to enzymatic-based processes.[13] On the other hand, most of these pretreatments are characterized by low selectivity influencing negatively the production of diverse value commodities at competitive costs. Thus, beyond the need to find alternative sources of energy, the development of novel and more environmentally benign technologies for lignocellulosic biomass processing is still strongly required.

Recently, green technologies such as high-pressure CO_2–H_2O approach have been used in the valorisation of lignocellulosic and starch-based biomass to produce a wide-range of chemicals and others value-added products.[14-20] Recently, Morais et al. published a review where the applicability and effectiveness of high-pressure CO_2 and CO_2–H_2O technology for biomass pretreatment and its potential as alternative to conventional methods such as acid-catalysed and water-only reactions were demonstrated.[19] The presence of CO_2 in hydrothermal processes allows to the *in situ* formation of acidic environment ($CO_2 + H_2O \leftrightarrow (H_2CO_3)$, $2H_2CO_3 \leftrightarrow H_3O^+ + HCO_3^-$, $HCO_3^- \leftrightarrow H_3O^+ + CO_3^{2-}$), which promotes acid-catalysed hydrolysis

of biomass-derived hemicellulose[21] and simultaneously decreases cellulose crystallinity, [22] without the typical disadvantages of acid-catalysed reactions. In this respect, van Walsum et al. observed that the addition of CO_2 to water-only reactions allowed to hydrolyse pure xylan to produce xylose oligomers at lower temperatures and at shorter holding times in comparison to those obtained with autohydrolysis (water-only) technology.[23] Miyazawa and Funazukuri explored the effect of compressed CO_2 in the hydrolysis of carbohydrates to monosaccharides under hydrothermal conditions.[24] In water-only process, the final xylose yield was less than 5 % while in CO_2-assisted process a great improvement in the yield was achieved with lower production of degradation products in comparison to acid-catalysed processes.

In this work, high-pressure CO_2–H_2O technology was selected for the pretreatment and hydrolysis of wheat straw. Previous results demonstrated the potential of this technology in hydrolysis of hemicellulose fraction into both oligosaccharides and monosaccharides[25-27] concurrently with reduction of crystallinity of the processed materials. The kinetics of the wheat straw hemicellulose hydrolysis using high-pressure CO_2–H_2O is also reported in literature.[27] The objective of this work was to evaluate the effect of holding time and initial CO_2 pressures on the conversion of hemicellulose present in wheat straw to C_5 sugars (either in oligomeric or in monomeric form) and its simultaneous effect on other constituents of biomass such as cellulose and lignin.

4.2.Experimental section

4.2.1. Raw material and chemicals

Wheat straw harvested in 2009 in Elvas, Portugal, was used as feedstock and was kindly supplied by Estação Nacional de Melhoramento de Plantas (Elvas, Portugal). The raw material was ground using a knife mill (IKA® WERKE, MF 10 basic, Germany) to a particle size smaller than 1.5 mm and stored at room temperature. The moisture level of wheat straw was determined upon drying at 105 °C for at least 18 h and was 8 % w/w. CO_2 used in high-pressure experiments was purchased from Air Liquide, AlphaGaz™ gamma, Paris, France with purity higher than (99.9 % w/w). Distilled water (18.2 $M\Omega \cdot cm^{-1}$) was produced by Purelab Classic Elga system and ethanol (96 % v/v), used to recover the gas phase during CO_2 depressurization, was acquired from Carlo Erba Group, Arese, Italy. For infrared analysis, potassium bromide with >99.5 % purity was purchased from Sigma-Aldrich Co. (St. Louis, MO). For compositional characterization of materials, aqueous solution of 72 % w/w H_2SO_4 prepared from 96 % w/w H_2SO_4 supplied by Panreac Química, Barcelona, Spain was used.The chemical composition of wheat straw was presented elsewhere[25] and is as follows (w/w): 38.5 ± 0.1 cellulose (as glucan), 19.1 ± 0.1 xylan, 3.0 ± 0.1 arabinan, 2.7 ± 0.2 acetyl groups, 17.7 ± 0.1 Klason lignin, 4.7 ± 0.1 protein, 10.7 ± 0.1 ash.

4.2.2. High-pressure CO_2–H_2O procedure

The high-pressure CO_2–H_2O treatment of wheat straw was carried out in a 160 mL stainless steel high-pressure Parr 4655 reactor (Parr Instruments Company, Moline, Illinois, USA) with Parr 4842 unit to monitor the reaction parameters such as temperature, pressure and agitation. The treatments were performed

at isothermal conditions (180 °C) and fixed loading of 75 g of water and 7.5 g dry wheat straw with various holding times (from 0 min to 45 min) and CO_2 pressures, namely: 0 (water-only reaction – no CO_2 present), 20, 35 and 50 bar. Aiming to minimize the CO_2 density variations caused by the initial temperature changes, the reactor was pressurized with CO_2 at an initial temperature of −9 °C resulting in the reaction starting temperature of 17 °C. Next, the reaction mixture was heated up and stirred to the moment when the required temperature was achieved (180 °C). The reaction was continued for determined period of time (holding time) with continuous stirring and after that, the high-pressure reactor was rapidly cooled down using ice bath to quench the hemicellulose hydrolysis reaction. When temperature of the reaction mixture was 20 °C, the reactor was slowly depressurised and the gas phase was collected to a flask containing a known amount (5 g) of ethanol immersed in ice bath (0 °C). The depressurisation was performed at controlled temperature to minimize loss of volatile compounds in the gas phase. This procedure allowed to capture all potentially volatile compounds in ethanol for further qualitative and quantitative analyses. Resulting liquid (liquor) and solid (processed materials) phases were separated by vacuum filtration. The qualitative and quantitative analyses of all fractions were performed using the procedures presented below.

4.2.3. Severity factor of high-pressure experiments

The severity factor was used with the aim to compare the data obtained at different reaction conditions. For water-only reaction, severity factor (R_0)[28] is described by the following equation: $R_0 = \int_0^t e^{\left(\frac{T(t)-100}{14.75}\right)} dt$, where t is time expressed in minutes, T abbreviates temperature expressed in °C and 14.75 is an empirical parameter related with temperature and activation energy. A combined severity factor (CS_pCO_2)[23] was applied to investigate the effect of high-pressure CO_2–H_2O technology on wheat straw pretreatment. For this purpose, $CS_pCO_2 = \log(R_0) - \text{pH}$ equation was used. The direct pH measurements were technically impossible due to elevated temperature and pressure used in this process. Thus pH was estimated using to the following expression: $\text{pH} = 8.00 \times 10^{-6} \times T^2 + 0.00209 \times \ln(pCO_2) + 3.92$, where T is the temperature in °C and pCO_2 is the partial pressure of CO_2 expressed in atmospheres.[23] In order to study the effect of CO_2 concentration on the severity of reaction (R_0), the CO_2 density was calculated according to Peng–Robinson equation of state[29] using both initial temperature and CO_2 pressure employed in each experiment. For the same calculations, the Henry's constant (H) was determined according to the literature[23] using the empirical equation $H(T) = -0.017037T^2 + 6.1553T + 78.227$. The CO_2 solubility in water was taken from literature[30] and modelled using PE software[31] for required temperature.

4.2.4. Chemical analysis

4.2.4.1.Characterisation of liquor and post-hydrolysate liquors

The liquid phases (liquors) produced in the high-pressure CO_2-H_2O treatments were analysed ccording to a method presented elsewhere[25]. For the determination of total sugars, either in oligomeric or in monomeric forms, an acid hydrolysis procedure was applied as described in literature[32].

4.2.4.2.Characterisation of processed solids

The processed solids were characterized according to the method described elsewhere[25]. Glucan, xylan, arabinan and acetyl groups contents were determined using quantitative acid hydrolysis with 72 % w/w H_2SO_4 according to the standard procedure published elsewhere[33]. Klason lignin was determined gravimetrically after correction for the acid insoluble ash. The ash content was established using NREL/TP-510- 42622 protocol.[34]

4.2.4.3.Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of produced samples were recorded using a spectrometer Spectrum BX, Perkin Elmer, Inc. (San Jose, CA, USA). This instrument was equipped with a DTGS detector and a KBr beam splitter. The used operating system was the Spectrum software (Version 5.3.1, Perkin Elmer, Inc., San Jose, CA, USA). FTIR spectra were recorded in the 4000–400 cm^{-1} region, with a total of 64 scans and a resolution of 4 cm^{-1} with strong apodisation. For each analysis, the spectrum of the air background was subtracted. The areas of absorption bands at 1437 and 898 cm^{-1} were analysed to calculate the cellulose crystallinity index according to the following equation: $\mathrm{LOI} = \frac{A_{1437}}{A_{898}}$,[35] where LOI is lateral order index and A is the absorbance value of the corresponding band.

4.2.4.4.Error analysis

Standard uncertainty (u) was determined for all the obtained results. Each weighing was made considering a u(m)=0.1 mg. All pretreatments were made with a u (T) =1 ºC and a u (p) =1 bar. An arbitrary error of 10 % of measured value was defined for all the FTIR measurements and HPLC analyses.

4.3.Results

4.3.1. Chemical composition of liquors

High pressure CO_2–H_2O and water-only reactions resulted in liquors containing products from hemicellulose hydrolysis such as XOS and arabino-oligosaccharide (AOS) or their respective monosaccharides (xylose and arabinose), aliphatic acids (acetic and formic acid) and trace amounts of furfural (the main product of pentose degradation). Additionally, products of cellulose hydrolysis such as glucose, mainly as gluco-oligosaccharide (GlcOS), and 5-hydroxymethylfurfural (5-HMF) (glucose degradation product) were found as well. The respective yield of each product is presented in Tables 4.1 and 4.2.

Table 4.1. Yield of each product present in liquors obtained in high-pressure CO_2-H_2O processes performed at 50 and 35 bar of initial CO_2 pressure with respective severity factor and both estimated and measured pH values.

t (min)	0	4	6	12	18	20	25	30	35	45	0	4	8	12	16	20	25	30	40
$p_{initial}$ (bar)	50										35								
CS_{PCO2}[a]	-1.16	-0.64	-0.49	-0.25	-0.09	-0.05	0.07	0.14	0.19	0.30	-1.25	-0.70	-0.45	-0.30	-0.19	-0.10	0.00	0.08	0.19
Estimated pH	3.72										3.78								
Final pH	4.46	4.38	4.37	4.11	3.94	3.99	3.92	3.62	3.73	3.64	4.5	4.33	4.23	4.22	3.92	3.9	3.65	3.66	3.58
Yield (g per 100 g of initial amount present in the raw material)																			
XOS	38.9	55.3	60.1	79.6	73.7	66.1	64.2	40.7	27.5	18.6	36.8	60.0	67.9	73.0	72.4	57.7	50.4	33.5	31.9
AOS	53.0	62.3	54.6	39.9	39.3	15.3	29.7	8.0	9.7	7.3	49.1	57.0	47.7	35.5	44.4	29.4	19.9	10.1	15.3
GclOS	11.5	9.5	9.2	10.4	11.3	9.5	9.9	9.3	6.7	7.5	11.6	9.9	11.3	12.3	12.0	11.0	10.7	7.2	8.7
Xylose	6.1	7.1	6.4	9.0	12.6	12.0	13.9	23.9	21.4	26.9	5.8	6.5	7.9	9.8	9.9	14.5	15.6	21.1	21.8
Arabinose	30.2	32.5	27.4	34.8	34.0	35.9	27.6	41.0	18.6	20.4	20.6	28.9	34.8	41.4	29.8	34.5	23.0	23.2	20.3
Glucose	1.9	1.9	1.3	1.5	1.5	1.5	1.2	3.0	1.4	2.1	0.9	1.5	1.5	1.3	0.6	1.4	0.8	1.6	1.5
5-HMF	0.0	0.1	0.1	0.3	0.4	0.4	0.4	0.8	0.7	1.4	0.1	0.1	0.1	0.2	0.3	0.4	0.5	0.6	0.9
Furfural	0.0	0.0	0.7	3.3	5.3	5.1	7.7	16.4	17.8	25.2	0.0	0.7	0.7	1.6	4.7	6.4	6.4	9.7	16.4

[a] calculated according to literature.[23] XOS – xylo-oligossacharides; AOS – arabino-oligosaccharides; GclOS - gluco-oligosaccharides; 5-HMF – 5-hydroxymetilfurfural.

Table 4.2. Yield of each product present in liquors obtained in high-pressure CO_2-H_2O reactions performed at 20 bar of initial CO_2 pressure and water-only experiments with respective severity factor and both estimated and measured pH values.

t (min)	0	4	8	12	16	20	25	30	40	0	4	8	12	16	20	25	30	40
$p_{initial}$ (bar)	20									0								
CS_{PCO2}[a]/log R_0	-1.28	-0.70	-0.45	-0.30	-0.19	-0.09	0.00	0.07	0.20	2.74[b]	3.20[b]	3.39[b]	3.52[b]	3.63[b]	3.71[b]	3.80[b]	3.87[b]	3.99[b]
Estimated pH	3.78									-								
Final pH	4.35	4.04	3.95	3.85	3.8	3.6	3.6	3.6	3.53	4.48	4.4	4.15	4.02	4.07	3.93	3.78	3.8	3.73
Yield (g per 100 g of initial amount present in raw material)																		
XOS	34.4	55.0	65.6	67.5	63.6	60.3	52.1	40.7	22.7	28.9	46.3	60.5	70.8	73.1	72.5	70.5	66.6	50.3
AOS	49.8	56.6	41.0	36.4	35.2	26.8	23.2	20.7	14.9	53.2	55.3	56.9	55.7	47.6	40.7	30.6	28.7	20.1
GclOS	10.0	11.3	11.0	9.5	12.3	9.1	9.3	9.3	8.0	11.9	11.6	12.5	11.8	11.0	12.1	10.7	10.7	10.0
Xylose	5.7	6.4	7.9	10.9	16.2	15.7	18.0	20.5	25.1	5.8	6.0	6.3	6.2	8.0	10.9	11.6	13.9	19.9
Arabinose	21.6	30.8	39.7	32.4	29.7	28.3	25.1	20.7	18.9	18.0	28.9	32.0	34.1	32.0	33.7	31.2	31.7	27.4
Glucose	1.6	1.2	1.0	1.1	1.7	1.5	1.5	1.3	1.8	0.9	1.2	1.5	0.8	0.8	0.8	0.7	0.9	1.16
5-HMF	0.0	0.1	0.2	0.3	1.7	0.4	0.5	0.7	1.0	0.0	0.1	0.1	0.1	0.2	0.3	0.3	0.4	0.6
Furfural	0.1	0.6	1.3	4.1	4.4	8.8	7.7	11.8	23.0	0.0	0.2	0.7	1.1	2.7	4.3	5.9	8.8	14.0

[a] calculated according to literature[23], [b] log R_0 determined according to literature[28]. XOS – xylo-oligossacharides; AOS – arabino-oligosaccharides; GclOS - gluco-oligosaccharides; 5-HMF – 5-hydroxymetilfurfural

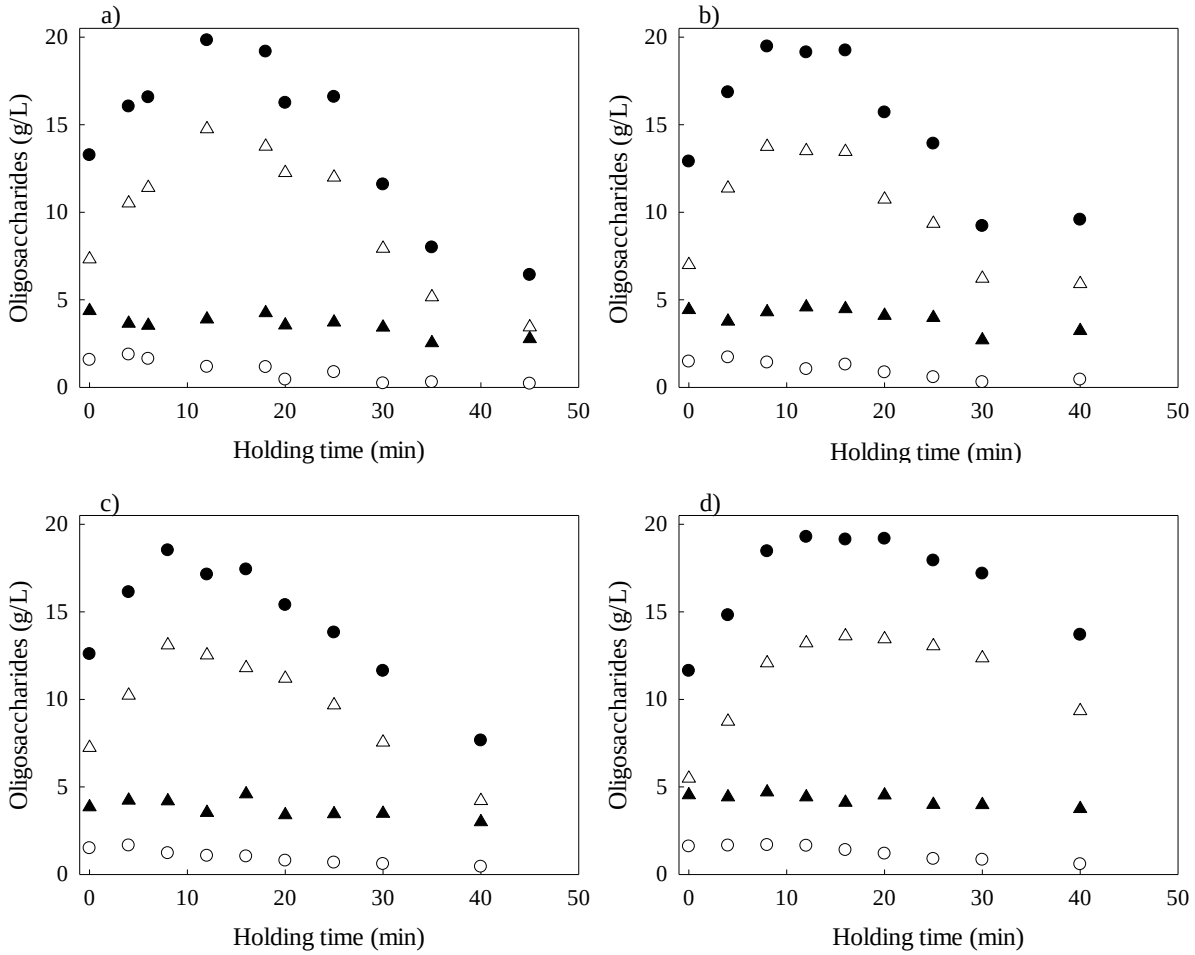

Figure 4.1 Composition of liquors in terms of oligosaccharides (OS) (● – total OS, ∆ – XOS, ▲ – GlcOS, ○ - AOS) obtained from high-pressure CO_2-H_2O experiment performed at a) 50 bar, b) 35 bar, c) and 20 bar of initial CO_2 pressure and d) water-only reaction as function of holding time.

The formation of all these compounds is highly dependent on the reaction conditions namely holding time and initial CO_2 pressure, as it is clearly depicted in Figures 4.1 and 4.2. Xylo-oligosaccharides were found to be the main products present in produced liquors. The processing of wheat straw using high-pressure CO_2–H_2O, performed with 50 bar of initial CO_2 pressure for 12 min of holding time ($CSpCO_2$ = -0.25), yielded a 79.6 % xylan conversion to XOS with corresponding concentration of XOS as high as 14.8 g/L.

As increase of reaction severity (namely holding time), a decline in XOS content was observed, achieving its minimum (3.6 g/L or 18.6 % of xylan conversion to XOS) for 45 min of reaction time ($CSpCO_2$ = 0.30). Under this condition, an extended xylan hydrolysis to xylose and furfural (26.9 % and 25.2 %, respectively) coupled with loss of 77 % of XOS yield were observed.

Additionally, interesting is that comparing the reactions with different initial CO_2 pressures, the XOS concentration was 18 % higher for reactions performed with 50 bar of initial pressure of CO_2 than this obtained at 20 bar of initial CO_2 pressure. On the other hand, higher CO_2 pressures favoured the quick decay of XOS yield along the reaction time than in the case of lower CO_2 pressures. Considering the effect of CO_2

presence, it can be stated that, the addition of CO_2 (initial pressure of 50 bar) to water-only reaction improved the XOS concentration by almost 10 % and at the same time the highest XOS concentration was observed at shorter holding reaction time (shift from 16 to 12 min) as presented in Tables 4.1 and 4.2. Xylose was the main monosaccharide present in liquors as depicted in Figure 4.2. Under the best condition for XOS production ($CSpCO_2$ = -0.25), the concentration of released xylose corresponded to 9 % of the initial xylan content. The concentration of xylose increased with the progress of reaction severity achieving a maximum concentration of 5.7 g/L (26.9 % xylan yield) at severest condition. Evaluating the influence of CO_2 presence, the xylose concentration increased 71 % with an initial CO_2 pressure of 50 bar than in water-only reactions for the same holding reaction time (30 min).

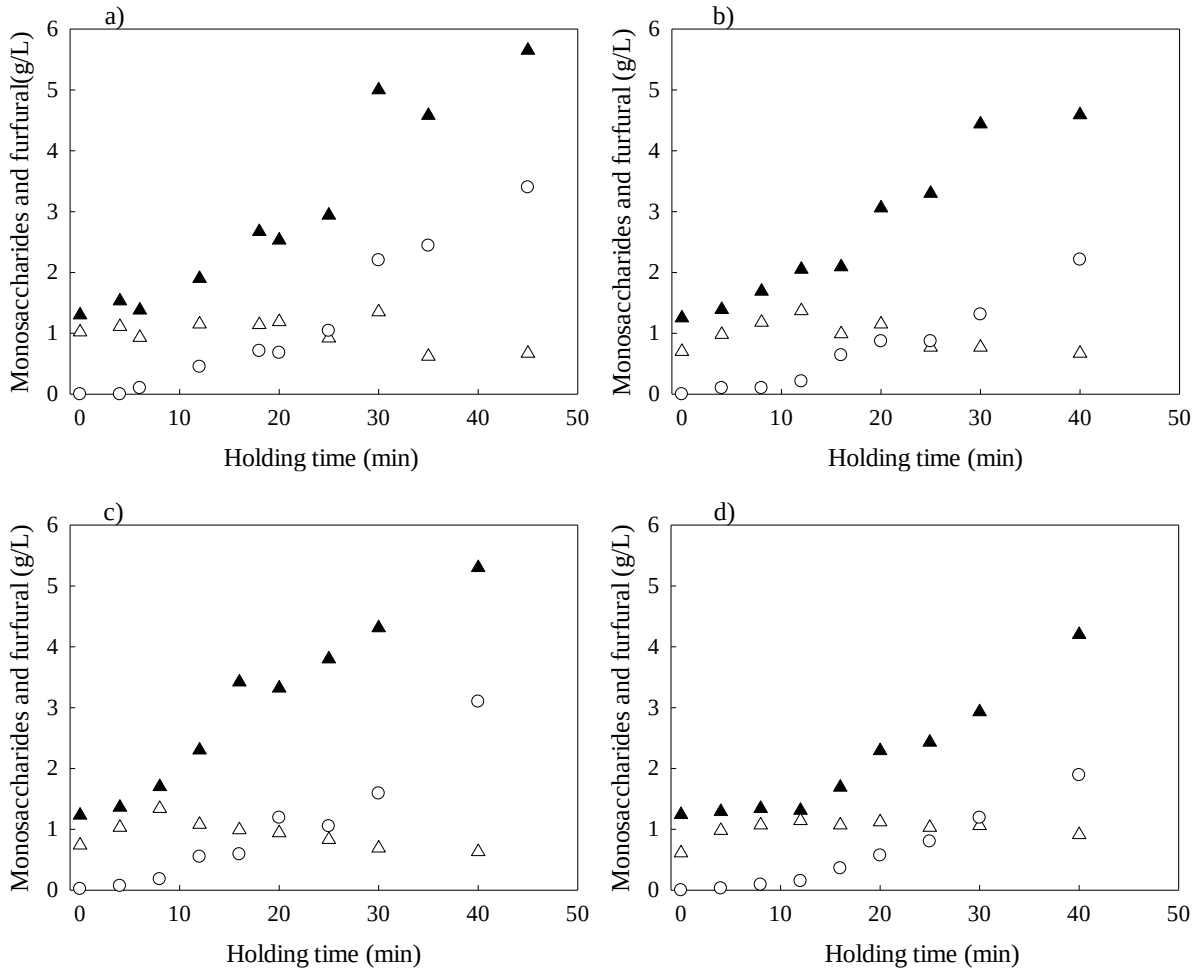

Figure 4.2. Composition of liquors in terms of (▲ – xylose, □ Δ– arabinose) and ○ – furfural obtained from high-pressure CO_2-H_2O experiment performed at a) 50 bar, b) 35 bar, c) and 20 bar of initial CO_2 pressure and d) water-only reaction as function of holding time.

Other hemicellulose-derived products such as arabino-oligosaccharides and arabinose exhibited similar profiles to those found for XOS and xylose. Under, the best condition for XOS production, the yield of released AOS and arabinose corresponded to 39.9 % and 34.8 % of initial arabinan content, respectively. Due to low content of arabinan in the raw material, the maximum concentration of AOS

was only 1.9 g/L and it was achieved at the shortest examined holding time (4 min at 50 bar of initial CO_2 pressure). For longer holding times, the concentrations of AOS and arabinose decreased rapidly and reached a minimum of 0.2 g/L and 0.7 g/L, respectively. The obtained liquors also contained free acetic acid and acetyl groups linked to oligosaccharides. As expected, the concentration of acetic acid increased along the reaction progress but demonstrated a tendency to stabilize (2.7 g/L) for prolonged reactions. The major C_5-sugar degradation product, furfural, was detected in almost all experiments. The formation of furfural is highly influenced by either initial CO_2 pressure or holding time. The increase of holding time from 12 min ($CSpCO_2$ = -0.25) to 45 min ($CSpCO_2$ = 0.30), increased furfural concentration almost 7.5-fold reaching even 25.2 % xylan conversion yield. Furthermore, an increase of 85 % of furfural concentration was observed in case of high-pressure CO_2–H_2O with 50 bar of initial CO_2 pressure for 30 min in comparison to water-only reaction at the same holding time. Among C_6-derived products, gluco-oligosaccharides and glucose were found in the liquors. The formation of glucose in either oligomeric or monomeric form may have its origin in hydrolysis of amorphous cellulose, which is highly prone to hydrolysis even at very mild conditions. Analysing the produced data it is clear that both GlcOS and glucose generally followed patterns of XOS and xylose. Even more, scrutinizing the effect of CO_2, it can be concluded that in high-pressure CO_2–H_2O reactions performed at 50 bar of initial CO_2 pressure for 30 min ($CSpCO_2$ = 0.14) and in water-only process, the obtained GlcOS concentrations were very similar (3.4 g/L and 4.0 g/L, respectively). On the other hand, the concentration of glucose was relatively different and in the case of high-pressure CO_2–H_2O was two times higher than in water-only reaction.

4.3.2. The composition of processed residues

The severity of reaction conditions (addition of CO_2, various initial CO_2 pressures and holding time) influenced either the liquor composition discussed above or the chemical composition of processed solids. The chemical composition of processed materials and respective solid recovery yields obtained from high-pressure CO_2–H_2O and water-only experiments under various experimental conditions are depicted in Tables 4.3 and 4.4. For all experiments, the lowest observed solid recovery yield was 60.4 %. The water-only reactions demonstrated lower biomass dissolution resulting in solid recovery yield of 66.9 % (for 30 min of reaction). This 11 % of difference is mainly caused by more extensive CO_2-assisted hydrolysis of hemicellulose, in particularly xylan, arabinan and acetyl groups. The presence of CO_2 led to an efficient decrease of hemicellulose content in the processed materials and consequently in lower solid recovery yield. Considering the holding time effect, it is clear that it played a great role on biomass recovery yield either for CO_2-assisted or for water-only reaction. As increase of the reaction time, the solid recovery yield decreased by 1/3 in comparison to the initial solid recovery yield found for 0 min holding time. The performed pretreatments (high-pressure CO_2–H_2O and water-only reaction) also resulted in noticeable changes in chemical composition of processed solids.

Table 4.3. The composition and recovery yields (g per 100 g of raw material) of processed solids obtained in high-pressure CO_2-H_2O experiments performed at initial CO_2 pressure of 50 and 35 bar.

t (min)	0	4	6	12	18	20	25	30	35	45	0	4	8	12	16	20	25	30	40
$p_{initial}$ (bar)	50										35								
CS_{PCO2}[a]	-1.16	-0.64	-0.49	-0.25	-0.09	-0.05	0.07	0.14	0.19	0.30	-1.25	-0.70	-0.45	-0.30	-0.19	-0.10	0.00	0.08	0.19
Solid yield	90.0	92.8	91.0	67.2	73.8	66.9	72.3	60.4	60.1	65.5	90.7	89.2	87.0	63.9	68.8	69.7	67.4	67.2	66.9
Composition																			
Glucan	36.8	41.3	41.5	33.9	37.6	33.0	36.4	31.0	32.3	31.6	37.5	41.7	43.1	31.4	35.1	34.4	35.0	35.3	32.2
Xylan	14.2	13.9	12.8	6.4	5.8	5.4	4.8	2.5	2.4	1.8	16.0	11.3	8.9	5.2	4.9	4.7	3.2	2.5	2.3
Arabinan	1.2	1.0	1.0	0.3	0.4	0.3	0.4	0.1	0.3	0.0	1.2	0.7	0.3	0.1	0.1	0.1	0.0	0.0	0.0
Acetyl groups	2.4	2.4	2.3	1.2	1.2	1.1	1.0	0.7	0.7	0.7	2.7	2.1	1.9	1.2	1.2	1.1	0.9	0.9	0.9
Klason lignin	17.7	19.1	20.2	17.6	20.3	18.5	20.3	19.4	19.1	22.8	18.5	20.7	21.7	17.3	19.6	20.8	20.9	21.6	22.1

[a] calculated according to literature.[23]

Table 4.4. The composition and recovery yields (g per 100 g of raw material) of processed solids obtained in high-pressure CO_2-H_2O reactions performed at initial CO_2 pressure of 20 bar and water-only reactions.

t (min)	0	4	8	12	16	20	25	30	40	0	4	8	12	16	20	25	30	40
$p_{initial}$ (bar)					20									0				
CS_{PCO2}[a]/log R_0	-1.28	-0.70	-0.45	-0.30	-0.19	-0.09	0.00	0.07	0.20	2.74[b]	3.20[b]	3.39[b]	3.52[b]	3.63[b]	3.71[b]	3.80[b]	3.87[b]	3.99[b]
Solid yield	92.9	70.9	72.5	67.0	64.6	68.7	67.7	65.8	67.6	92.8	83.7	75.4	74.4	71.4	65.5	67.0	66.9	69.0
Composition																		
Glucan	37.5	31.6	34.4	32.0	31.4	35.1	33.4	33.7	35.1	35.9	35.6	34.2	37.2	31.1	32.6	34.4	33.4	35.9
Xylan	15.5	9.1	8.0	5.3	4.6	4.2	3.2	2.6	2.5	17.8	13.3	10.6	9.3	7.3	6.4	5.2	5.0	4.0
Arabinan	1.3	0.4	0.2	0.1	0.1	0.0	0.0	0.0	0.0	1.6	0.7	0.4	0.2	0.2	0.2	0.1	0.0	0.0
Acetyl groups	2.6	1.6	1.4	1.1	1.0	1.0	0.8	0.7	0.7	2.5	1.8	1.3	1.0	0.9	0.7	0.5	0.5	0.4
Klason lignin	19.4	16.0	17.1	17.6	17.8	19.5	21.0	20.7	22.1	19.5	18.3	17.9	18.8	18.4	18.4	19.0	20.1	21.2

[a] calculated according to literature,[23] [b] log R_0 calculated according to literature[28].

For the most severe CO_2-assisted reaction ($CSpCO_2$ = 0.30), up to 90.2 % of hemicelluloses were removed. Despite the extensive hemicelluloses removal, an incomplete hydrolysis of xylan and minor amounts of acetyl groups present in processed solids were observed. For example, the content of xylan in processed solids gradually decreased with an increase of reaction severity reaching only 2.5 % for the severest condition ($CSpCO_2$= 0.30). For water-only process at similar holding reaction time, the xylan content was 2-fold higher. Similarly to the composition of the raw material, glucan is the major constituent of all processed solids, and for two the highest pressures examined its concentration decreased by less than 10 % along the reaction time. For 20 bar of initial CO_2 pressure and for water-only reaction, the glucan content was kept constant and varied within the experimental error. Another component of lignocellulosic biomass remaining in the processed materials is lignin. The lignin recovery was found to be between 16.0 and 22.8 %. The reactions performed at two the highest initial CO_2 pressures led to an increase of the lignin content in the processed materials to values above the lignin content in raw material. This fact could be explained by the formation of solid carbonaceous species (i.e. humins) due to lignin condensation reactions.[36-39]

4.4.Discussion

4.4.1. Production of oligosaccharides

The hemicellulose fraction is the most susceptible polymer to hydrothermal treatment due to lack of crystalline and resistant structure.[40] For instances, Liu et al. observed a total hydrolysis of hemicellulose (99 %) into its sugar constituents for 15 min at 220 ^{0}C in compressed water process.[41] Also Laakso and co-workers found a total sugar release of 66 % from arabinoxylan at optimal autohydrolysis conditions $logR_0$ = 3.50.[42] On the other hand, data presented herein indicates that presence of CO_2 promotes the hydrolysis of xylan to XOS. This beneficial effect occurs due to the *in-situ* formation of carbonic acid in the presence of water.[23,26,43] The dissociation of unstable carbonic acid increases the concentration of hydronium ion, which helps to lower the pH value of the reaction medium (slightly above 3), promoting dissolution and hydrolysis of biomass constituents.[25] Furthermore, the addition of CO_2 enhances the conventional hydrothermal reactions since it permits to use lower temperatures and shorter holding times. Similar effect of carbonic acid was found by Van Walsum who dissolved CO_2 in water to obtain a higher pentose yield in the liquid fraction in comparison to CO_2-free reactions.[23] Comparing the results obtained in this work to those achieved by Carvalheiro et al. it can be also stated that the presence of CO_2 guided to almost 50 % more production of XOS than a autohydrolysis process at maximal XOS concentration conditions (215 ºC at 0 min holding time).[5] For similar reaction conditions ($logR_0$ = 3.50), the XOS concentration obtained with a high-pressure CO_2-H_2O is even more pronounced because it is 142 % higher than that presented in literature.[5] Even at highest initial CO_2 pressure (50 bar) conditions, the solubility of CO_2 in water phase is very low (0.01 mole fraction of CO_2).[30] Nevertheless, this limited solubility is high enough to contribute to lower pH value of the medium promoting the hydrolysis of hemicellulose. In addition,

the obtained results clearly show that even lower pressure of CO_2 (*e.g.* 35 bar) is sufficient to play an important role in hemicellulose hydrolysis. For instance, at maximal XOS concentration ($CSpCO_2$ = -0.45), the solubility of CO_2 in aqueous phase is as low as (x_{CO_2}= 0.007). For the lowest initial CO_2 pressure conditions, XOS concentration remains lower because the solubility of CO_2 in water is null creating a system with three immiscible phases constituted by gaseous CO_2, aqueous liquid phase and solid biomass. This explains why the three phase system formed by 20 bar of initial CO_2 pressure allowed to obtain a pH of liquor and the concentration of XOS very similar to those obtained in the water-only reaction. This result also demonstrates the beneficial catalytic effect of CO_2, which can only be achieved when CO_2 is added at determined pressures. Contrary to hemicellulose, cellulose is a very resistant polymer to hydrolysis, since it is mainly composed of a crystalline structure with just some amorphous regions.[44,45] Hydrothermal technologies proved the ability to hydrolyse hydrogen-bond linked- structure of cellulose and its glycosidic bonds into glucose monomers. However, due to harsher conditions required for the cellulose hydrolysis, both GlcOS and glucose undergo quick conversion to degradation products such as 5-HMF. The conditions employed in this work are relatively mild to perform the hydrolysis of crystalline cellulose, thus it can be expected that the presence of GlcOS and glucose observed in all experiments was rather originated from the hydrolysis of amorphous cellulose than crystalline as it was also already reported in the literature.[5,26]

4.4.2. Formation of monosaccharides and furanic products

The high-pressure CO_2–H_2O processing of wheat straw allowed to produce C_5-sugars namely xylose and arabinose. The relation between production of xylose and furfural at various initial CO_2 pressures throughout the reaction time is depicted in Figure 4.2. The highest concentration of xylose and furfural (5.7 and 3.4 g/L, respectively) were found in liquors produced from highest CO_2 pressure condition, while for water-only reaction these values were 67 % and 80 % lower, correspondingly. In addition, the concentration of xylose increased steadily over reaction times showing that severer conditions are needed to promote the hydrolysis of XOS into monomers and later to furfural. These results are in agreement with those presented in literature.[23,46,47]As presented by van Walsum it was found that carbonic acid acts as catalyst in hydrolysis of pure xylan permitting to obtain oligosaccharides with lower depolymerisation degree in comparison to those obtained in water-only reaction.[23] Zhang and Wu investigated the influence of subcritical CO_2 in sugarcane pretreatment and found that the highest xylose yield obtained was 15.8 % (g per 100 g of feedstock), among which 45.2 % corresponded to XOS.[46] Gurgel et al. studied the addition of high-pressure CO_2 to water-only reaction in the production of *D*-xylose from sugarcane bagasse.[47] The maximum xylose concentration (115 ºC, 68 bar of initial CO_2 pressure for 60 min) was only 9.8 g/L. Thus, bearing in mind the profile of XOS and xylose as well as their C_6 homologs, it can be stated that the addition of CO_2 to water-only reaction promotes the hydrolysis of oligosaccharides to monomer analogs.

4.4.3. Formation of aliphatic acids and their influence on pH

One of important aspects examined in this work was the pH of the produced liquors. As mentioned above, due to the technical reasons the pH of liquors could only be measured after reaction ends. In the case of CO_2-assisted reactions pH was also estimated according to the equation presented elsewhere.[23] The estimated pH values were lower than the measured ones for shorter holding times as it is demonstrated in Tables 4.1 and 4.2. This can be explained by dissolution of CO_2 in water, which promotes the *in situ* carbonic acid formation responsible for pH lowering. The removal of CO_2 during the depressurisation led to the increase of pH which is reflected in the measured values. Hence, it can be concluded that the measured pH does not demonstrate the acidity of the medium during CO_2-assisted reactions. For this reason analysing the pH listed in Tables 4.1 and 4.2, the pH values measured after depressurization at room temperature vary between 4.5 and 3.6. Therefore, after depressurisation, the "acidification" effect of CO_2 dissolved in liquor is minimal, hence there must be another factor influencing the acidity of the medium especially so much noticeable for longer holding times. The answer lies in the composition of liquors and it is clear that along the reaction time the organic acids, such as acetic and formic, were formed which are responsible for pH decays (Figure 4.3). Various literature reports show that extensive hydrolysis of hemicellulosic acetyl groups after autohydrolysis experiments guided to lower pH.[5,48,49] van Walsum et al. discovered very similar behaviour by founding that the pH of liquors from corn stover and aspen wood treatment were quite different (3.68 and 4.95, respectively). This difference in final pH can be explained by the autocatalytic hydrolysis effect of acetyl groups of aspen wood in comparison to those of corn stover.[43] McWilliams et al. did not report any beneficial effect of CO_2 addition to water-only reaction on hydrolysis of aspen wood at 180–220 ºC since the formation of carbonic acid improved neither xylose nor furfural compounds yield.[50] Although this work does not show any benefits in the use of CO_2 it is important to understand that aspen wood contains highly acetylated hemicelluloses and these compounds are highly susceptible to autohydrolysis at temperatures above 170 ºC, thus no additional of CO_2 was required[51] as the formed acetic acid catalyses the hydrolysis of the hemicellulose.

4.4.4. Effect of high-pressure CO2–H2O on composition of processed solids

During high-pressure CO_2–H_2O treatments, the extension of hemicellulose hydrolysis is highly influenced by the process severity, while cellulose and lignin are retained in the processed solids. This data matches with the increasing sugar content in liquors up to a point that production of degradation products such as furfural and 5-HMF started to dominate. Tables 4.3 and 4.4 show the composition of the processed solid in function of pretreatment severity. This data is in a good agreement with literature because Morais et al.[26] found a xylan content in processed material of 5.2 % while in this work was found 5.8 %, for similar combined severity factor ($CSpCO_2$ = 0.19).

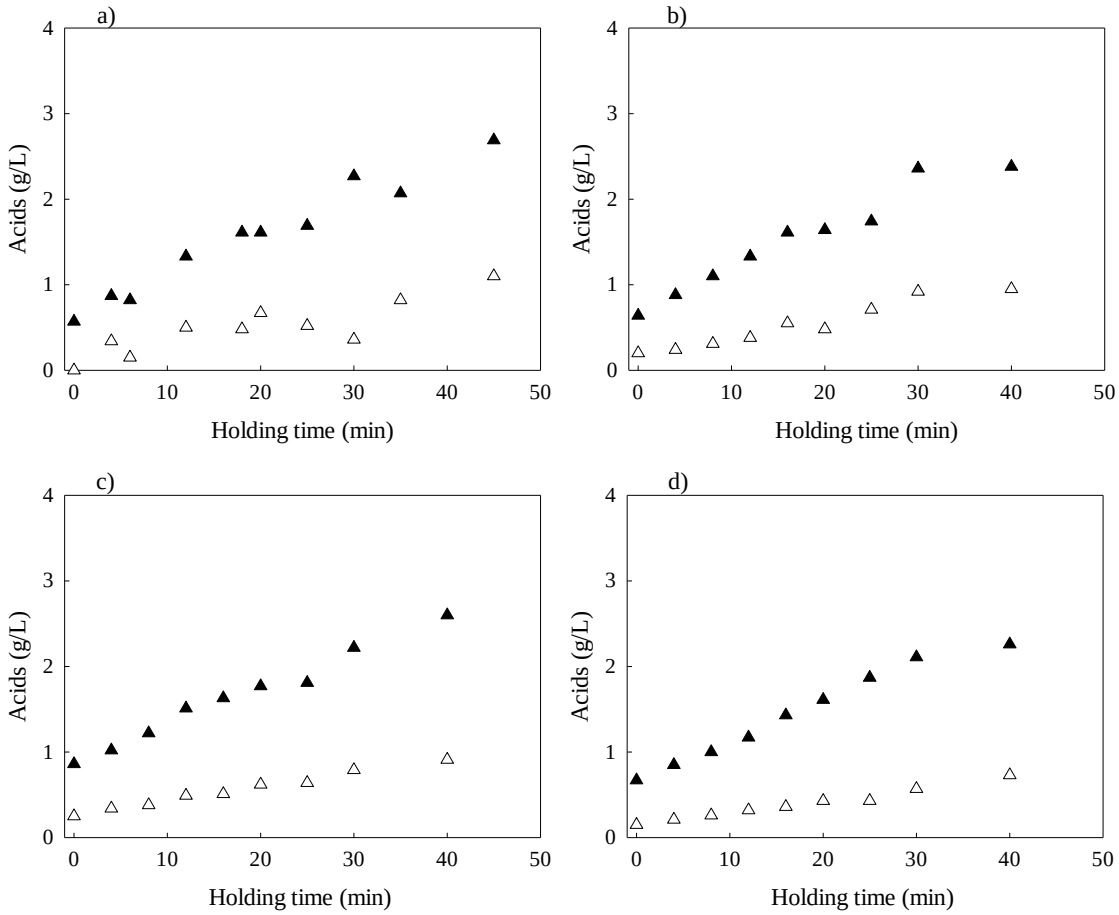

Figure 4.3. Composition of liquors in terms of aliphatic acids (▲ –acetic acid, ∆ – formic acid) obtained from high-pressure CO_2–H_2O experiments performed at (a) 50 bar, (b) 35 bar, (c) and 20 bar of initial CO_2 pressure and (d) water-only reaction as function of holding time.

It is known that arabinan is one of the easiest hydrolysable fractions of hemicellulose[5] and arabinan content in the processed solids decreased with the increase of the reaction severity. Similarly, the content of acetyl group in produced solids decreased accordingly to the increase of acetic acid concentration in the liquor reaching the content as low as 0.7 % at $CSpCO_2$ = 0.30 similarly to literature reports where comparable range of acetyl groups content was found in processed solids.[5,25,26] At the severest condition examined ($CSpCO_2$ = 0.30), the processed solids presented high cellulose and Klason lignin contents. Among all polysaccharides present in lignocellulosic biomass, cellulose is the least prone fraction for hydrolysis and this was also observed in liquor composition in which the concentration of GlcOS, glucose and 5-HMF were lower than products derived from hemicellulose. This cellulose characteristic is even more visible considering the relative amount of glucan in processed solids that was strongly enriched in comparison to the untreated biomass. Nevertheless, there are other parameters, such as both biomass and cellulose crystallinity that constitute a hurdle to achieve high enzymatic hydrolysis yields. To evaluate the effect of high-pressure CO_2–H_2O on cellulose crystallinity, native biomass and two processed solids samples (produced from high-pressure

CO_2–H_2O performed at $CSpCO_2$ = 0.14 and water-only reaction carried out with $logR_0$ = 3.87), underwent the FTIR analysis. Two absorption bands were selected for analysis of cellulose-rich fraction crystallinity. A band at 1437 cm^{-1} is characteristic to the scissoring vibration assigned to CH_2 in the crystalline cellulose and the band at 898 cm^{-1}, assigned to C–O–C bonds of β-1,4-glycosidic bonds is typical for amorphous fractions i.e. amorphous cellulose and hemicellulose.[52] To compare the cellulose-rich fraction crystallinities, the LOI index, which is the ratio between absorption bands at 1437 cm^{-1} and 898 cm^{-1}, was calculated.[35] The LOI results for native wheat straw and processed solids by water-only reaction and high-pressure CO_2–H_2O are given in Table 4.5.

Table 4.5. LOI index for native, water-only reaction and high-pressure CO_2-H_2O processed wheat straw samples.

	A_{1437}	A_{898}	LOI (A_{1437}/ A_{898})
Native wheat straw	0.239	0.104	2.30
Water-only reaction (log R_0 = 3.87)	0.217	0.061	3.56
High-pressure CO_2-H_2O ($CSpCO_2$ = 0.14)	0.183	0.044	4.16

The analysis of produced data shows that LOI for the untreated biomass has lower value (LOI = 2.30) than processed biomasses from water-only reaction (3.56) and high-pressure CO_2–H_2O processed solid (4.16). However, close inspection of obtained data shows that water-only process removed amorphous fractions (either cellulose or hemicellulose) as the absorption of the band at 898 cm^{-1} was reduced by 41 % in comparison to untreated wheat straw while at the same time crystalline cellulose was affected insignificantly. In the case of high-pressure CO_2–H_2O, both "amorphous" and "crystalline" bands were affected because both were reduced significantly. Although the LOI data does not reflect directly the reduction of crystallinity but the understanding of the vibrations resulting in creation of both bands allows to state that the water-only reaction in comparison to high-pressure CO_2–H_2O is less severe, reduces the crystallinity less and is more selective for hemicellulose hydrolysis.

Klason lignin is the second major component of processed solids and its content increased with the reaction progress and with the increase of exerted CO_2 pressure. Analogously to cellulose, the Klason lignin content in processed solids increased due to enhancement of a xylan removal. The Klason lignin content in processed materials is typical either for autohydrolysis process or for high pressure CO_2-assisted autohydrolysis as reported in literature.[5,25,26,49]

4.5.Conclusions

This work shows the potential of high-pressure CO_2–H_2O approach as effective and more sustainable pretreatment method of lignocellulosic biomass. The opted methodology was highly selective towards hydrolysis of wheat straw-derived hemicellulose resulting in liquors rich in XOS. A high recovery of XOS with minimal co-production of degradation products was obtained under high initial pressures of CO_2 and short holding times. Besides the production of value-added XOS, the obtained processed materials rich in cellulose and can be used in saccharification and lignin conversion processes towards other value-added commodities.

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Chapter V

Integrated conversion of agro-industrial residue with high-pressure CO_2 within the biorefinery concept

This chapter is based on the published manuscript: Ana Rita C. Morais, Ana C. Mata and Rafal Bogel-Lukasik, *Green Chem*. 2014, 16, 4312-4322.

5.1.Introduction

Gre**e**n reagents, such as CO_2 and water, used in the valorisation of lignocellulosic residues towards valuable products make the pre-treatment processes more environmentally acceptable. Recently, the CO_2–H_2O approach at high temperature (180–210 °C) and pressure of CO_2 (60 bar) demonstrated to be an interesting alternative[1] to conventional technologies principally in comparison with dilute acid hydrolysis, since no additional chemicals were used. The high-pressure CO_2–H_2O showed to be selective in the hemicellulose dissolution,[1] resulting in cellulose-rich processed solids. In this process, CO_2substituted the commonly used organic solvents and acted as a green catalyst. The presence of H_2O in the medium is an advantage, since it allows achieving higher sugar yields, increasing, at the same time, the effectiveness of the pre-treatment.[2] One of the major advantages of the use of CO_2–H_2O binary system is the *in situ* formation of carbonic acid which dissociates in two stages, promoting acid-catalysed dissolution of the biomass, namely hemicellulose,[1] into corresponding sugars, leading at the same time to higher cellulose enzymatic digestibility.[3] The *in situ* formed acidic environment helps in the pre-treatment, and additionally, unlike in the acid-hydrolysis, the acidity of CO_2 produced medium does not represent an environmental problem since after the depressurisation, CO_2 is no longer present in the reaction environment. Besides the chemical CO_2 effect on the pre-treatment, the CO_2 pressure (physical effect) is important because CO_2 under high pressure can easily penetrate small pores of the recalcitrant lignocellulosic structure, resulting in structural changes in feedstock. Furthermore, the synergetic attack of CO_2 and H_2O promotes fibre separation exposing the surface, leading meanwhile to elevated enzymatic digestibility of the processed solids. The use of enzymes to catalyse cellulose conversion is considered a greener alternative[4,5] in comparison to acid hydrolysis which mostly requires a two-step hydrolysis.[6]

In this study, for the first time, a methodology aiming to use high pressure CO_2 assisted pre-treatment followed by enzymatic hydrolysis is presented. The use of a green solvent such as CO_2 in the integrated approach of biomass valorisation by providing the benefits to polysaccharide conversion is presented. The high-pressure CO_2–H_2O process is employed to take advantage of the influence of temperature and CO_2 presence on the biomass depolymerisation reaction and on predisposition of the processed solid for enzymatic hydrolysis. This synergetic formation of value-added products such as oligosaccharides and later glucose after the enzymatic hydrolysis is a little jigsaw needed to advance towards a more sustainable bio-economy.

5.2.Materials and methods

5.2.1. Raw material and chemicals

Wheat straw was kindly provided by INIAV, I.P. – Estação Nacional de Melhoramento de Plantas (Elvas, Portugal). The material was milled to a particle size smaller than 1.5 mm using a knife mill (IKA® WERKE, MF 10 basic, Germany) and stored at room temperature. The moisture level in the dry wheat straw was

determined and was 8% w/w. CO_2 used in the high-pressure pre-treatments was purchased from Air Liquide, AlphaGaz™ gamma, Paris, France with ≥ 99.99% w/w purity. The paper filters (Ø = 150 mm, no. 1238) used for post-processing filtrations were purchased from Filter-Lab, Microchip Technology Inc., Arizona, USA. For all experiments and chemical analyses the following reagents were used: distilled water (18.2 MΩ/ cm) was produced by the PURELAB Classic Elga system, and ethanol with 96 % v/v purity for gas phase capturing was acquired from Carlo Erba Group – Arese, Italy. An aqueous solution of sulphuric acid 72 % w/w originated from 96 % w/w H_2SO_4 supplied by Panreac Química, Barcelona, Spain. For the enzymatic assays, sodium citrate buffer 0.05 M at pH 4.8 was prepared using citric acid monohydrate (99.7 % v/v purity) acquired from VWR International Ltd – Leicester, England. Sodium azide (99 % v/v purity) bought from Merck – Darmstadt, Germany was used to prepare 2% aqueous solution of sodium azide. Commercial enzyme cocktail of Celluclast® 1.5 L (cellulases from *Trichoderma reesei*) and Novozyme-188 (β-glucosidases from *Aspergillus niger*) was acquired from Sigma Co.

5.2.2. High-pressure CO_2-H_2O approach

The reactions were carried out according to the methodology presented elsewhere.[1] The reactions were performed at non-isothermal conditions (130, 215 and 225ºC) and the initial CO_2 pressure used was: 0 (water-only reaction - autohydrolysis), 15, 30, 45, 54 and 60 bar at room conditions. A 150 g of H_2O/ 15 g dry wheat straw and 100 g of H_2O/ 10 g dry wheat straw mixture loading were used in order to diverse the headspace and thus concentration of CO_2 in the reactor. Analogous methodology was applied to reaction with N_2 at 54 bar at 225°C. This reaction was carried out to verify the influence of physical effect on the biomass processing and subsequent enzymatic hydrolysis.

5.2.3. Severity factor

In order to evaluate the effect of high-pressure CO_2-H_2O system on hydrolysis of wheat straw, a combined severity factor ($CS_{p_{CO_2}}$)[3] was applied to examine the influence of the pH value on the reaction results according to the following equation: $CS_{PCO_2} = \log(R_0) - pH$. Due to the technical limitation, the acidity of the medium during the process is impossible to be measured, thus the pH was estimated according to the following expression: $pH = 8.00 \times 10^{-6} \times T^2 + 0.00209 \times T - 0.216 \times \ln(P_{CO_2}) + 3.92$, where T is temperature in ºC and p_{CO_2} is the partial pressure of CO_2 expressed in atmospheres. For the calculations of partial pressure of CO_2, the Henry's constant for binary CO_2-H_2O system was used.[3] In addition, the solubility of CO_2 in water for different reaction temperatures and pressures were taken from literature[7] and predicted for 215 and 225°C using PE package.[8]

5.2.4. Chemical characterisation of raw material and processed solids

Wheat straw and processed solids were characterised according to the previously established method.[1] Cellulose, xylan, arabinan and acetyl groups contents were determined after treatment with a 72% (w/w) H_2SO_4 according to the standard methods.[9] The acid insoluble residue was considered as Klason

lignin after correction for the acid insoluble ash. The ash content was established at 550ºC using NREL/TP-510-42622 protocol.[10]

5.2.5. Liquor and post-hydrolysate characterisation

The liquid phase (liquor) resulted from the high-pressure treatment was analysed as presented in the literature.[1] For the determination of total sugar content in the liquor, an acid hydrolysis procedure was applied as described in literature.[11]

5.2.6. Enzymatic hydrolysis

Cellulose digestibility of untreated and pre-treated washed solids were evaluated based on the NREL/TP-510-42629 protocol.[12] The cellulase activity of Celluclast® 1.5 L was 105.89 FPU/ mL[13] and the β-glucosidase activity of Novozyme-188 was 798.56 pNPGU/mL.[14] 1.0 mL of sample was taken at 6, 24, 48, 76 and 96 h of the digestion and heated at 90ºC for 5 min in a water-bath in order to quench the hydrolysis. All assays were performed at least in triplicate.

5.2.7. FTIR spectroscopy

The FTIR analysis was performed according to the procedure presented elsewhere.[15] All spectra were obtained with a FTIR spectrometer Spectrum BX, Perkin Elmer, Inc. (San Jose, CA, USA). This instrument was equipped with a DTGS detector and KBr beam splitter. The operating system used was Spectrum software (Version 5.3.1, Perkin Elmer, Inc., San Jose, CA, USA). FTIR spectra were acquired in the $4000 – 400\ cm^{-1}$ region, with a total of 64 scans and a resolution of $4\ cm^{-1}$ with a strong apodization. These spectra were subtracted against background air spectrum and recorded as absorbance values.

5.2.8. Scanning electron microscopy

Scanning electron microscopy (SEM) (XL30 FEG Philips) at 15 keV was used to monitor the changes in structural fibre morphology of wheat straw samples. For this study untreated and pre-treated with autohydrolysis and high-pressure CO_2-H_2O binary system processed solids were used. Samples were prepared in the following manner. Any volatile fractions of solid samples were removed by vacuum. Next, to provide the conductivity of the sample, samples were sputter-coated with gold-palladium in EMITECH k575x. The parameters of sputter current and time were set to 100 mA and 30 seconds in an inert atmosphere of argon and hydrogen.

5.3.Results and discussion

5.3.1. Chemical characterisation of wheat straw

The chemical analysis disclosed in the raw material (dry weight basis) was as follows: 38.8 ± 0.1 % glucan, 19.5 ± 0.4 % xylan, 2.9 ± 0.01% arabinan, 2.7 ± 0.03 % acetyl groups, 17.6 ± 0.1 % Klason lignin, 9.7 ± 0.0 % protein and 4.5 ± 0.1 % ash. The obtained data of chemical composition are in

good agreement with previous reports where an analogous relation between the main fractions (cellulose, hemicellulose, lignin and extractives) was found.[1,16,17]

5.3.2. Effect of reaction severity on the biomass processing

5.3.2.1.The liquor composition

Series of hydrolysis experiments have been carried out under variable conditions such as reaction temperature (215 and 225 °C), initial pressure of CO_2 (0, 15, 30, 45 and 54 bar) at non-isothermal conditions in order to assess the efficiency and selectivity of xylan conversion to xylo-oligosaccharides (XOS). The $CSpCO_2$ was applied with the objective to encompass all these variables and to facilitate the comparison of the obtained data. Both high-pressure CO_2–H_2O and water-only reaction (autohydrolysis) resulted in liquors containing a mixture of hemicellulose constituents such as xylose and arabinose namely in the oligomeric form, acetic acid and furfural (the main decomposition product of pentoses) and glucose as oligosaccharides obtained from cellulose. As is shown in Table 5.1, is the main compound present in liquors produced in all experiments. The amount of XOS recovered was highly dependent on the reaction conditions. When wheat straw was processed at $CSpCO_2$ = -0.33, a xylan to XOS yield of 61.7% was obtained and it corresponds to a concentration of XOS as high as 11.4 g/L. Under these conditions high quality liquor, rich in pentose sugars with low amounts of degradation products (1.7 g/L), was obtained. With the increase of severity, a decrease of XOS concentration was detected and the lowest value of 4.1 g/L was found for $CSpCO_2$ = 0.19. Under these conditions, an extended xylan hydrolysis (24.2% of xylose and 12.8% of furfural) coupled with the loss of XOS yield (64% lower in comparison with the best XOS yield conditions) was observed. Additionally, interesting is that by comparing the XOS yield at CO_2–H_2O processing at $CSpCO_2$ = -0.33 with the autohydrolysis, a 54 % higher XOS yield was produced in CO_2 coupled treatment. This may indicate that presence of CO_2 helps to promote the hydrolysis of xylan to XOS. The relation between XOS concentration and $CSpCO_2$ is shown in Figure 5.1.

Xylose is the main monosaccharide present in liquor, followed by the second monosaccharide – arabinose. Under the best XOS yield conditions, the concentration of released xylose and arabinose corresponds to 15 % and 46.5 % of initial xylan and arabinan contents, respectively. The concentration of xylose increased with the severity of the reaction up to $CSpCO_2$ = 0.11, at which a maximum concentration of 5.3 g/L was obtained in all other reactions under less severe conditions, furfural was formed almost in undetectable concentrations as depicted in Figure 5.1.

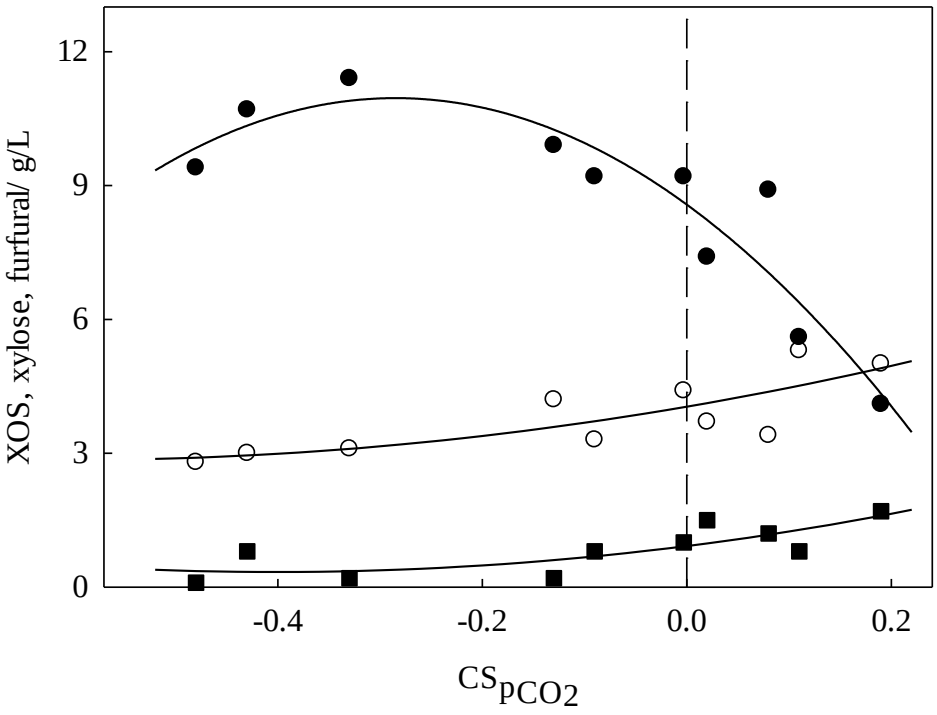

Figure 5.1. The XOS (●), xylose (○) and furfural (■) concentrations as a function of $CS_{p_{CO_2}}$. The solid lines are provided as a guide for the eye.

Gluco-oligosaccharides (GlcOS) and glucose follow similar patterns to XOS and xylose. The concentration of GlcOS tends to decrease and the glucose monomers' concentration increases with reaction severity. The formation of GlcOS might have its origin in the effect of the reaction conditions on cellulose dissolution, especially in the case of amorphous cellulose which is more susceptible to hydrolysis even under milder conditions. To confirm this concept, the crystallinity index from FTIR measurements[18-20] was calculated. LOI[21] is a tool to measure the degree of crystallinity of cellulosic material and is defined as the ratio of absorption bands at 1437 and 838 cm^{-1}. A band at 1437 cm^{-1}, assigned to a symmetric CH_2 bending vibration is the "crystallinity band", indicating that a decrease in its intensity reflects a reduction in the degree of crystallinity of the samples. The FTIR absorption band at 898 cm^{-1}, assigned to C–O–C stretching at β-(1→4)-glycosidic linkages, is an "amorphous" absorption band and an increase in its intensity happening in the amorphous samples.[20] The results obtained for samples untreated and pre-treated by autohydrolysis ($CSpCO_2$ = 0.02) and with CO_2 ($CSpCO_2$ = 0.08) are given in Table 5.2 and Figure 5.2.

The analysis of the produced data shows that LOI for the untreated sample is 3.28, while for autohydrolysis and CO_2 pre-treated solids it is 4.56 and 3.92, respectively. Surprisingly, the process, either autohydrolysis or CO_2-assisted, increases the LOI value or in other words crystallinity. However, close inspection of the obtained data shows that autohydrolysis removes amorphous cellulose as the band at 898 cm^{-1} was reduced by 27 % in comparison with untreated biomass and 1437 cm^{-1} remains intact.

Table 5.1. Composition of liquors (g/L) and yields of each product present in the liquors (g/100 g of the initial amount present in the feedstock) for all studied conditions.

Reaction conditions	High-pressure H_2O		High pressure CO_2-H_2O															
T (ºC)	225		225		225		215		215		225		225		225		225	
$p_{CO2init.}$(bar)	0		15		15		30		54		30		30		45		45	
p_{final}(bar)	23.6		32.6		36.4		61.0		126.3		61.9		62.1		83.7		93.0	
CO_2dissol./biomass (w/w)	-		0.039		0.060		0.198		0.471		0.208		0.195		0.303		0.344	
Log (R_0)	3.79		3.83		3.80		3.57		3.58		3.87		3.96		3.95		3.95	
$CS_{p_{CO_2}}$/pH[a]	0.02[b]/-		-0.48/4.31		-0.43/4.22		-0.33/3.90		-0.13/3.71		-0.09/3.96		0.00/3.96		0.08/3.87		0.11/3.84	
pH[c]	3.77		3.97		4.04		3.87		3.80		4.03		3.78		3.74		3.55	
Composition/ yields	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g	g/ L	g/ 100g
XOS	7.4	42.6	9.4	51.7	10.7	57.9	11.4	61.7	9.9	53.8	9.2	52.9	9.2	49.8	8.9	48.4	5.6	30.6	4.1	22.6
GlcOS	4.0	11.6	4.8	13.2	5.7	15.7	4.5	12.4	4.5	12.3	4.3	12.4	5.0	13.6	4.0	13.6	3.6	9.8	3.5	9.5
AcOS	0.4	-	0.8	-	0.6	-	2.7	-	1.9	-	2.1	-	0.6	-	0.2	-	1.7	-	1.7	-
Xylose	3.7	18.9	2.8	13.4	3.0	14.4	3.1	15.0	4.2	20.1	3.3	16.7	4.4	21.2	3.4	21.2	5.3	25.4	5.0	24.2
Arabinose	0.8	25.7	1.2	39.5	1.2	37.5	1.4	46.5	1.3	40.8	0.78	17.9	1.2	23.5	1.1	23.5	1.0	26.4	0.8	24.4
Glucose	0.8	2.1	1.2	2.9	0.8	1.9	0.9	2.2	1.0	2.6	0.7	1.8	0.9	2.3	1.2	2.3	1.1	2.7	1.2	2.9
Acetic Acid	3.5	-	3.2	-	3.0	-	2.5	-	3.3	-	3.0	-	3.5	-	3.5	-	3.6	-	4.0	-
Furfural	1.5	12.2	0.1	1.0	0.8	6.3	0.2	1.3	0.2	1.7	0.8	6.0	1.0	7.1	1.2	7.1	0.8	6.0	1.7	12.8
5-HMF	0.2	0.8	0.2	0.5	0.2	0.2	0.1	0.4	0.2	0.7	0.2	0.7	0.2	0.6	0.3	0.6	0.3	0.9	0.3	1.1

[a] predicted according to ref. [3] [b] calculated with measured pH value. [c] measured pH value of hydrolysate after reactions. XOS – xylo-oligosaccharides; GlcOS - gluco-oligosaccharides; AcOS - acetyl groups linked to oligosaccharides; 5-HMF – 5-hydroxymethylfurfural

Table 5.2. The LOI index for untreated, autohydrolysis and CO_2-treated biomass.

Sample	A_{898}	A_{1437}	$LOI = \frac{A_{1437}}{A_{898}}$
untreated	0.115	0.376	3.27
autohydrolysis ($CS_{p_{CO_2}}$=0.02)	0.084	0.383	4.56
CO_2 processed ($CS_{p_{CO_2}}$=0.08)	0.079	0.311	3.94

The analysis of the FTIR spectrum of the CO_2–H_2O treated sample shows that crystallinity reduction is more extensive than in the case of autohydrolysis as it reduces either amorphous cellulose or the crystalline one by 31 % and 17 %, respectively.

Therefore, the LOI for both processes is higher than LOI for the untreated sample while in fact crystallinity seems to be lower as in both cases amorphous cellulose was removed and in the CO_2 process some portion of the crystalline one was expelled as well.

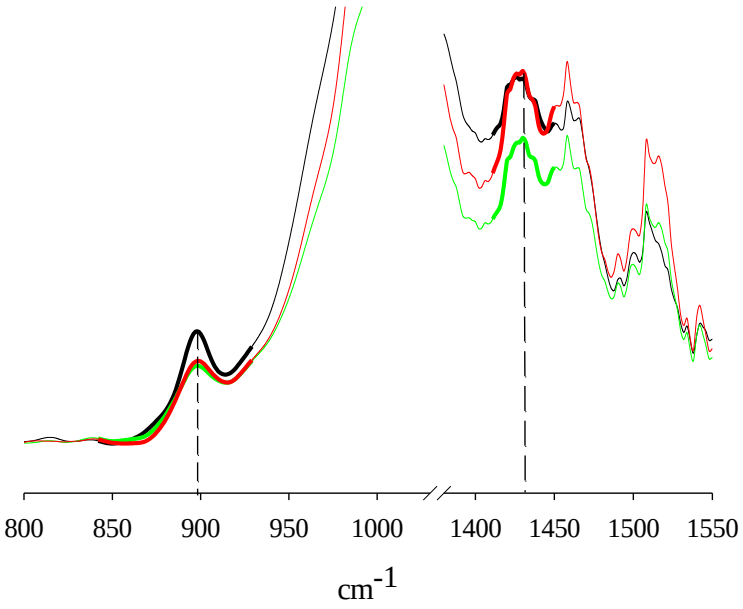

Figure 5.2. The FTIR spectra of untreated (black line), autohydrolysis (red line) and CO_2 processed wheat straw (green line) showing the regions for LOI determination. The adequate bands (898 and 1437 cm^{-1}) are marked by dashed lines.

The FTIR results confirm that autohydrolysis is a less severe process than the CO_2. GlcOS is mostly formed from amorphous cellulose, while in the case of the CO_2 coupled process, amorphous cellulose is hydrolysed to an even greater extent than in autohydrolysis, and crystalline cellulose is also affected allowing a progressive formation of GlcOS and glucose.

The obtained liquor in the studied processes contained acetic acid in both forms: as free acetic acid and as acetyl groups bound to OS (AcOS). As expected the maximum acetic acid (4.0 g L^{-1}) was achieved under the severest conditions while the highest AcOS concentration was obtained at moderate $CSpCO_2$ equals to -0.33. The mechanism of this process is analogous to the previously discussed conversion of XOS to xylose. More severe conditions favour hydrolysis of AcOS to acetic acid; therefore the concentration of acetic acid increases with an increase of severity of the reaction conditions. King and co-workers observed a similar trend for experiments based on hydrolysis of switchgrass with carbonated water to produce carbo-chemicals in the range of temperatures from 220 to 310 °C and commensurate higher pressure (68 bar) in a semi-continuous batch flow system. The aliphatic acids (acetic and formic) were produced at levels of 3–6 g per 100 g of feedstock while furfural was rapidly produced at 310 °C within 10 min.[22] The reaction conditions examined in this work permitted to produce liquors rich in oligosaccharides at a total concentration of 18.6 g L^{-1} at $CSpCO_2$ = -0.33. The produced solution was mostly constituted by XOS which is a major product among oligosaccharides present and corresponds to 61 % of them. One of the most important aspects examined in this work is the pH of the produced liquors. The measured and predicted pH values of hydrolysates from either autohydrolysis or high-pressure CO_2–H_2O experiments are presented in Table 5.1. As can be seen, the measured pH values (after CO_2 released at room temperature) vary between 3.55 and 4.04 for experiments carried out with CO_2. The processes with CO_2 for $CSpCO_2 \geq 0.08$ gave pH of the hydrolysate lower than that for the autohydrolysis reaction (pH = 3.77). Considering that no additional amount of acetic acid was formed during these reactions, as is shown in Table 5.1, it indicates that *in situ* formed carbonic acid acidifies the medium, leading to a decrease of the final pH of the liquor. The obtained results are in contrast to those presented by van Walsum and co-workers, in which addition of carbonic acid increased the pH of the liquor produced.[23] These differences can be elucidated by the fact that at reduced CO_2 pressure, the solubility of CO_2 in the aqueous phase is much lower (even one order of magnitude); therefore under these conditions the acidification of liquor by carbonic acid is practically negligible. For instance, for a reaction at 225 °C and 15 bar of initial CO_2 pressure (32.6 bar final total pressure) for $CSpCO_2$ = -0.48, the solubility of CO_2 in water is almost null (x_{CO_2}=0.0016)[8] resulting in a very low CO_2 dissolved/water ratio equal to 0.039. In contrast, the solubility of CO_2 in water for the harshest examined conditions $CSpCO_2$ = 0.19 is x_{CO_2}=0.00196 giving 0.49 g of CO_2 dissolved in water per 1 g of biomass. These data clearly demonstrate that CO_2 pressure, and by this the severity of the reaction, plays an important role as under less severe conditions, reactions occur in fact in a three phase system involving solid biomass, water mostly in liquid state and gaseous phase constituted almost exclusively by CO_2. Therefore these three phase type reactions give a pH of liquor similar to the pH of liquor for autohydrolysis $CSpCO_2$ = 0.02.

Another important aspect to be scrutinised is that under the harshest conditions, degradation products with acidic characteristics are formed in a larger amount. Their presence also contributes to biomass hydrolysis and consequently guides to lower pH values of generated liquors. For example, lower pH values were found

also in hydrothermal processes reported in the literature, where an extensive hydrolysis of hemicellulosic acetyl groups was found.[16,24,25]Another very important factor influencing the pH of the liquor is the composition of biomass explored. Biomass rich in high acetyl group may lead to a decrease of pH during the reaction. A comparison of the results obtained in this work with those reported in in the literature [26] shows that the pH value of liquors from aspen wood processing with carbonic acid for $CSpCO_2$ = 0.17 is 3.95[26], and is not very different from that obtained under similar reaction conditions s ($CSpCO_2$ = 0.19, pH = 3.68) presented in this work. On the other hand, corn stover for $CSpCO_2$ close to that obtained in this work gives the final pH very different (pH = 4.95). According to van Walsum *et al.*, differences between the final pH can be explained by the extended autocatalytic hydrolysis of acetyl groups of aspen wood in comparison with those in corn stover.[26] Although the CO_2 experiments resulted in different pH of liquors, in fact the decrease of pH does not show a significant effect on hemicellulose dissolution. For example, the difference in hemicellulose dissolution between all reactions with $CSpCO_2 \geq$-0.13 was only around 6%. Brunner and co-workers also found similar conclusions, where no substantial relation between hemicellulose dissolution and the decrease of pH was determined.[27] Even a decrease of pH to around 2 caused by the addition of sulphuric acid did not have any effect on biomass dissolution.[27]

5.3.2.2.The processed residue composition

The pH of the medium produced during the process and the reaction severities do not only influence the liquor composition but also affect the processed solid composition too. Table 5.3 summarises the composition and yield of the processed solid residues obtained by either autohydrolysis or CO_2–H_2O under various severity conditions. For all conditions of high-pressure CO_2–H_2O processes, the solid dissolution was high and superior to 50 %. For comparison, the autohydrolysis reaction, similarly to CO_2–H_2O experiments, demonstrated still a high solid dissolution (46.7 % of initial biomass) with hemicellulose removal close to 75 %. Another important aspect is that the processed solids suffered significant changes in composition in comparison with the raw material. The degree of biomass dissolution increases almost linearly with a severity factor reaching values of hemicellulose removal up to 86.4 %. This demonstrates an incomplete hydrolysis of xylan and presence of a minor amount of acetyl groups. With an increase of the severity of the reaction, a faster cleavage of the linkages of hemicellulose and cellulose was observed.[27] At elevated temperatures (above 200 °C) a similar catalytic effect of carbonic acid on pure xylan hydrolysis was observed. An increase of pentose release and a decrease of polymerisation degree of xylan oligomers in comparison with autohydrolysis were found.[3] The obtained data show that cellulose, despite being partially affected by either autohydrolysis or the CO_2–H_2O process, is a dominant constituent of the processed solid and its relative concentration increases with the severity of the reaction because of the dissolution of hemicellulose. The maximum glucan content of 74.9 % in the processed solid was found at $CSpCO_2 \geq 0.19$. The increase of severity had minor effects on glucan dissolution, with a maximum of glucan loss of 14.3 % for the most severe conditions. The third principal component of biomass remaining in the processed solid is lignin. The recovery of lignin in the solid phase was found to be between 27.0 % and 31.2 %.

Table 5.3. The solid phase composition (g/ 100 g processed solids) and solid yield (g/ 100 g feedstock) obtained after the examined wheat straw experiments.

Reaction conditions	High-pressure H_2O	High-pressure CO_2-H_2O								
T (ºC)	225	225	225	215	215	225	225	225	225	225
$CSpCO_2$[a]	0.02[b]	-0.48	-0.43	-0.33	-0.13	-0.09	0.00	0.08	0.11	0.19
Solid yield	53.28	48.33	49.02	49.83	49.13	48.87	49.16	45.03	48.26	44.53
Glucan	65.85 ± 1.15	69.93 ± 0.17	70.11 ± 1.0	70.62 ± 0.46	71.26 ± 0.81	72.15 ± 0.52	73.83 ± 1.06	74.39 ± 0.73	74.63 ± 0.30	74.88 ± 1.71
Xylan	9.09 ± 3.05	8.40 ± 1.66	8.64 ± 3.65	10.15 ± 2.67	8.80 ± 3.32	7.93 ± 2.61	7.38 ± 2.03	6.71 ± 2.16	6.91 ± 1.03	5.95 ± 1.41
Arabinan	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces	Traces
Acetyl groups	1.33 ± 0.08	0.67 ± 2.61	0.51 ± 0.91	1.26 ± 0.02	0.94 ± 0.54	0.56 ± 0.33	0.43 ± 1.60	0.51 ± 5.45	0.34 ± 0.15	0.28 ± 0.00
Klason lignin	31.15± 0.33	27.0 ± 0.71	27.15 ± 0.81	28.03 ± 2.23	28.62 ± 4.55	28.82 ± 0.45	29.30 ± 1.35	29.51 ± 1.16	29.07 ± 1.72	27.60 ± 4.63

[a] Predicted according to ref[29;] [b] Calculated with measured pH value after the reaction

The treatments resulted in an increase of the total amounts of lignin due to lignin condensation reactions, which makes even worse the digestibility of the remaining cellulose fraction.[28,29] The aforementioned hemicellulose removal and the influence on the morphology of biomass are visible and were analysed by the SEM technique. The SEM technique allowed to investigate the effect of the process on the ultrastructure and possible disruption of the cell walls. Figure 5.3 shows an SEM analysis of native wheat straw and wheat straw after either autohydrolysis (225 °C) or CO_2–H_2O reactions (225 °C and 54 bar of CO_2).

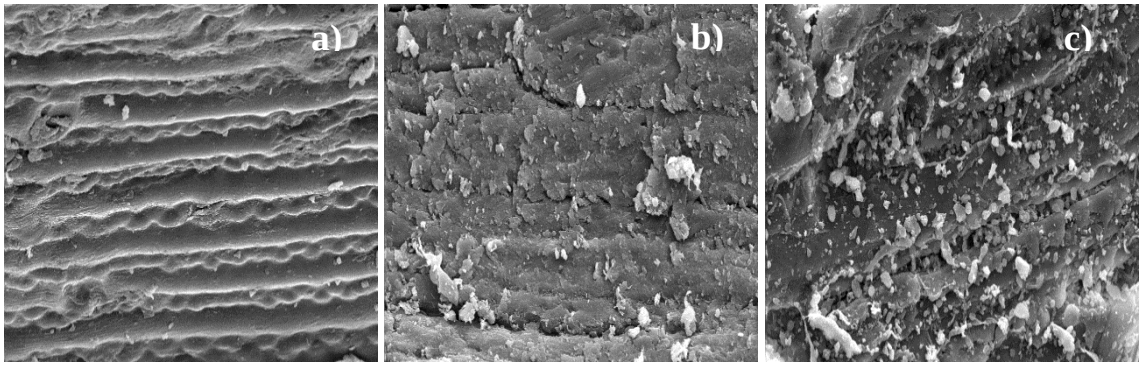

Figure 5.3. SEM micrographs of (a) untreated wheat straw and after (b) autohydrolysis and (c) CO_2–H_2O reactions obtained with amplification 1200×.

After both processes were applied, physical changes of the raw material surface were noticeable. The ground untreated wheat straw exhibited a rigid, tight and contiguous surface while fibres of treated samples have anomalous porosity and the lamellar structures became fleecy. The pre-treated solids are significantly more heterogeneous in structure than the untreated one. This indicates that the surface of the raw material was subjected to severe conditions during both processes with the dominant effect visible in the case of the CO_2 involved. These morphological changes find an explanation in the previously discussed results. The extended hemicellulose removal from middle lamella caused by the reaction conditions led to the structural changes visible in Figure 5.3. In other words, the synergetic attack of CO_2 and H_2O promotes fibre separation exposing the surface, leading also to an elevated enzymatic digestibility of the processed solid[30] as will be discussed later. Furthermore, the interaction between biomass with hot liquid water and high dense CO_2 leads to an increase of diffusivity of the gas into biomass, promoting the swelling of biomass.[31] Similar conclusions regarding the effect of supercritical CO_2 on the physical structure of lignocellulosic materials were presented in the literature.[32,33]Zheng *et al.* studied the effects of different gases such as nitrogen, helium and CO_2 and the latter has shown higher glucose yields from enzymatic hydrolysis of lignocellulosic materials.[34] Narayanaswamy *et al.* reported that supercritical CO_2 (150 °C, 241 bar, 1 h and moisture of 75%) had a significant effect in opening pores and exposing the internal areas of corn stover.[32] On the other hand, no effect of supercritical CO_2 on switchgrass was found, probably due to the rigid structure of this biomass[32] Gao *et al.* discovered that the reaction with supercritical CO_2 (110 °C, 300 bar for 30 min and a liquid–solid ratio of 1 : 1) promotes changes in porosity, and fibres became more susceptible to enzymatic attack, increasing their digestibility.[33] Contrary to the results presented in this work, both the referred

literature studies used the CO_2 explosion technique that has a great impact on biomass pore rupture. Presumably, a rapid release of CO_2leads to explosion and the effect on pore opening is more evident. Benazzi *et al.* reported that the depressurisation rate of 50–200 kg m^{-3} min^{-1} after ultrasound assisted supercritical CO_2 pre-treatment did not result in a significant increase of glucose yield obtained by enzymatic hydrolysis. This might be explained by the slow CO_2 release in comparison with CO_2 explosion pre-treatment.[35] Ferreira-Leitão and co-workers found that CO_2-explosion pre-treatment at 205 °C for 15 min resulted in less pronounced structural modifications of the material than SO_2-explosion at 190 °C for 5 min which can be directly related to the combined severity of each pre-treatment.[36] Especially, SO_2-pre-treatment resulted in more extensive hemicellulose removal equal to 68.3% in comparison with the 40.5% obtained from CO_2-pre-treatment.

5.3.3. Enzymatic hydrolysis

To survey the influence of the examined processes on the monosaccharides production, the processed solids were subject to enzymatic hydrolysis. As was already mentioned, the enzymatic attack is affected by several factors of the pre-treatment process, which are essential to make glucan much more accessible. The most important parameters influencing the rate of enzymatic hydrolysis are hemicellulose and lignin content, cellulose crystallinity, degree of polymerisation[37] as well as the liquid–solid ratio. All of these factors are strongly related with the choice of pre-treatment and the conditions employed.

5.3.3.1.Pressure effect

The wheat straw samples pre-treated at various CO_2 pressures at a fixed temperature were hydrolysed by the addition of cellulase and Novozyme 188 at a concentration of 60 FPU per 1 g of glucan and 64 pNPGU per 1 g of glucan, respectively, for a maximum of 96 h. Figure 5.4 shows the effect of CO_2 pressure on glucose yield from glucan for the high-pressure CO_2–H_2O reaction of wheat straw performed at constant temperature (225 °C). The glucose yield increased with time of hydrolysis, which indicates that enzymatic digestibility of the pre-treated solids increases. The glucose yields rise to a maximum of 56.02, 57.77, 63.93, 75.39 and 82.21% at 96 h of enzymatic hydrolysis for 0, 15, 30, 45 and 54 bar of initial CO_2 pressures, respectively. For comparison, the untreated biomass showed a maximum glucose yield of 34.31% after 96 h of processing. The obtained data are shown in Figure 5.4. The glucose yield was increased by 46.8% with an increase of the pre-treatment pressure (from 0 to 54 bar of the initial or from 23.6 to 127.4 bar of the final total pressure). This shows that CO2 pressure plays an important role in improving the enzymatic digestibility. Zheng et al. studied the effect of supercritical CO2 on hydrolysis of Avicel (commercial form of cellulose). They concluded that the disruption of cellulosic structure was caused by CO2 under supercritical conditions, followed by quick depressurisation, which increases the rate of enzymatic hydrolysis, resulting in a glucose yield of around 50%.[38]Another report of Alinia *et al.* demonstrates that the pressure changes from 80 to 120 bar in the wheat straw pre-treatment promote an increase of yield of reducing sugars. At the same time, further pressure increase above 120 bar does not change the final sugar

yield.[39] Kim *et al.* studied the hydrolysis of aspen and southern yellow pine pre-treated at 214 and 276 bar of CO_2 and did not observe any effect on the enzymatic digestibility of both materials.[40] The conclusion drawn by Kim *et al.* might be burdened by the high lignin content present in both aspen and southern yellow that may have a negative effect on enzymatic hydrolysis.

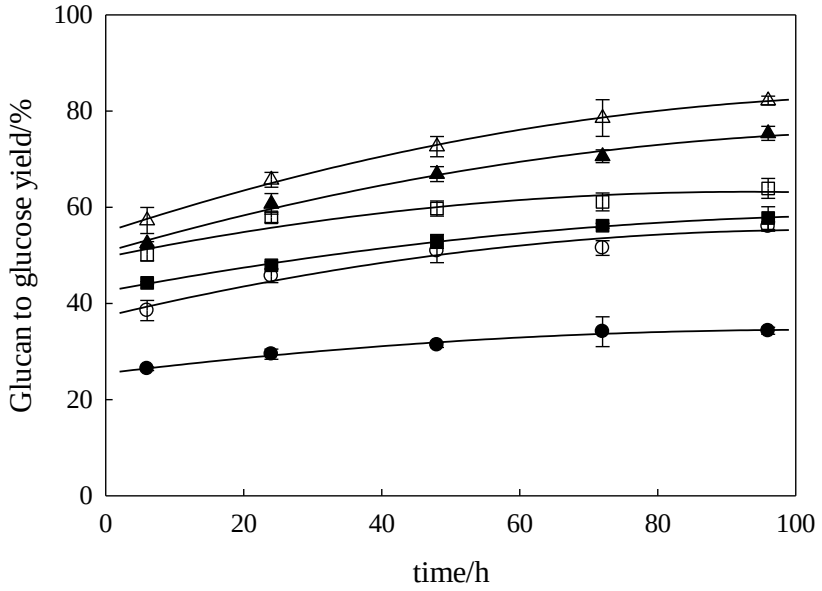

Figure 5.4. The glucan to glucose yield/% (with the respective error bars) for (●) untreated, (○) autohydrolysis and treated under (■) 15 bar, (□) 30 bar, (▲) 45 bar or (△) 54 bar of initial CO_2 pressure. The solid lines provided as guide for an eye.

It is also important to mention that not only supercritical CO_2 affects the enzymatic hydrolysis. The CO_2 used under subcritical conditions also improves the enzymatic hydrolysis. For instance, sugarcane bagasse treated with 70 bar of CO_2 at 160 °C for 60 min showed an increase of 55% in glucose yield in comparison to the pre-treatment without CO_2.[41] Puri *et al.* studied the effect of steam and CO_2 under sub- and supercritical conditions on the cellulose hydrolysis of wheat straw.[42] A maximum glucose yield of 81% was obtained at 200 °C in the range of CO_2 pressures of 34.5–138 bar. The acquired results clearly show that enzymatic hydrolysis is strongly influenced by chemical and physical effects of the process. To investigate the effect of CO_2 on the more favourable enzymatic hydrolysis of the processed solid, by the removal of hydrolysis inhibitors (hemicellulose) as well as by the physical cellulose structure opening, a reaction in the presence of a neutral gas – nitrogen – was carried out under conditions analogous to the reaction with $CS_{p_{CO_2}}$ = 0.19 (225 °C and 54 bar of N_2). The processing of wheat straw with N_2 gave 9.8, 3.0 and 1.3 g L^{-1} of XOS, xylose and furfural, respectively. At the same time, the formed processed solid contained 61.2% of glucan, which in the enzymatic hydrolysis process was converted to glucose, giving after 72 h 63% of glucan to glucose yield. Comparing these data with the autohydrolysis (51.6%) and the analogous CO_2 process (78.6%), it can be concluded that presence of neutral gas in the headspace of the reactor and, by

this, creation of the pressure influence positively the enzymatic digestibility of the processed solid. On the other hand, the increase of enzymatic hydrolysis from 63% for N_2 to 78.6% for the CO_2 process indicates the strong chemical effect of CO_2 on removal of hemicellulose and, by this, a more favourable enzymatic digestibility of glucan present in the processed solid. Figure 5.5 depicts the influence of both effects on the enzymatic hydrolysis of processed solid produced in autohydrolysis (H_2O process), N_2 (N_2–H_2O process) and CO_2 (CO_2–H_2O process).

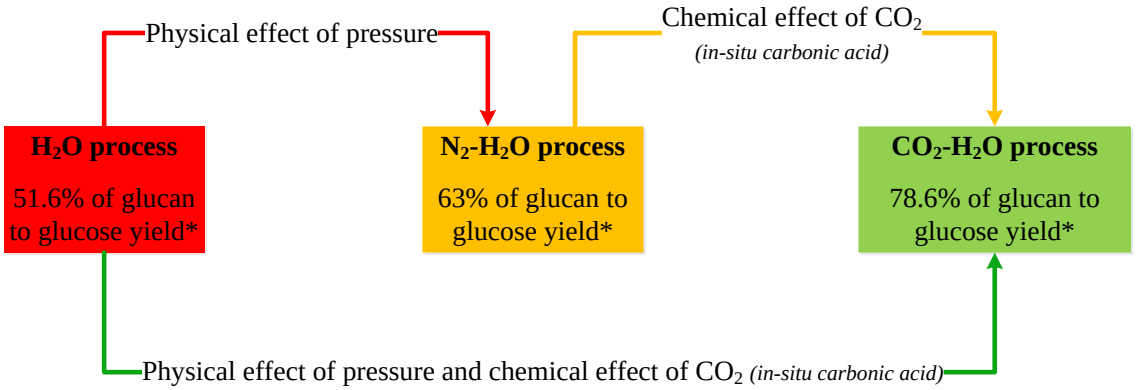

Figure 5.5. The schematic representation of both physical and chemical effects of high pressure processes of wheat straw valorisation. *The value of the glucan to glucose yield obtained after 72 h of hydrolysis.

5.3.3.2. Temperature effect

Another variable influencing the hydrolysis of processed solid is the temperature of the process. In order to demonstrate the effect of temperature on high-pressure CO_2–H_2O, wheat straw was subjected to reactions at three different temperatures (130, 215 and 225 °C) and fixed initial CO_2 pressure (30 bar). Both 215 and 225 °C were previously examined for hemicellulosic-sugar production, while experiments at 130 °C were carried out to demonstrate the influence of temperature on the enzymatic hydrolysis. Figure 5.6 demonstrates this relation and the maximum glucose yields for 130, 215, and 225 °C were 39.97, 62.64 and 63.93%, respectively. In comparison, enzymatic hydrolysis of untreated material after 96 h revealed a 34.31% yield of glucose. The obtained results show that the process temperature of 130 °C seemed to have a low impact on glucose yield. The obtained result can be explained by the low diffusivity of CO_2 at inferior temperatures and thus temperature and CO_2 pressure are important factors in the efficient conversion of wheat straw. Kim and Hong[40] tested the effect of $scCO_2$ at different temperatures (112–165 °C). They found that at temperatures below 120 °C and 214 bar for 60 min the pre-treatment has no significant effect on the sugar yield from enzymatic hydrolysis. However, when a temperature of 160 °C was used, a higher glucose yield was achieved. Similar conclusions were presented by Narayanaswamy *et al*., who reported that an increase of temperature from 120 °C to 150 °C led to the release of 24 and 30 g glucose per 100 g of dry biomass, respectively.[32] Gao *et al*.also investigated the influence of the temperature of rice straw pre-treatment on the glucose yield from enzymatic hydrolysis.[33] The maximum glucose yield obtained was only 32.4% at 110 °C and 300 bar of CO_2 as, according to the conclusions given, the low yield may be caused by the low

temperature used in the experiments as hemicellulose and lignin start to dissolve under neutral conditions at 180 °C.[33]

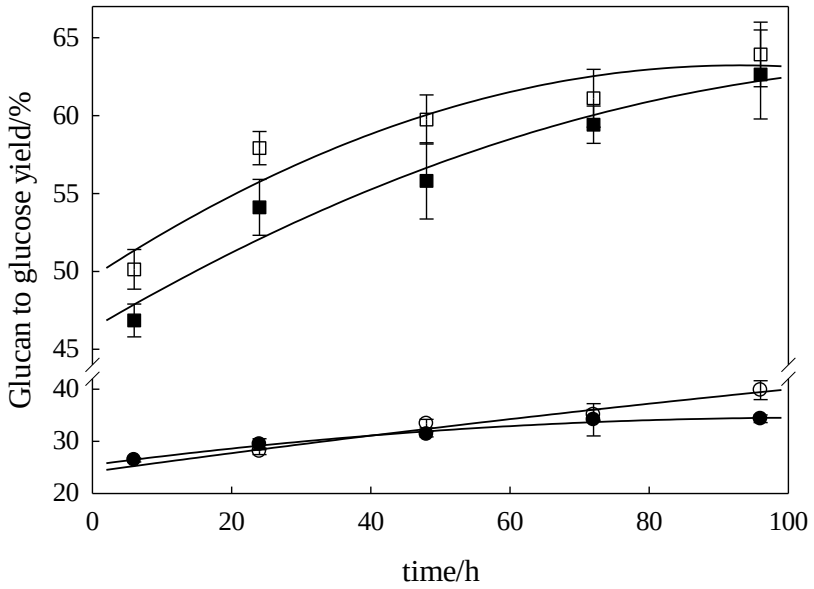

Figure 5.6. The glucan to glucose yield/% (with respective error bars) for (●) untreated wheat straw and treated under 30 bar of CO_2initial pressure at (○) 130 °C, (■) 215 °C, or (□) 225 °C. The solid lines are provided merely as a guide for the eye.

The presented results clearly show that higher temperature promotes more efficient conversion of glucan to glucose. For example, the increase of temperature from 130 °C to 215 °C gives an increase of glucose yield by more than 50% (from 39.97 to 63.93%), helping produce much monosaccharide rich solution.

Comparing the obtained data for different temperatures and CO_2 pressures with those achieved by the classical methods shows a more favourable result in the case of reactions with CO_2. Hsu *et al.* investigated the effect of dilute-acid hydrolysis on enzymatic hydrolysis of rice straw, and a maximum sugar yield of 83% was achieved when rice straw was pre-treated with 1% (w/w) of sulphuric acid with a reaction time of 5 min at 180 °C.[43] Also, Henk and co-workers found that corn stover pre-treated with 2% (w/v) sulphuric acid showed a cellulose digestibility higher than 80%.[44] The found results are similar to those obtained in this work for 225 °C and 54 bar of initial CO_2 pressure without an additional chemical catalyst such as sulphuric acid. Considering that the spent acid and consequently its neutralisation results in gypsum, which must be eliminated, making the overall process environmentally and economically unfeasible, it is important to underline that the CO_2 process seems to be an interesting and more environmentally friendly method of bio-waste pre-treatment, enhancing the enzymatic hydrolysis of the processed solid. Furthermore, dilute-acid hydrolysis requires substrate washing with water and often with alkaline solution prior to enzymatic hydrolysis in order to elevate the pH to the value of optimal acidity of enzymes. Unlike acid-catalysed

reactions, the CO_2–H_2O does not require additional water amounts and prevents the residue formation, retaining the advantages gained from employing this technology in biomass processing.

5.3.4. Polysaccharide wheat straw valorisation

The valorisation of both hemicellulosic and cellulosic fractions of wheat straw polysaccharides aims to convert these inaccessible saccharides to easily transformable sugars. The integrated polysaccharide conversion to sugars in either oligomer or monomer form was analysed for all performed reactions. Figure 5.7 depicts the selected best case for a CO_2-assisted reaction. The data for this process show that either xylan or glucan in the integrated (pre-treatment with CO_2–H_2O and the next enzymatic hydrolysis) is converted to xylose- or glucose-derived sugars with a yield of 86.3% and 83.5%, respectively. For comparison, the analogous calculations for the autohydrolysis process reveal yields of xylan or glucan to sugars as high as 79.6 and 61.3%, respectively. The mass balance of xylan and glucan is depicted in Figure 5.8.

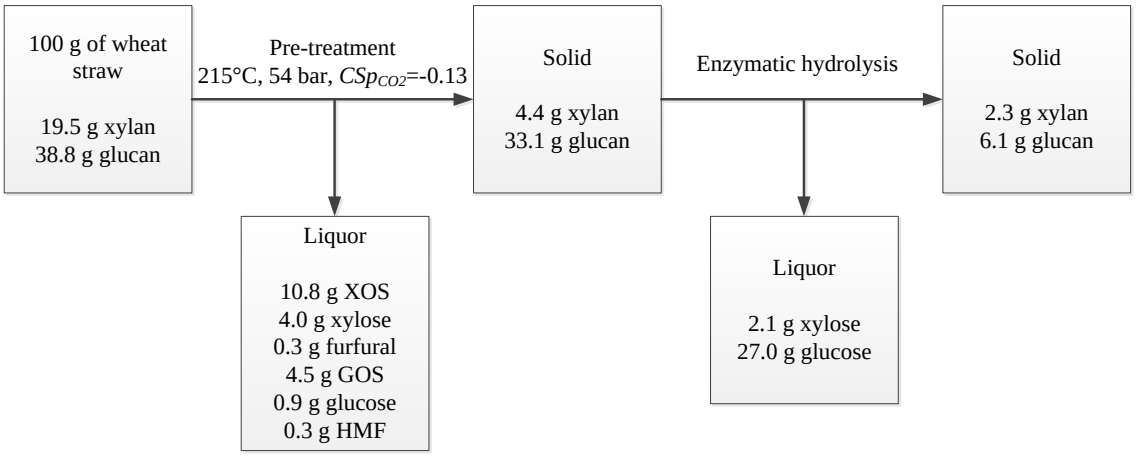

Figure 5.7. The mass balance of integrated polysaccharide conversion.

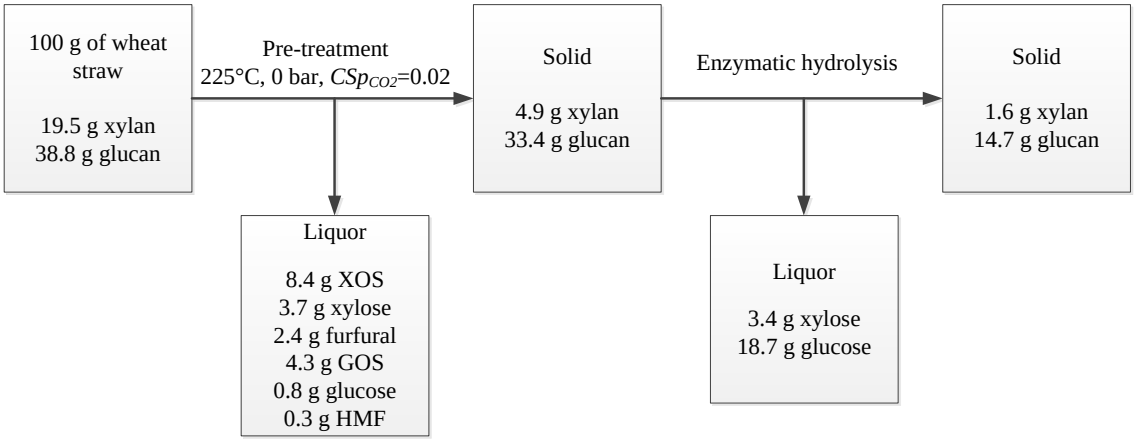

Figure 5.8. The mass balance of integrated polysaccharide conversion for the autohydrolysis process.

The obtained data confirm that CO_2-assisted autohydrolysis integrated with subsequent enzymatic hydrolysis of the processed solid gives much higher total sugar yields (84.4 %) than this for autohydrolysis alone (67.4 %).

5.4.Conclusions

This work shows the potential of the high-pressure CO_2–H_2O process in both hydrolysis and pre-treatment of lignocellulosic biomass. The high-pressure CO_2–H_2O process results in liquors rich in xylose oligomers, which in contrast to other valorisation methods, *e.g.* acid hydrolysis, produces monomers of xylose, can be an advantage due to the prebiotic activities of XOS. The integrated valorisation of polysaccharides, using a green solvent, permits to achieve an 84.7% of total sugar yield (from xylan and glucan present in the raw feedstock) in the form of mono- or oligosaccharides. The obtained results confirm that maximal exploitation of the hemicellulose fraction in the form of XOS and xylose, together with glucose production during the enzymatic hydrolysis, are the best approaches of the wheat straw polysaccharide valorisation method. The incorporation of CO_2 into hydrothermal technologies was shown to be successful, allowing (i) to carry out processes at lower temperatures than autohydrolysis, (ii) to obtain hemicellulose-rich solutions without degradation products' formation and (iii) to produce pre-treated solids which were highly susceptible to enzymatic hydrolysis as well.

5.5.References

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Chapter VI

Highly efficient and selective CO_2-adjunctive dehydration of xylose to furfural in aqueous media with THF

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6.1.Introduction

Furans including furfural are examples of building blocks[1] which can be used as biomass-originated substitute of fossil-based pivot compounds in the production of diverse chemicals (*e.g.* tetrahydrofuran (THF)).[2-5] Currently, furfural is exclusively produced by mineral acid catalysed dehydration of C_5-sugars present in the hemicelluloses of agricultural residues and hardwoods. It has been reported that at industrial practice, the furfural yield originated in batch processes has remained at or below 50 mol % of theoretical[6] due to occurrence of undesired parallel reactions leading to formation of humins.[7,8]

Up to date, the production of furfural from hemicellulose-derived sugars has been reported to be carried out in both monophasic and biphasic systems.[5,9,10] It is highly desired to produce furfural *via in-situ* generated acid catalyst conditions, without typical drawbacks for both homogeneous and heterogeneous catalysts. The basics of inorganic chemistry teach that CO_2 is little soluble in water, however even this negligible solubility forms unstable carbonic acid. This weak acid promotes the medium acidification helping biomass processing[11-15] and dehydration of sugars into furans as presented in this work. The proposed approach has great environmental benefits since the acidity of the medium can be easily turned off by simple depressurisation and removal of CO_2 from environment allowing its recovery and reuse.[14]

In this work, D-xylose was converted into furfural. The examined approach takes the benefits from the use of auto-generated acidic environment due to high-pressure CO_2/H_2O biphasic mixture and the phases 'separation caused by addition of CO_2 to aqueous/THF mixture, guiding to the enhancement of final furfural yield and reaction selectivity.

6.2.Results and Discussion

Series of runs presented in Table 6.1 were carried out to scrutinise the role of THF (entries 1 and 2), CO_2 (*e.g.* entries 2 and 7) and temperature in the range of 160 - 180 °C for 30 and 60 min of reaction time (entries 3 - 6). Additionally, variation of water ($V_{aq.}$) and THF (V_{THF}) volumetric ratios were examined (entries 7 - 9) in an effort to decrease the reactor headspace to analyse the effect of the CO_2 amount loaded into the reactor.

The analysis of results obtained in entries 1 and 2 confirms the expected adjunctive effect of THF presence. This data demonstrates that albeit lower xylose concentration was examined in entry 2, the furfural yield and selectivity of reaction raised expressively reaching values of 65.2 mol % and 41.4 %, respectively. This is in a good agreement with previous reports about THF effect on furfural production. For example, Wyman and co-workers found that in acid catalysed reaction with THF as co-solvent, furfural yield was as high as 86 mol % due to "protecting" effect of THF on furfural.[16] However, it is important to state that Wyman and co-workers did not clarify how THF, as co-solvent in a monophasic system, is able to protect furfural from further degradation reactions. Beyond THF, another equally important agent in the formation of furfural is CO_2. The comparison of results

obtained in entries 2 and 7 demonstrates that the presence of CO_2 enhances the furfural yield by 2/3. It indicates that in the presence of water, high-pressure CO_2 produces unstable carbonic acid leading to generation of acidic environment and consequently to more favourable xylose dehydration boosting the furfural yields in comparison to reactions without CO_2 (entries 2 and 7).

Table 6.1. Furfural production by dehydration of D-xylose using high-pressure CO_2 as catalyst.[a]

Entry	T (°C)	t (min)	[Xylose] (g/L)	$V_{aq.}$ mL	V_{THF} mL	X_{Xylose} (mol %)[b]	$Y_{furfural}$ (mol %)[b]	$S_{furfural}$ (%)[b]
1[c]	180	60	18.8	15	0	54.0	24.5	45.5
2[c]	180	60	12.5	10	5	65.2	41.4	63.5
3	160	30	18.8	25	0	42.1	21.0	49.9
4	160	60	18.8	25	0	44.8	25.1	56.0
5	180	30	18.8	25	0	73.1	31.2	42.7
6	180	60	18.8	25	0	88.0	42.7	48.6
7	180	60	12.5	10	5	82.9	69.4	83.7
8	180	60	12.5	20	10	81.9	66.9	81.7
9	180	60	12.5	40	20	68.3	17.9	26.3

[a] All CO_2-assisted dehydration experiments were carried out at 50 bar of initial CO_2 pressure. [b] For xylose conversion (X_{xylose}), furfural yield ($Y_{furfural}$) and reaction selectivity to furfural ($S_{furfural}$) definitions please refer to Appendix A. [c] Reactions performed without CO_2.

Also the selectivity of xylose dehydration is driven by the presence of CO_2. A maximum 83.7 % selectivity was achieved when CO_2 and THF were used together (entry 7). This result again confirms the dual adjunctive character of CO_2 and THF on dehydration of xylose to furfural. The formed carbonic acid accelerated the conversion of sugars and THF acting as extracting solvent reduces the occurrence of secondary loss reactions (*e.g.* products of condensation – humins).[17] It is possible because as little as 30 bar of CO_2 makes a phase splitting separating furfural from water to THF-expanded CO_2-rich phase.[18] This, due to an advantageous partition of furfural between THF- and water-phases, helps to extract furfural from aqueous to organic gaseous phase protecting furfural from further conversion and degradation reactions in aqueous phase.

The preliminary tests showed that the reaction temperature is another important factor influencing the furfural production. Considering the results from entries 3-6, the xylose conversion doubled and furfural yield increased from 25.1 mol % to 42.7 mol % with an increase of temperature by only 20 °C from 160 °C (entry 4) to 180 °C (entry 6) for 1 h reaction. One the other hand, the increase of furfural yield was accompanied by a decrease of reaction selectivity to furfural from 56.0 % to 48.6 %. The obtained results indicate that higher reaction temperature increased the xylose conversion, either to furfural or other products (*i.e.* formic acid and/or humins) often reported in literature.[17]

Entries 7-9 show that either furfural yield or selectivity of the reaction is sensitive on the amount of feed charged in the reactor. It seems to be obvious, because the headspace of the reactor diminishes by

increasing the amount of feed. Hence, at fixed initial temperature and pressure, less CO_2 was charged into the reactor and consequently less acidic environment could be generated. The highest furfural yield of 69.4 mol % was obtained for the lowest amount of feed charged, which corresponds to 83.7 % of reaction selectivity. Increasing 4 times the amount of feed charged into reactor either furfural yield or selectivity decreased to only 17.9 mol % and 26.3 mol %, respectively.

The effect of volumetric aqueous to organic ratio on the CO_2-assisted dehydration of D-xylose to furfural was investigated as is shown in Table 6.2. Varying the amount of H_2O and THF notable changes in composition of system occur, which influences the composition of both gaseous and liquid phases. The analysis of these changes and the knowledge of phase behaviour of H_2O:THF:CO_2 system (the phase envelopes for examined systems were obtained following the modelling of the reaction mixture according to procedure given in the Appendix A) confirm that the increase of V_{THF} in the reactive system favours CO_2 solubility in aqueous phase. This promotes more favourable conditions of acid-catalysed dehydration of xylose into furfural. For the highest $V_{aq.}/V_{THF}$ ratio (entry 11), the xylose conversion was relatively high (73.8 mol %) but the furfural yield was only 45 mol %. Decreasing the $V_{aq.}/V_{THF}$ ratio from 12:3 to 10:5 (entry 11 and 7), both xylose conversion and furfural yield increased up to 82.9 mol % and 69.4 mol %, respectively. Conversion of xylose reached its maximum of 100 mol % when $V_{aq.}/V_{THF}$ ratio was 3:12 (entry 14) but simultaneously only 45.4 mol % of furfural yield was achieved. Hence, higher V_{THF} in reactive system adjuncts to achieve higher xylose conversion, however about certain value the excessive amount of THF has negative effect on furfural yield and reaction selectivity as both decreases. Similar observation was made by Li et al. who found that when the amount of organic phase was higher than the aqueous phase, the production of humins was increased.[19]

Table 6.2. Furfural production by dehydration of D-xylose using high-pressure CO_2 as catalyst at various volumetric aqueous to organic solvent ratios.[a]

Entry	$V_{aq.}$	V_{THF}	X_{Xylose}	$Y_{furfural}$	$S_{furfural}$
	mL		(mol %)[b]	(mol %)[b]	(%)[b]
10	15.0	0.0	60.6	28.4	46.9
11	12.0	3.0	73.8	45.0	60.9
7	10.0	5.0	82.9	69.4	83.7
12	7.5	7.5	86.2	54.8	63.6
13	5.0	10.0	87.8	51.0	58.1
14	3.0	12.0	100.0	45.4	45.4

[a] All experiments were performed at the following conditions: 50 bar of initial CO_2 pressure, 12.5 g/L of xylose concentration in the feed, at 180 °C during 60 min of reaction time. [b] For xylose conversion (X_{xylose}), furfural yield ($Y_{furfural}$) and reaction selectivity to furfural ($S_{furfural}$) definitions please refer to Appendix A.

The behaviours found for 12.5 g/L of xylose concentration in the feed were similar also for two other studied (9.4 and 6.3 g/L) xylose concentrations as can be seen in Figure 6.1.

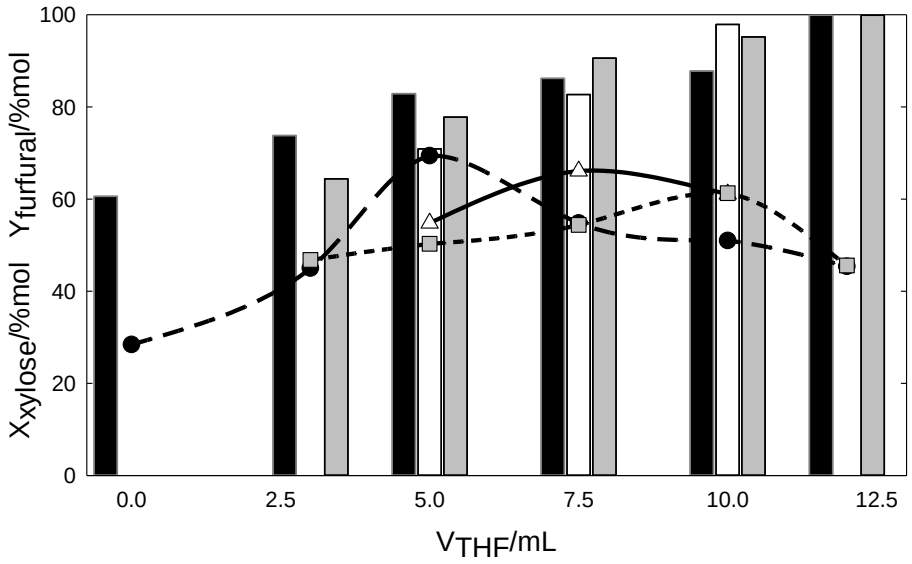

Figure 6.1. The evolution of xylose conversion (bars) and furfural yield (symbols) for various V_{THF} as a function of initial xylose concentration in the feed (black – 12.5 g/L, white – 9.4 g/L, grey – 6.3 g/L). All experiments were performed at the following conditions: 50 bar of initial CO_2 pressure, at 180 °C during 60 min of reaction time and at total volume ($V_{aq.}$+V_{THF}) of 15 mL. Lines are given as guide for the eye.

Besides the above discussed parameters, the furfural yield also depends on reaction time and initial xylose concentration in the feed as it is depicted in Figure 6.2. The obtained results show that at the initial stage of reaction, the furfural production was the fastest for the lowest xylose concentration; however the differences between concentrations especially for longer reaction times (*e.g.* 90 min) are negligible. However, prolonged reaction time had a negative effect on the furfural yield, especially for more concentrated solutions, because for 120 min of the reaction time, a decrease of furfural yield was observed mostly due to possible aforementioned side reactions.

The furfural yields presented in this work are in the similar range to those reported for xylose dehydration using mineral acid (HCl) as catalyst.[20] Gairola and Smirnova used different approach to investigate the hydrothermal dehydration of D-xylose to furfural with simultaneous furfural extraction with supercritical CO_2.[21] The maximal furfural yield from xylose was similar to those obtained in this work (68 mol % *vs.* 69 mol %) but it was achieved at severer reaction conditions (230 °C and 120 bar of CO_2 pressure) than those presented in this work.

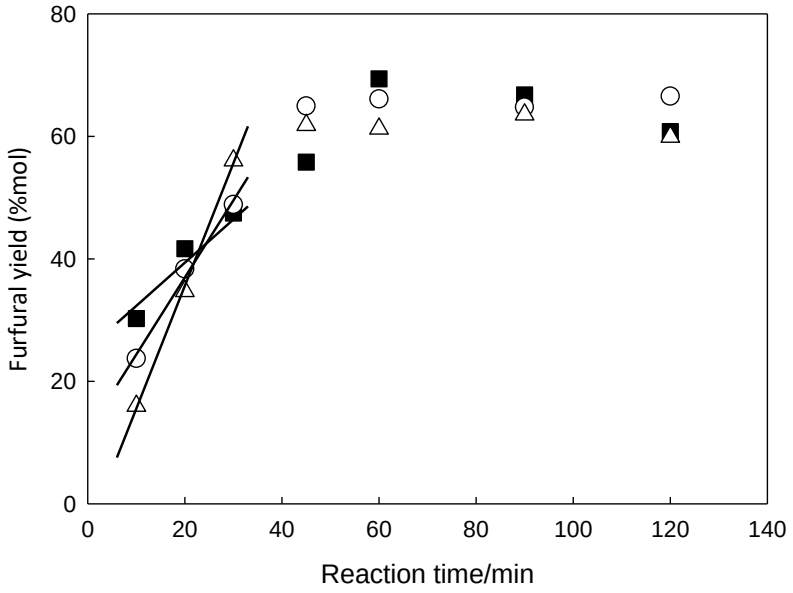

Figure 6.2. The evolution of furfural yield for various initial xylose concentrations (■ -12.5 g/L, ○ – 9.4 g/L, ∆ – 6.3 g/L) achieved over time in reactions performed at 50 bar of initial CO_2 pressure, 180 °C and $V_{aq.}/V_{THF}$ ratio of 10:5 mL/mL. Lines merely demonstrate the difference in the initial furfural production rate for various xylose concentrations.

6.3.Conclusions

Concluding, the efficient production of furfural in selective way using high-pressure CO_2 as catalyst in H_2O/THF system was reported. The combined adjunctive character of CO_2 in aqueous media and THF as extracting solvent enabled a simple operational procedure for xylose dehydration into furfural. This methodology led to obtain a xylose conversion above 83 mol % resulting in a final furfural yield of 70 mol %. The used approach does not require post-reaction neutralisation typical for acids and uses the most environmentally friendly solvents, such as CO_2 and H_2O, to obtain furfural yields similar to those achieved with mineral acids and with combined extraction systems.

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Chapter VII

New Approach to Ammonia Pre-treatment Integrates Better Feedstock Logistics with Improved Sugar Conversion

This chapter is based on the on the patent application in preparation and manuscript Ana Rita C. Morais, Thapelo Mokomele, Hui Dang, Venkatesh Balan, Bruce Dale, Rafal M. Lukasik and Leonardo da Costa Sousa. New Approach to Ammonia Pre-treatment Integrates Better Feedstock Logistics with Improved Sugar Conversion. Energy Environ. Sci., 2018. (in preparation)

7.1.Introduction

All over the world, non-renewable fossil resources (crude oil, coal and natural gas) have been used to produce a wide-range of fuels, chemicals and materials. However, the extensive consumption of petroleum-based feedstocks has been driving our planet to serious environmental problems.[1] To mitigate this issue, the "biorefinery concept" has been receiving a growing interest as one of the most important sustainable options for the replacement of petro-chemical technologies.[2] Herein, renewable biomass must be used in an effective and sustainable way that is *sine qua non* to guarantee a smooth transition from fossil to a more competitive bio-economy. Lignocellulosic biomass has been typically acknowledged as one of the most promising renewable carbon feedstocks for the delivery of biofuels, biochemicals and biomaterials on a feasible scale.[3,4] To convert lignocellulosic biomass into any of these commodities, a complex matrix of highly functionalised bio-based (macro)molecules, *i.e.* cellulose, hemicellulose and lignin, must be disrupted so that catalysts (*e.g.* enzymes) can easily depolymerise the polysaccharides into a stream of fermentable C_6- and C_5-sugars. However, the successful conversion of lignocellulosic biomass is highly dependent on the development of an effective pre-treatment technology aiming to produce fermentable sugars at affordable costs. Numerous pre-treatments have been developed and some of them are able to disrupt the biomass matrix and decrease the crystallinity of cellulose prior to enzymatic hydrolysis.[5] Unfortunately, most of these pre-treatments are energy-demanding, requiring high chemical inputs, while promoting moderate sugar yields.[6] These bottlenecks, which often jeopardize the economic viability of the biomass conversion process, have led to extensive research on more advanced pre-treatment methods. Among these, ammonia-based technologies have been playing an important role. Ammonia fibre expansion (AFEX™) is one of the leading technologies with potential to be used at commercial scale in the biorefinery facility.[7] The major drawbacks of this technology are the considerable operating pressures and the need for ammonia recycling, which represent a significant CAPEX (Capital Expenditure) and OPEX (Operating Expenditure) that may hinder its commercialisation. In addition, AFEX™ pre-treatment is only effective on grasses (mainly monocots), which limits the usage of this technology in areas where woody biomass or mixed herbaceous dicots are present. AFEX™ does not require the addition of external heat during pre-treatment, as it takes advantage of an exothermal reaction between the water soaked into the biomass and ammonia to reach temperatures of about 140 ºC. As this mechanism requires a considerable amount of water (typically 60% of biomass dry weight), AFEX™ pre-treatment is not able to convert native cellulose I (CI) into the highly digestible cellulose III (CIII) allomorph. In the view of ammonia´s potential to decrease the recalcitrance of lignocellulosic biomass, extractive ammonia (EA) technology has been developed by da Costa Sousa et al.[8] This pre-treatment, characterised by the use of liquid ammonia at low water content (~10 %), combines the benefits of ammonolysis of cell wall ester cross-linkages, conversion of CI to CIII and removal of lignin from the biomass plant cell wall. The authors have reported higher fermentable sugar yields over those obtained during AFEX™, whilst using 60 %

lower enzyme loading.[8] However, the need of external heating coupled with high pressures (~1250 psi), required to maintain ammonia in liquid state, raises concerns about the economic feasibility of EA pre-treatment. Furthermore, a common drawback associated to EA is the need of high ammonia-to-biomass loadings, which leads to an increase of OPEX (Operational Expenditure) for energy expenses during ammonia recovery.[8] Also, Beckham's group has developed two-step process in which corn stover was pre-treated with anhydrous ammonia followed by mild NaOH extraction.[9] The authors reported great glucan and xylan enzymatic conversion yields approaching 100% and 75 %, respectively, at relatively low enzyme loading (4.5 mg/g glucan) for 72 h at 1 % solid loading. Despite the unquestionable good results, it should be highlight that there is no information regarding the pressures applied in ammonia pre-treatment step. In addition, very low solid loading experiments during enzymatic hydrolysis makes the obtained results unclear at industrially relevant conditions. Furthermore, a second step, NaOH extraction, is translated into higher chemical input, which is a clear disadvantage of this process.

Herein, we propose using a new technology, called Compacted Biomass with Reduced Ammonia (COBRA) pre-treatment, to pre-treat pelletised sugarcane bagasse under operational conditions that allow conversion of native crystalline cellulose I_β into highly digestible CIII. This technology aims to increase pre-treatment solid loading through the usage of pelletised biomass, which has significant influence on reducing ammonia loading, reactor sizing and associated energy and chemicals costs. In this work, we subjected pelletised sugarcane bagasse to a wide range of COBRA pre-treatment conditions, to determine the effect of temperature, ammonia loading and residence time on sugar yields. A set of selected conditions that maximised total sugar yields, whilst minimising ammonia and enzyme loadings as well as pressures applied to the system, were used to investigate the performance of COBRA pre-treated biomass under high-solid loading enzymatic hydrolysis conditions followed by fermentation. The obtained results, in terms of fermentable sugar and ethanol yields, were compared to those achieved by mature technologies *i.e.* AFEX™ and Steam-Explosion (StEx), using the same exact substrate. Lastly, the robustness and efficiency of COBRA to handle different sources of biomass, including hardwoods, herbaceous dicots and monocots, for the production of fermentable sugars was also addressed.

7.2.Materials and Methods

7.2.1. Raw materials and chemicals

Sugarcane bagasse composed of 39.5 ± 0.4 % glucan, 25.2 ± 0.1 % xylan and 19.4 ± 0.1% lignin was collected from two industrial South African sugarcane sources located in Malelane (TSB Sugar, Mpumalanga) and Mount Edgecombe (SASRI, Kwazulu Natal). The bagasse was milled through a disk mill (Condux LV15M, Netzch-Condux GmbH, Germany) equipped with a 20mm screen. The size-reduced bagasse samples were sieved in a stacked-sieve system to remove mineral impurities (*e.g.*,

sand), bagasse pith and fines smaller than 600μm x 600μm. Prior to pelletisation, the sugarcane bagasse was milled through a 40 mesh screen.

Corn stover (Pioneer 36H56) composed of 33.7 ± 0.6 % glucan, 25.4 ± 0.5 % xylan and 14.4 ± 0.8 % lignin was harvested from Michigan State University farms (Lansing, MI) in November 2014 and milled through a 40 mesh screen. *Miscanthus* x *giganteus* composed of 44.0 ± 0.1 % glucan, 17.9± 0.4% xylan and 21.8 ± 0.6% lignin, produced at Michigan State University farms (Lansing, MI), was harvested in the Spring of 2014 and milled through a 40 mesh screen prior to further usage. Prairie cord grass composed of 42.1 ± 1.0 % glucan, 25.1 ± 0.6 % xylan and 18.1 % ± 0.2 %lignin was harvested in Brookings, SD in 2009 and milled through a 4 mm screen. Hybrid poplar (*Populus nigra* var. *charkoviensis* x *caudina* cv. NE-19) composed of 34.9 ± 0.2 % glucan, 12.7 ± 0.1 % xylan and 25.3 ± 1.2 % lignin was harvested at the University of Wisconsin Arlington Agricultural Research Station in 2010 and milled through a 20-mesh screen prior to further usage. All the feedstocks were stored at 4 ºC in zip-lock bags before usage.

Anhydrous liquid ammonia cylinders equipped with a dip tube were procured from Airgas (Radnor, PA, USA) for ammonia pre-treatment. Solvents, sugar standards, acids and bases were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cellic® CTec3 (197 mg protein/mL, batch number VDNI0002) and Cellic® HTec3 (171 mg protein/mL batch number VIN00001) enzymes were kindly donated by Novozymes North America, Inc. (Franklinton, NC, USA) and Multifect Pectinase (72 mg/mL, batch number 4861295753) enzyme was kindly donated by DuPont Industrial Biosciences (Palo Alto, CA, USA). The protein concentration in enzyme solutions was determined using Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Cooperative Inc., Ithaca, NY, USA).[10]

7.2.2. Biomass densification

All untreated biomasses, including sugarcane bagasse, corn stover, poplar, Miscanthus and prairie cord grass, were pelletised using a Buskirk Engineering PM810 (Ossian IN) flat die pellet mill. Firstly, both roller and dye were heated up to 70 ºC by passing AFEX™ pre-treated corn stover through the die. The untreated biomasses were mixed with water until they reached a moisture content of 25% (total weight basis). The moist biomass was stored in a closed container and placed at 4 ºC overnight so that the moisture could be fully absorbed by the biomass. The moist biomass was allowed to reach room temperature before being pelletised. No external binder was added as pellet adhesive. The pellets were collected into a plastic container and cooled down at room temperature. Next, they were oven dried at 50 ºC for 48 h and stored at room temperature in sealed plastic bags before usage.

7.2.3. COBRA pre-treatment of sugarcane bagasse

COBRA pre-treatment of sugarcane bagasse was performed in 33 mL in-house designed reactors coupled to a control unit to monitor and to control temperature. The details of the reaction system are given elsewhere.[8] The reactors were filled in with the desired amount of pelletised sugarcane bagasse (10% of moisture content (total weight basis)) along with ammonia charged into the reaction with a high-pressure syringe pump (Harvard Apparatus, model PHD 2000, Holliston, MA, USA). Once ammonia was loaded, the reactors were heated up to the desired temperature and maintained according to the reaction time. Both temperature and time were established by the experimental design. After reaching the desired reaction time, a slow (~2 min) release of ammonia out of the system was performed. Next, the pre-treated materials were transferred out of the reactor and left under the fume hood overnight to remove any residual ammonia. After drying, the moisture content of the pre-treated sugarcane bagasse was determined using a moisture analyser (A&D MX-50, A&D Engineering, Inc., San Jose, CA, USA).

COBRA pre-treatment for high-solid-loading enzymatic hydrolysis was carried out using an in-house built reactor of 700 mL with a similar design as the one of 33 mL. In the case of these reactors, a desired amount of sugarcane bagasse (dry weight basis) was added into the reactor and the ammonia was loaded gravimetrically by weighing the ammonia transferred from a pre-weighed vessel to the reactors. Immediately after filling the system with ammonia, the reactors were heated up and kept at the desired temperature for defined reaction time. All the subsequent steps were identical to those described for the small-scale reactors.

To assess the influence of lignin removal on enzymatic hydrolysis yields, COBRA pre-treatment was performed with lignin extraction, hereinafter referred to as COBRA – LE. COBRA – LE was carried out at the same operational conditions as regular COBRA pre-treatment. In COBRA – LE pre-treatment, the bottom of the reactor was connected to a high-pressure lignin collection vessel, whilst the top of the reactor was connected to a nitrogen line. After reaching the required reaction time, the ammonia was drained along with the dissolved lignin from the reactor to the lignin collection vessel. The exhaust valve from the lignin collector was slowly opened to remove ammonia from the system. Right after, the nitrogen was introduced through the top of the reactor to keep the pressure in the system approximately at 300 psi. This procedure allowed the nitrogen flowing through the system, and helped to flow the liquid ammonia with the dissolved lignin down to the lignin collector. After lignin extraction, the nitrogen flow was cut off to allow system releasing the pressure slowly. For mass balance purposes, the pre-treated sugarcane bagasse was transferred from the reactor to a pre-weighted tray, which was placed under the fume hood for 48 h to remove any potential traces of ammonia. The pre-treated sugarcane bagasse was weighted and its respective moisture content was measured as described above.

7.2.4. Experimental design for COBRA pre-treatment

A statistical design of experiments (DoE) was applied to assess the effect of temperature (X_1), reaction time (X_2) and ammonia-to-biomass ratio (NH_3:BM) (X_3) on conversion of glucan and xylan enzymatic into their respective monomers at 72 h. To achieve this, a Box-Behnken DoE was employed using software (Minitab Inc., State College, PA, USA) with 30 experimental points, including replicates and four centre point replicates with high and low values of temperature (100 ºC and 50 ºC), residence time (1 h and 6 h) and NH_3:BM (0.5:1 and 1:1 (g/g)), respectively.

A quadratic response was carried out on the experimental data as a function of temperature, residence time and NH_3:BM ratio (g/g) as independent variables. The interactions between all independent variables were considered in the response surface design. The parameters describing the effect of those variables were considered according to their statistical significance, *i.e. p*-value ($p<0.05$) and model predictive ability ($R^2_{predicted}$). The regression equations describing the response surface design were used to predict the responses of the various effects within the range of experimental domains.

7.2.5. Low-solid-loading enzymatic hydrolysis

Low-solid-loading enzymatic hydrolysis was performed aiming to evaluate the effect of COBRA operational conditions on both glucan and xylan conversion into their respective monomers. For this purpose, enzymatic hydrolysis was performed in 20 mL screw-cap scintillation vials with 1 % glucan loading (w/w, glucan) and 15 mg of protein/g of glucan in 50 mM citrate buffer (pH 4.8), with 15 mL of reaction volume, and incubated at 50 ºC in an orbital shaking incubator (New Brunswick, USA) at 250 rpm for 72 h. Sodium azide (0.02 % w/v) was added as antibiotic to prevent any microbial contamination during the enzymatic reaction. The enzymes used herein were Cellic® CTec3, HTec3 and Multifect Pectinase. The enzyme ratios (dry weight basis) were 68 wt.%, 22 wt.% and 10 wt.% for CTec3, HTec3 and Multifect Pectinase, respectively. These ratios were previously optimised to maximise total sugar conversion on AFEX™-pre-treated sugarcane bagasse as described elsewhere.[11] After 72h of enzymatic hydrolysis, the hydrolysates were filtered through a 0.2 µm filter, and soluble sugars, mainly glucose and xylose, were determined using an HPLC equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as previously reported.[12]

7.2.6. High-solid-loading enzymatic hydrolysis

For the purpose of evaluating the pre-treatment potential under industrially relevant conditions, the enzymatic hydrolysis experiments of COBRA, COBRA – EL and EA pre-treated sugarcane bagasse were performed at high solid loading (6 % glucan loading, w/w). The experiments were carried out in duplicate in 250 mL Erlenmeyer flasks with 100 mL reaction volume, in 50 mM sodium buffer (pH 4.6), and incubated at 50 ºC in an orbital shaking incubator (New Brunswick, USA) at 250 rpm for 96 h. Chloramphenicol (50 µg/mL) was added to prevent any microbial contamination during the enzymatic

and fermentative reaction as well. Previously optimised cocktail of CTec3, HTec3 and Multifect Pectinase was used on a dry protein weight basis for COBRA-pre-treated sugarcane bagasse at various enzyme loadings of 15, 10 and 7.5 mg protein/g glucan (data not shown). In the first 6 h of enzymatic hydrolysis, the pH was monitored and if needed adjusted to 4.8 using 12.1 M HCl at every 2 h. The blank reactions for both substrate and enzyme complexes were carried out at the same experimental conditions. At the desired enzymatic hydrolysis time (24, 48, 72 and 96 h), 0.5 mL of sample was taken, incubated at 95 ºC for 10 min (Eppendorf, Westbury, USA) to denature the enzymes, centrifuged for 4 min at 3500 rpm. The supernatant was sampled, diluted (10-fold), filtered through a 0.2 µm filter and analysed for monomeric sugars as described elsewhere.[12] After 96 h of enzymatic hydrolysis, the slurry was centrifuged at 10000 g for 30 min to separate the remaining solids from the hydrolysate. The solid streams were washed with 100 mL of water, centrifuged at 10000 g for 30 min and the washing water was analysed in terms of sugar content for mass balance closure purposes. The washed solids were then dried in a freeze-dryer for 72 h before being subjected to compositional analysis. A sample of the hydrolysate was taken, processed and analysed for monomeric and oligomeric sugar content. Due to the presence of soluble oligosaccharides in hydrolysates, an acid hydrolysis procedure for estimating the oligomeric sugar content was performed as recommended by NREL/TP-510-42623.[13] The oligosaccharide content was determined from the increase in concentration of the monomeric sugars after acid hydrolysis. In preparation for fermentation, the pH of the hydrolysates was adjusted to 5.5 using 10 M KOH, sterilised using a 0.22µm filter and stored at 4 °C.

7.2.7. Fermentation

The genetically modified xylose-fermenting strain *Sacchromyces cerevisiae* 424A (LNH-ST) used in the fermentation experiments was kindly provided by Prof. Nancy W.Y. Ho, Purdue University. The seed culture of this strain was prepared in 250mL Erlenmeyer flasks containing 100 mL YPDX (75 g/L glucose, 25 g/L xylose, 10 g/L yeast extract, 20 g/L tryptone) seed culture medium. A frozen glycerol stock stored at -80 ºC was used for seed culture inoculation at an initial optical density of 0.1. The seed culture was incubated at 30 °C and 150rpm under micro-aerobic conditions for 18 hours. The seed culture reached at optical density (OD600) of about 12 within 18 hours. This seed culture was harvested and used as inoculum for fermentations of the various hydrolysates. The fermentations experiments were initiated with an initial OD_{600} of 2 (or initial yeast density of 0.96 g/L). Samples were taken at various time points during the fermentation and cell-free supernatants were submitted for HPLC analysis. The total ethanol yield was determined based on the sugar yield during enzymatic hydrolysis, the sugar consumption and metabolic yield during fermentation.

7.2.8. Chemical analysis

High-solid loading un-hydrolysed sugarcane bagasse solids were milled in a knife mill to a particle size of 0.5 mm and characterised for their carbohydrate and lignin content according to the NREL/TP-510-

42618.[14] The composition of carbohydrates was determined using Shimadzu HPLC system equipped with an Aminex HPX-87-H (Bio-Rad, Hercules, CA, USA) column at 50 ^{0}C that was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The same HPLC analysis conditions were used for the chemical analysis of water-soluble fraction after enzymatic hydrolysis and fermentation. Shimadzu refractive index detector (RID) was used to identify and to quantify glucose, xylose, arabinose, lactic acid and ethanol by means of external calibration. The acid insoluble lignin obtained after acid hydrolysis was quantified gravimetrically and then corrected for the acid insoluble ash that was determined by igniting the content at 550 °C for 5 h. The acid soluble lignin was determined by ultraviolet spectrophotometry of biomass acid hydrolysates at 320 nm and using the absorptivity of 30 $L\cdot(g\cdot cm)^{-1}$ as recommended in the literature.

7.2.9. X-ray powder diffraction (XRD)

XRD experiments were carried out on an X-ray powder diffractometer with its beam parallelised by a Gobel mirror (D8 Advance with Lynxeye detector; Bruker, Bruker AXS Inc., Madison, WI, USA). CuKα radiation (wavelength = 1.5418 Å) was generated at 40 kV and 40 mA. The detector slit was set to 2.000 mm. Sample was analysed using a coupled 2θ/θ scan type with a continuous PSD fast scan mode; 2θ started at 8.000° and ended at 30.0277° with increments of 0.02151°, 136 while θ started at 4.0000° and ended at 15.0138° with increments of 0.01075°. Step time was 1.000 sec (i.e., 1025 total steps, effective total time 1157 sec per run). Cellulose samples (approximately 0.5 g) were placed in a specimen holder ring made of PMMA with 25 mm diameter and 8.5 mm height, rotating at 5 degrees per minute during analysis.

7.3.Results and Discussion

7.3.1. Influence of COBRA pre-treatment on the performance of low-solid enzymatic hydrolysis

The release of sugars obtained during enzymatic hydrolysis of COBRA-pre-treated sugarcane bagasse is highly affected by the pre-treatment conditions, including temperature, residence time and ammonia-to-biomass ratio (NH_3:BM, g/g). Contour plots and regression models depicting the influence of operational conditions on glucan and xylan conversion into glucose and xylose, respectively, are shown in Figure 7.1A and B. The extent of glucan and xylan conversion yield was calculated based on the release of glucose and xylose after 72h of enzymatic hydrolysis.

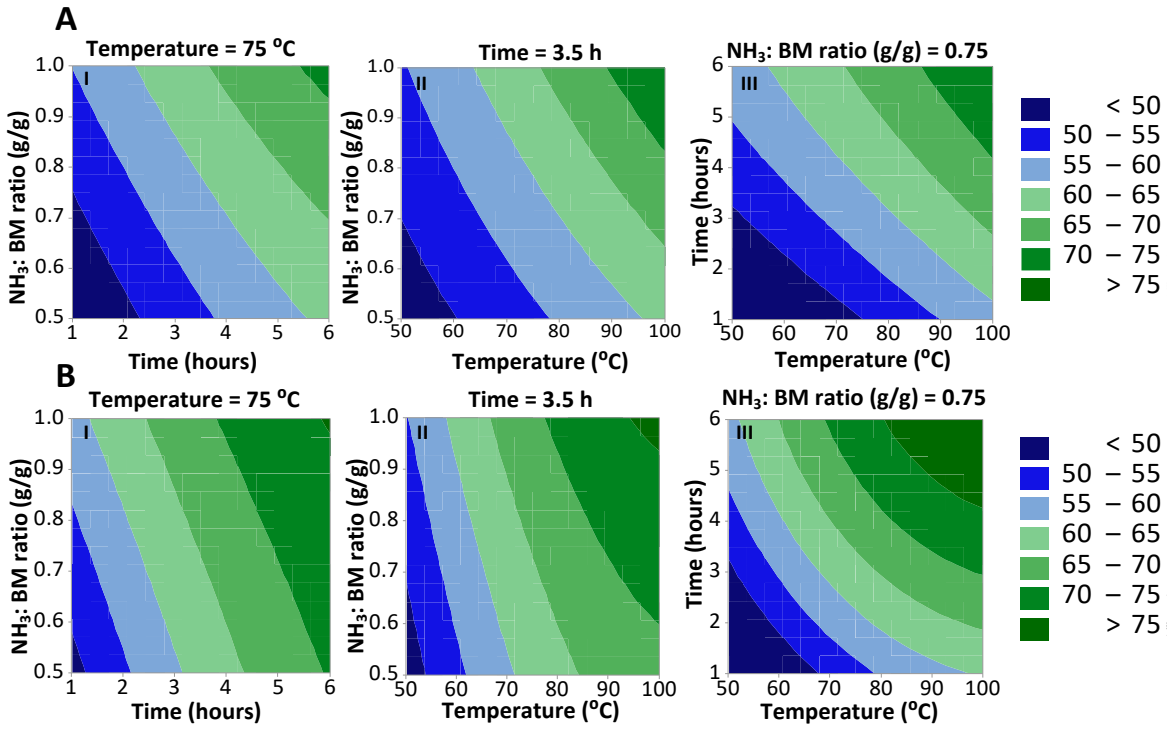


Figure 7.1. Enzymatic hydrolysis performance for pelletised sugarcane bagasse pre-treated under various operational conditions. Contour plots showing the influence of pre-treatment parameters (temperature, residence time and ammonia: biomass (NH_3: BM (g/g)) loading on 72h of enzymatic conversion of glucan to glucose (a) and xylan to xylose (b). Enzymatic hydrolysis experiments were carried out at 15 mg protein/g glucan with 1% glucan loading (w/w, glucan). The enzymatic cocktail composed of 68 wt.% CTec3, 22 wt.% HTec3 and 10 wt.% Multifect Pectinase, on a protein basis, as previously optimised by Mokomele et al.[11] for AFEX™-pre-treated sugarcane bagasse.

All pre-treatment variables studied were found to affect the release of fermentable sugars during enzymatic hydrolysis (Figure B1). Figure 7.1A and B depict that COBRA-pre-treated SCB at 75 ^{0}C for 3.5 h at 0.75:1 NH_3:BM (g/g) loading allowed up to 55-60% glucan and xylan enzymatic hydrolysis into their monomers. Significant improvements in carbohydrate susceptibility to enzymatic hydrolysis were observed at 100 ^{0}C, NH_3: BM loading of 1:1 (g/g) for 3.5 h of reaction time resulting in 78.6 ± 1.8% glucan and 80.6 ± 1.0% xylan conversion yields. While higher temperatures have been reported to speed up the de-esterification reactions, higher ammonia loading promotes the cleavage of ester linkages as well as the conversion of cellulose CI into CIII increasing the biomass susceptibility to enzymatic attack.[8,15]

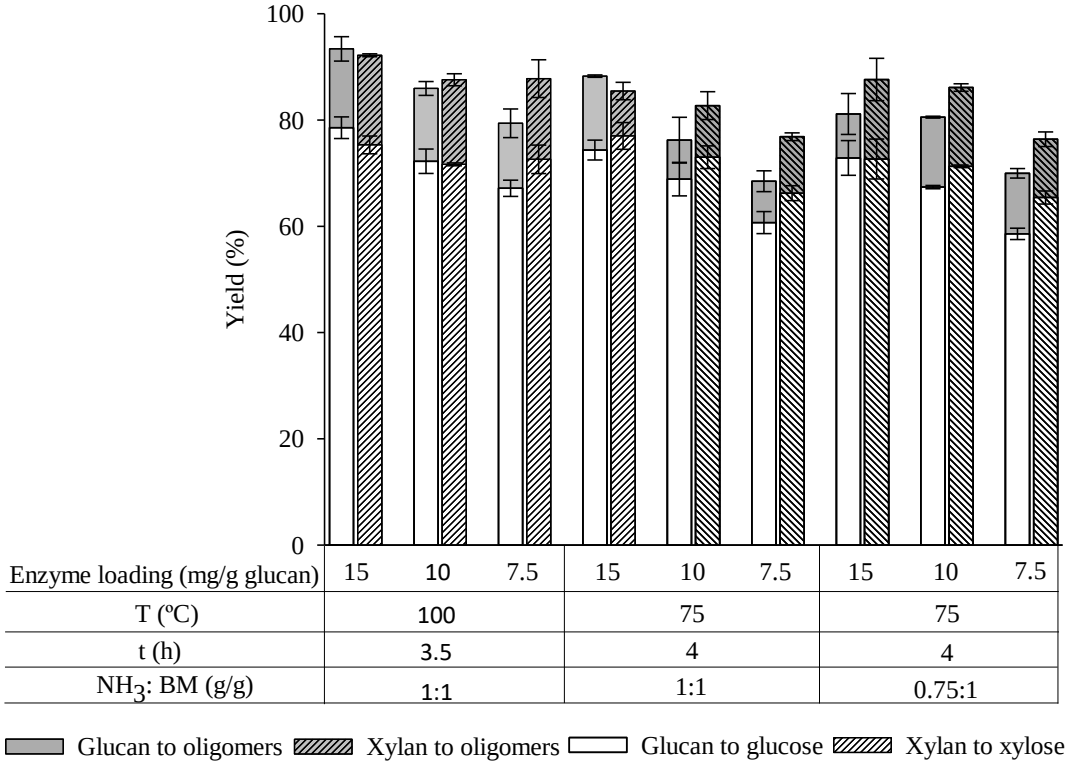
7.3.2. Potential of COBRA pre-treatment

7.3.2.1. Enzymatic conversion of sugarcane bagasse to fermentable sugars at high-solid loadings

To understand the potential benefits of COBRA pre-treatment for enzymatic hydrolysis, it is necessary to assess the pre-treated sugarcane bagasse under industrially-relevant conditions. Herein, various pre-treatment conditions were selected based on: i) operational parameters defined in Figure 7.1A and 7.1B (100 ^{0}C, 3.5 h with 1:1 NH_3:BM (g/g)); ii) usage of low temperatures and pressures in order to decrease both CAPEX and OPEX (75 ^{0}C, 4 h with 1:1 NH_3:BM (g/g)) and iii) low ammonia loadings (75 ^{0}C, 4 h with 0.75:1 NH_3:BM (g/g)), which are beneficial in terms of energy savings for ammonia recovery operations. Furthermore, for COBRA pre-treatment, optimal commercial enzyme cocktail (71 wt.% CTec3: 23 wt.% HTec3: 6 wt.% Multifect Pectinase) was used to fully exploit the enzymatic hydrolysis performance in terms of combined sugar (glucose + xylose) yields. Figure 7.2 depicts the influence of COBRA pre-treatment conditions on glucan and xylan conversion over various enzyme loadings. It is well known that pre-treatment temperature impacts both enzymatic hydrolysis rates and yield (Figure B2 in Appendix B). High temperature COBRA pre-treatment with 1:1 NH_3:BM loading (g/g) at 15 mg enzyme/g glucan resulted in 78.6 ± 2.0 % glucan to glucose and 75.3 ± 1.7 % xylan to xylose conversion yields, which correspond to a total monomeric yield of 56.0 ± 1.9 kg/ 100 kg of SCB. The obtained results showed benefits of performing COBRA at high temperatures; however, high temperatures are defeat due to higher OPEX and CAPEX, which are associated to high energy inputs and operating pressures (~850 psi at 100 ºC), respectively. In addition, high pressures raise concerns about the COBRA pre-treatment feasibility in a continuous operation mode.[8] Thus, the optimal temperature for the enzymatic conversion of SCB must be a "compromise" between a temperature high enough to promote de-esterification reactions and, at the same time, a temperature as low as possible to minimise both OPEX and CAPEX.

Another important aspect of ammonia-based technologies is the amount of ammonia used in the pre-treatment step. SCB pre-treated with 0.75:1 NH_3:BM loading (g/g) resulted in a slight reduction of 1-3 kg of monomeric sugar/ 100 kg of SCB over those obtained when SCB was pre-treated with 1:1 NH_3:BM loading at 75 ^{0}C and 100 ^{0}C, even when not all native cellulose was converted into CIII (Figure B3 in Appendix B). When restricting COBRA pre-treatment temperature to 75 ^{0}C (~450 psi), longer residence times (~4 h) were needed to guarantee that all pelletised sugarcane bagasse was totally submerged and soaked in liquid ammonia, so that all ammonia-related reactions can take place effectively and high enzymatic hydrolysis yields are assured. COBRA-SCB pre-treated at 75 ^{0}C with 1:1 NH_3:BM loading for 4 h at 15 mg enzyme/g glucan, resulted in a total monomeric yield of 54.7 kg ± 3.1/ 100 kg of SCB, which corresponds to a very similar yield to that found for COBRA-SCB pre-treated at 100 ^{0}C (56.0 ± 1.9 kg/ 100 kg of SCB).

A.



B.

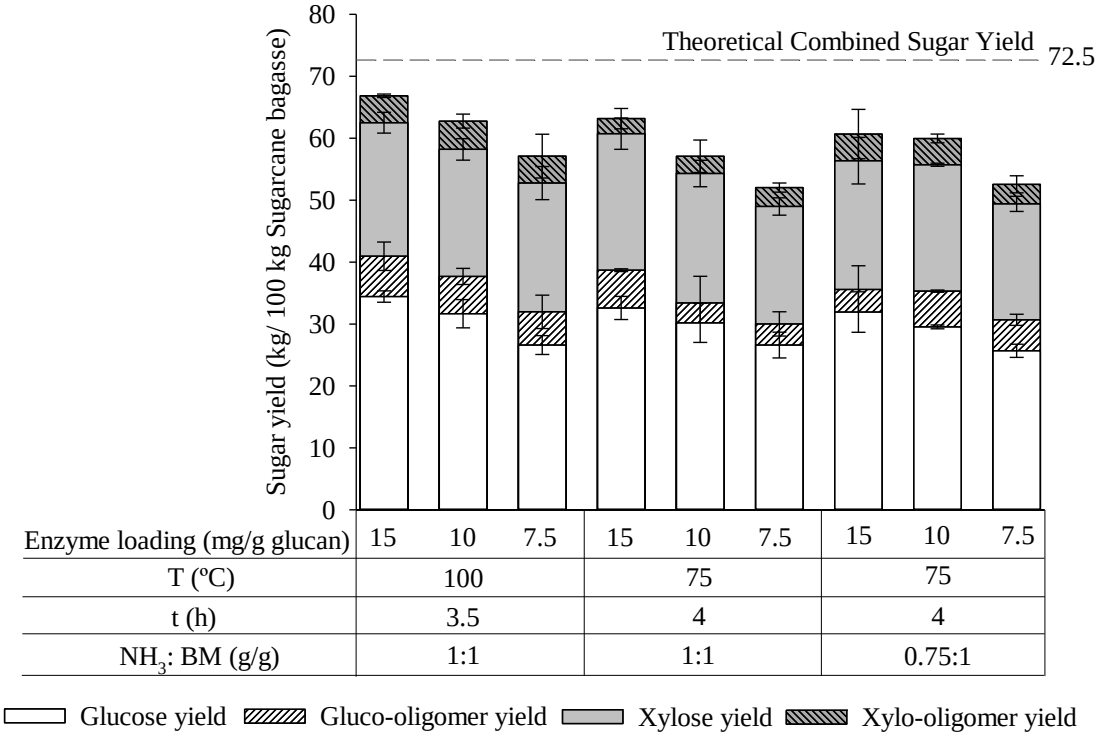


Figure 7.2. Influence of COBRA pre-treatment conditions on sugarcane bagasse enzymatic hydrolysis performed under various enzyme loadings based on (A) glucan (glucose and gluco-oligomers) and xylan

(xylose and xylo-oligomers) conversion yields and (B) total sugar yield on the basis of 100 kg of untreated sugarcane bagasse. All enzymatic hydrolyses were performed using the optimised enzyme cocktail (71 wt.%, 23 wt.% and 6 wt.% for CTec3, HTec3 and Multifect Pectinase, respectively). The solid loading was kept at 6% glucan loading (w/w glucan)), pH 4.8, and incubated at 50 ºC for 96 h.

Another important aspect is the substantial oligosaccharide accumulation, which represents a potential yield loss. High oligomeric sugar yields, reaching up to 15% and 17% for gluco- and xylo-oligomers, respectively, were found at 100 ^{0}C, 1:1 NH_3:BM loading (g/g) for 3.5 h. This is equivalent to a sugar yield loss of about 8-14 kg of monomeric sugars per 100 kg of dry sugarcane bagasse when compared to theoretical yield if oligosaccharides would be converted to respective monosaccharides. Similar results were found for other operational conditions indicating that commercial enzymatic cocktail is devoid of some complementary activities that might be required to further improve the total monomeric yields.[16] A similar phenomenon has been reported in literature, for ammonia-based technologies such as AFEX™, ionic liquids and dilute-acid hydrolysis.[16] Thus, more extensive research to understand the nature of these oligosaccharides, which remain after pre-treatment and enzymatic hydrolysis, is highly desired.

The use of high enzyme loadings enhances the fermentable sugar yields. However, considering the economics of the process it is undesirable. Thus, the effect of different enzyme loadings (15, 10 and 7.5 mg/ g glucan) was explored at the same enzymatic conditions. For all reaction conditions studied, the performance of high-solid loading enzymatic hydrolysis was greatly impaired by the decrease of enzyme loading. For example, at 7.5 mg enzyme/g glucan, a decrease of about 15 % on total sugar yield per 100 kg of sugarcane bagasse was observed over those found for 15 mg enzyme/ g glucan. However, to achieve similar sugar yields as those obtained under high enzyme loadings, higher pre-treatment temperature or ammonia loadings could be needed. Therefore, the ultimate choice between pre-treatment options and enzyme loading will depend on a techno-economic analysis.

The strategy for improving carbohydrate conversion, without using higher enzyme loadings and/or more severe pre-treatment conditions, was to perform COBRA with lignin extraction (COBRA-LE). The process relies on the pre-treatment of SCB with subsequent lignin removal as described in the Experimental section. COBRA-pre-treated SCB under 100 ^{0}C and 75 ^{0}C with 1:1 NH_3:BM (g/g) loading were used as control. For COBRA-LE conducted at 100 ^{0}C, an effective lignin delignification of 30% was achieved, whist at 75 ^{0}C a substantial decrease on the delignification yield up to 19% was observed. Figure 7.3. depicts the influence of delignification extension on glucan and xylan conversion yields obtained for COBRA and COBRA-LE under different pre-treatment conditions.

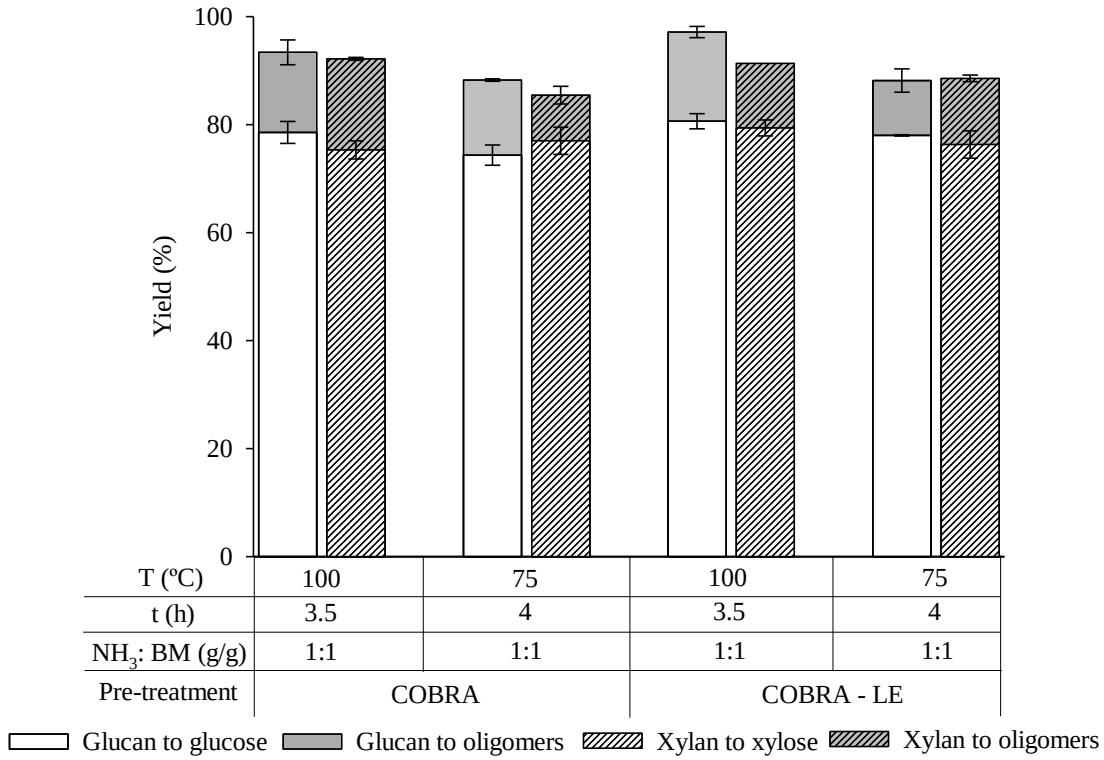


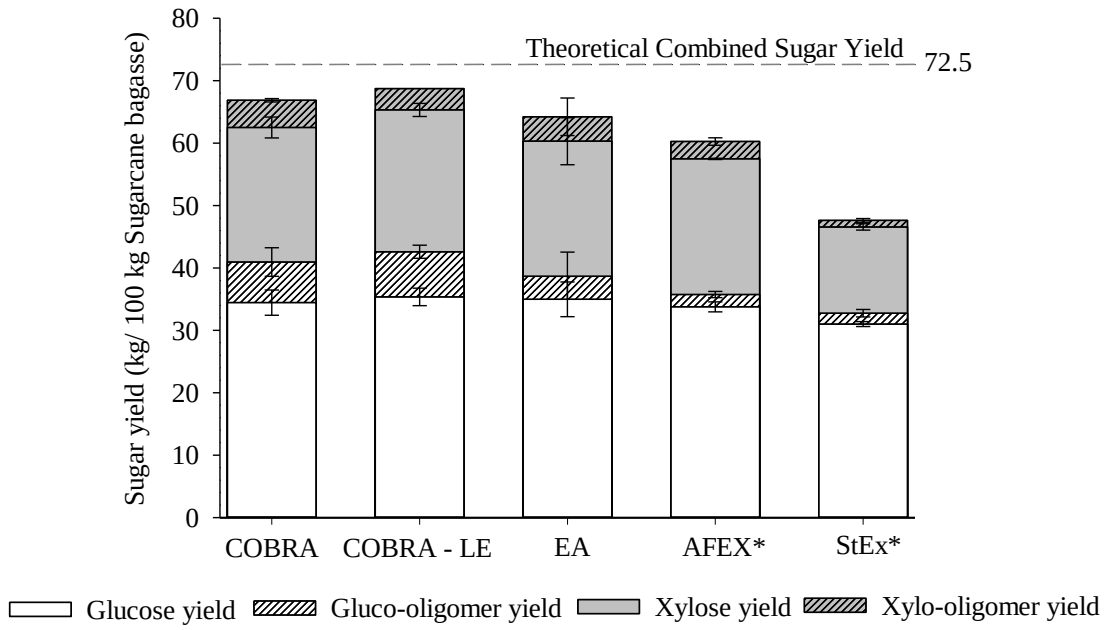
Figure 7.3. Effect of lignin extraction on total glucan (glucose and gluco-oligomers) and xylan (xylose and xylo-oligomers) conversion yield. COBRA was used as a control to evaluate the benefits of lignin extraction in high-solid loading enzymatic hydrolysis of pelletised sugarcane bagasse.

At 100 °C, COBRA-LE resulted in an improvement of total glucan conversion by 4 % over that found for COBRA, yielding 97 % overall glucan conversion. Regarding COBRA-LE performed at 75 °C, no significant improvement on total glucan conversion was found, probably due to low delignification yield. This comparison demonstrates the advantages of extracting lignin to obtain higher glucan conversion yields without increasing enzyme and/or ammonia loadings. The high sugar yields associated with COBRA-LE can be attributed to relatively good ability of ammonia to dissolve lignin, which contributes to an increase of the susceptibility of biomass surface to enzymatic hydrolysis. The achieved glucan conversion improvements, *via* lignin extraction, are in good agreement with those reported for corn stover pre-treated with EA. For instance, da Costa Sousa et al. reported an improvement of 6 % on glucan conversion over that found for EA (without lignin extraction), yielding 89 % overall glucan conversion from corn stover.[8]

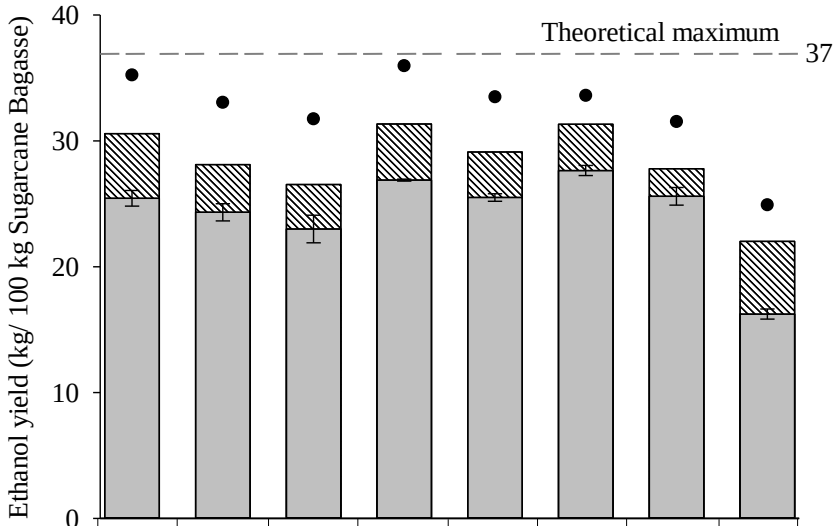
7.3.2.2.Benchmarking with other mature technologies

COBRA-LE pre-treatment was benchmarked with other technologies, *i.e.* EA, AFEX™ and StEx on the basis of the production of fermentable sugars (Figure 7.4A) and ethanol (Figure 7.4B). Loose sugarcane bagasse was used as feedstock for EA and COBRA was used as pre-treatment control.

A.



B.



Enzyme loading (mg/g)	15	15	15	15	15	15	25	25
T (ºC)	100	75	75	100	75	120	140	205
t (h)	3.5	4	4	3.5	4	0.5	1	0.225
NH_3: BM (g/g)	1:1	1:1	0.75:1	1:1	1:1	6:1	1:1	-
Pre-treatment	COBRA			COBRA - LE		EA	AFEX*	StEx*

Figure 7.4. Comparison of COBRA-LE with EA, AFEX™ and StEx (whole slurry) pre-treatments in terms of sugar and ethanol (EtOH) yields. (A) Total sugar yields (considering glucose, gluco-oligomers, xylose and xylo-oligomers). (B) Ethanol yields were calculated on the basis of 100 kg of untreated sugarcane bagasse input. COBRA was used as pre-treatment control. The theoretical maximum for sugar and ethanol yields was calculated based on the initial glucan and xylan contents in untreated sugarcane

bagasse. AFEX* and StEx* sugar and ethanol yields were obtained by Mokomele et al.[11] ¤The potential ethanol yield from oligomers was estimated based on the metabolic yields and sugar consumption obtained in each operational condition (Table B1 in Appendix B). ¥The potential ethanol yield from soluble sugars was estimated considering the complete conversion of soluble sugars into ethanol with the highest metabolic yield obtained (97.5%). All the enzymatic liquors were produced at 6% glucan loading (w/w, glucan) for 96 h of hydrolysis time. COBRA, COBRA-LE and EA enzymatic hydrolysis were performed with 15 mg/ g glucan, while AFEX™ and StEx were carried out with 25 mg/ g glucan.

These pre-treatments were chosen taking into consideration that: EA is an alkaline-type pre-treatment with a similar reaction mechanism to COBRA-LE, *i.e.*, extracts lignin and modifies the cellulose crystalline structure. In addition, EA has been reported as the most effective ammonia-based pre-treatment for fermentable sugars and ethanol production.[8] AFEX™ is an alkaline-based technology with established maturity and, unlike COBRA and EA, does neither lead to CIII formation nor lignin removal. Finally StEx is another industrially used technology, has been selected to benchmark alkaline and acidic pre-treatments. Remarkably, COBRA-LE-pre-treated SCB resulted in the highest total sugar yield of approximately 68.8 ± 1.8 kg / 100 kg of SCB. This sugar yield represents a pronounced improvement as the theoretical maximum sugar yield for is 72.5 kg/ 100 kg of SCB. However, 10.1 ± 1.0 kg of 100 kg of SCB (out 68.8 kg/ 100 kg SCB) corresponded to oligomeric sugars. Although EA showed good performance in the removal of lignin and modification of CI into CIII at a temperature of 120 ^{0}C with 6:1 NH_3:BM loading (g/g), it did not perform as well as COBRA-LE. EA pre-treatment generated a total sugar yield of 64.2 kg/ 100 kg of SCB, which corresponds to 7% lower yield than that found for COBRA-LE. This interesting result can be explained by the densification impact on biomass structure, which subsequently affects pre-treatment and enzymatic hydrolysis processes.

Though densification helped to improve the pre-treatment effectiveness, the densification process alone could not improve the enzymatic yields, as the untreated loose (used as a control) and untreated pelletised SCB revealed similar glucan conversion yields (15% vs. 16%, respectively) (data not shown). Thus, understanding the potential synergies between densification and pre-treatment processes is crucial to unveil improved methods of deconstructing lignocellulosic biomass. It should be remarked that literature data available demonstrates that pelleting at high temperatures improves sugar conversion for either alkaline or hydrothermal pre-treatments.[17] For instance, Guragain et al. reported greater sugar yields with alkali-pre-treated densified biomass than with loose biomass.[18] However, high temperature densification entails additional processing costs, which shall be minimised to contribute for the economy of the biorefinery.

Unlike the results obtained with COBRA-LE and EA, both AFEX™ and StEx showed lower ability for the production of fermentable sugars. The AFEX™-treated SCB with 25 mg/ g glucan of enzyme loading resulted in a total sugar yield of 60.3 ± 1.1 kg / 100 kg of SCB, which corresponds to a decrease of 14 % over that obtained for COBRA-LE. This result stresses the importance of CIII formation along

with lignin extraction for the improvement of sugar yields, while decreasing the enzyme loadings. Lastly, StEx is characterised by moderate sugar yield (47.6 ± 0.9 kg/ 100 kg SCB), even with 25 mg/ g glucan of enzyme loading. This low sugar yield is mainly due to lower polysaccharide recovery after the pre-treatment step. StEx requires high reaction severities for improving cellulose digestibility leading to the degradation of sugars into, *e.g.*, furans, hampering the production of sugars at high yields. Besides, StEx-pre-treated leftover solids require substantial washing which leads to soluble sugar losses. Furthermore, unlike COBRA-LE and EA, there is neither formation of CIII nor lignin removal during StEX pre-treatment to help improve the susceptibility of polysaccharides to enzyme attack.

The ethanol yields from COBRA-LE-SCB at 100 °C with 1:1 NH_3:BM loading (g/g) and 15 mg enzyme/ g glucan was comparable to that found for EA at 120 ^{0}C with 6:1 NH_3:BM (g/g) (26.9 ± 0.0 kg vs. 27.6 ± 0.4 kg of ethanol/ 100 kg SCB, respectively). Both COBRA-LE and EA-pre-treated SCB hydrolysates are highly fermentable due to extraction of lignin-based inhibitors, while preserving the microbial nutrient availability. Although COBRA-LE offers slightly lower experimental biofuel potential, based on the available sugars, it has better performance than EA. It was performed at lower ammonia loadings (83 % reduction relative to EA) and pressures (850 psi vs. 1200 psi). This contributes to lower OPEX (ammonia recovering costs) and lower CAPEX (lower-pressure resistant pre-treatment unit). Taking into consideration that most soluble sugars obtained in COBRA-LE were fermented to ethanol with a metabolic yield of 97.5 %, the ethanol yield was as high as 36.0 kg/ 100 kg SCB, which represents a remarkable achievement in terms of pre-treatment performance. This hypothetically improved ethanol yield stresses the importance of development of yeast strains able to successfully convert sugar oligomers into ethanol. In addition, comparing COBRA-LE to AFEX™ and StEx, the removal of lignin-derived inhibitory compounds is one of the most significant advantages for the production of ethanol as well. For instance, for all COBRA-LE experiments an improvement of approximately 6% in ethanol yield was found. The importance of lignin extraction can also be observed when comparing COBRA to COBRA-LE. Interestingly, at 75 °C, COBRA-LE offered comparable ethanol yield to that found for COBRA at 100 ^{0}C. Hence, by simple lignin extraction, a decrease in the operational temperature (from 100 ^{0}C to 75 ^{0}C) and pressure (from 850 psi to 450 psi) could be achieved which, in turn, contributes to lower OPEX and CAPEX of the process, without compromising the ethanol yield.

As above-mentioned, the selection of pre-treatment operation conditions for effective fermentable sugar production and their conversion into ethanol will depend on the techno-economic analysis. Notably, COBRA and, ultimately COBRA-LE, lead to a remarkable improvement in ethanol yields over mature technologies such as AFEX™ and StEx, with enzyme loading savings of *ca.* 40 %. AFEX™ gave relatively high ethanol yield (25.6 ± 0.7 kg / 100 kg of SCB), while StEx hydrolysate showed very low performance (16.2 ± 0.4 kg ethanol/ 100 kg SCB) for the production of targeted biofuel. Although the liquid stream generated by StEx is composed by relatively high sugar concentration that could

potentially be fermented, the presence of inhibitory compounds, *e.g.*, furans, requires detoxification and nutrient supplementation to be effective for ethanol production.

7.3.3. COBRA as a feedstock- independent technology

Ideally, biorefineries for a large-scale bio-economy requires highly available, consistent and year round available source of feedstocks.[19] However, there is no single feedstock able to meet affordable prices at fixed high availability to guarantee the productivity and profitability of the biorefineries. In this sense, various biomass sources, including agro-industrial residues, woody and dedicated energy crops, are required to feed those large-scale biorefineries in most places across the globe. Yet, differences in chemical and physical properties between different biomass types have driven to limited development of such versatile industries.[20] Thus, the goal of this work was also to validate the robustness of COBRA pre-treatment to efficiently handle different types of feedstocks, regardless of their macromolecular composition and morphological structure, to produce high yields of fermentable sugars. Since one of the most important bottlenecks for the development of a commercially feasible pre-treatment is the potential need for feedstock-specific operational conditions, it is highly desirable to have a practical operational window for a wide-variety of feedstocks. In this respect, the COBRA pre-treatment conditions used herein for SCB were used without optimisation for other scrutinised feedstocks. Figure 7.5 summarises the impact of known COBRA operational condition (100 °C, 6 h and 1:1 NH_3: BM (g/g)) on the enzymatic digestibility of corn stover, sugarcane bagasse, prairie cord grass, miscanthus and poplar after 96 h of enzymatic hydrolysis. It indicates that COBRA generated combined sugar yields higher than 80 % for all the biomasses, despite their differences in terms of cell wall chemical composition and polysaccharide linkages presented in grasses and woody biomasses. As one of the few feedstock-independent pre-treatment technologies known, COBRA is capable to effectively process agricultural residues, energy crops and hardwoods. Additionally, COBRA works well with densified pellets of different types of biomasses, a requirement that only few pre-treatments can meet. To address the importance of developing appropriate enzymatic cocktails to maximise combined sugar yields, the effect of enzyme ratio on the performance of COBRA-pre-treated poplar for the production of fermentable sugars has been investigated as well. The highest combined sugar yield of 91.5 ± 1.0 % was obtained for “Enzyme 3” condition, which corresponds to an improvement of approximately 9 % in comparison to that found for “Enzyme 2” condition. These key findings suggest that it is the unquestionable importance of tailor-made enzymatic cocktail for a specific type of biomass. However, to accomplish large-scale biorefineries, intensive research should be focus on developing an effective and robust enzymatic cocktail capable of handling biomasses from a variety of sources, with minimal negative impact in terms of overall performance, fermentable sugar yield and biofuel production.

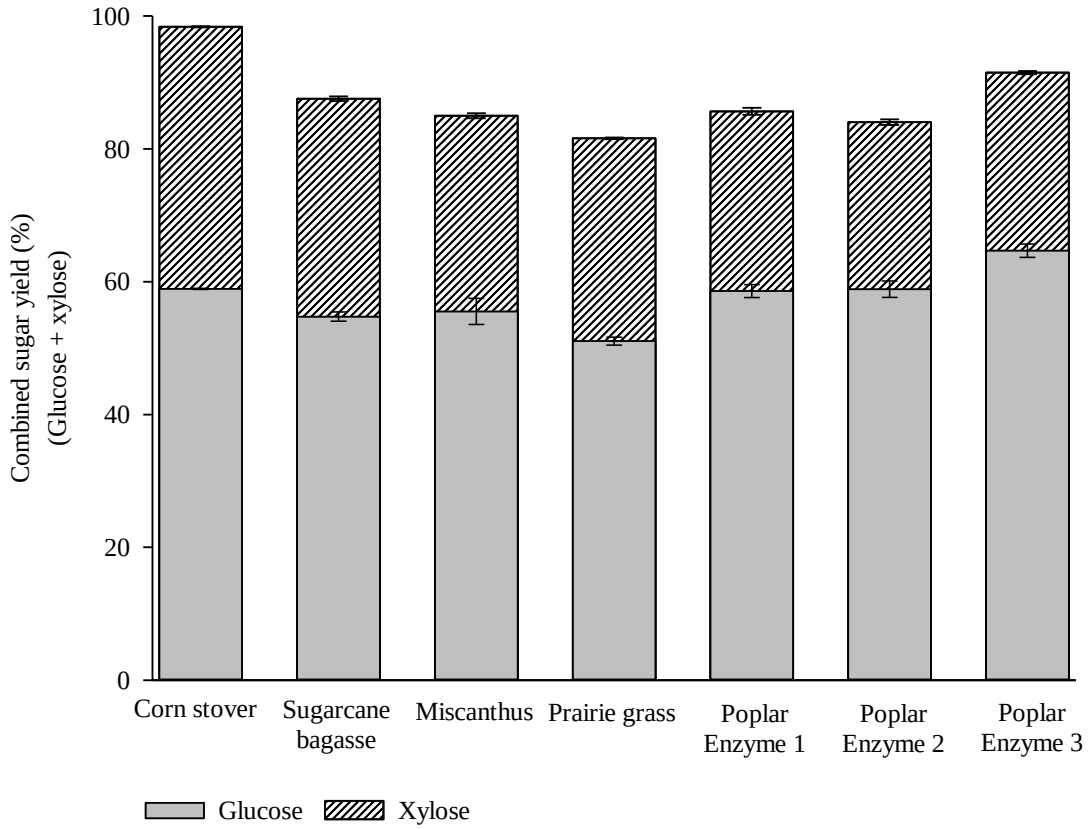


Figure 7.5. Performance of COBRA pre-treatment, at 100 ^{0}C for 6 h and with 1:1 NH_3:BM loading (g/g), for a wide-range of feedstocks on 96 h of enzymatic hydrolysis. Enzymatic hydrolysis experiments were carried out with 30 mg protein/ g glucan at 1% glucan loading (w/w, glucan). The enzymatic cocktails used for corn stover, sugarcane bagasse and miscanthus enzymatic hydrolysis experiments were composed of 71% CTec3: 23% HTec3: 6% Multifect Pectinase, on a protein basis, as previously optimised for COBRA-pre-treated sugarcane bagasse. In addition, the effect of changing enzyme ratio on enzymatic cocktails was also examined for poplar. Enzyme 1- 71 wt.% CTec3: 23 wt.% HTec3: 6 wt.% Multifect Pectinase. Enzyme 2 – 75 wt.% CTec3: 25 wt% HTec3: 0 wt.% Multifect Pectinase. Enzyme 3 – 50 wt.% CTec3: 20 wt.% HTec3: 30 wt.% Multifect Pectinase.

7.4.Conclusions

The present work takes a major step forward providing an effective and robust ammonia-based pre-treatment in terms of its performance of converting pelletised sugarcane bagasse to fermentable sugars for biofuels production. COBRA was developed to take full advantage of using densified biomass to reduce ammonia loadings, while simultaneously converting crystalline native cellulose I_β into highly digestible CIII allomorph. COBRA can be coupled with lignin extraction (COBRA – LE) to produce highly digestible sugarcane bagasse. This affects the COBRA-pre-treated biomass contributing to enzyme reductions *ca.* 40 % in comparison to two mature pre-treatment technologies – AFEX™ and StEx. COBRA-LE pre-treated sugarcane bagasse led to sugar yields comparable to EA pre-treatment, and achieved up to ~ 68.8 kg of fermentable sugars per 100 kg of untreated sugarcane bagasse (dry

weight basis). COBRA pre-treatment contribute to ammonia reduction of about 83 % compared to the most effective ammonia-based pre-treatment – EA. Furthermore, COBRA - LE pre-treatment can be effectively conducted at reduced temperatures (75 - 100 ^{0}C) and operating pressures lower than those used in EA (from 1200 psi down to 450 psi). COBRA-LE allows the production of hydrolysate with fermentabilities comparable to those produced by EA, and a maximum of 27.6 kg of ethanol per 100 kg of untreated sugarcane bagasse (dry weight basis). Ultimately, COBRA demonstrated to be a feedstock-independent technology capable to handle a wide range of biomass types, regardless of their macromolecular chemical composition and morphological structure, to produce high fermentable sugar yields.

7.5.References

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Chapter VII

Conclusions and Perspectives

8.1.Conclusions

Making a complex matrix of lignocellulosic biomass available for upgrading in posterior processes is one of the most cost- and energy-demanding step in the biorefinery. This dissertation aims to provide some potential solutions that in the future may help to overcome or at least minimise these significant hurdles. Therefore, two distinct methodologies of biomass processing are presented in this thesis. These technologies have a great potential as drivers for the valorisation of lignocellulosic polymeric fractions. Thus, the proposed solutions can aid to make the shift from fossil- to bio-based economies.

The main conclusions from the work embed in this thesis are presented below.

8.1.1. High-pressure CO_2/H_2O mixture

- Due to the additional acidic character of formed *in-situ* carbonic acid, high-pressure CO_2/H_2O confirmed to be advanced in comparison to liquid hot water pre-treatment in the hydrolysis of hemicellulose fraction of biomass.
- Higher yield of pentoses, either in monomeric or oligomeric form, over those found for liquid hot water was found. Furthermore, these higher yields were observed at less severe reaction conditions, namely temperature and reaction times.
- The very selective catalytic potential of high-pressure CO_2/H_2O towards hemicellulose fraction produces leftover enriched in cellulose and Klason lignin.
- The produced solid leftovers are highly susceptible to enzymatic hydrolysis, increasing the potential for more advantageous valorisation of null- or low-price feedstocks, such as wheat straw, in comparison to *e.g.* liquid hot water.
- The aforementioned catalytic effect of formed *in-situ* carbon acid is universal as it acts efficiently on the hydrolysis of pentose sugar, *i.e.* xylose. The effective dehydration of xylose to pivot compound, *i.e.* furfural, catalysed by high-pressure CO_2/H_2O mixture confirmed the applicability of this technology in a broader context of biomass valorisation.
- In addition to the catalytic effect of high-pressure CO_2/H_2O, high-pressure CO_2, in a presence of an organic solvent, plays also an important role of a stripping agent for freshly produced furfural. Thus, the reaction might be driven towards high yield of furfural with high selectivity of xylose conversion.

8.1.2. High-pressure ammonia

- One of the main limitations in the industrial biomass processing, namely the biomass loading per operation unit, could be solved by the biomass densification.

- The COBRA process takes full advantage of using densified biomass to reduce ammonia loadings. Simultaneously, the chemical character of ammonia converts crystalline native cellulose I_β into highly digestible allomorph form of cellulose CIII.
- The integration of lignin extraction to COBRA (called COBRA-LE) produces pre-treated sugarcane bagasse with improved enzymatic digestibility. This allows achieving significant enzyme reductions in comparison to mature pre-treatment technologies.
- Biomass densification in COBRA helps to reduce significantly, by 4/5 of ammonia loading, in comparison to the most effective ammonia-based pre-treatment, *i.e.* Extractive Ammonia.
- COBRA - LE pre-treatment produces a hydrolysate with fermentabilities comparable to those produced by Extractive Ammonia, whilst decreasing the temperatures and pressures of the process.
- COBRA demonstrated to be a feedstock-independent technology capable to handle a wide range of biomass types, regardless of their macromolecular chemical composition and morphological structure, to produce high fermentable sugar yields.

8.2.Perspectives[1]

This thesis contributes to the knowledge about the use of high-pressure fluids in the biomass processing. The results presented in this dissertation demonstrate a great potential for the use of these technologies in more advanced scale. However, some improvements are still required. The most relevant aspects that have to be addressed or are still insufficiently explored are: i) use of high-pressure fluids in a direct hydrolysis of untreated biomass; ii) employment of CO_2/H_2O approach for formation of value-added products from all biomass fractions; iii) the need for more advanced enzymes; iv) integrated valorisation of biomass; v) use of densified biomass; vi) the flexibility and robustness of the process for a multiple and diverse feedstock; vii) the analysis of the process taking into consideration all three pillars of sustainability.

i) A direct hydrolysis of untreated biomass in the presence of high-pressure fluids, such as CO_2 would allow the integration and intensification of the biomass valorisation. In this way, biomass would not require a cost-demanding pre-treatment step allowing the use of biomass directly for glucose production, which later could be converted to energy sources, such as biofuels or value-added products. Up to now, this challenging task has been barely tackled and only cellulose samples have been examined. It is important to stress out that this is one of the most important aspects in the economic and technological development of biomass processing, which also would

[1] The perspectives related to the use of high-pressure CO_2/H_2O mixture has been adapted from Ana Rita C. Morais, André M. da Costa Lopes and R. Bogel-Lukasik, Chem. Rev., 2015, 115, 3-27.

require advances in the enzyme engineering to develop more resistant and efficient biological catalysts.

ii) Lignin has been considered as a less valuable fraction of biomass. However, it serves as a source of polyphenols showing the great potential in the CO_2-assisted production of phenolic compounds directly from biomass. It should be also said that this approach is different from the already well-known and examined extraction of value-added phenolic-like compounds from specific plants for pharmaceutical or food purposes. Hemicellulose is also very often considered as a fraction with lower value. The reason for this is its diverse composition, which is strongly dependent on the biomass type and other external factors. This diversity makes the hemicellulose fraction valorisation challenging and less versatile but it gives room for numerous products that can be obtained, depending on the technique applied. Cellulose is mostly used for ethanol production but there are many other chemicals that can be produced from this feedstock. Some examples, *i.e.* 5-HMF, levulinic acid, *etc.*, show still unrevealed potential of high-pressure fluids' application in cellulose processing.

iii) Enzyme technologies made a significant progress since the development of use of ethanol as energy carrier. Although a great advance has been carried out, the reduction of enzyme charge per unit of processed biomass, still cost of such enzymes cocktails is rather high. This is still considered as one of the main drawbacks and limitations in the successful implementation of biorefinery concept. Besides, other important aspect might be the need for new enzymes able to hydrolyse some saccharides, mostly those in oligomeric form. The presence of these potentially upgradable sugars may not justify their extraction and valorisation as separate products as it was observed in case of COBRA. Thus, they could be hydrolysed to provide a monosaccharide stream richer in upgradable sugars.

iv) Lignocellulosic biomass is chiefly constituted by three main fractions: cellulose, hemicellulose and lignin. Up to now, the integrated valorisation of these three fractions at the industrial scale does not exist. The need for more research in this direction is crucial to provide the required knowledge in this field and to acquire enough maturity to advance toward pilot or demonstration scales of biorefineries.

v) One of the main and relevant limitations of the biomass valorisation is their low density, which hampers an efficient supply chain as well as requires bigger reactor volumes contributing to greater CAPEX (capital expenditure). The results of COBRA demonstrate that biomass densification is an option to overcome this bottleneck. However, due to the specific plasticizer properties of lignin, or the densification method used, the biomass pelletisation should be made in the way that does not obstruct the posterior valorisation of biomass.

vi) The scale of the biorefinery affects the techno-economic analysis of the process. In the normal practice, an increase of the scale works positively on the economics. However, this indicates that more feedstock is required. In many cases the availability of one type of feedstock is limited, *e.g.* in Europe, thus, the multi-feedstock biorefinery turns to be the only option. This requires versatile but robust technologies. COBRA process showed that there is a potential in this field and the use of multiple feedstocks can be applied with success. Nevertheless, research in this field is still required either for COBRA or for high-pressure CO_2/H_2O mixture.

vii) SWOT (Strengths, Weaknesses, Opportunities and Threats) analysis of any process is crucial to find out pros and cons of each technology. Besides, a comprehensive techno-economic analysis as well as Life Cycle Analysis are crucial to provide answers for fundamental questions about the future of high-pressure CO_2/H_2O technology in the biorefinery. Especially that in some sense, high-pressure CO_2/H_2O technology can take benefits from the successful industrial application of ammonia-based processes.

Appendix A to Chapter VI

Highly efficient and selective CO_2-adjunctive dehydration of xylose to furfural in aqueous media with THF

Experimental and material section

Material and chemicals

The CO_2 used in experiments was purchased from Air Liquid, AlphaGaz™ gamma, Paris, France with ≥ 99.99 % purity (w/w). For all reactions and chemical analyses the following reagents were used: distilled water (18.2 MΩ/cm) was produced by the PURELAB Classic Elga system and ethanol with 96 % purity (v/v) for gas phase capturing was acquired from Carlo Erba Group – Arese, Italy. The aqueous solution of xylose was originated from D-xylose (Sigma Aldrich) with 99.99 % of purity. Reagent THF (Merck, D-6100 Darmstadt) with purity of 99.5 % was used in all THF-assisted dehydration experiments.

Dehydration experiments

The acid-catalysed dehydrations were carried out in aqueous media in presence of THF as extracting solvent. The reaction system contained a mixture of aqueous solution of D-xylose and THF consisted of 15:0, 12:3, 10:5, 7.5:7.5, 5:10, 3:12 and 2:1 (v/v). The reactions were performed in a stainless steel 160 mL reactor (series 4655, Parr Instruments Company, Moline, Illinois, USA) coupled with Parr 4842 unit used to control and monitor reaction parameters (pressure, temperature and stirring). An external fabric mantle was used to heat the reactor, while an internal stainless steel loop was used to cool the system with cold water. The xylose dehydration experiments were carried out at established isothermal conditions (160 °C and 180 °C) and various holding times (from 10 min to 120 min). In all experiments where CO_2 was used as catalyst, the initial CO_2 pressure was 50 bar. All solutions were mixed at constant speed (70 rpm) using a magnetic drive. In an effort to decrease the CO_2 density variations due to changes of initial temperature, the reactor was pressurised with CO_2 with initial temperature of -9 °C and the reaction was started when the temperature of mixture was 22 °C. When the final holding time was reached, the reactor was immediately cooled down to quench the reaction. A slow depressurisation of reaction mixture was performed when temperature was lower than 25 °C. The depressurised gaseous phase passed through a vial filled into 5 g of ethanol placed in the ice bath and later analysed as described below.

Chemical analysis

The liquid and gaseous phases were analysed separately by running on High-Performance Liquid Chromatography (HPLC) using an Agilent 1100 series HPLC system, Santa Clara, CA, USA equipped with a Bio- Rad Aminex HPX-87H column (Hercules, CA, USA). The set conditions of the column were as follow: 50 °C and mobile phase was 5 mM of H_2SO_4 flowing at a rate of 0.6 mL/min. A refractive index (RI) detector was used to examine xylose content. The furfural analyses were performed using a UV/Vis detector at 280 nm. All samples were analysed in duplicated. All

experimental errors related with measurements described above pertain solely to the calibration technique used to quantify the concentrations of products.

Phase equilibria prediction

The phase equilibria of systems constituted by THF, H_2O and CO_2 were predicted using ADF 2014 software of Scientific Computing & Modelling. For this purpose COSMO-SA 2013-ADF model was used. The predicted compositions of solid and liquid phases for all examined reactions are given in Tables A1-A4.

Table A1. The composition of liquid (x) and vapour (y) phase together with the composition of the feed (x_{feed}) for reactions listed in Table 6.1.

Entry	T_{final}	$t_{at\ final\ T}$	$p_{inicialCO2}$	[xylose]	$V_{aq.}:V_{THF}$	x_{H2O}	x_{THF}	x_{CO2}	x_{H2O}	x_{THF}	x_{CO2}	y_{H2O}	y_{THF}	y_{CO2}
	ºC	min	bar	g/L	mL/mL	feed	feed	feed						
1	180	60	-	18.8	15:0	1.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00
2	180	60	-	12.5	10:5	0.90	0.10	0.00	0.95	0.05	0.00	0.05	0.94	0.01
3	160	30	50.7	18.8	25:0	0.37	0.00	0.63	0.98	0.00	0.02	0.00	0.00	1.00
4	160	60	51.7	18.8	25:0	0.37	0.00	0.63	0.98	0.00	0.02	0.00	0.00	1.00
5	180	30	51.5	18.8	25:0	0.37	0.00	0.63	0.98	0.00	0.02	0.00	0.00	1.00
6	180	60	51.2	18.8	25:0	0.38	0.00	0.62	0.97	0.00	0.03	0.00	0.00	1.00
7	180	60	48.2	12.5	10:5	0.56	0.06	0.38	0.87	0.08	0.05	0.00	0.03	0.97
8	180	60	50.8	12.5	20:10	0.69	0.08	0.24	0.83	0.09	0.08	0.00	0.02	0.98
9	180	60	49.4	12.5	40:20	0.81	0.09	0.10	0.85	0.10	0.05	0.00	0.04	0.96

Table A2. The composition of liquid (x) and vapour (y) phase together with the composition of the feed (x_{feed}) for reactions listed in Table 6.2.

Entry	T_{final}	$t_{at\ final\ T}$	$p_{inicialCO2}$	[xylose]	$V_{aq.}:V_{THF}$	x_{H2O}	x_{THF}	x_{CO2}	x_{H2O}	x_{THF}	x_{CO2}	y_{H2O}	y_{THF}	y_{CO2}
	ºC	min	bar	g/L	mL/mL	feed	feed	feed						
10	180	60	50.2	12.5	15:0	0.63	0.00	0.37	0.98	0.00	0.02	0.00	0.00	1.00
11	180	60	49.4	12.5	12:3	0.58	0.03	0.39	0.93	0.04	0.03	0.00	0.02	0.98

7	180	60	48.2	12.5	10:5	0.56	0.06	0.38	0.87	0.08	0.05	0.00	0.02	0.98
12	180	60	49.9	12.5	7.5:7.5	0.44	0.10	0.47	0.71	0.15	0.15	0.00	0.02	0.98
13	180	60	49.6	12.5	5:10	0.36	0.16	0.49	0.56	0.24	0.20	0.00	0.02	0.98
14	180	60	49.5	12.5	3:12	0.23	0.20	0.57	0.31	0.28	0.42	0.00	0.01	0.99

Table A3. The composition of liquid (x) and vapour (y) phase together with the composition of the feed (x_{feed}) for reactions depicted in Figure 6.1.

T_{final}	$t_{at\ final\ T}$	$p_{inicialCO2}$	[xylose]	$V_{aq.}:V_{THF}$	$x_{H2O\ feed}$	$x_{THF\ feed}$	$x_{CO2\ feed}$	x_{H2O}	x_{THF}	x_{CO2}	y_{H2O}	y_{THF}	y_{CO2}
ºC	min	bar	g/L	mL/mL									
180	60	49.6	9.4	10:5	0.53	0.06	0.41	0.85	0.09	0.06	0.00	0.02	0.98
180	60	49.6	9.4	7.5:7.5	0.45	0.10	0.45	0.73	0.15	0.12	0.00	0.02	0.98
180	60	48.9	9.4	5:10	0.33	0.15	0.52	0.49	0.21	0.30	0.00	0.01	0.98
180	60	50.1	6.3	12:3	0.57	0.03	0.40	0.92	0.04	0.04	0.00	0.02	0.97
180	60	50.1	6.3	10:5	0.51	0.06	0.44	0.84	0.09	0.07	0.00	0.02	0.98
180	60	50.3	6.3	7.5:7.5	0.42	0.09	0.49	0.70	0.15	0.15	0.00	0.02	0.98
180	60	50.6	6.3	5:10	0.33	0.15	0.52	0.50	0.22	0.28	0.00	0.01	0.99
180	60	49.1	6.3	3:12	0.22	0.20	0.58	0.31	0.28	0.41	0.00	0.01	0.98

Table A4. The composition of liquid (x) and vapour (y) phase together with the composition of the feed (x_{feed}) for reactions depicted in Figure 6.2

T_{final}	$t_{at\ final\ T}$	$p_{inicialCO2}$	[xylose]	$V_{aq.}:V_{THF}$	x_{H2O}	x_{THF}	x_{CO2}	x_{H2O}	x_{THF}	x_{CO2}	y_{H2O}	y_{THF}	y_{CO2}
ºC	min	bar	g/L	mL/mL	feed	feed	feed						
180	10	50.4	12.5	10:5	0.54	0.06	0.40	0.84	0.09	0.08	0.00	0.03	0.97
180	20	52.1	12.5	10:5	0.53	0.05	0.42	0.85	0.09	0.07	0.00	0.03	0.97
180	30	51.4	12.5	10:5	0.54	0.06	0.40	0.86	0.08	0.06	0.00	0.03	0.97
180	45	50.9	12.5	10:5	0.55	0.06	0.39	0.84	0.09	0.07	0.00	0.03	0.97
180	60	48.2	12.5	10:5	0.56	0.06	0.38	0.87	0.08	0.05	0.00	0.03	0.97
180	90	49.5	12.5	10:5	0.56	0.06	0.38	0.85	0.09	0.06	0.00	0.03	0.97
180	120	50.0	12.5	10:5	0.56	0.06	0.38	0.83	0.09	0.08	0.00	0.03	0.97
180	10	50.6	9.4	7.5:7.5	0.45	0.10	0.45	0.73	0.16	0.12	0.00	0.02	0.98
180	20	51.1	9.4	7.5:7.5	0.44	0.10	0.46	0.72	0.15	0.13	0.00	0.02	0.98
180	30	51.8	9.4	7.5:7.5	0.44	0.10	0.46	0.71	0.15	0.15	0.00	0.02	0.98
180	45	49.8	9.4	7.5:7.5	0.44	0.10	0.46	0.71	0.15	0.14	0.00	0.02	0.98
180	60	49.6	9.4	7.5:7.5	0.45	0.10	0.45	0.73	0.15	0.12	0.00	0.02	0.98
180	90	50.1	9.4	7.5:7.5	0.46	0.10	0.44	0.71	0.15	0.14	0.00	0.02	0.98
180	120	50.9	9.4	7.5:7.5	0.46	0.10	0.44	0.73	0.15	0.12	0.00	0.02	0.98
180	10	49.5	6.3	5:10	0.35	0.14	0.51	0.52	0.23	0.29	0.00	0.01	0.99
180	20	50.1	6.3	5:10	0.33	0.14	0.53	0.50	0.21	0.29	0.00	0.01	0.99

180	30	50.7	6.3	5:10	0.32	0.14	0.53	0.51	0.22	0.27	0.00	0.01	0.99
180	45	49.8	6.3	5:10	0.33	0.15	0.52	0.48	0.21	0.31	0.00	0.01	0.99
180	60	50.6	6.3	5:10	0.33	0.15	0.52	0.50	0.22	0.28	0.00	0.01	0.99
180	90	49.2	6.3	5:10	0.33	0.15	0.52	0.49	0.21	0.30	0.00	0.01	0.99
180	120	49.6	6.3	5:10	0.33	0.15	0.53	0.48	0.21	0.31	0.00	0.01	0.99

The example of the obtained phase envelopes is given in the Figure A1.

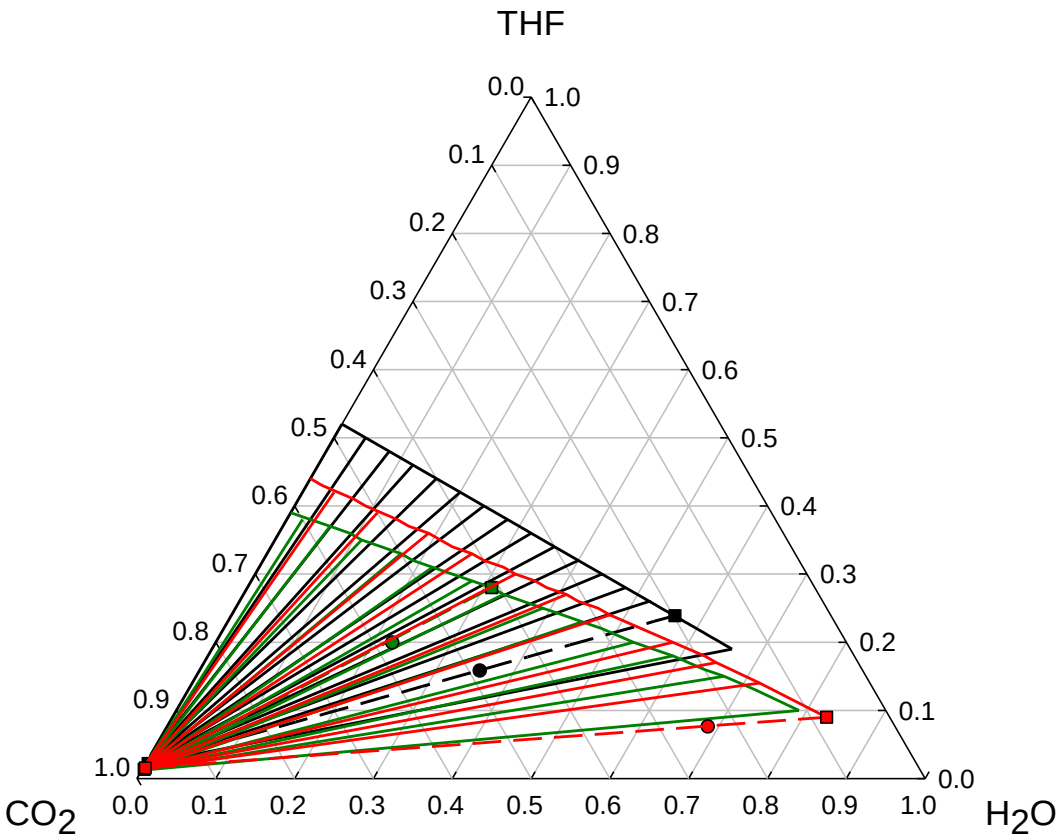

Figure A1. The phase envelopes of system containing CO_2 (50 bar), THF and H_2O for 180 °C and for final pressure of 99 bar (black – entry 13) or 117 bar (red – entry 8) or 127 bar (green – 6.3 g/L xylose concentration in feed, $V_{aq.}$:V_{THF} ratio of 3:12 mL/mL). The closed circle represents the overall composition of the reaction mixture and the dash line depicts tie-line connecting points (closed squares) describing either liquid or gas phase compositions.

Experimental error analysis

The randomly selected dehydration experiments were performed at least in duplicate to examine the reproducibility of the performed works. This approach was not employed in case of all experiments due to the impossibility to reproduce exactly the same experimental conditions, namely the amount of CO_2 (number of moles of CO_2) placed in the reactor. Even with the methodology presented above regarding the conditions at which CO_2 was introduced to the system, the number of CO_2 moles varies producing every time different conditions. The only method allowing to establish the correctness of the produced data is the obtained tendency observed analysing the entire set of data.

Mathematic formulas

Xylose conversion and furfural selectivity were calculated using the following formulas:

$Xylose\ conversion\ (X_{Xylose}) = \frac{[xylose]_{feed} \times V_{feed} - [xylose]_{final} \times V_{final}}{[Xxlose]_{feed} \times V_{feed}} \times 100\%$ and

$Furfural\ yield\ (Y_{furfural}) = \frac{[furfural] \times V_{final}}{[xylose]_{feed} \times V_{feed}} \times 100\%$ and

$Selectivity\ to\ furfural\ (S_{furfural}) = \frac{Y_{furfural}}{X_{xylose}}$, where $[xylose]_{feed}$ means initial xylose concentration (mol/mL), $[xylose]_{final}$ represents xylose concentration after reaction (mol/mL), V_{feed} represents the volume of xylose solution (mL), V_{final} indicates the total volume of xylose solution and THF after reaction (mL).

Appendix B to Chapter VII

New Approach to Ammonia Pre-treatment Integrates Better Feedstock Logistics with Improved Sugar Conversion

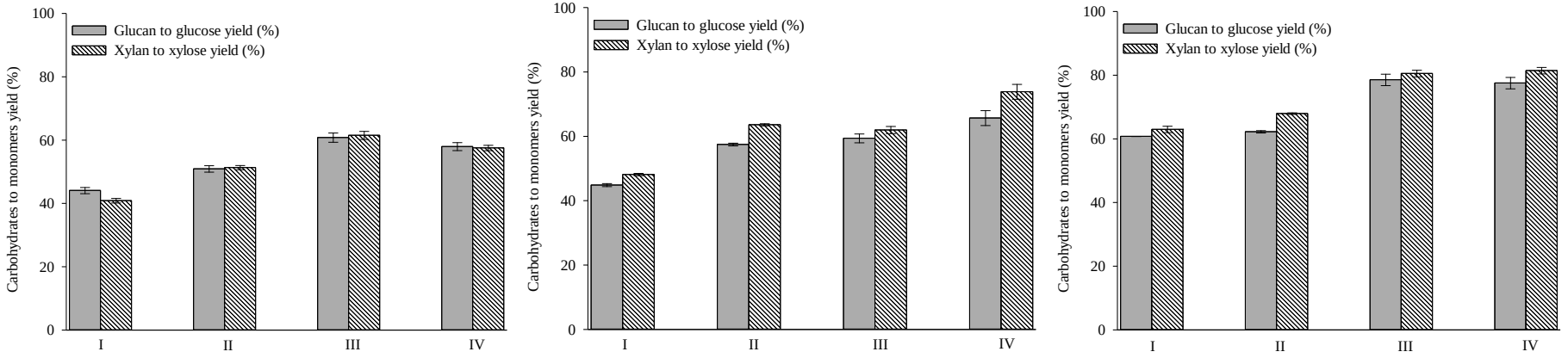

Figure B1. Effect of COBRA conditions on 72 h low-solid loading enzymatic hydrolysis of pelletised sugarcane bagasse. The enzymatic hydrolysis experiments were conducted with 1% glucan loading and 15 mg enzyme/ g glucan.

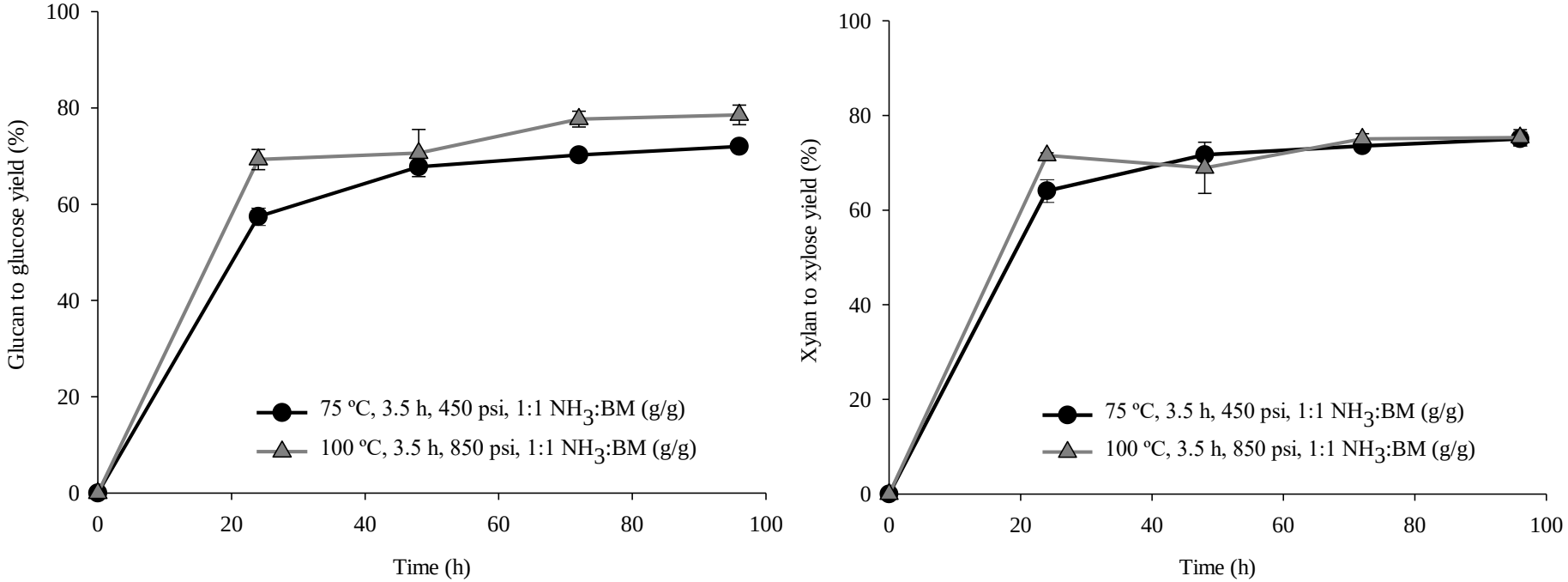

Figure B2. Effect of COBRA pre-treatment temperature on conversion rates of glucan and xylan into glucose and xylose, respectively. High-solid loading enzymatic hydrolysis experiments were carried out at 6% glucan loading (w/w, glucan), 15 mg protein/ g glucan and using the optimised enzymatic cocktail (71 wt.% CTec3: 23 wt.% HTec3: 6 wt.% Multifect Pectinase) for 96 h.

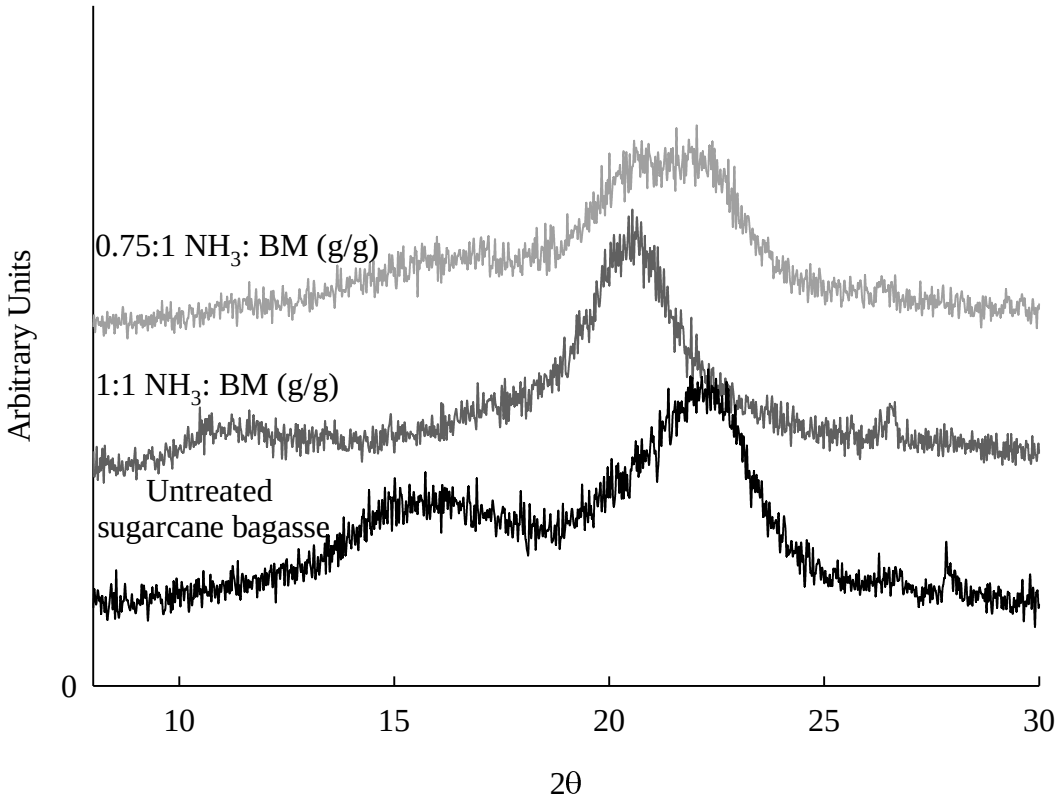

Figure B3. Influence of COBRA ammonia loading on crystallinity of pelletised sugarcane bagasse by X-ray diffraction.

Table B1. Summary of *Saccharomyces cerevisiae* 424A (LNH-ST) fermentation performance in hydrolysate produced from sugarcane bagasse subjected to various pre-treatments and operational conditions. All enzymatic hydrolysates from COBRA, COBRA – LE and EA were produced at 6% glucan loading (w/w, glucan), 15 mg of protein/ g glucan for 96 h of hydrolysis time, whilst AFEX^{TM} and StEx-derived liquors were produced at 25 mg protein/ g glucan. Prior to fermentation, all enzymatic liquid streams were supplemented with 0.25 % (w/w) of corn steep liquor. Fermentations were carried out with an initial inoculum concentration of 1.072 g/L at 30 °C, pH 5.5, and a shaking speed of 150 rpm for 120 h.

Pre-treatment	Operational conditions	Initial concentration		Consumption (%)		$Y_{x/s}$ (g_{CDW}/g_{sugar})	Metabolic Yield (%)	$Y_{p/s}$ (g_{EtOH}/g_{sugar})	EtOH		Process Yield (%)
		Glucose (g/L)	Xylose (g/L)	Glucose	Xylose				Concentration (g/L)	Productivity (g/L/h)	
COBRA	100 °C, 3.5 h, 850 psi, 1:1 NH_3:BM (g/g)	57.8	36.9	98.7 ± 0.0	83.5± 0.5	0.0461	96.0± 0.4	0.454	38.9	25.4± 0.6	69
	75 °C, 4 h, 450 psi, 1:1 NH_3:BM (g/g)	57.5	38.1	98.7± 0.1	81.7± 0.5	0.0458	96.2± 0.4	0.445	37.8	24.3± 0.7	66
	75 °C, 4 h, 450 psi, 0.75:1 NH_3:BM (g/g)	53.7	36.6	98.7± 0.1	85.8± 2.0	0.0444	92.8± 0.9	0.436	38.7	23.0± 1.1	62
COBRA-LE	100 °C, 3.5 h, 850 psi, 1:1 NH_3:BM (g/g)	60.9	36.2	99.0± 0.1	89.5± 0.4	0.0446	97.5± 1.8	0.468	42.5	26.9± 0.1	73
	75 °C, 4 h, 450 psi, 1:1 NH_3:BM (g/g)	58.5	37.3	98.9± 0.1	84.3± 0.1	0.0454	96.2± 0.3	0.451	39.5	25.5± 0.3	69

EA	120 °C, 0.5 h, 1200 psi, 6:1 NH_3:BM (g/g)	54.8	36.3	99.2± 0.0	93.6± 1.0	0.0454	93.1± 1.0	0.454	41.0	27.6± 0.4	75
AFEX™*	140 °C, 1 h, 400 psi, 1:1 NH_3:BM (g/g)	59.0	37.01	100	96	0.0493	92	0.461	44.2	25.6	69
StEx*	200 °C, 0.1 h	69.7	26.1	98	37	0.0291	87	0.362	34.6	16.2	44

EA – Extractive Ammonia; AFEX™- Ammonia Fiber Expansion; StEx – Steam-Explosion; EtOH – Ethanol; CDW – Initial inoculum concentration.* Results obtained by Mokomele et al.[11]